

***In vivo* high content analyses of
protein-protein interactions at budding yeast kinetochores
using a wide-field time-gated FLIM-based platform
*openFLIM-HCA***

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Abstract

This thesis describes my work to develop and apply an automated fluorescence lifetime imaging (FLIM) microscopy-based method for high-content analysis (HCA) of protein-protein interactions (PPIs) at the budding yeast kinetochore in live cells via Förster resonance energy transfer (FRET). FLIM provides a robust readout of FRET, since the fluorescence lifetime is independent of the fluorophore concentration, excitation power and the spectral properties of the sample and instrument, but it requires relatively high signal to noise. Here I demonstrated, for the first time, that FLIM HCA could be applied in live cells to study endogenous proteins labelled with fluorescent proteins and developed new protocols and data acquisition and analysis tools to address the challenges associated with the low copy number of kinetochore proteins (KPs) and high (autofluorescence) background.

The *openFLIM-HCA* platform developed at Imperial College London implements automated multiwell plate FLIM using wide-field, optically-sectioned, time-gated imaging with open source software to analyse FLIM data. I adapted the instrumentation and software tools to map (sub-10 nm) co-localisation of KPs using FRET in live yeast cells and validated this methodology by showing that it can detect known KP interactions. I then explored the potential of this FLIM HCA methodology to query the kinetochore localisation of Rdh54p as a means to investigate how this DNA repair protein promotes the adaptation to the DNA damage-induced cell cycle arrest. I also forced the associations of Rdh54p (and mutants) with most of the proteins in the proteome using the high-throughput synthetic physical interaction (SPI) method to identify associations that affect cell growth. Both the FLIM and SPI data converged on the CTF19/COMA complex at the kinetochore, suggesting that Rdh54p constitutively localises adjacent to this complex, which may have functional significance. The SPI screens also identified hits related to the DNA damage checkpoint that are interesting for future investigations.

Author declaration

All the work presented in this thesis was performed by the author with the following exceptions:

- The initial set-up of the wide-field time-gated FLIM microscope and the *μManager* plug-in for automated data acquisition with the microscope were developed by a number of previous and current members of the Photonics Group at Imperial College London, including Dr. Douglas Kelly, Dr. Frederik Görlitz, and Dr. Sunil Kumar.
- The implementation of the frequency-doubled Ti:Sapphire laser as the new excitation source for the FLIM microscope was performed in collaboration with Dr. Sunil Kumar (Imperial College London).
- The testing of using the fluorescence decay of quenched fluorescein and the Delta function convolution method for FLIM data analysis was performed in collaboration with Dr. Edwin Garcia (Imperial College London).
- The *FLIMfit* software is developed by Dr. Sean Warren (previously Imperial College London).

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List of abbreviations

°C	Degree Celsius
Δ	ORF deletion
5-FOA	5-Fluoroorotic Acid
AC	Alternating current
ADE	Adenine
Amp	Ampicilin
APC/C	Anaphase promoting complex/cytosome
CCAN	Constitutive centromere-associated network
CCD	Charge-coupled device
CDE	Centromere-determining element
CDK	Cyclin-dependent kinase
CFP	Cyan fluorescent protein
ChIP-Seq	Chromatin immunoprecipitation followed by sequencing
CNPMK	Copy number per metaphase kinetochore
CPC	Chromosome passenger complex
cyro-EM	Cryogenic electron microscopy
cyro-ET	Electron cryotomography
DC	Direct current
DDC	DNA damage checkpoint
DFCM	Delta function convolution method
DNA	Deoxyribonucleic acid
DSB	Double-strand break
EM	Electron microscopy
FAD	Flavin adenine dinucleotide
FEAR	Cdc-fourteen early-anaphase release
FLIM	Fluorescence lifetime imaging
FOV	Field-of-view
FRAP	Fluorescence recovery after photobleaching

FRET	Förster resonance energy transfer
γ-H2A	H2A phosphorylated on S129
G1-phase	Gap 1 phase
G2-phase	Gap 2 phase
GAL	Galactose
GBP	GFP-binding protein
GFP	Green fluorescent protein
GO	Gene ontology
GOI	Gated optical intensifier
GUI	Graphic user interface
H3K79	H3 methylated on K79
HCA	High-content analysis
His	Histidine
HR	Homologous recombination
HYG	Hygromycin
IRF	Instrument response function
KI	Potassium iodide
KP	Kinetochore protein
LB medium	Luria-Bertani medium
Leu	Leucine
LGR	Log growth ratio
M-phase	Mitotic phase
MAP	Microtubule-associated proteins
<i>MATa</i>	Yeast mating type a
<i>MATα</i>	Yeast mating type alpha
MCC	Mitotic-checkpoint complex
MCP	Microchannel plate
MEN	Mitotic exit network
MMS	Methyl methanesulfonate
mTq2FP	mTurquoise2 fluorescent protein
NADH	Reduced nicotinamide adenine dinucleotide

ND filter	Neutral density filter
NHEJ	Non-homologous end joining
NPC	Nuclear pore complex
NTH algorithm	Nonlinear top-hat algorithm
ORF	Open reading frame
PALM	Photo
PCNA	Proliferating-cell nuclear antigen
PCR	Polymerase chain reaction
PMT	Photomultiplier tube
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP4	Protein phosphatase 4
PPI	Protein-protein interaction
PSF	Point spread function
Ref(s).	Reference(s)
RFP	Red fluorescent protein
ROI	Region-of-interest
ROS	Reactive oxygen species
S-phase	Synthesis phase
S/N	Signal-to-noise ratio
SAC	Spindle assembly checkpoint
SC medium	Synthetic complete medium
(s)CMOS camera	(Scientific) Complementary metal-oxide-semiconductor camera
SD	Synthetic defined medium
SGA	Synthetic genetic array
SIM	Structure illumination microscopy
SPA	Selective ploidy ablation
SPB	Spindle pole body
SPI	Synthetic physical interaction
SPO medium	Sporulation medium

SPOC	Spindle positioning checkpoint
SRM	Super-resolution microscopy
ssDNA	Single-stranded DNA
START	Restriction checkpoint
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
TCSPC	Time-correlated single photon counting
TVB	Time-varying background
UDS	Universal donor strain
UK	United Kingdom
US	United States
YFP	Yellow fluorescent protein
YPD medium	Yeast extract peptone dextrose medium
ZDC module	Z-drift compensation module

Chapter 1. Introduction

The research reported in this thesis was motivated by the ultimate goal of studying the protein interactions of Rdh54p at the kinetochore of the budding yeast *Saccharomyces cerevisiae*, which play a key role in promoting the escape of a cell from a prolonged checkpoint-induced cell cycle arrest. To this end, I developed a methodology for assaying of protein-protein interactions (PPIs) *in vivo* at the budding yeast kinetochore, which is a macromolecular multi-protein complex that plays key roles in ensuring faithful chromosome segregation. The methodology utilises automated Fluorescence Lifetime Imaging (FLIM) microscopy to implement a high-content analysis (HCA) modality to read out Förster resonance energy transfer (FRET) between fluorescent proteins labelling endogenous proteins at the kinetochores of live yeast cells. In this introductory chapter I provide an overview of the cell cycle and the cell cycle checkpoints of budding yeast, I outline the architecture and the functions of the budding yeast kinetochore, and I briefly review the FLIM FRET-based techniques that have been applied to study PPIs.

1.1 The budding yeast cell cycle and cell cycle checkpoints

The budding yeast *Saccharomyces cerevisiae* has long served as a useful model organism for studying the functional roles of genes and proteins in eukaryotic cells¹. It is one of the simplest eukaryotic organisms and also the first eukaryote to have a fully sequenced genome². It can grow as a haploid with 16 chromosomes and a mating type of *MAT α* or *MATa*, or as a diploid with 16 pairs of chromosomes. It is fast growing (divides every ~100 min with sufficient nutrients³), can be genetically manipulated with relative ease, and is naturally compatible with high-throughput genome-scale studies⁴. More importantly, despite its simplicity, it shares highly conserved features (including molecular functions, cellular components, and biological processes) with metazoans, including humans⁴.

One process that has particularly benefited from yeast-based studies is the cell cycle. The work of Leland Hartwell and Paul Nurse, who discovered the key regulators of the cell

cycle using the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*⁵⁻⁹, was recognised by the Nobel Prize in Physiology or Medicine in 2001 (together with the sea urchin egg-based work of Tim Hunt¹⁰). In this section, I start with an overview of the budding yeast cell cycle and cell cycle checkpoints, and progress to describe the two cell cycle checkpoints that are most relevant to my project, the DNA damage checkpoint (DDC) and the spindle assembly checkpoint (SAC), in terms of their activation, recovery, and adaption. I will also review the existing literatures on the potential crosstalk between these two checkpoints.

1.1.1 Overview of the budding yeast cell cycle and cell cycle checkpoints

The cell cycle is a process consisting of a series of events that a cell goes through for proliferation, i.e. the division of one cell into two. In the case of budding yeast, cells divide through the budding of a mother cell that generates a nascent daughter cell. In order to divide, the mother cell needs to go through the four stages of the cell cycle: G1-, S-, G2-, and M-phases (Figure 1-1).

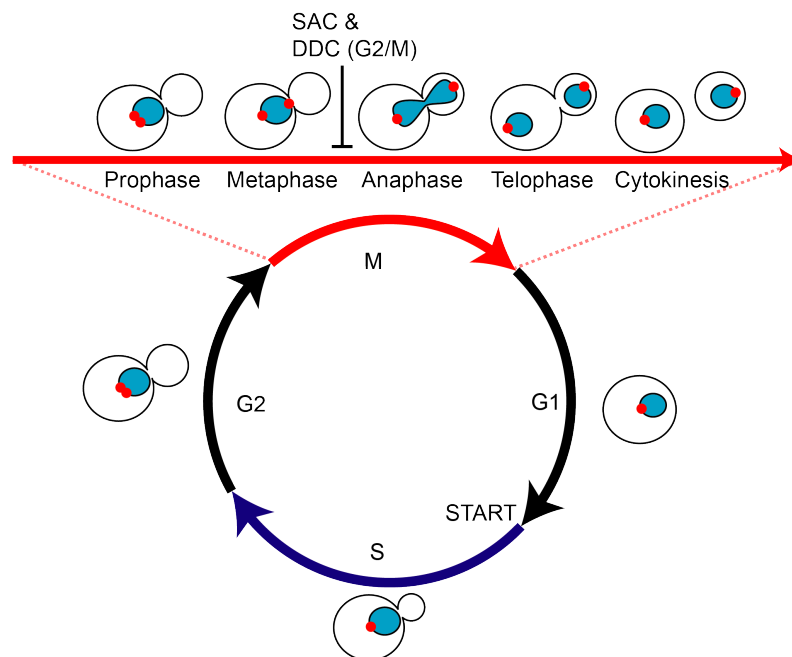


Figure 1-1 The budding yeast cell cycle

The cell cycle of the budding yeast *Saccharomyces cerevisiae* consists of four stages: G1, S, G2, and M. The M-phase is when mitosis and cytokinesis occur. A cell commits to the cell cycle after satisfying the restriction checkpoint (START). The spindle assembly checkpoint (SAC) and the

G2/M DNA damage checkpoint (DDC) can arrest the cell cycle at the metaphase-to-anaphase transition for ensuring genome integrity. The budding yeast cells show the nucleus (blue) and the SPBs (red).

The G1-, S-, and G2-phases are sometimes referred to as the interphase. In G1 (Gap 1), the cell grows in size to prepare for the subsequent S-phase (Synthesis-phase), during which DNA replication, spindle-pole body (SPB; the microtubule-organising centre in yeast) duplication and bud emergence occur. The cell then grows again in G2 (Gap 2) before entering M-phase (Mitotic-phase) for cell division.

M-phase comprises two steps: mitosis and cytokinesis. Mitosis is the process where sister chromatids are segregated and it can be further divided into four phases:

- *prophase*, during which the DNA replicated in S-phase condenses and microtubules emanating from the two SPBs make attachments to the sister chromatids through the kinetochores (which is introduced later in Section 1.2);
- *metaphase*, during which the two kinetochores on each pair of sister chromatids are attached by microtubules from opposite SPBs, respectively, and sister chromatids are bi-oriented on the spindle equator under the poleward pulling force from depolymerising microtubules and the resisting force from the ring-shaped cohesin complexes that hold sister chromatids together;
- *anaphase*, during which cohesin is cleaved and each pair of sister chromatids are segregated and pulled respectively towards to the two SPBs;
- *telophase*, during which the mitotic spindle is disassembled.

As cells exit from mitosis, cytokinesis ensues, upon which the mother cell divides through budding, resulting in a mother cell and a daughter cell, each with an identical set of chromosomes. After cell division, both the mother and the daughter cells can enter a new cell cycle to produce more offspring.

The cell cycle of eukaryotic cells is highly regulated, principally by phosphorylation events mediated by cyclin-dependent kinases (CDKs), to drive the relevant cellular events in a timely manner. In budding yeast, there is only one CDK responsible for the cell cycle progression, Cdc28p, and it realises its function through interacting specifically with nine

different cyclins, its “activators”, at different cell cycle stages¹¹ (Figure 1-1). There are two classes of cyclins: G1 cyclins (Cln1p, Cln2p, Cln3p), which are responsible for the kinase activity of Cdc28p in G1, and B-type cyclins (Clb1p to Clb6p), which function with Cdc28p in the rest of the cell cycle. In G1 as the cell grows, the accumulation of Cln3p activates Cdc28p by the formation of the Cln3p-Cdc28p complex, which then phosphorylates Whi5p, an inhibitor of the SBF complex that is required for the transcription of *CLN1* and *CLN2*. Cln3p-Cdc28p-dependent phosphorylation of Whi5p drives Whi5p’s nuclear export, and therefore promotes the synthesis of Cln1p and Cln2p^{12,13}. Cln1p and Cln2p, when bound to Cdc28p, also promote the synthesis of themselves through a positive feedback loop^{14–16}. The association of the three G1 cyclins with Cdc28p further promotes the degradation of a Cln-Cdc28p inhibitor, Far1p¹⁷, and a Clb-Cdc28p inhibitor, Sic1p¹⁸, and eventually promotes the irreversible transition from G1 to S¹⁹.

From S phase, the six B-type cyclins start to be expressed and associate with Cdc28p in three successive waves²⁰ and, while there are some overlaps in their functions (i.e. not all cyclins are essential), each wave of B-type cyclins have their specific cell cycle roles. The first wave, Clb5p-Cdc28p and Clb6p-Cdc28p, achieve their peak activity in early S-phase. They are involved in both the initiation of DNA replication^{21–24} and the prevention of re-replication²⁵, and also drive the degradation of the G1 cyclins²⁶. The concentrations of Clb3p-Cdc28p and Clb4p-Cdc28p then reach their peaks after DNA replication starts, and they are required for not only the completion of DNA replication but also the assembly of interphase spindle and the G2 to M transition²⁷. Finally, Clb1p-Cdc28p and Clb2p-Cdc28p function prior to, and in, mitosis to promote the entry into mitosis together with Clb3p-Cdc28p and Clb4p-Cdc28p^{27,28}, and to drive the metaphase to anaphase transition by activating the anaphase-promoting complex/cyclosome (APC/C)-Cdc20p, which degrades the securin Pds1p and leads to the cleavage of cohesin by the separase Esp1p, and by promoting spindle elongation²⁹.

Upon mitotic exit, the phosphates that were added by Cdc28p need to be removed. This process is under the coordination of phosphatases, principally Cdc14p, whose release from the nucleolus and accumulation in anaphase requires the CdcFourteen Early Anaphase Release (FEAR) network³⁰ and the Mitotic Exit Network^{31,32} (MEN). The

FEAR network triggers the release of Cdc14p in early anaphase via the phosphorylation of the nucleolar protein Net1p by Clb1p-Cdc28p and Clb2p-Cdc28p³³, and also promotes the activation of the MEN through the Cdc14p-mediated dephosphorylation of the MEN component Cdc15p³⁴. The MEN activation also requires the Clb-Cdc28p-dependent mitotic spindle formation and orientation³⁵. In turn, the Cdc14p released from the FEAR network and the MEN represses the activity of Clb-Cdc28p, by activating the APC/C-Cdh1p complex to degrade B-type cyclins and by promoting the synthesis and maintenance of the aforementioned Clb-Cdc28p inhibitor Sic1p^{36,37}. Cdc14p also works in partnership with protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) to dephosphorylate key phosphosites in the cell as cells exit mitosis. How these phosphatases are coordinated remains elusive³⁸. Collectively, these mechanisms render Cdc28p inactive when the cell finishes the current cell cycle and re-enters G1.

Alongside the CDK Cdc28p and the cyclins, there are several surveillance mechanisms called cell cycle checkpoints that act at critical transition points to halt cell cycle progression until certain requirements are fulfilled, so as to ensure the faithful replication and segregation of the genetic materials. These checkpoints include:

- the restriction point (START), which arrests the cell in G1 in the absence of an appropriate cell size and sufficient nutrients, or in the presence of mating pheromone. Once it is satisfied, the cell enters S-phase and commits to an irreversible cell cycle^{6,39}.
- the DNA damage checkpoint (DDC), which in the presence of damaged DNA can arrest the cell in either G1, S, or G2/M using cell cycle stage-dependent signalling pathways, offering time for DNA repair. It eventually directs the cell to resume the cell cycle through checkpoint recovery/adaption or to apoptosis when the damage is too severe^{40–46} (see Section 1.1.2 for details).
- the DNA replication checkpoint, which partially shares its pathway with the DDC and specifically monitors the progress of DNA replication and the presence of stalled replication forks in S-phase^{47,48}.
- the morphogenesis checkpoint, which makes sure that the cell only enters M-phase after proper bud formation^{49,50}.

- the spindle assembly checkpoint (SAC), which halts the cell cycle prior to anaphase until all the kinetochores are attached by spindle microtubules and chromosome bi-orientation is established or until SAC adaptation occurs^{51–54} (see Section 1.1.3 for details).
- the spindle positioning checkpoint (SPOC), which functions prior to mitotic exit and ensures that the mitotic spindle is properly aligned for the faithful partitioning of the chromosomes between the mother and the daughter cells^{35,55}.

While they respond to different unfavourable scenarios, the checkpoints ultimately arrest the cell cycle through signalling pathways that inhibit or maintain the activities of Cdc28p and cyclins. For example, in the presence of pheromone, START inhibits the transition from G1 to S by repressing the activity of Cln3p-Cdc28p via the Cln-Cdc28p inhibitor Far1p, which then inhibits the downstream positive feedback loop that drives the transcription of *CLN1* and *CLN2* and the irreversible transition into S-phase³⁹. For detailed reviews on the checkpoints act on the cyclin-Cdc28p activities, please refer to Refs.^{11,56}.

1.1.2 DNA damage checkpoint (DDC)

The DDC is a surveillance mechanism that swiftly reacts to the detection of DNA damage and arrests cell cycle progression, offering time to repair the damaged DNA. DNA damage can be induced by endogenous factors (e.g. replication errors, free radicals produced by cellular metabolism, and programmed nuclease activities for events like meiotic recombination) as well as exogenous factors (e.g. ionising radiation, ultraviolet radiation, and chemical agents like methyl methanesulfonate (MMS) and cisplatin). It can appear in the form of mutations in the bases or the sugar-phosphate backbone of nucleotides, DNA strand breaks, and DNA-DNA or DNA-protein crosslinks; the most severe one of these is DNA double-stranded break (DSB)^{57–60}. Unrepaired or improperly repaired, DNA DSBs can lead to genome instability with various types of chromosomal rearrangements, affecting the viability of yeast cells and causing diseases, including cancer, in humans^{45,58,61,62}. Hence, the evolutionarily highly conserved DDC and DNA damage repair pathways are critical for the preservation of genome integrity during cell proliferation⁶³. In budding yeast, DNA DSB is primarily repaired by non-homologous

end joining (NHEJ) in G1 and homologous recombination (HR) in S and G2/M⁶⁴. Without going into details about the differences between the two pathways, I focus here on introducing the DDC in response to DNA DSB in G2/M that is most relevant to my project, as it arrests the cell at the metaphase-to-anaphase transition^{65,66}, which is the same as the SAC (see Section 1.1.3) that is regulated at the kinetochore (see Section 1.2) and indeed has some modes of crosstalk with the SAC that are yet to be well characterised (see Section 1.1.4).

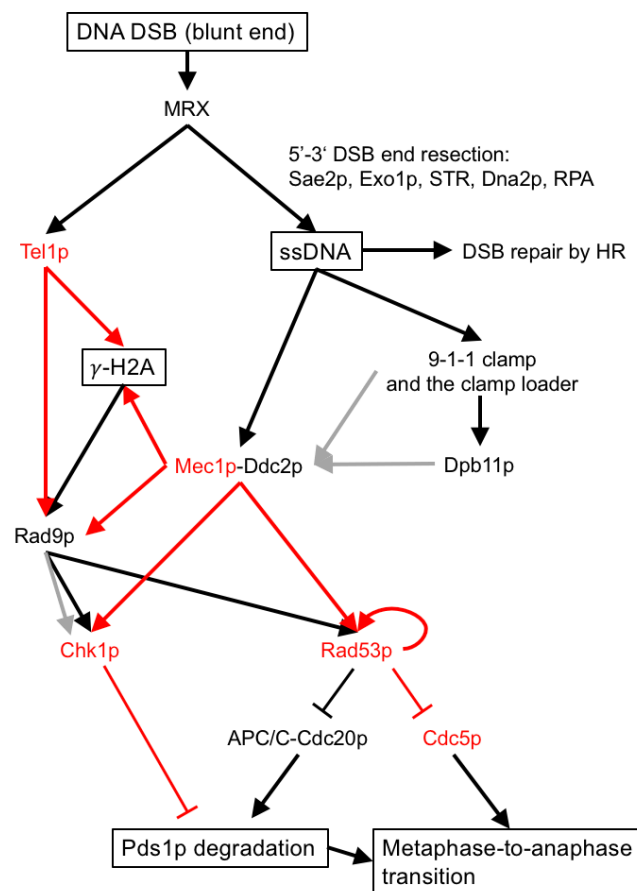


Figure 1-2 Simplified schematic for the activation of the G2/M DDC

Black arrow: recruits the downstream protein(s) or promotes the downstream event . Grey arrow: contributes to the activation of the downstream kinase. Proteins marked as red: kinases. Red arrow: phosphorylation of the downstream protein. Black line terminated with a bar: inhibition. Red line terminated with a bar: inhibition by phosphorylation.

Activation of the DDC at G2/M

The activation of the DDC in response to DNA DSB starts with the detection of DSB ends. This triggers a complex cell cycle-dependent kinase cascade to transduce the checkpoint signal to the downstream effectors of cellular processes, and eventually results in a cell cycle arrest as well as other cellular responses⁶⁷; for the G2/M DDC, the cell cycle arrest is at the metaphase to anaphase transition⁶⁸.

Blunt DNA DSB ends are first detected by the MRX (Mre11p-Rad50p-Xrs2p) complex^{69,70}. MRX competes with the Ku (Yku70p-Yku80p) complex for binding to unprocessed DSB ends, the latter of which promotes the repairing of the DSB by NHEJ, i.e. by directly ligating the two DSB ends, but is only predominant in G1^{71,72}. Upon binding to the DSB ends, MRX recruits Tel1p (ATM in humans), a key protein kinase for initiating the DDC, to the DSB site, primarily via the interaction between Xrs2p and Tel1p⁷³. The Mre11p-Rad50p subcomplex may facilitate the Tel1p recruitment independently, as is supported by a study in fission yeast⁷⁴, and its ATP-bound conformation is required for the activation of Tel1p⁷⁵. MRX also promotes the 5'-3' resection of DSB ends in a two-step manner: it first works with the endonuclease Sae2p to generate short 3' ssDNA (single-stranded DNA) tails⁷⁶, and then stimulates the extensive degradation from the 5' ends to generate long 3' ssDNA overhangs in two pathways, one involving the exonuclease Exo1p, the other involving the STR (Sgs1p-Top3p-Rmi1p) helicase-topoisomerase complex, the flap endonuclease Dna2p, and the trimeric RPA (replication protein A) complex (composed of three subunits: Rfa1p, Rfa2p, Rfa3p)⁷⁷⁻⁸⁰. RPA molecules bind to the ssDNA tails, and subsequently function in protecting the ssDNA from being degraded by nucleases and forming fold-back hairpin structures⁸¹, recruiting Rad52p and other proteins required for DNA repair via HR⁸², and recruiting Mec1p (ATR in humans), another protein kinase critical for the DDC, through the interaction between RPA and the Mec1p's interacting partner Ddc2p⁸³. The recruitment of Mec1p-Ddc2p to the RPA-coated ssDNA channels the transition from the initial Tel1p-dependent DDC signalling to the Mec1p-dependent one^{84,85}, as it is followed by the dissociation of MRX and Tel1p from the DNA break sites⁷⁰ and the Mec1p-Ddc2p-independent recruitment of the 9-1-1 checkpoint clamp (Ddc1p-Rad17p-Mec3p; Rad9p-Rad1p-Hus1p in humans) and the clamp loader Rad24p-RFC(Rfc2p-Rfc3p-Rfc4p-Rfc5p)

to the 5'-junctions of the ssDNA⁸⁶⁻⁸⁸. The 9-1-1 checkpoint clamp, in turn, stimulates the kinase activity of Mec1p, by directly interacting with Mec1p via its Ddc1p subunit and by recruiting the replication initiation protein Dpb11p, another activator of Mec1p⁸⁹⁻⁹¹. The 9-1-1 checkpoint clamp also coordinates the resection at DSB ends, by both promoting resection through stimulating the activity of Exo1p and Sgs1p-Dna2p and inhibiting resection through recruiting Rad9p (a mediator protein for the DDC; see below) to DSB ends^{92,93}.

Tel1p and Mec1p initiate the transduction of the DDC signal, in two partially overlapped but non-redundant pathways respectively, via their kinase activities. Central to these pathways is the phosphorylation of histone H2A on its serine 129 (termed γ -H2A). Upon its early recruitment to the unprocessed break sites by MRX, Tel1p phosphorylates the histone H2A molecules, resulting in γ -H2A spreading ~50 kb from each DSB end⁹⁴⁻⁹⁶. Mec1p subsequently promotes the late spreading of γ -H2A as it is recruited to the ssDNA generated by the DSB end resection⁹⁶. γ -H2A is responsible for the recruitment of cohesin to the DSB sites⁹⁷, the exact role of which is still under examination⁹⁸. More important for the DDC signalling, γ -H2A recruits a key DDC mediator, Rad9p, which is constitutively associated with chromatin via interacting with methylated histone H3 at lysine 79 (H3K79)^{99,100}. Rad9p acts as an adaptor between Telp1/Mec1p and the downstream DDC effectors, Rad53p and Chk1p. Rad9p is hyperphosphorylated by Tel1p and/or Mec1p, and this hyperphosphorylation enables the binding of Rad53p to Rad9p^{101,102} and consequently the Mec1p-dependent phosphorylation of Rad53p¹⁰³. Rad9p also brings the Rad53p molecules in close proximities of each other, facilitating the *in trans* autophosphorylation of Rad53p that leads to the full activation of Rad53p and its release from Rad9p¹⁰⁴. The Mec1p-dependent activation of Chk1p is promoted by Rad9p as well, although the exact mechanism behind this is not well characterised¹⁰⁵⁻¹⁰⁷. In addition, the oligomerisation of Rad9p via the interaction between its BRCT domain and its SCD domain is essential for amplifying and maintaining the DDC signalling, but it is constrained by a feedback loop that weakens the oligomerisation by the Rad53p-mediated phosphorylation of Rad9p's BRCT domain¹⁰⁸.

After activation, the effector kinases Rad53p and Chk1p work in separate pathways to inhibit both the metaphase-to-anaphase transition and the mitotic exit^{105,109}. The two

kinases contribute separately to inhibiting the APC/C-Cdc20p-mediated degradation of Pds1p, the securin protein that protects cohesin from being cleaved^{110–112}. Specifically, Rad53p prevents the interaction between Pds1p and Cdc20p, whereas Chk1p phosphorylates Pds1p to render it a poor target for the APC/C-Cdc20p-mediated ubiquitination¹¹². The persistent Pds1p and cohesin therefore prevent anaphase entry, i.e. the separation of sister chromatids. The Chk1p-mediated phosphorylation of Pds1p further inhibits the separase Esp1p from downregulating the PP2A-Cdc55p phosphatase, hence preventing the Cdc28p-dependent phosphorylation of Net1p that is required for the FEAR network¹¹³. As the downregulation of PP2A-Cdc55p is required for the activation of MEN as well, the contribution of Chk1p in preventing mitotic exit also includes the inhibition of MEN¹¹⁴. Rad53p also acts on keeping the MEN inactive, by facilitating the phosphorylation of Bfa1p, a MEN component¹⁰⁹. Additionally, the Rad53p-dependent phosphorylation of the Polo-like kinase Cdc5p prevents the Cdc5p-mediated degradation of Clb-Cdc28p and again inhibits mitotic exit^{105,115}. Collectively, these partially redundant functions of Rad53p and Chk1p in the DDC ensure a robust G2/M arrest in response to DNA damage.

DDC recovery

After completing the repair of the damaged DNA, a cell can “recover” from the DDC-mediated arrest and resume the cell cycle. While a systematic understanding of the DDC recovery process is currently lacking, it has been shown that the process relies on the disruption of the DDC signalling pathway, from the inactivation of Mec1p and Tel1p to the removal or dephosphorylation of various DDC-related proteins. For example, Sae2p, an endonuclease involved in initiating DSB end resection (see above), also functions in down-regulating the DDC by removing MRX and Tel1p from DSB ends and by limiting Rad9p accumulation at DSB ends, both of which down-regulate the activation of Rad53p^{116,117}. The DNA helicase Srs2p is also likely to promote the removal of DDC-related proteins, besides its role in DNA repair, contributing to not just DDC recovery but also DDC adaptation^{118,119} (see below). Another example is Asf1p, a chromatin assembly factor, which on one hand works redundantly with another chromatin assembly factor CAF-1 to drive DDC recovery (likely by restoring chromatin structure after DNA repair)¹²⁰, and on the other hand promotes the dephosphorylation of Rad53p upon its

dissociation from histone H3 mediated by the acetylation of H3K56 by Rtt109p and the subsequent histone H3 ubiquitination by Rtt101p¹²¹. The dephosphorylation events during DDC recovery are mainly carried out by the PP2C phosphatases, Ptc2p and Ptc3p, as well as the PP4 phosphatase Pph3p-Psy2p. All these phosphatases act by dephosphorylating Rad53p, leading to Rad53p inactivation^{122–124}, whilst Pph3p-Psy2p also dephosphorylates γ -H2A, which inactivates Rad53p indirectly via Rad9p inactivation¹²⁵.

DDC adaptation

If the DNA damage cannot be repaired after a prolonged cell cycle arrest, there is a conserved mechanism called DDC adaptation that allows the cell to bypass the arrest despite the persistence of the damaged DNA^{41,126}. Similar to the case of DDC recovery, the exact signal pathways that drive DDC adaptation remain to be fully understood. It is also unclear why metazoan cells adopt this mechanism: while it may be beneficiary for unicellular organisms like budding yeast to override the arrest for the survival of the entire population, DDC adaptation in metazoan cells leads to genome instability and could eventually lead to tumorigenesis¹²⁷.

Studies on DDC adaptation in budding yeast have often employed a system where one or several irreparable DSBs can be induced by the HO endonuclease^{41,126,128}. The occurrence of adaptation was often characterised by the loss of the otherwise persistent phosphorylation and kinase activity of Rad53p¹²⁹, which also implies that cells may bypass the cell cycle arrest by switching off the DDC, perhaps via some common pathways with DDC recovery. Indeed, deleting or mutating several of the genes encoding the aforementioned recovery-related proteins (Sae2p, Srs2p, Ptc2p, and Ptc3p) also leads to defects in adaptation^{118,122,130}, and the Mec1p-interacting partner, Ddc2p, is at least partially removed from the DSB sites upon adaptation⁸⁶. Proteins involved in DSB repair (Yku70p, Sgs1p, the RPA subunit Rfa1p, Rad51p, Rdh54p, Srs2p, Fun30p, Rtt107p-Slx4p) are also required for DDC adaptation^{118,128,131–134}, suggesting that the decision to adapt might depend on the status of repair. The discovery that *yku70* Δ , which increases DSB end resection, causes an adaptation defect that can be suppressed by deleting either of the MRX components, Mre11p or Rad50p, which represses the end resection¹²⁸, led to

the idea that the extent of ssDNA at the break sites determines whether a cell can adapt. However, this was later contradicted by the findings that deletions of Sae2p, Sgs1p, Fun30p, or Rdh54p, which either reduce or do not affect the DSB end resection rate, are also defective in adaption^{130,131,133}. Other factors contributing to DDC adaption include the polo-like kinase Cdc5p^{135,136} and the cytoplasm-to-vacuole autophagy pathway¹³⁷. Interestingly, the permanent arrest phenotype caused by deleting some of the adaptation-related proteins mentioned above can be rescued by deleting the SAC component Mad2p¹³⁸. This indicates that the SAC somehow contributes to the DDC-induced arrest, which is described in more details in Section 1.1.4.

1.1.3 Spindle assembly checkpoint (SAC)

The SAC is a well-conserved mechanism that can arrest the cell cycle at the metaphase-to-anaphase transition similarly to the G2/M DDC introduced above, but it responds to defects in the kinetochore-microtubule attachments instead of to damaged DNA⁵³. For the accurate partition of genetic materials during cell division, the two kinetochores on each pair of sister-chromatids must be respectively attached by microtubules emanating from the two opposite SPBs before entering anaphase, i.e. before chromosome segregation. When all the kinetochores are properly attached, the chromosomes achieve bi-orientation in metaphase with the kinetochores under tension, i.e. being stretched by the poleward force generated by depolymerising microtubules and the resisting force from the cohesin complexes linking each pair of sister-chromatids. Upon entry to anaphase, the linkages between sister-chromatids are cleaved and sister-chromatids are segregated to opposite poles. When there is even one erroneous kinetochore-microtubule attachment that lacks the intra-kinetochore tension, however, the erroneous attachment is disrupted by the Aurora B kinase Ip11p, which generates an unattached kinetochore and allows another attempt to make a correct attachment (see Section 1.2.2 for details). Importantly, such an unattached kinetochore also triggers the SAC, which halts the cell in metaphase to provide time for establishing a correct attachment by preventing the cleavage of cohesin and the degradation of B-type cyclins^{53,139,140}. The SAC is crucial for ensuring faithful chromosome segregation; defects in the SAC in both yeast and metazoan cells could lead to chromosome instability and aneuploidy, both of which are hallmarks of cancer cells^{141–143}.

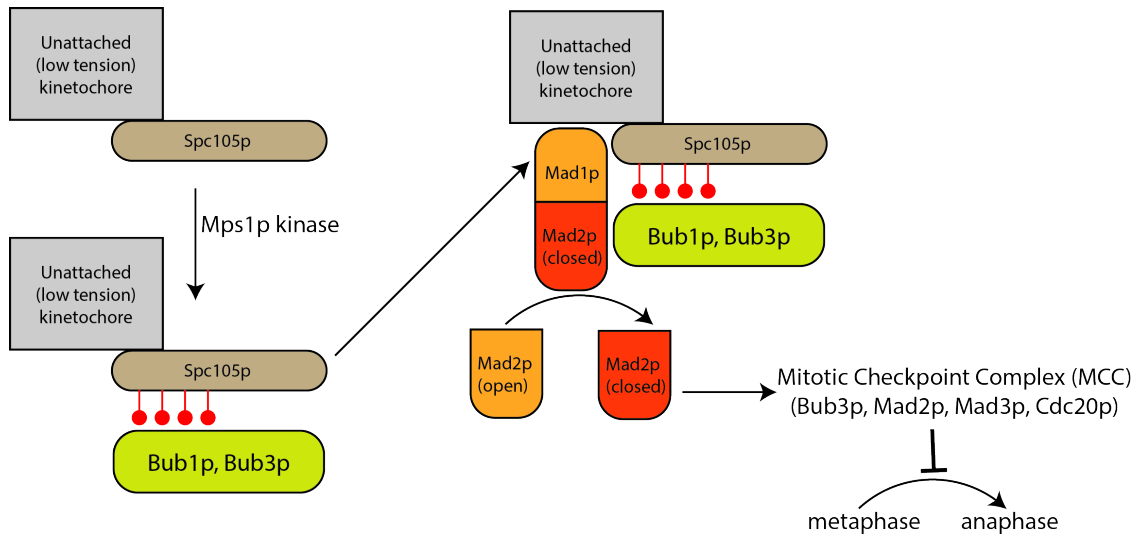


Figure 1-3 Simplified schematic for the activation of the SAC

In the presence of an unattached kinetochore with low intra-kinetochore tension, the kinetochore protein Spc105p is phosphorylated by Mps1p, promoting the subsequent recruitment of the SAC components Bub1p, Bub3p, Mad1p, and Mad2p (closed conformation) to the kinetochore. These components that act as a platform for converting the diffusive Mad2p molecules from the open conformation to the closed conformation, and as such promote the assembly of the MCC that inhibits the metaphase-to-anaphase transition.

SAC activation

It is evident that unattached kinetochores can lead to the activation of the SAC^{144,145}, and it has also been suggested that the lack of intra-kinetochore tension is sufficient to activate the SAC regardless of the attachment status^{144,145}. Although it remains unclear which one of the two scenarios is the primary trigger of the SAC, the signalling pathway governing the SAC activation has been well studied. SAC signalling starts from the unattached or improperly attached kinetochore(s), with the phosphorylation of the protein Spc105p at the outer kinetochore (see Section 1.2.1) on one or several of its conserved MELT repeats by the Mps1p kinase^{146–148}. The kinetochore receptor for Mps1p may be Ndc80p, another protein at the outer kinetochore^{149,150}. Phosphorylated Spc105p recruits the Bub1p-Bub3p complex^{147,151}, and subsequently Bub1p is phosphorylated by Mps1p to enable the subsequent recruitment of the heterotetrameric Mad1p-Mad2p complex^{152,153}. The kinetochore-bound Mad1p-Mad2p complex functions as a platform for converting the soluble Mad2p molecules from the open conformation (Mad2p-O) to the closed conformation (Mad2p-C)¹⁵⁴, which then cycle off the kinetochore to form the mitotic

checkpoint complex (MCC) together with Bub3p, Mad3p, and Cdc20p^{155–157}. The formation of the MCC inhibits the activity of the E3 ubiquitin ligase APC/C, which requires Cdc20p for activation, and therefore prevents the APC/C-Cdc20p-mediated degradation of the securin Pds1p (which protects cohesin via inhibiting the separase Esp1p¹¹⁰) and B-type cyclins^{157–165}, resulting in a cell cycle arrest prior to anaphase entry. Notably, while MCC inhibits APC/C-Cdc20p from ubiquitinating its substrates, it stimulates the autoubiquitination of Cdc20p, which, on one hand maintains a low Cdc20p level for maintaining the SAC-induced arrest, and on the other hand allows prompt activation of the APC/C-Cdc20p when the SAC is off with the rapid turnover of Cdc20p^{166,167} (see below).

SAC recovery

Similar to the case of the DDC, a cell can recover from a SAC-induced cell cycle arrest when all kinetochores are properly attached. This SAC recovery process involves the reversal of the SAC activating activities, including the inhibition of further Mps1p-mediated phosphorylation of Spc105p, the dephosphorylation of Spc105p to disrupt the assembly of Bub1p-Bub3p-(Mad1p-Mad2p)₂ at the kinetochore, and the disassembly of the MCC to activate the APC/C-Cdc20p. Upon the establishment of an end-on attachment, Mps1p is likely to be physically separated from its substrates on Spc105p by its binding to the calponin-homology domain of Ndc80p, with the DAM1 complex acting as an additional barrier¹⁵⁰. It is also possible that the microtubule competes with Mps1p for binding to Ndc80p, and therefore removes Mps1p from the kinetochore upon binding^{168,169}. In addition, it has been shown that Mps1p is degraded by the APC/C after anaphase entry¹⁷⁰. The PP1 protein Glc7p is the key player in reversing the SAC-induced phosphorylation events. The predominant target of Glc7p for SAC recovery is Spc105p, the dephosphorylation of which promotes the release of Bub1p from the kinetochore and therefore inhibits the assembly of the MCC^{147,171}. Glc7p also interacts with Fin1p, which leads to the kinetochore recruitment of Glc7p in anaphase that further promotes the removal of Bub1p¹⁷². In addition, Glc7p reverses the Ipl1p-mediated phosphorylation for promoting chromosome bi-orientation, which indirectly contributes to SAC recovery^{173–175}. The disassembly of the existing MCC is likely to rely on the autoubiquitination of Cdc20p¹⁶⁶, as well as the ubiquitination of Mad3p¹⁷⁶, and Bub3p enhances the binding of

the newly-synthesised Cdc20p to APC/C to promote the activation of APC/C-Cdc20p and hence anaphase entry¹⁷⁷.

SAC adaptation

The SAC-induced cell cycle arrest is not permanent; after being arrested at metaphase for several hours, a budding yeast cell can proceed into anaphase via a mechanism called SAC adaptation in spite of the presence of defective kinetochore-microtubule attachments (e.g. when being treated with microtubule-depolymerising agents such as nocodazole or benomyl) and/or persistent SAC signalling (e.g. when induced by overexpressing Mad2p)^{178–180}. The molecular mechanisms behind SAC adaptation are yet to be fully understood. Several pieces of evidence suggest that the SAC adaptation is regulated by the interplay between the kinase activity of Cdc28p-Clb2p and the phosphatase activity of PP2A-Cdc55p^{54,178,181}. Specifically, it has been shown that Cdc28p is responsible for activating the APC/C by phosphorylating Cdc16p and Cdc27p, which are two APC/C components that promote SAC adaptation¹⁸¹. These phosphorylation events are, however, antagonised by PP2A-Cdc55p and presumably some other phosphatases⁵⁴. The observation of the Clb2p accumulation during the SAC-induced arrest then supports an attractive model, where the accumulation of Cdc28p-Clb2p during the arrest eventually reaches a threshold for activating APC/C-Cdc20p, which subsequently drives anaphase entry by functioning in both a positive feedback loop for the further activation of itself by inhibiting PP2A-Cdc55p and a negative feedback loop for rapid Clb2p degradation⁵⁴. Besides this relatively simple model, there seem to be other factors affecting SAC adaptation. The phosphorylation of Bub1p by the activated Cdc28p, for example, is required for SAC adaptation, likely by promoting Bub1p degradation¹⁸². It has also been reported that the RSC chromatin-remodelling complex contributes to SAC adaptation via its role in the FEAR network that promotes the early release of Cdc14p¹⁷⁹. However, this does not agree with the finding that Cdc14p is not required for anaphase entry when the SAC is activated⁵⁴. Moreover, it seems that, at least under certain circumstances (e.g. strong Cdc28p inhibition), Cdh1p, another activator of APC/C that normally functions in anaphase/telophase, can activate APC/C to overcome the SAC^{54,183,184}. Importantly, human cells can also escape from SAC-induced arrest, which not only causes aneuploidy but also leads to resistance to anti-mitotic drugs that aim to induce programmed cell death

via inhibiting mitotic progression for cancer treatment^{185–187}. This adaption-like process is often referred to as “mitotic slippage”, as it involves the gradual degradation of cyclin B mediated by the APC/C that eventually drives mitotic progression¹⁸⁸.

1.1.4 Crosstalk between the DDC and the SAC

While the DDC and the SAC respond to different types of defects during cell cycle progression and therefore were thought to function independently, there is increasing evidence acquired from various organisms in support of the existence of crosstalk between the two checkpoints¹⁸⁹. In budding yeast, the initial findings of the contributions of the SAC components Mad1p, Mad2p, and Bub1p to the DNA damage-induced arrest led to the proposal that DNA damage might trigger the SAC by generating detached kinetochore(s), where the SAC components are subsequently assembled^{190–193}. This idea was supported by a study that demonstrated the dependence of the SAC-related arrest on the spreading of the DNA damage-induced γ -H2A to the centromeric region, which might trigger the SAC by reducing the intra-kinetochore tension via affecting the normal centromeric enrichment of cohesin¹³⁸. Another study further revealed that the inner kinetochore protein Cep3p is phosphorylated in a Mec1p- and Rad53p-dependent manner in the presence of a DNA DSB and promotes a Mad2p-dependent arrest¹⁹⁴. However, results from two other studies complicated the situation, as one showed that in the presence of DNA damage the SAC-induced arrest persisted in cells devoid of functional kinetochores¹⁹⁵ and the other showed that neither of the two kinases initiating the DDC (i.e. Mec1p and Tel1p) were required for the Mad2p-dependent protection of Pds1p in response to DNA damage¹⁹⁶. Interestingly, the DDC adaptor Rad9p and effector Rad53p have been shown to be phosphorylated by SAC components with the SAC activated by the microtubule depolymerising drug nocodazole¹⁹⁷. It is also possible that the two checkpoints converge at the nuclear pore complex (NPC), as persistent DNA lesions have been found to relocalise to the NPC for proper repair (reviewed in Refs.^{198,199}) whilst the SAC components Mad1p and Mad2p also constitutively localise at the NPC throughout interphase^{200–202}, yet it is unclear how these facilitate the crosstalks. Additionally, the SAC component Bub1p and the SPOC component Bub2p have been shown to be recruited to DSBs for promoting DSB repair by NHEJ²⁰³.

1.2 The budding yeast kinetochore

The kinetochore is an evolutionarily-conserved protein complex assembled on each centromere of the sister chromatids. It plays a pivotal role in facilitating accurate chromosome segregation during mitosis, specifically in:

- maintaining a load-bearing connection between the centromere of each chromatid and a dynamic spindle microtubule, and coupling the force generated by depolymerising microtubules to pull apart sister chromatids during chromosome segregation;
- destabilising erroneous kinetochore-microtubule attachment(s) for the reestablishment of proper one(s);
- ensuring that each pair of sister chromatids are attached by microtubules originating from opposite spindle poles prior to chromosome segregation, by activating the SAC to arrest the cell cycle at the metaphase of mitosis in the presence of any erroneous kinetochore-microtubule attachment.

The budding yeast kinetochore is comprised of >60 proteins that are either stably-associated with, or transiently-presented at, the kinetochore throughout the cell cycle, and most of these proteins have homologs not just in other yeast species (e.g. *S. pombe*) but also in human cells²⁰⁴ (Table 1-1). Unlike its counterpart in humans, which can typically attach to ~20-30 microtubules²⁰⁵, each budding yeast kinetochore is either unattached or attached by 1 microtubule²⁰⁶. The evolutionary conservation in its composition and the simplicity in the kinetochore-microtubule attachment status make the budding yeast kinetochore a useful model for studying the kinetochore's functions.

In this section, I provide an overview on the current knowledge of the composition and the architecture of the budding yeast kinetochore, and also review the experimental methods that were utilised to acquiring the knowledge.

Table 1-1 Homologs of kinetochore proteins

	Subcomplex in <i>S. cerevisiae</i>		<i>S. cerevisiae</i>	<i>S. pombe</i>	Human	Subcomplex in human
Inner kinetochore	CBF3		Ndc10p	-	-	-
			Ctf13p	-	-	
			Cep3p	-	-	
			Skp1p	-	-	
			Scm1p	Scm1p	HJURP	
			Cse4p	Cnp1p	CENP-A	CCAN
			Cbf1p	Abp1p	CENP-B	
			Mif2p	Cnp3p	CENP-C	
	CTF19	COMA	Ctf19p	Fta2p	CENP-P	
			Okp1p	Fta7p	CENP-Q	
			Mcm21p	Mal2p	CENP-O	
			Ame1p	Mis17p	CENP-U	
		CTF3	Mcm16p	Fta3p	CENP-H	
			Ctf3p	Mis6p	CENP-I	
			Mcm22p	Sim4p	CENP-K	
			Nkp1p	Fta4p	-	
			Nkp2p	Chl2p	-	
			Chl4p	Mis15p	CENP-N	
			Iml3p	Fta1p	CENP-L	
	CNN1		Cnn1p	Cnp20p	CENP-T	
			Wip1p	New1p	CENP-W	
			Mhf1p	Mhf1p	CENP-S	
			Mhf2p	Mhf2p	CENP-X	
Outer Kinetochore	MIND		Mtw1p	Mis12p	Mis12p	KMN
			Dsn1p	Mis13p	Dsn1p	
			Nnf1p	Nnf1p	Nnf1p	
			Nsl1p	Mis14p	Nsl1p	
	SPC105		Spc105p	Spc7p	Kn11p	
			Kre28p	Sos7p	Zwint-1p	

	NDC80	Ndc80p Nuf2p Spc24p Spc25p	Ndc80p Nuf2p Spc24p Spc25p	Hec1p Nuf2p Spc24p Spc25p	
	DAM1	Ask1p Dad1p Dad2p Dad3p Dad4p Dam1p Duo1p Hsk3p Spc19p Spc34p	Ask1p Dad1p Dad2p Dad3p Dad4p Dam1p Duo1p Hsk3p Spc19p Spc34p	Ska1p* Ska2p* Ska3p* - - - - - - -	Ska (evolutionarily distinct from but functionally analogous to DAM1)
	MAPs	Stu1p Stu2p Bik1p Bim1p	Peg1p Dis1p/Alp14p Tip1p Mal3p	CLASP1/CLASP2 XMAP215 CLIP-170 EB1	MAPs
	Motors	Cin8p Kip2p Kip3p Kar3p	Cut7p Tea2p Klp6 Pkl1	Kif11p Kif22p Kif18p Kifc1p	Motors
	MAP- or motor- related proteins	Kar9p Slk19p	- -	- -	MAP- or motor- related proteins
Regulation	CPC	Bir1p Ipl1p Nbl1p Sli15p	Bir1p Ark1p Mug118p Pic1p	Survivin Aurora B Borealin INCENP	CPC
	SAC	Mps1p Cdc20p Bub1p Bub3p	Mph1p Slp1p Bub1p Bub3p	Mps1p/TTK Cdc20p Bub1p Bub3p	SAC

		Mad1p Mad2p Mad3p	Mad1p Mad2p BubR1p	Mad1p Mad2p Mad3p	
		Sgo1p	Sgo1p	Sgo1p	
		Cdc28p	Cdc1p	Cdk1p	
		Cdc5p	Plk1k	Plk1p	
	PP1	Glc7p Sds22p	Dis2p Sds22p	PP1 PP1	PP1
	PP2A	Cdc55p Pph21p Pph22p Rts1p Tpd3p	Pab1p Ppa1p Ppa2p Par2p Paa1p	B55 PP2A PP2A B56 PP2A	PP2A

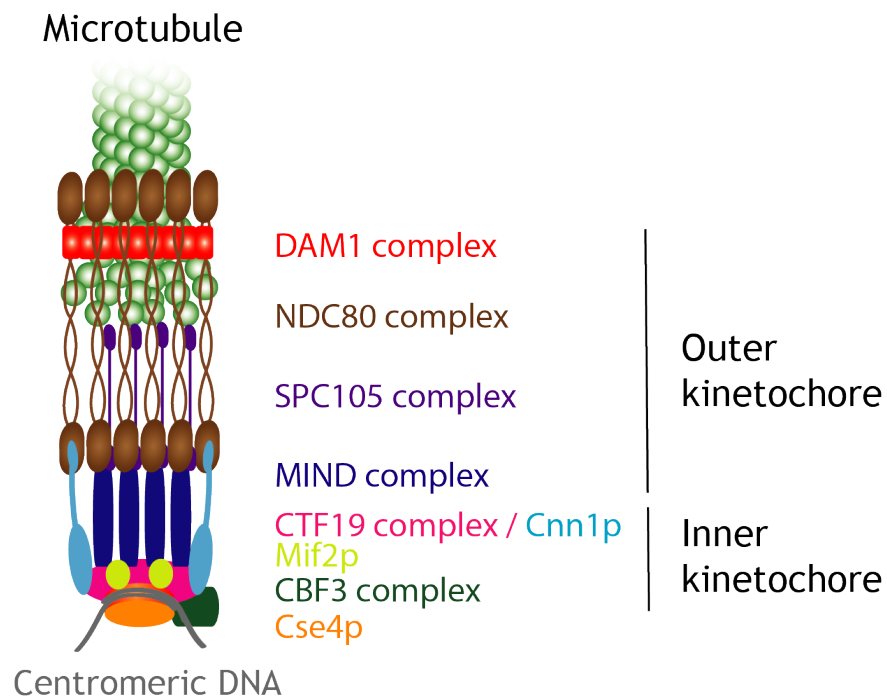


Figure 1-4 Schematic of a budding yeast kinetochore with its constitutive components

Proteins and subcomplexes are colour-coded. Please refer to Table 1-1 or the main text for the components in each subcomplex.

1.2.1 Kinetochore composition and architecture

The budding yeast kinetochore can be regarded as a complex with a two- or three-layer architecture along the kinetochore-microtubule axis. While there is no universal agreement on which model best describes the architecture and on how the various proteins and subcomplexes at the kinetochore should be respectively categorised into each layer, here I discuss kinetochores based on a two-layer model. In this model, as shown in Table 1-1 and Figure 1-4, the proteins/subcomplexes in contact with the centromere comprise the inner kinetochore whereas the other constituent proteins/subcomplexes, as well as the microtubule-associated proteins (MAPs) and the motor proteins, are considered as the outer kinetochore. I also provide a brief introduction on the regulators of the kinetochore's functions.

The centromere and the inner kinetochore

The budding yeast centromere is defined by a specific DNA sequence of ~125 bp on each sister chromatid and is therefore termed a “point” centromere, in contrast to the “regional” centromere found in human cells that is defined epigenetically and spans up to several Mb with alpha-satellite DNA repeats²⁰⁷. The centromeric DNA consists of three centromere-determining elements (CDEs)²⁰⁸. CDEI (8 bp) and CDEIII (26 bp) bind to the protein Cbf1p^{209,210} and the CBF3 complex (composed of a homodimer of Cep3p, a homodimer of Ndc10p, and a Skp1p-Ctf13p heterodimer)^{211–213}, respectively, whereas at the AT-rich CDEII (78-86 bp) resides Cse4p, a histone H3 variant specific to the centromeric nucleosome^{214–216} (in replacement of the canonical histone H3). The deposition of Cse4p into the centromeric nucleosome requires both the chaperone Scm3p and the interaction between Scm3p and the CBF3 component Ndc10p^{217–219}. The precise number and composition of the Cse4p-containing centromeric nucleosome at the centromere are controversial (reviewed in Ref.²⁰⁴); the current understanding is that the centromeric DNA wraps around a single centromeric nucleosome through the interaction between the CDEIII-bound Ndc10p and the CDEI-bound Cbf1p²¹³, and the nucleosome is composed of a (Cse4p-H4)₂ tetramer and possibly a (H2A-H2B)₂ tetramer^{220,221} throughout the cell cycle (except a brief period in S-phase when Cse4p turnover occurs). As the number of centromeric Cse4p has been used as a reference for estimating the copy

numbers of kinetochore proteins (KPs) - based on fluorescence intensity of the green fluorescent protein (GFP) labelling the KPs (see Section 1.2.3 for details), according to this model the copy numbers of different KPs range from 1 to ~20 in metaphase and in general the outer kinetochore KPs have more copies than the inner kinetochore ones²²².

From the centromere, the budding yeast kinetochore is assembled by its constituent proteins and subcomplexes in a hierarchical manner. Once assembled, it remains intact and is attached by spindle microtubules throughout the cell cycle, only disassembled briefly in the early S-phase of the next cell cycle for the replication of the centromeric DNA²²³. In contrast, in human and other metazoan cells where kinetochore-microtubule attachments are only established after nuclear envelope breakdown in early mitosis, only the inner kinetochore components, collectively referred to as the CCAN (constitutive centromere-associated network), remain at the centromere for most of the cell cycle, and the outer kinetochore components are only assembled during mitosis (reviewed in Ref.²²⁴). Most budding yeast KPs at the inner kinetochore are homologs of the human CCAN components (Table 1-1), including Mif2p, the CTF19 complex, and the CNN1 complex.

The binding of the Mif2p dimer to the centromere via its interaction with Cse4p and CBF3 is the starting point of the assembly^{222,225–228}. Mif2p facilitates the recruitment of the CTF19 complex via its interaction with the Ame1p-Okp1p dimer²²⁹. The CTF19 complex is formed by several subcomplexes: the COMA complex (Ctf19p, Okp1p, Mcm21p, Ame1p), the CTF3 complex (Ctf3p, Mcm16p, Mcm22p), Chl4p-Iml3p, and Nkp1p-Nkp2p^{230,231}. While all of them are required for accurate chromosome segregation, only the Ame1p-Okp1p dimer in the COMA complex are essential for viability. Ame1p-Okp1p directly associates with both Mif2p and the centromeric DNA for its recruitment to the kinetochore, and also interacts with Mtw1p in the outer kinetochore MIND complex via Ame1p's N-terminus²²⁹. Ame1p-Okp1p also recruits Nkp1p-Nkp2p, which stabilises the COMA complex but is dispensable for the recruitment of other KPs²³². The other dimer in the COMA complex, Ctf19p-Mcm21p, recruits the other CTF19 components, i.e. the Chl4p-Iml3p dimer and the CTF3 complex^{230,233}. Apart from linking CTF3 to COMA²³³, Chl4p-Iml3p interacts with Mif2p and Sgo1p, likely playing a role in maintaining sister-chromatid cohesion by recruiting Sgo1p that protects cohesin from being cleaved by Esp1p²³⁴. CTF3 is also involved in promoting the establishment of

pericentromeric sister-chromatid cohesion, specifically by recruiting the Dbf4p-dependent kinase, which then phosphorylates Ctf19p's N-terminus to promote the recruitment of the cohesin loader complex Scc2p-Scc4p^{235–237}. CTF3 has yet another role: it forms a complex with the CNN1 components, Cnn1p and Wip1p, and provides an additional connection between the centromere and the outer kinetochore NDC80 complex, alongside or in competition to the CTF19-MIND-mediated connections described below. This Cnn1p-mediated connection to NDC80, specifically to Spc24p-Spc25p, is cell cycle-dependent, and the phosphorylation-regulated Cnn1p activity peaks at anaphase, presumably to reinforce the kinetochore-microtubule attachment during chromosome segregation^{230,238–242}.

The outer kinetochore and the microtubule-binding proteins

The outer kinetochore consists of the MIND complex, the SPC105 complex, the NDC80 complex, the DAM1 complex, as well as some microtubule-binding MAPs and motor proteins. It facilitates the attachment to the spindle microtubule and is crucial for the regulation of this attachment. It is also involved in the activation of the SAC (see Section 1.1.3).

The MIND, SPC105, and NDC80 complexes (MIS12, KNL1, and NDC80 in humans, respectively, collectively known as the KMN network) are highly conserved between yeast and human. The MIND complex comprises two heterodimers, Mtw1p-Nnf1p and Dsn1p-Nsl1p. The crystal structure of this complex has been reported for both *Kluyveromyces lactis*, another yeast species, and humans, and both studies revealed that the complex has an asymmetric elongated Y-shaped structure, with two globular domains and a coiled-coil rod^{243,244}. The N-termini of Mtw1p and Nnf1p are in one of the globular domains, and they are responsible for the binding of MIND to Ame1p and Mif2p in the inner kinetochore. This binding is promoted by the phosphorylation of the N-terminal tail of Dsn1p from the other globular domain by the Aurora B kinase Ipl1p (see Section 1.2.2), which releases the N-terminal domain of Mtw1p-Nnf1p from inhibition by Dsn1p's unphosphorylated N-terminus^{243,245}. The C-terminus of Dsn1p, on the other hand, binds to the C-terminal domain of the Spc24p-Spc25p heterodimer in the NDC80 complex. MIND has also been shown to interact to the C-terminus of Spc105p²⁴⁶, a component of

the SPC105 complex. SPC105 is an elongated heterotrimeric complex comprising one Spc105p and two Kre28p²⁴⁷. As described in Section 1.1.3, it plays a key role in the activation of the SAC, through the Mps1p-mediated phosphorylation of the MELT motifs on Spc105p that recruits the SAC proteins. Spc105p is also required for the kinetochore recruitment of some MAPs and motors²⁴⁷, and its N-terminus has also been shown to have a relatively low affinity to the microtubule plus-ends²⁴⁷. The major microtubule-binding capability of the kinetochore is provided by the NDC80 complex, which has an elongated rod-like structure, with a Spc24p-Spc25p heterodimer on the centromere-proximal end connected to a Ndc80p-Nuf2p heterodimer on the microtubule-proximal end via a tetrameric junction^{248–251}. The C-termini of Spc24p and Spc25p form two globular domains on the centromere-proximal end, which interacts with either Dsn1p in the MIND complex or Cnn1p in the CNN1 complex, as mentioned above. The N-terminal end of the Ndc80p-Nuf2p heterodimer confers the microtubule-binding activity, via both the calponin-homology domains in the globular domains of the two proteins at this end and the unstructured N-terminal tail of Ndc80p^{252–254}. Additionally, Ndc80p-Nuf2p interacts with the microtubule-binding DAM1 complex. This interaction was initially thought to be mediated by a “loop” region in the middle of Ndc80p’s rod-like coiled-coil structure²⁵⁵, but evidence from recent studies suggests that the interaction is associated with a more extensive region while the “loop” may affect the interaction indirectly^{254,256}.

The DAM1 complex is another component in the budding yeast kinetochore that possesses a microtubule-binding activity. All the subunits of this decameric structure (Ask1p, Dad1p, Dad2p, Dad3p, Dad4p, Dam1p, Duo1p, Hsk3p, Spc19p, Spc34p) are essential in budding yeast^{257–261}, but their homologs are not found in humans (Table 1-1); instead, the trimeric SKA complex acts as the functional homolog of DAM1 in human cells^{262–265}. Multiple copies (~16) of DAM1 are likely to form a ring around a microtubule *in vivo*, as suggested by *in vitro* studies that visualised the ring structure using electron microscopy (EM)^{260,261,266} or cryogenic EM (cryo-EM)^{267,268} and *in vivo* quantitative fluorescence microscopy studies that measured sufficient copies of DAM1 at the kinetochore to form one²²² or two²⁶⁹ such rings. Remarkably, a recent *in vivo* study using electron cryotomography (cryo-ET) has visualised DAM1 complexes forming both complete and partial rings around microtubules in metaphase cells²⁷⁰. It has also been shown that a faithful kinetochore-microtubule attachment requires the binding of two

DAM1 rings to the NDC80 component Ndc80p²⁵⁶. Ndc80p is required for the *in vivo* recruitment of DAM1 to the kinetochore²⁵⁵, which then works together with NDC80 to maintain the attachment of the kinetochore to the dynamic (polymerising or depolymerising) microtubule plus-end^{271,272}. The maintenance of this attachment when the kinetochore is under tension from the shrinking microtubule (and therefore the establishment of chromosome bi-orientation) requires the oligomerisation of DAM1, which presumably results in the formation of the microtubule-encircling ring(s)²⁷³. Notably, the microtubule-binding activities of both NDC80 and DAM1 are regulated by the Ipl1p kinase, which is discussed in Section 1.2.2.

There are also some accessory proteins present at the outer kinetochore besides the SAC components introduced in Section 1.1.3. These include four MAPs/MAP-related proteins (Stu1p, Stu2p, Bik1p, Bim1p), four motor/motor-related proteins (Cin8p, Kip2p, Kip3p, Kar3p), and two MAP- or motor-related proteins (Kar9p, Slk19p). While they only interact with the kinetochore transiently, unlike the constitutive KPs introduced above, these microtubule-related proteins are important, not only for the establishment and maintenance of the kinetochore-microtubule attachment but also for the dynamics of the mitotic spindle. For example, the MAP Stu1p preferentially localises to unattached kinetochores in an Ndc80p- and Spc105p-dependent manner - likely to promote the capture of kinetochores by microtubules - and relocalises to spindle microtubules in anaphase to stabilise the spindle^{274,275}. Stu1p is also responsible for the kinetochore recruitment of another MAP Stu2p²⁷⁶. Kinetochore-localised Stu2p facilitates the generation of kinetochore-derived microtubules²⁷⁷, which could facilitate the kinetochore capture by spindle microtubules²⁷⁶. Stu2p also preferentially stabilises tension-bearing kinetochore-microtubule attachments and promotes kinetochore detachment and/or microtubule disassembly at the plus-end when the kinetochore is not under tension^{278,279}, and therefore could be important for correcting erroneous attachments in cooperation with the Ipl1p-dependent mechanisms (see below in Section 1.2.2).

1.2.2 Regulation of the kinetochore-microtubule attachment

As discussed above, one of the kinetochore's key functions is regulating the kinetochore-microtubule attachment. This regulation can be subdivided into three aspects: (i)

establishing attachments before metaphase, (ii) correcting erroneous attachments and (iii) maintaining tension-bearing attachments before chromosome segregation and continuing to maintain the attachments during chromosome segregation when the tension is lost (reviewed in Ref.²⁰⁴).

Shortly after its assembly following the replication of the centromeric DNA in early S-phase, the budding yeast kinetochore is captured by a spindle microtubule emanating from the “old” SPB²²³ (i.e. the one inherited from the previous cell cycle). The kinetochore is initially attached to the lateral surface of the microtubule, and is subsequently transported towards the SPI, by sliding along the microtubule and converting the lateral attachment to an end-on attachment²⁸⁰. A number of MAPs and motors are involved in these steps, e.g. Stu2p, Bik1p, Bim1p, and Kar3p^{276,277,281,282}. All the kinetochore subcomplexes introduced in Section 1.2.1 except DAM1 are required for establishing the lateral attachment²⁸¹. DAM1 is needed only after the kinetochore slides to the microtubule plus-end and attaches to the microtubule in an end-on manner that allows the poleward pulling of the kinetochore by the depolymerising microtubule²⁸². After the kinetochore is transported to the old SPB and the new SPB is matured and separated from the old one, the two kinetochores on a pair of sister-chromatids are attached by microtubules from the two SPBs, respectively, and eventually achieve chromosome bi-orientation in metaphase²⁸⁰.

Achieving bi-orientation in metaphase requires the activity of the conserved Aurora B kinase Ipl1p at the kinetochore^{283–285}. Ipl1p is a component of the chromosome passenger complex (CPC) that also contains Bir1p, Sli15p, and Nbl1p²⁸⁶. Its functions include disrupting tensionless end-on kinetochore-microtubule attachments by phosphorylating the outer kinetochore proteins Dam1p and Ndc80p^{258,271,287–289}, which enables the correction of erroneous syntelic attachments (i.e. sister kinetochores attached to microtubules from the same SPB) to bipolar ones, and also triggers the SAC that provides more time for correct attachments to be made (see Section 1.1.3). The ability of Ipl1p to preferentially disrupt tensionless attachments is likely to be related to its localisation at the centromere/inner kinetochore (mediated independently by the CPC components Bir1p and Sli15p) during metaphase^{290–293}. With this configuration, Ipl1p is spatially separated from its substrates at the outer kinetochore when the kinetochore is stretched by the

poleward movement of the attached microtubule and the opposite resistance imposed by the pericentromeric cohesin²⁹⁴. Sgo1p, which is recruited to the centromere via Bub1p-mediated histone H2A phosphorylation^{295–297}, is required for maintaining Ipl1p at the centromere, and also recruits condensin (a ring-like complex similar to cohesin that alters chromatin conformation)²⁹⁸ to the pericentromeric region via recruiting the PP2A subunit Rts1p; both of these processes promote bi-orientation^{299,300}. There are some additional regulators involved in the process of establishing bi-orientation. For example, the PP1 phosphatase Glc7p is responsible for dephosphorylating Ipl1p substrates^{301,302}. Another example is the Mps1p kinase, which has been found to be important for bi-orientation *per se* besides its role in activating the SAC^{303,304}.

After achieving bi-orientation, i.e. satisfying the SAC, sister chromatids are separated and pulled towards opposite SPBs by shrinking microtubules, respectively. During this process, the kinetochore-microtubule attachments need to be maintained in spite of the lack of intra-kinetochore tension. This requires the relocation of the CPC from the kinetochores to the spindle, which in turn depends on the phosphatase activity of Cdc14p released in anaphase^{305,306}. As the linkage between the inner and the outer kinetochore via COMA and MIND relies on the phosphorylation of Dsn1p by Ipl1p, the kinetochore itself needs to undergo a conformational change after the relocation of the CPC in order to maintain the attachment, presumably through increasing the connections between CNN1 and NDC80^{239,242,307} (see above in Section 1.2.1).

1.2.3 Methods for studying the budding yeast kinetochore

As outlined above, the budding yeast kinetochore has been extensively studied, in terms of its composition, its hierarchical assembly, its architecture (including the stoichiometry and the molecular structures of its components), and the regulation of its functions via the interactions with non-constitutive proteins and microtubules, etc. A wide range of experimental methods have contributed to these studies.

The initial identification of the KPs and subcomplexes constituting the budding yeast kinetochore largely relied on a combination of genetic, biochemical, biophysical, and cytological methods. For example, the four components of the COMA complex (Ctf19p,

Okp1p, Mcm21p, Ame1p) were identified using one-hybrid assays for assessing their proximity to the centromeric DNA, co-immunoprecipitations and two-hybrid assays for showing their interactions with each other and with Cse4p, Mif2p, and the CBF3 complex, affinity purifications followed by mass spectroscopy for identifying their interacting partners in cell extracts and hydrodynamic analyses for showing that they form a stable complex. In addition, live cell fluorescence microscopy has been used to study their kinetochore localisations and the phenotypes caused by genetic mutations^{226,308}. These and similar methods were also employed to examine the interdependency among KPs for kinetochore assembly (e.g. Refs.^{229,238}). Particularly, *in vitro* experiments utilising reconstituted kinetochore particles have provided remarkable insights, not only into the kinetochore's structure and assembly, but also its function and interactions with the microtubule and in SAC signalling (e.g. Refs.^{147,153,242,256,273,278,289,309–312}). In recent years, the development of cryo-EM-based technologies has enabled the visualisation of the molecular structures of various kinetochore components with sub-nanometre resolution^{231,240,243,268,270,313}. Fluorescence microscopy has also played crucial roles in revealing the kinetochore architecture besides examining phenotypes (reviewed in Ref.³¹⁴). *In vitro* studies using total internal reflection microscopy have elucidated the binding of purified kinetochores to microtubules (e.g. Ref.³¹¹). Quantitative fluorescence microscopy techniques, including fluorescence recovery after photobleaching (FRAP), have been applied to measure the copy numbers of KPs at different stages of the cell cycle (e.g. Refs.^{220–222,307}). High-resolution fluorescence microscopy has utilised centroiding of the diffraction-limited kinetochore foci to reveal the *in vivo* localisations of various KPs along the kinetochore-microtubule axis in metaphase^{314,315}. Förster resonance energy transfer (FRET), which is discussed in the following section, has been used to establish proximities between various KPs, for example to study the architecture of the kinetochore-microtubule attachment interface at the outer kinetochore^{316,317}.

1.3 Fluorescence microscopy and the application of FLIM FRET to study PPIs

Fluorescence-based techniques, including fluorescence microscopy, tomography, and spectroscopy, have become indispensable tools for biological sciences over the past few decades, owing to the molecular specificity of fluorescence labelling techniques and the advances in excitation sources and detectors that can provide spectroscopic and spatial resolution with single molecule sensitivity. This thesis concerns the application of quantitative fluorescence microscopy to study protein-protein interactions (PPIs) at the budding yeast kinetochore, taking advantage of the ease of constructing budding yeast strains that have specific endogenous proteins labelled with genetically expressed fluorophores such as fluorescent proteins. In particular, I have exploited Förster resonance energy transfer (FRET) to detect the close proximity of potentially interacting proteins labelled with fluorescent proteins, utilising fluorescence lifetime imaging (FLIM) microscopy to provide a robust readout of FRET. This section begins with an introduction to fluorescence microscopy, followed by an overview of the FRET phenomenon and various approaches that have been used to measure FRET. An overview of the commonly-used techniques for FLIM measurements is then provided, including their implementations for high-content analysis (HCA), followed by a brief review of the applications of FLIM FRET for budding yeast-related studies.

1.3.1 Introduction to fluorescence microscopy

Photophysics of fluorescence

Fluorescence is the spontaneous emission of a photon associated with the relaxation of an electron from an excited state in a fluorophore that has been excited by a photon of higher energy. The fluorescent species, or “fluorophore”, can be a molecule intrinsic to a cell (e.g. cellular metabolites, structural proteins, amino acids), for which the emission is termed “autofluorescence”. More frequently in biology research, specific structures or proteins are labelled with a specific extrinsic fluorophore that fluoresces with high efficiency. Such fluorophore labels include small-molecule organic dyes, fluorescent proteins, quantum dots, and other nanoparticles³¹⁸. The transitions of electrons between

electronic states in such a fluorophore can be represented using a Jablonski diagram, a simplified version of which is presented in Figure 1-5.

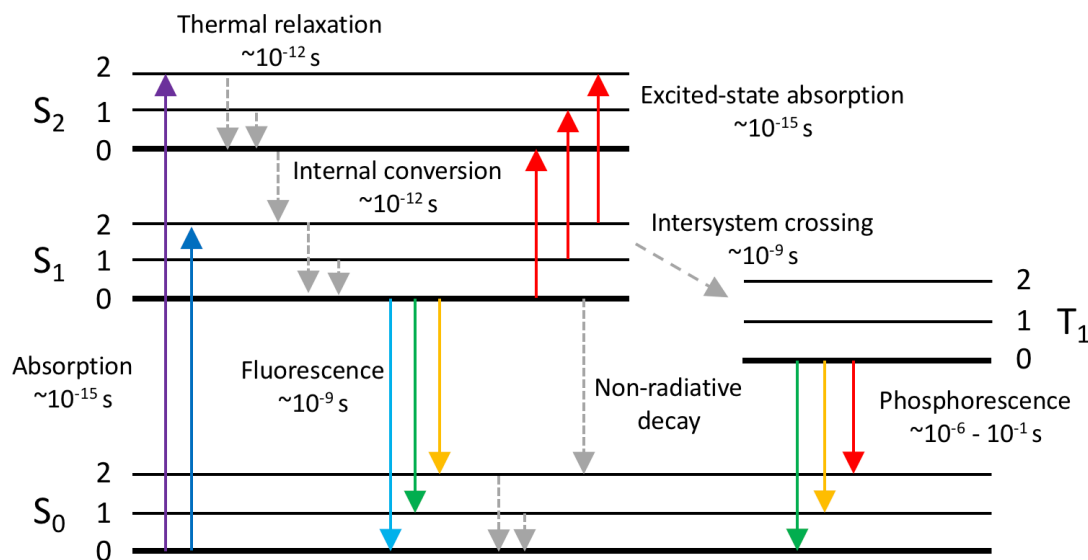


Figure 1-5 A simplified Jablonski diagram depicting the electronic transitions involved in fluorescence and their typical transition rates

S_0 : the ground singlet state. S_1 : the first singlet state. S_2 : the second singlet state. T_1 : the first triplet state. The first three vibrational levels for each state are labelled as 0, 1, and 2. The rotational levels for each vibrational level are not depicted. The approximate timescale for each transition is given in units of seconds.

Upon the absorption of a photon, an electron in a fluorophore can be excited from the ground singlet state (S_0) to an excited singlet state (S_1 or S_2). If being excited to the second singlet state (S_2) or a high vibrational level of the first singlet state ($S_{1,n}$; $n \neq 0$), the electron typically undergoes rapid internal conversion and/or vibrational relaxation to $S_{1,0}$. The excited electron can then relax to a vibrational level of the ground singlet level ($S_{0,n}$) by emitting a photon (resulting in fluorescence) or by a non-radiative decay pathway. As internal conversion and vibrational relaxation occur much faster than fluorescence after excitation ($\sim 10^{-12}$ s vs $\sim 10^{-9}$ s), fluorescence is generally generated from the transitions of electrons from the lowest vibrational level of the first singlet state ($S_{1,0}$). Besides relaxing to the $S_{0,n}$ energy levels, an excited electron in S_1 can also undergo a spin conversion to the excited triplet state T_1 via intersystem crossing, albeit with lower probability. The relaxation of an electron in T_1 back to S_0 is, however, spin-forbidden,

and therefore occurs much slower than the other processes mentioned above ($\sim 10^{-6}$ to $\sim 10^{-1}$ s); the corresponding photon emission from this process is termed phosphorescence^{319,320}. Electrons “trapped” in the relatively long-lived T_1 state are associated with deleterious effects, such as irreversible photobleaching that converts an excited fluorophore into a non-fluorescent species and phototoxicity, which can result from reactive oxidative species (ROS) generated by the interaction between excited fluorophores and molecular oxygen^{321,322}.

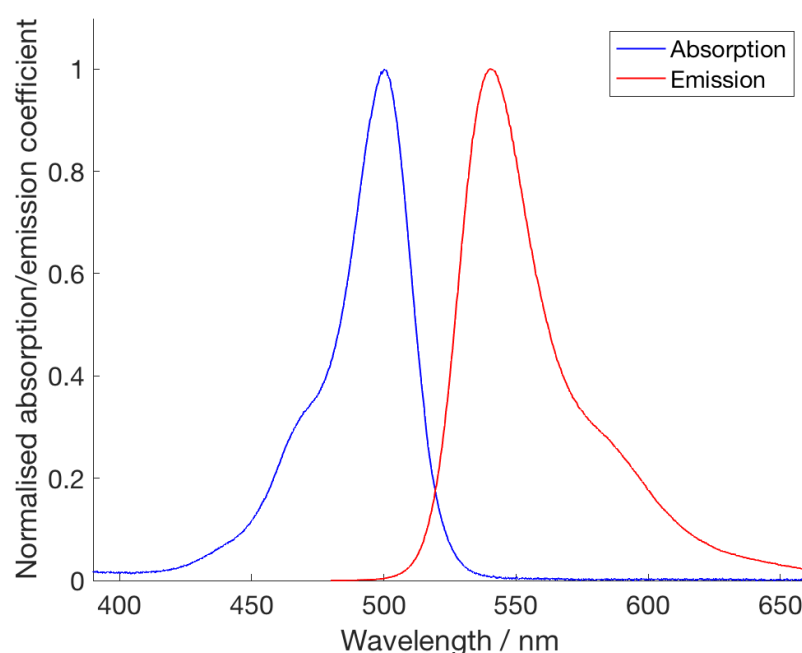


Figure 1-6 Absorption and emission spectra of fluorescein in ethanol

Spectral data are acquired from the PhotochemCAD database³²³.

Figure 1-6 shows the absorption and emission spectra of the fluorescent dye fluorescein (dissolved in ethanol). As indicated by the Jablonski diagram shown in Figure 1-5, most excited electrons relax to the lowest vibrational level of the first singlet state $S_{1,0}$ before emitting photons and so the emission spectrum is independent of the excitation wavelength; this is known as Kasha’s rule³²⁴. The broad emission spectrum originates from the existence of a multiplicity of vibrational and rotational levels associated with each electronic energy level, to which the excited electrons can transit. Similarly, the broad absorption spectrum is accounted by the vibrational and rotational levels associated

with S_1 . As the energy level structures of S_0 and S_1 closely resemble each other, the emission spectrum of a fluorophore is typically similar to the mirror image of the excitation spectrum - the so-called “mirror-image rule”³¹⁹. Owing to the energy lost during internal conversion and vibrational relaxation, the energy of the emitted photon is generally lower than that of the absorbed photon. As the frequency ν and wavelength λ of a photon is determined by the photon’s energy following the equation:

$$E = h\nu = \frac{hc}{\lambda} \quad (1.1)$$

where h is the Planck constant and c is the speed of light. The lower energy of the emitted photon means that fluorescence is emitted at a longer wavelength than the wavelength of the excitation light. This difference between the absorbed and emitted photon energies results in the Stokes shift between the absorption and emission spectra³¹⁹. The existence of the Stokes shift enables the separation of the emitted fluorescence from the scattered excitation light by using spectral filters, and is critical for most practical applications of fluorescence.

Fluorescence intensity and spectroscopic parameters of fluorescence

Various parameters of fluorescence can be a source of contrast and have been widely exploited for extracting qualitative and/or quantitative information. These parameters include fluorescence emission spectrum, intensity, lifetime, and anisotropy, which are introduced below.

- Fluorescence excitation and emission spectra are characteristics determined by the energy level structures of a fluorophore species. Different excitation and emission spectra can be used to distinguish different fluorophores and spectral unmixing algorithms have enabled the quantification of the relative concentrations of multiple fluorophore species presented in the same sample³²⁵. As the energy level structures can be affected by the local molecular environment surrounding the fluorophores, measurements of the fluorescence emission spectrum can also be applied to report parameters of the environment, such as pH and solvent polarity³²⁶. Fluorescence spectroscopy has been widely exploited in cancer diagnosis for its capability to discriminate normal and abnormal tissues

that are different in the molecular level, by sensing the local environment and the fluorophore species that generate autofluorescence³²⁷.

- Fluorescence intensity describes the power of fluorescence per unit area, and is a function of both the absorbance and the quantum yield of the fluorescent sample (assumed to contain a population of fluorophores) under examination. The absorbance, A , of the sample is the logarithmic ratio of the incident intensity I_{ex} to the transmitted intensity I_{ex-out} . According to the Beer-Lambert law³¹⁹, it can be denoted by the following equation:

$$A = \log\left(\frac{I_{ex}}{I_{ex-out}}\right) = \varepsilon(\lambda)CL \quad (1.2)$$

where $\varepsilon(\lambda)$ is the wavelength-dependent molar extinction coefficient for the fluorophore species, C is the molar concentration of the fluorophore molecules, and L is the path length of the incident light transmitting through the sample. The quantum yield of the fluorophore species η describes how efficiently the absorbed excitation light is converted to fluorescence emission, and is determined by the transition rates of the radiative and the non-radiative decays from S_1 to S_0 (k_r and k_{nr} , respectively):

$$\eta = \frac{N_{em}}{N_{ab}} = \frac{k_r}{k_r + k_{nr}} \quad (1.3)$$

where N_{ab} is the number of the absorbed photons and N_{em} is the number of the emitted photons. Taking into account the difference in the energy of the absorbed and emitted photons (owing to the difference in their wavelengths λ_{ab} and λ_{em} ; see Equation 1.1), the relationship of the fluorescence intensity I_{em} to the excitation intensity I_{ex} can then be written as:

$$I_{em} = \frac{\lambda_{ab}}{\lambda_{em}} [1 - 10^{-A}] \eta I_{ex} = \frac{\lambda_{ab}}{\lambda_{em}} [1 - 10^{-\varepsilon(\lambda_{ab})CL}] \eta I_{ex} \quad (1.4)$$

Therefore, with the absorbance A staying constant, fluorescence intensity measurements can monitor changes in the quantum yield η , which may infer changes in the fluorophores' status (in terms of their conformations and

interactions with other molecules) or in their surrounding environment (e.g. pH, viscosity, ion concentration) that affect the radiative and/or non-radiative decay rates^{328,329}. Alternatively, knowing the molar extinction coefficient ε and the quantum yield η of a fluorophore species, the product of which provides information about the relative brightness of the fluorophore, the fluorophore concentration C can be estimated using fluorescence intensity measurements.

- Fluorescence lifetime describes how long on average a fluorophore stays in the excited state before decaying back to the ground state. It is typically of the order of nanoseconds and is determined by both the radiative and non-radiative decay rates as:

$$\tau = \frac{1}{k_r + k_{nr}} = \frac{\eta}{k_r} \quad (1.5)$$

where τ is the fluorescence lifetime and the other parameters are as previously defined. For a homogeneous population of fluorophores in the excited state, the temporal behaviour of their decay is determined by:

$$\frac{\partial N(t)}{\partial t} = -(k_r + k_{nr})N(t) = -\frac{N(t)}{\tau} \quad (1.6)$$

where $N(t)$ is the number of fluorophores remaining at the excited state at a given time. Solving this differential equation yields:

$$N(t) = N_0 e^{-\frac{t}{\tau}} \quad (1.7)$$

where N_0 is the initial number of molecules in the excited state following an impulse of excitation radiation. As Equation 1.6 shows, the number of photons generated by the decay at any instant is proportional to $N(t)$. The instantaneous fluorescence intensity $I(t)$ can then be denoted as:

$$I(t) = I_0 e^{-\frac{t}{\tau}} \quad (1.8)$$

where I_0 is the initial fluorescence intensity immediately after excitation. Therefore, the intensity of the fluorescence from a homogeneous population of

fluorophores exhibits an mono-exponential temporal decay following impulse excitation, and the fluorescence lifetime τ is the amount of time taken for the intensity to drop to $1/e$ of its initial value.

More often than not, the fluorescence signal from a sample under examination follows a more complex decay profile, especially for biological samples where there could be fluorophores being affected differently by neighbouring molecules/environment or belonging to different species. This type of complex decay is often represented as a linear sum of mono-exponential lifetime components:

$$I(t) = I_0 \sum_{i=0}^l \beta_i e^{-\frac{t}{\tau_i}} \quad (1.9)$$

where l is the total number of the mono-exponential lifetime components, and τ_i and β_i are the fluorescence lifetime and the pre-exponential factor of the i -th component, respectively. The average fluorescence lifetime $\bar{\tau}$ for such a decay can be calculated as:

$$\bar{\tau} = \sum_{i=0}^l \beta_i \tau_i \quad (1.10)$$

As with the fluorescence emission spectrum and intensity, fluorescence lifetime measurements can be utilised for monitoring changes in the molecular environment, as it is also dependent on the radiative and non-radiative rates that can be sensitive to these changes. Quantitatively unmixing the multiple mono-exponential components in a complex measured decay profile via fitting-based or fit-free algorithms can help quantify emission from a number of fluorescent components, e.g. providing the relative concentrations of fluorophores with similar spectral properties^{330,331}. Furthermore, fluorescence lifetime provides a way for detecting and quantify FRET, which is discussed in more detail in Section 1.3.2.

- When excited by linearly polarised light, a homogenous solution of fluorophores emits fluorescence that is also partially polarised, as the fluorophores in the solution whose transition dipole moment are parallel to the electric vector of the excitation light are preferentially excited. The degree of polarisation of the emitted fluorescence can be described by the fluorescence anisotropy r_{iso} as below:

$$r_{iso} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (1.11)$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the fluorescence signals parallel to and perpendicular to the polarisation direction of the linearly polarised excitation light.

The fluorescence anisotropy r_{iso} can be used to quantify the depolarisation of the fluorescence emission relative to the excitation. This depolarisation can be due to the rotation of the fluorophores, which is characterised by the rotational correlation time (the characteristic decay time of r_{iso} , analogous to the fluorescence lifetime) and is affected by the size of fluorophores (together with any rigidly attached molecules) as well as environmental factors like the viscosity and temperature of the solvent surrounding the fluorophores. This feature has made fluorescence anisotropy a useful tool for various applications, ranging from biochemical assays to drug discovery^{332,333}. Depolarisation of fluorescence can also be caused by FRET, and therefore provides a readout for FRET measurements, especially for measuring “homo-FRET”, i.e. FRET between fluorophores of the same species³³⁴ (see Section 1.3.2).

Fluorescence microscopy

Fluorescence microscopy has played important roles in biological studies since early last century. Fluorescence microscopy techniques can be classified as wide-field techniques, which use cameras to acquire all the image pixel data in parallel, and point-scanning techniques, which typically focus a laser beam to a diffraction-limited spot on the sample and scan either the sample or the laser beam to build up an image from the sequential acquisition of single pixel data using a detector such as a photomultiplier or a photodiode.

Wide-field imaging techniques typically acquire images more quickly than point-scanning techniques and can utilise lower instantaneous excitation intensities for a given image acquisition time, resulting in lower photobleaching and phototoxicity. Point scanning systems can provide optically sectioned images using a “confocal” pinhole placed in front of the detector so that only photons emitted from the focused excitation spot are imaged efficiently through the pinhole, with fluorescence originating from above or below the focal plane being mostly imaged to outside the pinhole. However, the sequential pixel acquisition results in slower imaging rates and the higher intensities associated with the focused laser beam result in higher photobleaching and phototoxicity, which can scale nonlinearly with excitation power.

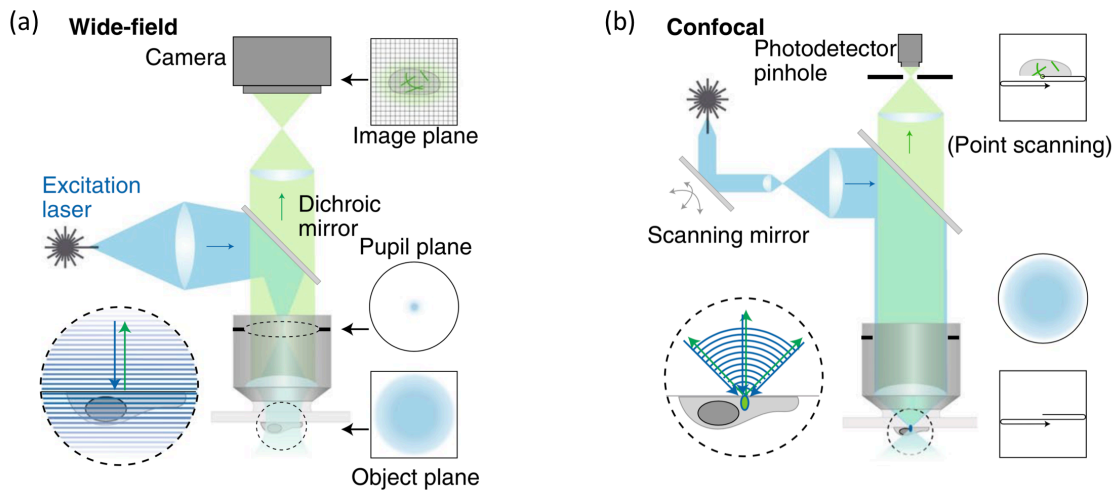


Figure 1-7 Schematics of two typical set-ups for fluorescence microscopy

(a) A wide-field microscope. (b) A confocal point-scanning microscope. Note that for the wide-field microscope, the excitation light is typically focused at the pupil plane (i.e. the back focal plane) of the objective lens for achieving a homogenous excitation pattern at the sample plane (i.e. Köhler illumination³³⁵). Adapted from Ref.³³⁶ with permission.

The work in this thesis utilised a quasi-confocal Nipkow disk microscope that excites the sample with multiple focused laser beams in parallel and the excited fluorescence is imaged to an array of detector pinholes in front of a wide-field detector. The parallel laser beams are focused by an array of microlenses mounted on a spinning disk and the corresponding confocal pinholes are arrayed on a parallel spinning disk, as depicted in Figure 1-8. The lenslets and pinholes are arranged on a spiral pattern on the disk so that, as it rotates, the focused beams are swept across all the pixels in the field of view. This

spinning disk concept was originally proposed by Paul Nipkow to realise a mechanically scanned television system and the quasi-confocal scanning optical microscope is often described as a “Nipkow disk microscope” or a “spinning disk microscope”. Originally the quasi-confocal scanning optical microscope utilised incoherent white light sources that illuminated the disk of pinholes and the transmission through the disk was inefficient³³⁷. The instrument that I have used (Yogogawa Electric Co., CSU-X) utilises an array of microlenses to efficiently focus the incident laser beam through the array of pinholes³³⁸. This approach combines many of the advantages of wide-field imaging (faster, less phototoxic due to parallelised pixel acquisition) with laser scanning confocal microscopy (optical sectioning).

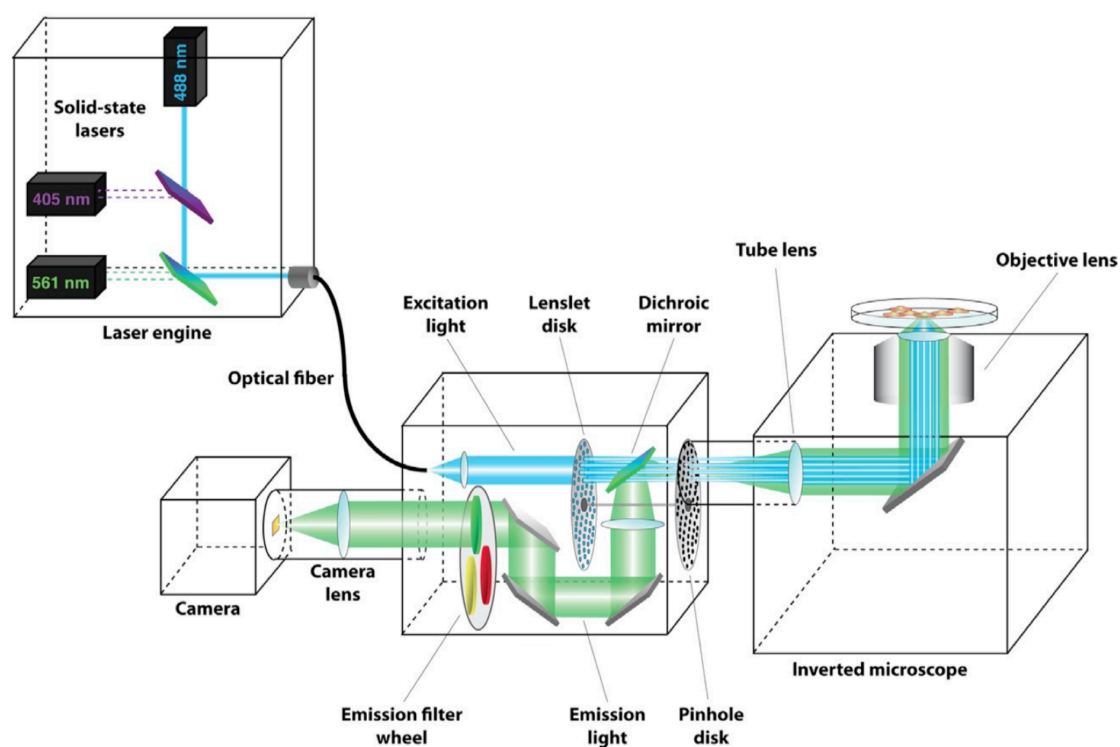


Figure 1-8 Schematic of a fluorescence microscope set-up employing a spinning-disk unit

Adapted from Ref.³³⁹ with permission.

There are many other fluorescence microscopy techniques that can provide optical sectioned images, some of which are discussed Refs.^{340,341}. These include multiphoton

microscopy, light-sheet microscopy, total internal reflection fluorescence (TIRF) microscopy and super-resolved fluorescence microscopy techniques.

Multiphoton microscopy, which is analogous to laser scanning confocal microscopy, utilises multiphoton excitation of the sample where a single excitation photon does not have sufficient energy to excite the fluorophore but two or more identical photons arriving at the same time can achieve the excitation and resulting fluorescence emission³⁴². The efficiency of multiphoton excitation depends on the incident excitation intensity (for two-photon excitation the fluorescence signal is proportional to the square of the excitation intensity) and, for tightly focused excitation beams, this means that in practice, fluorescence is only detected from the focal volume. Thus, multiphoton microscopy provides a similar optical sectioning to confocal microscopy (albeit using longer excitation wavelengths) but does not need a confocal pinhole in front of the detector and there is no fluorescence excited out of the focal plane – this makes it more suitable for imaging in biological tissue where the scattering of the fluorescence from the focal volume greatly reduces the signal that would pass through a confocal pinhole. The lower excitation energy photons are centred at longer wavelengths (typically in the near infra-red) and thus they scatter less than the shorter wavelength light used for single-photon excitation, which is also beneficial for imaging in biological tissue. To provide the required high intensities without thermally damaging the sample, multiphoton microscopy is implemented using ultrashort excitation pulse trains with high peak power but low average power. This ultrashort pulsed excitation can be exploited for fluorescence lifetime imaging (FLIM – see Section 1.3.3). However, multiphoton excitation is less efficient than single photon excitation – resulting in slower imaging – and would not be advantageous for imaging the yeast cell cultures studied during this project.

Light sheet microscopy entails illuminating a sample with a thin sheet of light (or a scanned laser beam that synthesizes a light sheet) and imaging from a different angle (typically 90 degrees) using wide-field detection³⁴³. While it is a very powerful technique for rapid 3D imaging of cells, most implementations are not suitable for multiwell plate imaging. One approach that is compatible with multiwell plate imaging is oblique plane microscopy (OPM)³⁴⁴, but this is not commercially available and would have been more

difficult to implement than spinning disk microscopy and would have provided lower spatial resolution.

TIRF microscopy entails exciting the sample with an excitation beam that is internally reflected at the cell-coverslip interface³⁴⁵. The resulting fluorescence is excited by the evanescent wave that only extends ~100 nm into the sample and so TIRF microscopy provides images only of the regions of the cell next to the coverslip. TIRF microscopy is complicated to set-up, and particularly challenging for an automated multiwell plate microscope, and was not thought to provide any significant advantages for this project over spinning disk microscopy.

The recent three decades have witnessed the emergence of a variety of super-resolution microscopy (SRM) techniques that surpass the diffraction-limited optical resolution of ~200 nm, including structured illumination microscopy (SIM), stimulated emission depletion (STED) microscopy and single-molecule localisation-based microscopy (SMLM) techniques, such as photoactivation localisation microscopy (PALM) and stochastic optical reconstruction microscopy (STORM). For a recent review of SRM techniques, please refer to Ref.³³⁶. Of these SRM techniques, only SMLM can typically provide spatial resolution below ~50 nm and this is challenging to achieve in live cells and is still insufficient to spatially resolve protein-protein interactions.

1.3.2 FRET and FRET measurements

Principles of FRET

Förster resonance energy transfer (FRET) is a non-radiative energy transfer process that can occur between an excited “donor” fluorophore and an “acceptor” fluorophore in the ground state when they are in very close proximity such that energy can be transferred via a dipole-dipole interaction. It is named after Theodor Förster, who first proposed its existence in 1948^{346,347}.

The occurrence of FRET depends on a number of factors:

- the distance between the donor and the acceptor r ;

- the overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor J ;
- the orientation factor κ^2 , which is determined by the relative positions of the donor and the acceptor as well as the relative orientations of the transition dipole moments of the two fluorophores³⁴⁸;
- the quantum yield of the donor η_d ;
- the radiative and non-radiative decay rates of the donor in the absence of FRET k_{dr} and k_{dnr} .

The energy transfer rate of FRET, k_{FRET} , can be expressed as a function of the above parameters:

$$k_{FRET} = \frac{(k_{dr} + k_{dnr})\eta_d\kappa^2J}{r^6} \left(\frac{9000\ln 10}{128\pi^5 n^4 N_A} \right) \quad (1.11)$$

where n is the refractive index of the medium and N_A is the Avogadro constant. Alternatively, k_{FRET} can be re-expressed in a simpler way, as:

$$k_{FRET} = (k_{dr} + k_{dnr}) \left(\frac{R_0}{r} \right)^6 \quad (1.12)$$

where R_0 is the so-called Förster radius; when a donor and an acceptor are separated by a distance of R_0 , 50% of the total decay rate of the excited donor fluorophores is contributed by FRET.

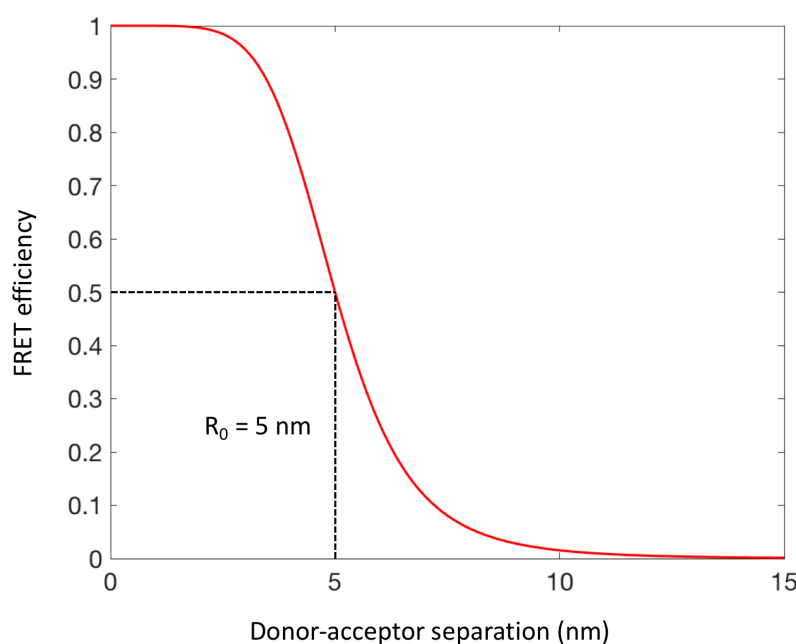


Figure 1-9 The sixth-power dependence of the FRET efficiency on the donor-acceptor separation

The FRET efficiency curve is simulated based on Equation 1.13 for a pair of fluorophores that an assumed Förster radius of 5 nm.

This percentage contribution of FRET to the total donor decay rate is termed “FRET efficiency”, E_{FRET} , which can be expressed as:

$$E_{FRET} = \frac{k_{FRET}}{k_{dr} + k_{dnr} + k_{FRET}} = \frac{R_0^6}{R_0^6 + r^6} \quad (1.13)$$

Equation 1.12 and Equation 1.13 demonstrate the strong (sixth-power) dependence of the FRET efficiency on the separation between the donor and the acceptor fluorophores, which is also illustrated in Figure 1-9. As the values of R_0 for typical donor and acceptor pairs used for FRET studies are on the order of 5 nm³⁴⁹, FRET enables the identification and/or the quantification of (appropriately labelled) protein separations that are on the scale of ~ 10 nm, which is far below the diffraction limited spatial resolution of an optical system at ~ 200 nm. This scale also matches the sizes of typical protein molecules and the separations between interacting molecules in biological systems, and therefore has resulted in the widespread applications of FRET to study PPIs and protein conformation changes³⁵⁰. While a variety of fluorophores have been utilised as FRET pairs in these

studies³⁵¹, fluorescent proteins and fluorescent protein-based biosensors are particular attractive, as they can be genetically expressed in cells and are compatible for live cell experiments^{352,353}.

Methods for measuring FRET

The presence of FRET between fluorophores can be measured using any of the fluorescence properties outlined in Section 1.3.1, namely fluorescence intensity, fluorescence emission spectrum, fluorescence lifetime, and fluorescence anisotropy^{354–356}. Such FRET measurements can be implemented in single point spectroscopic instruments or in fluorescence microscopes, where maps or images of FRET efficiency can be obtained.

As FRET effectively quenches the donor fluorescence signal by non-radiatively transferring the energy from the excited donor to the acceptor, and hence results in “sensitised” emission from the acceptor fluorophore, the presence of FRET can be monitored by measuring the intensities of the fluorescence signals from the donor and/or the acceptor. This is the foundation of both intensity-based and spectrum-based FRET measurements.

FRET can be detected by measuring only the donor fluorescence in the presence and absence of the acceptor molecules and then calculating the FRET efficiency E_{FRET} using the equation below that can be derived from Equation 1.4 and Equation 1.13:

$$E_{FRET} = 1 - \frac{I_{da}}{I_d} \quad (1.14)$$

where I_{da} and I_d are the donor fluorescence intensities in the presence and in the absence of the acceptor molecules, respectively. These intensity values can be acquired from two types of samples, one labelled with both the fluorophores of the FRET pair and the other with only the donor fluorophore³⁵⁷, or from a dual-labelled sample before and after photobleaching the acceptor molecules^{358,359}. While these methods are conceptually simple and can be realised with standard fluorescence microscopes, they have limitations in quantifying E_{FRET} on a cell-by-cell basis (for the two-sample method) and monitoring dynamic changes in E_{FRET} (for the acceptor photobleaching method). The acceptor

photobleaching typically requires a high-intensity laser beam that may also introduce some adverse effects such as phototoxicity. Importantly, these intensity-based FRET measurements are sensitive to fluctuations in the excitation power and to spectral “bleed-through” of the acceptor fluorescence into the detection channel of the donor fluorescence.

The FRET-induced sensitised emission from the acceptor can also be used for detecting FRET. However, similar to the case above, quantifying this intensity signal is sensitive to excitation power fluctuations as well as to spectral cross-talk, which in this case includes the donor fluorescence bleed-through into the acceptor channel and the acceptor fluorescence directly excited by the excitation radiation for the donor. Sensitised emission measurements are often used in combination with the simultaneous measurements of the donor fluorescence, e.g. with the donor and acceptor signals separated by a dichroic beamsplitter and then measured with two detectors. This type of approach that uses both the donor and acceptor signals for quantifying FRET is termed “ratiometric FRET”, and has the strength of being robust against excitation power fluctuations. Nevertheless, ratiometric FRET measurements still require sophisticated correction of spectral cross-talk, and cannot quantify the population fraction of FRET-ing donors and/or the true FRET efficiency unless information about either one of them is acquired with control constructs^{356,360}. Another spectrum-related method for detecting FRET is spectral imaging, which entails measuring the full spectrum of the fluorescence emitted from both the donor and the acceptor and subsequently unmixing the measured spectra to quantify the contributions from the FRET pair³⁶¹.

Considering Equation 1.5, FRET provides an additional non-radiative decay pathway for excited donor fluorophores, leading to a reduction in the donor fluorescence lifetime upon its occurrence. The FRET efficiency E_{FRET} can also be expressed in terms of the donor fluorescence lifetime, as:

$$E_{FRET} = 1 - \frac{\tau_{da}}{\tau_d} \quad (1.15)$$

where τ_{da} and τ_d are the donor fluorescence lifetimes in the presence and in the absence of the acceptor fluorophore. Hence, measuring these two fluorescence lifetime values can provide the FRET efficiency. In contrast to the aforementioned intensity-based methods,

lifetime-based measurements of FRET are intrinsically ratiometric and therefore are insensitive to fluctuations in the excitation power and also to variations in the concentrations of the donor and acceptor fluorophores, as well as to the spectral properties of both the fluorophores and the instrument, as the donor fluorescence lifetime is independent of these parameters. Compared with other methods for measuring FRET, fluorescence lifetime measurements can therefore provide more quantitative results, including the population fraction of FRETing donor molecules and the FRET efficiency when the donor fluorescence decay profile is fitted to an appropriate complex decay model. However, this method requires relatively complicated instruments to resolve the nanosecond fluorescence lifetimes and it requires many more detected photons than other methods if donor fluorescence is to be fitted to complex decay models, which limits the measurement speed. This issue can be mitigated by the use of non-fitting-based or global-fitting-based strategies^{330,362} as discussed in Section 1.3.3 and Section 3.1.3. The implementation of fluorescence lifetime imaging (FLIM) microscopy is described in Section 1.3.3.

Fluorescence anisotropy provides a further means to detect FRET. Specifically, it allows the detection of not only hetero-FRET, which occurs between fluorophores of two different species, but also homo-FRET, which occurs between fluorophores of the same species³⁵⁶.

As described in Section 1.3.1, when excited with a linearly polarised light in the donor channel, the fluorescence from the donor molecules that have a rotational correlation time much longer than the fluorescence lifetime (e.g. fluorescent proteins) should exhibit a relatively high fluorescence anisotropy in the absence of FRET, as should the directly-excited acceptor fluorescence. In the presence of hetero-FRET, which reduces the donor fluorescence lifetime and therefore reduces the extent of rotation a donor molecule can achieve before fluorescing, the donor fluorescence anisotropy should further increase. The acceptor fluorescence in such a case would become more depolarised, as FRET can enable sensitised emission from acceptor molecules whose dipole orientations are far from the polarisation direction of the light exciting the donor fluorophores³⁶³.

When homo-FRET occurs, both the fluorescence intensity and emission spectrum of the single fluorophore species that is both the donor and the acceptor remains unchanged (the

fluorescence lifetime is also unchanged unless homo-FRET occurs between two conformations of the same fluorophore species that exhibit different fluorescence lifetimes). However, the fluorescence anisotropy should show a reduction upon FRET and so fluorescence anisotropy provides a unique means to measure homo-FRET³⁶⁴.

Nevertheless, this approach to FRET measurement is sensitive to polarisation artifacts (e.g. depolarisation of the fluorescence by an objective lens with a high numerical aperture), requires relatively bright samples (as the emission needs to be split between vertical and horizontal components before detection), and usually only provides a qualitative indication of the presence of FRET³⁵⁶. Time-resolved fluorescence anisotropy measurements can be realised together with fluorescence lifetime measurements on the same instrument for more quantitative results³⁶⁵. However, this type of implementation shares the same drawbacks of the fluorescence lifetime-based methods, being demanding in terms of the instrumentation and the detected photon count.

1.3.3 Techniques for fluorescence lifetime imaging (FLIM) microscopy

Fluorescence lifetime-based FRET measurements have found extensive applications in microscopic imaging of biological samples. FLIM microscopy techniques can be broadly divided into two categories: frequency-domain FLIM and time-domain FLIM. Typical implementations of these techniques are introduced below.

Frequency-domain FLIM

Frequency-domain FLIM typically involves the excitation of a fluorescent sample with a sinusoidally modulated light and the detection of corresponding sinusoidal fluorescence emission³⁶⁶. The fluorescence emission is modulated at the same frequency as the excitation light, but with changes in magnitude and phase relative to the excitation light, due to the delay between excitation and fluorescence emission (Figure 1-10).

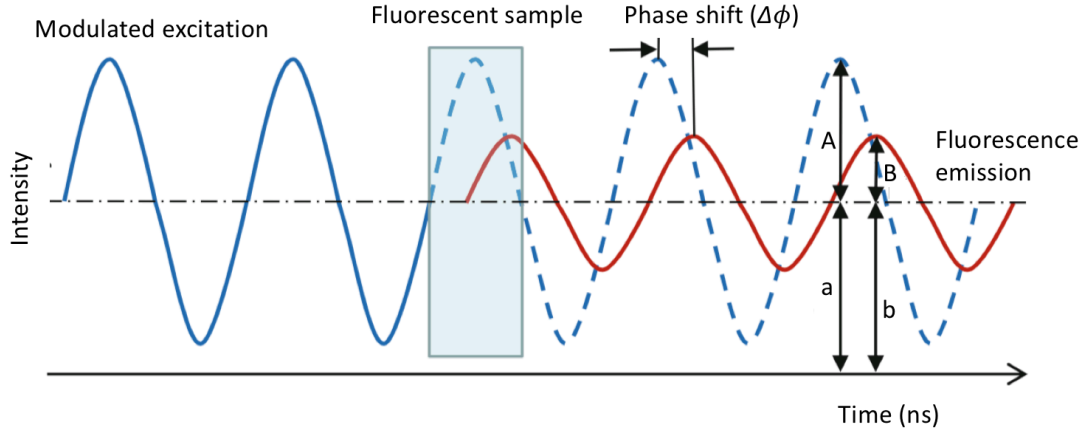


Figure 1-10 Schematic of frequency-domain FLIM measurements

Note the changes in the fluorescence emission's modulation depth and phase relative to those of the excitation light. Adapted from Ref.³⁶⁷ with permission.

According to Figure 1-10, the ratio of the modulation depths of the fluorescence emission to that of the excitation, M , can be calculated as:

$$M = \frac{B/b}{A/a} \quad (1.16)$$

where A and B are the amplitudes of the AC components of the excitation and the emission, respectively, whereas a and b are the amplitudes of the respective DC components. This ratio allows the calculation of a fluorescence lifetime parameter as:

$$\tau_M = \frac{1}{\omega} \sqrt{\frac{1}{M^2} - 1} \quad (1.17)$$

where ω is the modulation frequency.

Alternatively, the phase shift $\Delta\phi$ between the emission and the excitation (illustrated in Figure 1-10) can also be used to calculate a fluorescence lifetime parameter, as:

$$\tau_\phi = \frac{1}{\omega} \tan(\Delta\phi) \quad (1.18)$$

If the underlying decay of the fluorescence emission is mono-exponential, the lifetime values calculated using the above two equations would be equal. If $\tau_M \neq \tau_\phi$, it suggests that the fluorescence decay should be described by a complex exponential model. The multiple components in a complex decay can be determined by additional measurements at different modulation frequencies³⁶⁸. It is also possible to fit single-frequency FLIM data to a bi-exponential model using global analysis^{369,370}. It has also been demonstrated that quantitative information about two fluorophore species that each displays a multi-exponential decay profile can be acquired from a single-frequency FLIM measurement using “lifetime unmixing”, given the *a priori* knowledge of τ_M and τ_ϕ for both fluorophore species³⁷¹. Alternatively, single-frequency FLIM data can be analysed using the fit-free phasor approach^{331,372,373}, which discriminates pixels containing different fluorophore species by displaying them in a polar plot, where the radial and angular coordinates of each pixel are determined by M and $\Delta\phi$.

Frequency-domain FLIM has predominately been implemented in wide-field microscope systems, using the combination of a modulated image intensifier and a CCD camera^{374,375} or a modulated CMOS camera^{376–378} for wide-field detection, and therefore allowing rapid data acquisition (in comparison to point-scanning-based FLIM techniques). It has also been used in point-scanning confocal^{379,380} and multiphoton microscopes^{381,382}.

Time-domain FLIM

Time-domain FLIM directly measures the fluorescence decay profile.. This type of measurement has been realised using a range of techniques, including approaches based on streak cameras^{383,384}, fast digitizers³⁸⁵, and time-binned detection^{386–388}, but the most commonly used approaches today are time-correlated single photon counting (TCSPC) and wide-field time-gated detection, which are discussed below.

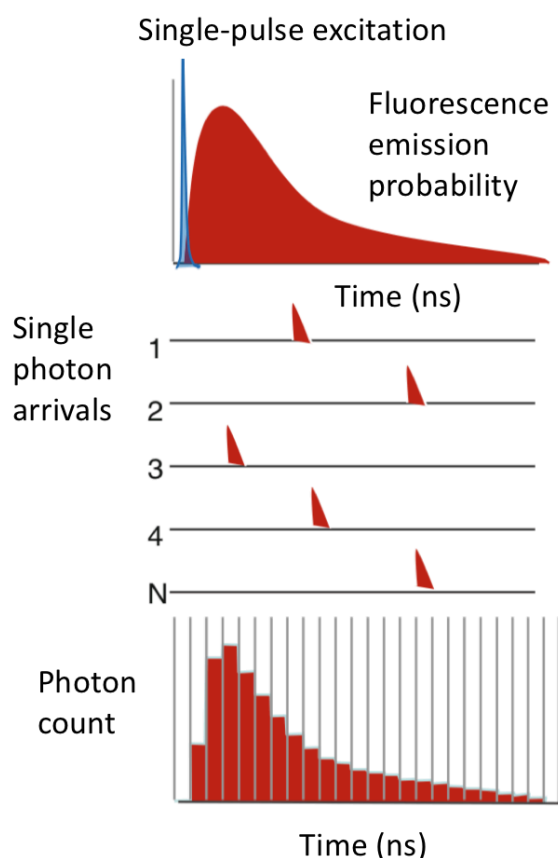


Figure 1-11 Schematics of the principle behind TCSPC

Adapted from Ref.³⁶⁷ with permission.

TCSPC is commonly implemented in laser scanning microscopes and is a point detection method using fast electronics that record the arrival time of each detected photon with respect to the corresponding single-pulse excitation for the pixel under interrogation³⁶⁶. As the probability of the emission of a single photon after an excitation pulse essentially reflects the fluorescence decay profile from a population of simultaneous excited fluorophores, repeatedly recording the arrival times of these single-photon events over multiple excitation pulses can generate a histogram whose envelope is the fluorescence decay profile, from which fluorescence lifetime information can be extracted (Figure 1-11). Practically, the excitation power from the pulsed excitation source needs to be set such that, on average, less than one photon is detected per excitation pulse (the typical number is less than 1 photon for every 100 excitation pulses), in order to avoid the pulse pile-up effect, which distorts the measured decay profile, as most current TCSPC

hardware has a dead time after the arrival of the first detected photon from each excitation pulse during which no further photons will be detected^{389,390}. Together with the need to sequentially acquire the data from all the pixels in a field-of-view (FOV) in a typical laser-scanning microscope set-up, this limits the acquisition speed of the TCSPC-based FLIM systems. Nevertheless, recent developments in algorithms correcting for the pulse pile-up effect^{391,392}, in detectors with shorter dead times³⁹³, and in parallelised TCSPC detection schemes^{394,395}, have to some extents mitigated the speed issue. TCSPC systems are effectively shot-noise limited and utilise every detected photon for building at the arrival time histogram. Compared to other FLIM techniques, TCSPC offers the highest signal-to-noise ratio (S/N) per detected photon, and is widely considered to be the “gold standard” for FLIM measurements³⁶⁶.

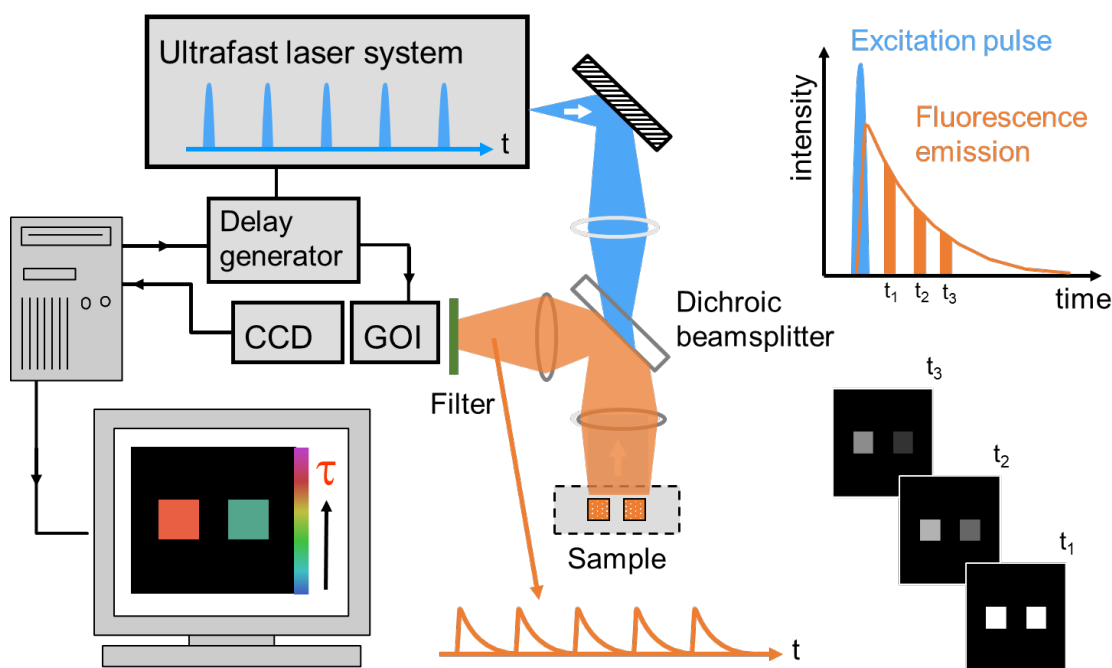


Figure 1-12 Schematic of wide-field time-gated FLIM using a gated imaging intensifier (GOI)

Adapted from Ref.³⁹⁶.

In contrast to TCSPC, time-gated detection is commonly implemented in wide-field microscope systems, and therefore can provide much faster readouts because all the pixels in the FOV are interrogated simultaneously³⁹⁷. As discussed in detail in Section 3.1.1, time-gated FLIM is typically realised with a gated optical intensifier (GOI)³⁹⁸, which

effectively functions as an ultrafast shutter that can be switched within 100 ps. In combination with a CCD or sCMOS read-out camera, the GOI enables the parallelised sampling of the fluorescence decay profiles from all the pixels in a FOV as the “shutter” function enables wide-field time-gated images to be acquired at a number of temporal delays relative to the excitation pulse, thereby sampling the fluorescence decay profiles, as represented in Figure 1-12. Time-gated FLIM is inherently less photoefficient than TCSPC because the fluorescence decays are sampled, rejecting photons outside the time-gate that would otherwise have been detected. This loss of photons can be mitigated by using long time-gates, however, noting that the precision of the lifetime measurement is determined by the edge of the time-gate rather than its duration – indeed, long time-gates provide superior lifetime precision due to the improved S/N ³⁹⁹ and this approach has demonstrated the ability to resolve lifetime changes with a precision of less than 10 ps⁴⁰⁰. The total FLIM data acquisition time depends on the number of delays at which time-gated images are acquired. For mono-exponential decays with no background, only two time-gate delays are required while, for complex decay profiles, it may be necessary to sample the fluorescence decays at up to 10 time gate delays.

In terms of data analysis, as time-domain FLIM directly samples the fluorescence decay profiles, lifetime information can generally be extracted by performing non-linear least squares fitting of the measured decay data to a mono-exponential or complex multi-exponential model, as described in Equation 1.8 and Equation 1.9. Fitting to a complex model can provide more quantitative information (e.g. the population fraction of the FRETing donor as well as the FRETing and non-FRETing donor fluorescence lifetimes), but it requires much more photons to achieve an adequate accuracy⁴⁰¹. This issue can be largely addressed by employing global fitting-based algorithms, as described in detail in Section 3.1.3. Alternatively, Bayesian analysis has been shown to be suitable for performing mono- or bi-exponential fittings with low photon counts^{402,403}. Fit-free methods like the phasor plot have also been applied to analyse time-domain FLIM data³⁷³.

FLIM implementations for high content analysis (HCA)

HCA refers to high-throughput assays that utilise automated imaging to provide data with subcellular resolution across a large number of FOVs of biological samples (usually

arrayed in multiwell plates) in a single experiment run. They typically provide image-based readouts based on cell morphology and intracellular localisation of signals and can include temporal information when assaying live cells. Automatically imaging a large number (typically hundreds to thousands) of FOVs enables experimental parameters, such as gene expression or silencing, signalling readouts, or drug application, to be systematically varied across cells prepared under near identical conditions and enables replicates of each measurement that make it possible to average over various type of noise to increase the statistical power of the assay. The automated sample preparation and data acquisition and analysis can also reduce the scope for operator bias in an experiment.

Since FLIM can provide robust and quantitative readouts of FRET, it is interesting to implement FLIM with automated microscopy to realise FLIM HCA. The first automated multiwell plate FLIM microscope that enabled FLIM HCA was presented in 2007, based on wide-field frequency-domain FLIM⁴⁰⁴. Wide-field frequency-domain FLIM was subsequently utilised in another HCA study that imaged cell arrays on multiwell chamber slides prepared by automated reverse transfection⁴⁰⁵. More recently an automated frequency domain FLIM microscope was reported that was assembled using commercial components⁴⁰⁶.

The first demonstration of time-domain FLIM HCA was reported in 2008 (at Imperial College London), utilising wide-field time-gated imaging at a rate of ~20 s per FOV⁴⁰⁷. TCSPC FLIM was later implemented in an automated multiwell plate microscope with an imaging speed of ~3 min per FOV following initial fast steady-state fluorescence anisotropy imaging that pre-identified “hits” as secondary (quantitative) measurements⁴⁰⁸. The Photonics Group at Imperial College London have continued to develop and optimise automated wide-field time-gated FLIM microscope systems for HCA^{399,409–412}, and recently presented the open source *openFLIM-HCA* platform that utilises open source *μManager*⁴¹³-based software to control automated FLIM HCA data acquisition³⁹⁶ and an open source *MATLAB*-based tool, *FLIMfit*, for data analysis³³⁰. This was the starting point for the work reported in this thesis. For a detailed description of this platform, please see Section 3.1.

1.3.4 Applications of FLIM FRET for studying PPIs in budding yeast

While FLIM FRET has been applied to a large number of studies on PPIs in fixed or live cells (e.g. Refs.^{414,415}), including a few studies utilising FLIM HCA^{412,416}, to the best of my knowledge FLIM FRET has not yet found much application to study PPIs in budding yeast cells, despite the relative simplicity in labelling endogenous proteins in live yeast cells with fluorescent proteins via genetic modifications, and FLIM HCA of yeast cells has not been reported. There have been a few reports of manual TSCPC FLIM microscopy applied to study PPIs between FP-labelled budding yeast proteins^{316,417–420}. In comparison, intensity/spectral-based FRET measurements have been more frequently employed in budding yeast-related studies, including some recent ones of the budding yeast kinetochore^{316,317,421}.

1.4 Project aims

Although the potential of FLIM HCA has previously been demonstrated to identify and quantify PPIs, most of the previous work has been undertaken with fixed cells and, where fluorescent proteins were used, the FRET constructs have been over-expressed using plasmid transfection. Before this project, FLIM HCA had not been applied to study *in vivo* PPIs at the budding yeast kinetochore and it was not clear that it would be possible using FP-labelled endogenous proteins of such low copy number. Therefore, the primary aim of this project was to develop and validate a FLIM HCA methodology for such studies (Chapter 3 and Chapter 4). Then, I aimed to employ this FLIM HCA methodology to screen for the interacting partners of a DNA repair protein Rdh54p at the budding yeast kinetochore (Chapter 5). Rdh54p is involved in the adaptation of the DDC-induced cell cycle arrest in the presence of persistent DNA damage. Because this cell cycle arrest is also dependent on the the SAC that is regulated at the kinetochore, and because Rdh54p is also constitutively localised at the kinetochore, identifying the interacting partners of Rdh54p at the kinetochore may shed light on the exact molecular mechanism governing Rdh54p's function in checkpoint adaptation.

Chapter 2. Materials & Methods

This chapter describes the materials and methods used for the work reported in this thesis.

2.1 Buffers, media, and solutions

Table 2-1 summarises the buffers, media, and solutions used for the work reported in this thesis.

Table 2-1 Buffers, media, and solutions

Name	Composition ¹	Usage
Luria-Bertani (LB) medium	1% bacto-tryptone, 0.5% yeast extract, 1% NaCl; for solid medium add 1.5% bacto-agar	<i>E.coli</i> growth medium
LB Amp medium	LB plus 0.05-0.1 g/L ampicillin	<i>E.coli</i> growth medium for plasmid selection
SOC medium	2% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl ₂ , 10 mM MgSO ₄ , 20 mM glucose	<i>E.coli</i> electroporation
YPD medium	1% yeast extract, 2% bacto-peptone, 2% glucose; for solid medium add 2% bacto-agar	Yeast growth medium
Synthetic complete (SC) medium	1.7 g/L yeast nitrogen base (without amino acids), 5 g/L ammonium sulphate, 109 µM adenine sulphate, 95 µM L-arginine sulphate, 95 µM L-histidine HCl, 229 µM L-isoleucine, 457 µM L-leucine, 164 µM L-lysine HCl, 134 µM L-methionine, 303 µM L-phenylalanine, 98 µM L-tryptophan, 166 µM L-tyrosine, 178 µM uracil, 1280 µM L-valine, 2% glucose; for solid medium add 2% bacto-agar	Yeast growth medium
Synthetic drop-out medium	SC medium without specific supplement(s)	Yeast growth medium
SC/drop-out+ADE medium	SC or drop-out medium with 100 mg/L additional adenine sulphate	Yeast growth medium for microscopy
SC/drop-out + Hygromycin (HYG)	SC or drop-out medium without the 5 g/L ammonium sulphate, plus 200 mg/L Hygromycin	Yeast growth medium

¹ All dissolved in MilliQ water.

TE/LiOAc solution	10 mM Tris-HCl pH7.5, 1 mM EDTA, 100 mM LiOAc	Yeast transformation
TE/LiOAc + 40%PEG solution	10 mM Tris-HCl pH7.5, 1 mM EDTA, 100 mM LiOAc, 40% (weight/volume) PEG ₃₃₅₀	Yeast transformation
SPO solid medium	20 g/L potassium acetate, 2.5 g/L yeast extract, 1 g/L glucose, 2% bacto-agar, and 75 mg/L of each: adenine sulphate, L-histidine HCl, L-leucine, L-lysine HCl, L-methionine, L-tryptophan, uracil	Yeast sporulation
Synthetic defined (SD) medium	1.7 g/L yeast nitrogen base (without amino acids), 5 g/L ammonium sulphate, 2% glucose; for solid medium add 2% bacto-agar	Tetrad scoring for mating types
Smash n' Grab buffer	1% SDS, 2% Triton X-100, 100 mM NaCl, 10 mM Tris-HCl pH8, 1 mM EDTA	Yeast genomic and plasmid DNA extraction
TE (1x) buffer	10 mM Tris-HCl pH7.5, 1 mM EDTA	Yeast genomic and plasmid DNA extraction
TAE buffer	40 mM Tris, 20 mM acetic acid, 1 mM EDTA	Agarose gel electrophoresis
GAL -Leu solid agar medium	20% (weight by volume) filter-sterilised galactose solution; 1.7 g/L yeast nitrogen base (without amino acids), 5 g/L ammonium sulphate, 41 mg -Leucine amino acid mix ^{II} , and 20 g/L bacto-agar, dissolved in water by stirring and then autoclaved for melting the agar and sterilising. NOTE: the two solutions above were mixed by gentle stirring when the latter had cooled to 55-60°C, and then the mixture were poured into plates.	SPI assay
GAL -Leu 5-FOA solid agar medium	For 1 L medium: 1.7 g yeast nitrogen base (without amino acids), 5 g ammonium sulphate, 41 mg -Leucine amino acid mix, 30 mg uracil, 750 mg 5-fluoro-orotic acid (5-FOA), and 2% (weight by volume) galactose, dissolved in 500 ml of water by stirring for ~1h at room temperature, filter-sterilised through a 0.22 µm filter, and placed in 60°C water bath; 20 g bacto-agar dissolved (melted) in 500 ml of water by autoclaving. NOTE: the two solutions above were mixed by gentle stirring when the latter had cooled to 55-60°C, and then the mixture were poured into plates.	SPI assay

^{II} The mix consists of the amino acids and uracil as in a -Leu dropout medium.

2.2 Bacterial methods

2.2.1 Bacterial strain

The electro-competent *E.coli* strain ElectroMAX DH10B (Invitrogen, US) was used for the bacterial work, which made use of the *E.coli* cells for the amplification of the yeast plasmids used in this thesis.

2.2.2 E.coli growth conditions

E.coli cells were incubated at 37°C in Luria-Bertani (LB) broth (with shaking at 300 rpm) or on LB agar. 0.05-0.1 mg/ml ampicillin was added to the liquid or solid media when plasmid selection was needed.

2.2.3 E.coli electroporation

Electroporation was utilised to transform a plasmid to be amplified into the electro-competent *E.coli* cells. For this procedure, first the cells were taken out of the -80°C freezer and thawed on ice, and then 10 µl of the cells were mixed with 5-10 µl of plasmid DNA and 20 µl of 10% glycerol. The mixture was transferred into a chilled 2 mm Gene Pulser cuvette (Bio-Rad, US), and then electroporated using a Gene Pulser Xcell device (Bio-Rad, US) with the settings of 2.5 KV, 200 Ω, and 25 µF. Immediately after electroporation 1 ml of SOC medium was added into the cuvette, and the culture was incubated at 37°C (with shaking at 300 rpm) for 1 hour. 100 µl of the culture was plated onto a LB Amp agar plate and incubated overnight at 37°C for single colonies of the transformants containing the plasmid. Finally, a couple of the resulting colonies were streaked out on fresh LB Amp plates for further purification.

2.2.4 Plasmid extraction from E.coli

For extracting plasmid DNA from the transformed *E.coli* cells, the cells were grown overnight in 5 ml LB Amp cultures and collected by centrifugation for 2 min at 8000 rpm. Subsequently, a GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used following

manufacturer's instructions. The extracted plasmid DNA was stored in 4°C (short-term) or -20°C (long-term).

2.2.5 E.coli storage

For long-term storage, 0.7 ml of an overnight LB Amp culture of the transformed *E.coli* strain of interest was mixed with 0.7 ml 50% glycerol in a 2 ml screw cap tube and stored in a -80°C freezer. For short-term storage, the cells were kept on agar plates at 4°C for up to 6 months.

2.3 Yeast methods

2.3.1 Yeast strains

Budding yeast strains used in this project are listed in Table 2-2. All these strains were of either W303⁴²² or S288C⁴²³ background.

Table 2-2 Yeast strains used in this project

Strain	Genotype ^{III}	Usage	Source
PT208	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100</i>	Wildtype based on W303	This work
PT192	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 ASK1-mTq2FP::HYGMX/ASK1-YFP::HIS3MX6</i>	FLIM	This work
PT194	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 MTW1-mTq2FP::HYGMX/MTW1-YFP::HIS3MX6</i>	FLIM	This work
PT197	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 NDC10-mTq2FP::HYGMX/NDC10-YFP::HIS3MX6</i>	FLIM	This work
PT244	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 SPC24-mTq2FP::HYGMX/SPC24</i>	FLIM	This work

^{III} Genotypes listed once are homozygous.

PT258	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 ASK1-mTq2FP::HYGMX/ASK1</i>	FLIM	This work
PT260	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 MTW1-mTq2FP::HYGMX/MTW1</i>	FLIM	This work
PT261	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 NDC10-mTq2FP::HYGMX/NDC10</i>	FLIM	This work
PT266	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 ASK1-mTq2FP::HYGMX/ASK1 NDC10-YFP::HIS3MX6/NDC10</i>	FLIM	This work
PT267	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 SPC24-mTq2FP::HYGMX/SPC24 SPC25-YFP::HIS3MX6</i>	FLIM	This work
PT268	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 MTW1-mTq2FP::HYGMX/MTW1 CTF19-YFP::HIS3MX6</i>	FLIM	This work
PT269	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 MTW1-mTq2FP::HYGMX/MTW1 NDC10-YFP::HIS3MX6</i>	FLIM	This work
PT270	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 NDC10-mTq2FP::HYGMX/NDC10 ASK1-YFP::HIS3MX6</i>	FLIM	This work
PT273	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 NDC80-mTq2FP::HYGMX/NDC80 NUF2-YFP::HIS3MX6/NUF2</i>	FLIM	This work
PT276	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 NUF2-mTq2FP::HYGMX/NUF2 ASK1-YFP::HIS3MX6/ASK1</i>	FLIM	This work
PT278	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 NDC80-mTq2FP::HYGMX/NDC80</i>	FLIM	This work
PT279	<i>MATα/MATa ADE2 RAD5 TRP1/trp1-1 LYS2/lys2Δ his3-11,15 leu2-3,112 ura3-1 can1-100 NUF2-mTq2FP::HYGMX/NUF2</i>	FLIM	This work
PT286	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 CTF19-GFP::HIS3MX6/CTF19 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT287	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 CTF19-GFP::HIS3MX6/CTF19</i>	FLIM	This work
PT288	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 OKP1-GFP::HIS3MX6/OKP1 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT289	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 OKP1-GFP::HIS3MX6/OKP1</i>	FLIM	This work

PT290	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 MCM21-GFP:HIS3MX6/MCM21 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT291	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 MCM21-GFP:HIS3MX6/MCM21</i>	FLIM	This work
PT292	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 AME1-GFP:HIS3MX6/AME1 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT293	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 AME1-GFP:HIS3MX6/AME1</i>	FLIM	This work
PT294	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 NDC80-GFP:HIS3MX6/NDC80 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT295	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 NDC80-GFP:HIS3MX6/NDC80</i>	FLIM	This work
PT296	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 NUF2-GFP:HIS3MX6/NUF2 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT297	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 NUF2-GFP:HIS3MX6/NUF2</i>	FLIM	This work
PT298	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 SPC24-GFP:HIS3MX6/SPC24 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT299	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 SPC24-GFP:HIS3MX6/SPC24</i>	FLIM	This work
PT300	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 SPC25-GFP:HIS3MX6/SPC25 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT301	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 SPC25-GFP:HIS3MX6/SPC25</i>	FLIM	This work
PT302	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 ASK1-GFP:HIS3MX6/ASK1 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT303	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 ASK1-GFP:HIS3MX6/ASK1</i>	FLIM	This work
PT304	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 DAD1-GFP:HIS3MX6/DAD1 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT305	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 DAD1-GFP:HIS3MX6/DAD1</i>	FLIM	This work
PT306	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 DAD3-GFP:HIS3MX6/DAD3 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT307	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 DAD3-GFP:HIS3MX6/DAD3</i>	FLIM	This work

PT308	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 CEP3-GFP:HIS3MX6/CEP3 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT309	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 CEP3-GFP:HIS3MX6/CEP3</i>	FLIM	This work
PT310	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 NKP2-GFP:HIS3MX6/NKP2 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT311	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 NKP2-GFP:HIS3MX6/NKP2</i>	FLIM	This work
PT312	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 MTW1-GFP:HIS3MX6/MTW1 RDH54-mCherry::HYGMX/RDH54</i>	FLIM	This work
PT313	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 MTW1-GFP:HIS3MX6/MTW1</i>	FLIM	This work
PT314	<i>MATα/MATa his3Δ1 leu2Δ0 ura3Δ0 LYS2/lys2Δ0 MET15/met15Δ0 SPC24-GFP:HIS3MX6/SPC24 SPC25-mCherry::HYGMX/SPC25</i>	FLIM	This work
W8161-2B	<i>MATα ADE2 RAD5 LYS2 trp1-1 his3-11,15 leu2-3,112 ura3-1 CEN1-16::Gal-KI-URA3</i>	SPI	Ref. ⁴²⁴
GFP library	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 XXX-GFP::HIS3MX6</i>	SPI	Ref. ⁴²⁵
E211	<i>MATa cyc3-3 his1</i>	Mating type tester	This work
PT163-11A	<i>MATα ADE2 HIS1 LEU2 URA3 TRP1 LYS2</i>	Mating type tester	This work

2.3.2 Yeast growth conditions

Yeast cells were typically grown in either rich YPD or synthetic drop-out media (with 2% glucose as the carbon source) unless stated otherwise. Cells growing on agar plates were incubated at 30°C, whereas liquid cultures were incubated at 30°C with shaking at 300 rpm. To select for cells with hygromycin resistance, 200 μ g/ml hygromycin B (Roche, Switzerland) was added to the appropriate growth media. Cells for microscopy were grown overnight at 23°C as liquid cultures in either synthetic complete (SC) or synthetic drop-out (e.g. for maintaining plasmids) media, with an additional 100 mg/ml adenine for suppressing autofluorescence originating from phosphor-ribosyl-amino-imidazole.

2.3.3 Yeast transformations

Yeast transformations for introducing exogenous DNA for genomic integration, for introducing plasmid DNA, or for gap repair ligation of plasmids were performed using a high-efficiency lithium acetate (LiOAc) method⁴²⁶.

For one transformation for genomic integration, the strain to be transformed was grown overnight in a liquid culture of YPD (or a drop-out medium when necessary) at 30°C (with shaking). The overnight culture was diluted 100-fold in 50 ml of fresh medium and grown for another ~3-5 hr until the optimal culture density was reached ($OD_{600} = \sim 0.5-0.7$). The cells were then harvested by centrifugation for 5 min at 4000 rpm, and the resulting cell pellet was washed with 10 ml TE/LiOAc solution. The cells were harvested again by centrifugation for 5 min and the resulting cell pellet was resuspended in 600 μ l TE/LiOAc. Next, 200 μ l of these cells were mixed with 150 μ g (15 μ l) salmon sperm DNA (Sigma-Aldrich, US), 200-500 ng of the transforming DNA, and 700 μ l of the solution TE/LiOAc + 40% PEG (polyethylene glycol, average molecular weight 3350 g/mol). The mixture was incubated at 30°C for 30 min, and then heat shocked at 42°C for 15 min. After heat shock, the transformed cells were either directly plated onto appropriate selection medium or, if drug selection was needed, the cells were collected by centrifugation, resuspended in YPD (or a drop-out medium when necessary), and incubated at 30°C (with shaking) for an additional 2-4 hr before plating on appropriate selection medium. The transformed-cell plates were incubated at 30°C for 2-3 days.

Transformations for introducing plasmid DNA into yeast cells were done using a scaled-down version of the procedure described above, with reduced amounts of cells (30 μ l), salmon sperm DNA (50 μ g, i.e. 5 μ l), plasmid DNA (50-100 ng), and TE/LiOAc + 40% PEG (140 μ l). Approximately, 50 μ l of this small-scale transformation was plated onto selection medium.

Transformations for gap repair ligation were also done in a similar manner as for genomic integration. The only differences were that instead of adding in 200-500 ng of the transforming DNA, ~50 ng of the cut plasmid and ~50 ng of the DNA insert were added; 150 μ l of this transformation was plated.

2.3.4 Yeast crosses

Yeast crosses were performed by mating two haploid yeast strains of opposite mating types (*MAT α* and *MATa*, respectively) together on YPD to create a diploid strain. For selection of the resulting diploids, the two haploid strains contained different auxotrophic markers, e.g. one with *MAT α trp1-1 LYS2* and the other with *MATa TRP1 lys2 Δ* . Thus, the resulting diploid cells were selected on a plate of the appropriate medium, e.g. a -Trp -Lys plate, at 30°C for two days via replica plating. Single colonies were further purified by streaking out onto the appropriate medium and incubated at 30°C for another 2 days.

2.3.5 Yeast sporulations, tetrad dissections, and scoring the tetrads

Yeast sporulations were achieved by patching single colonies of diploid yeast cells onto sporulation (SPO) solid medium and incubating the cells at 30°C for at least 3 days until the formation of tetrads, i.e. asci each containing four haploid spores.

For dissection, the tetrads were then picked from the SPO plate and suspended in 100 μ l 5% glusulase enzyme at room temperature for 5 min to digest the walls of the asci. After the digestion, 10 μ l of the tetrads were pipetted onto a line in the centre of a YPD plate and allowed to dry for a few minutes. The tetrads were then dissected into sets of 4 individual spores on the YPD plate via micromanipulation using an MSM300 dissection microscope (Singer Instruments, UK). The plate was incubated at 30°C for 2-3 days for colony formation.

The dissected tetrads were scored by replica plating. The colonies of the spores were replica plated onto several selection plates, each for one auxotrophic or drug resistance marker of interest. To score for the mating types, the colonies of the spores were first mated with the two mating type testers (E211 and PT163-11A) respectively via replica plating and overnight incubation at 30°C, and then replica plated again onto synthetic defined (SD) medium and incubated at 30°C overnight for new colony formation.

2.3.6 Yeast genomic and plasmid DNA extraction

The “Smash n’ Grab” method⁴²⁷ was mainly employed in my project for extracting yeast genomic and plasmid DNA. Yeast cells were first collected from 3 ml of overnight yeast culture in YPD by centrifugation at 4000 rpm for 5 min and discarding the supernatant. The pellet was resuspended in 200 µl Smash n’ Grab buffer, and then mixed with 200 µl phenol/chloroform (Invitrogen, US) and 250 µl glass beads (0.4-0.5 mm in diameter) (Sigma-Aldrich, US). The mixture was vortexed for 2 min to break up the cells and subsequently centrifuged at 4°C and 13000 rpm for 10 min to precipitate the cell debris. After centrifugation, 100 µl of the supernatant was transferred to a new Eppendorf tube together with 200 µl of phenol/chloroform, vortexed again for 2 min, and then centrifuged again at 4°C and 13000 rpm for 10 min. 50 µl of the resulting supernatant was transferred onto a nitrocellulose membrane filter disc (0.025 µm) (Millipore, US) and dialysed against TE for 20 min, before being transferred into a new Eppendorf tube for storage at -20°C.

2.3.7 Yeast storage

Yeast cells on agar plates were routinely stored at 4°C. Cells on rich YPD plates were kept for up to 6 months, whereas when on synthetic plates they were kept for only ~1 month. For long term storage, 0.7 ml of an overnight YPD (or an appropriate drop-out medium) culture of the yeast strain of interest was mixed with 0.7 ml 50% glycerol in a 2 ml cryo tube and then stored at -80°C.

2.4 Molecular biology methods

2.4.1 Polymerase chain reaction (PCR)

Q5-High fidelity polymerase (New England BioLabs, US) was used for high-fidelity PCRs whereas DreamTaq Green master mix (Thermo Scientific, US) was used for diagnostic PCRs. Both types of reactions were run in T100 Thermal Cyclers (Bio-Rad, US) following the manufacturer’s instructions.

Primers for PCRs were designed using the software tool SeqBuilder (DNASTAR, US) and ordered from Sigma-Aldrich. DNA templates used for PCRs were typically yeast genomic DNA, plasmid DNA, DNA fragments from other PCRs, or synthesised DNA fragments ordered from GeneArt Strings (Thermo Fisher Scientific, US).

PCR products were purified using a GeneJET PCR Purification Kit (Thermo Scientific, US) following manufacturer's instructions. After purification, the DNA concentrations of the products were measured using a NanoVue Plus spectrophotometer (General Electric, US).

2.4.2 Agarose gel electrophoresis

DNA gel electrophoresis was performed using 0.5-1.5% agarose gels (Invitrogen, US) made in TAE buffer. To stain the DNA, 0.1 µl/ml GelRed Nucleic Acid Gel Stain (Biotium, US) was added to the molten agar before casting. PCR products or other DNA fragments were loaded into separate wells on the gel after being mixed with 10x loading dye, alongside with the 1kb Gene Ruler DNA ladder (Thermo Scientific, US), and were run on a mini horizontal submarine Hoefer HE33 unit (Amersham Biosciences, UK) at 90 V with TAE buffer for 30-60 min. The resulting DNA bands were visualised using a Bio-Doc-It UV imaging system (UVP LLC, US).

2.4.3 DNA extraction from agarose gels

After agarose gel electrophoresis, sometimes DNA fragments in bands of the intended sizes were extracted from the gel, using a GeneJET Gel Extraction Kit (Thermo Scientific, US) following manufacturer's instructions.

2.4.4 Plasmid construction

Plasmids used in this project were listed in Table 2-3.

Table 2-3 Plasmids used in this project

Plasmid	Genotype ^{IV}	Usage	Source
pWJ1512	<i>pCUP1 LEU2</i>	Donor plasmid	Ref. ⁴²⁴
pX22	<i>pCUP1 LEU2 RDH54-YFP</i>	SPI	This work
pHT4	<i>pCUP1 LEU2 GBP-RFP</i>	SPI	Ref. ⁴²⁸
pHT38	<i>pCUP1 LEU2 RDH54-GBP-RFP</i>	SPI	This work
pHT39	<i>pCUP1 LEU2 rdh54_AA-GBP-RFP</i>	SPI	This work
pHT627	<i>pCUP1 LEU2 rdh54_K318R-GFP-RFP</i>	SPI	This work
pHT446	<i>pCUP1 LEU2 ASK1-mTurquoise2-YFP</i>	FLIM	This work
pHT448	<i>pCUP1 LEU2 MTW1-mTurquoise2-YFP</i>	FLIM	This work
pHT449	<i>pCUP1 LEU2 NDC10-mTurquoise2-YFP</i>	FLIM	This work
pHT522	<i>pCUP1 LEU2 SPC24-mTurquoise2-YFP</i>	FLIM	This work

The constructions of the plasmids made during this project were performed mainly via yeast transformations for gap repair (see Section 2.3.3). The sequence of each plasmid was first designed using the software tool SeqBuilder (DNASTAR, US), incorporating both the sequence of the donor plasmid pWJ1512 cut at the HpaI site and that of the DNA insert. Next, the DNA insert was acquired either via a PCR reaction or as a GeneArt Strings DNA fragment (Thermo Fisher Scientific, US), and then transformed into a yeast strain together with the donor plasmid cut by the restriction enzyme HpaI (New England BioLabs, US) for gap repair ligation in the yeast cells. Cells containing the gap repaired plasmids were selected on -Leu drop-out solid medium, and then the plasmids were extracted from the yeast cells, cloned in and amplified in *E.coli*, and transferred back into an appropriate yeast strain as described earlier in Sections 2.3.6, 2.2.3, 2.2.4, 2.3.3.

^{IV} All have *ARS209*, *CEN6*, and *AMP*.

2.5 Synthetic physical interaction (SPI) assays

2.5.1 Introduction

The SPI method was primarily developed in the Thorpe Lab by Dr. Guðjón Ólafsson and Dr. Peter Thorpe for large-scale screens of protein-protein associations that affect the growth of cells when forced⁴²⁹. In an SPI assay, a protein of interest, i.e. a target protein, is artificially tethered to a collection of GFP-tagged query proteins, respectively, via the use of a GFP binding protein (GBP, also known as nanobody, nanotrap, or chromobody when fused with a fluorescent protein)^{430,431} that is endogenously tagged to the target protein. The GBP is a heavy-chain antibody derived from the lymphocytes of alpacas *Lama pacos*. It is small in size (11-13 kDa, as compared to the 27 kDa for GFP) and binds to GFP with high specificity and high affinity *in vitro* ($K_d < 1$ nM)⁴³⁰, making it possible to associate the GBP to endogenous GFP-tagged proteins *in vivo* and to study the resulting phenotypes in terms of cell growth. An artificial association of two proteins that perturbs cell growth is termed a Synthetic Physical Interaction or SPI. Making use of the ROTOR robotic pinning platform (Singer Instruments Ltd., UK) together with the budding yeast GFP library consisting of >4000 strains⁴³², in each of which a different open-reading frame in the yeast genome is fused with GFP (S65T)⁴³³, the SPI method allows the systematic assessment of the cell growth phenotype when a GBP-labelled target protein is artificially and respectively associated with most of the proteins in the yeast proteome.

2.5.2 Materials, strains, and equipment for SPI assays

Media and solutions

Media and solutions used for SPI assays are summarised in Table 2-1. All solid media were made in Singer Plus square plates (Singer Instruments Ltd., UK) that are designed to be used on the ROTOR robotic pinning platform (see below).

Yeast strains and plasmids for SPI assays

A subset of the GFP collection of yeast strains was used in the SPI experiments⁴²⁵, alongside the universal donor strain (UDS) W8161-2B. The genotypes for these strains

are provided in Table 2-2. The use of the UDS made it possible to perform selective ploidy ablation (SPA)⁴²⁴, as each of the 16 chromosomes contain a counter-selectable *URA3::GAL* promoter cassette⁴³⁴ upstream of its centromere. All these chromosomes can be eliminated by selecting with galactose and 5-FOA, and therefore the diploid cells resulting from the mating of a strain in the GFP collection and the UDS can be reverted back to haploid cells, retaining only the GFP-tagged allele. Alternatively, the diploid cells can be maintained by growing the cells on -His -Leu media (for selecting the GFP gene and the plasmid of interest) with glucose as the carbon source. In this way, the cells will contain an untagged allele of the GFP-tagged gene, and any growth defect will reveal the dominant effect of the SPI.

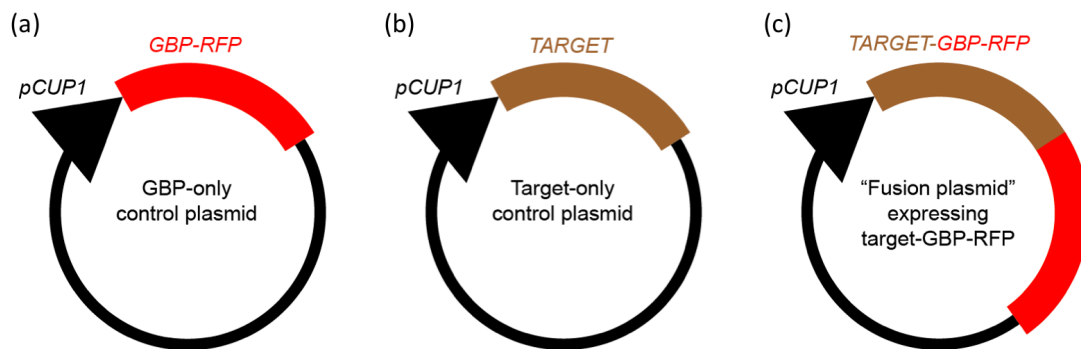


Figure 2-1 Schematic of the plasmids used for a typical SPI assay

(a) A control plasmid expressing only the GBP construct labelled with RFP. (b) A control plasmid expressing only the target protein without GBP-RFP. (c) A “fusion plasmid” expressing the target protein labelled with GBP-RFP.

Single copy CEN plasmids based on pWJ1512⁴²⁴ were transferred into the UDS W8161-2B, for the SPI assays, as listed in Table 2-3. As illustrated in Figure 2-1, two or three types of plasmids were used for each SPI assay: one “fusion plasmid”, expressing a target protein of interest fused to a GBP and a red fluorescent protein, and separately one or two “control plasmids”, expressing either the same target protein alone (with no GBP or RFP) or only the GBP-RFP construct (without the target protein). Expressions of the constructs from the plasmids were driven by a *CUP1* promoter, which is a constitutively active promoter⁴³⁵. All plasmids contained a *LEU2* gene for selection.

Pinning robot

A ROTOR robotic pinning platform (Singer Instruments Ltd., UK) was used for copying colonies in a high-throughput format, including mating the GFP collection of strains with a lawn of the UDS. Using this platform and sterile pins, yeast colonies can be pinned into 96-, 384-, or 1536-arrays on rectangular agar plates, and it is straightforward to convert between different array formats. For all the assays reported here, collections of strains stored in different array formats were all first converted into the 1536-array format. For the initial proteome-wide screen for Rdh54p SPIs, each strain was pinned with 4 replicates, whereas for the subsequent validation screens 16 replicates were used.

Plate imaging

Prior to data analysis, plates containing the final colonies of strains were scanned using a desktop flatbed scanner (Epson V750 Pro, Seiko Epson Corporation, Japan) at 300 dpi (dots per inch) resolution in transmission mode, with the plate lids removed and the plates placed upside down in the scanner. The resulting images were saved in the .tiff format for the subsequent analysis using the ScreenMill software.

2.5.3 Procedures for a typical SPI assay

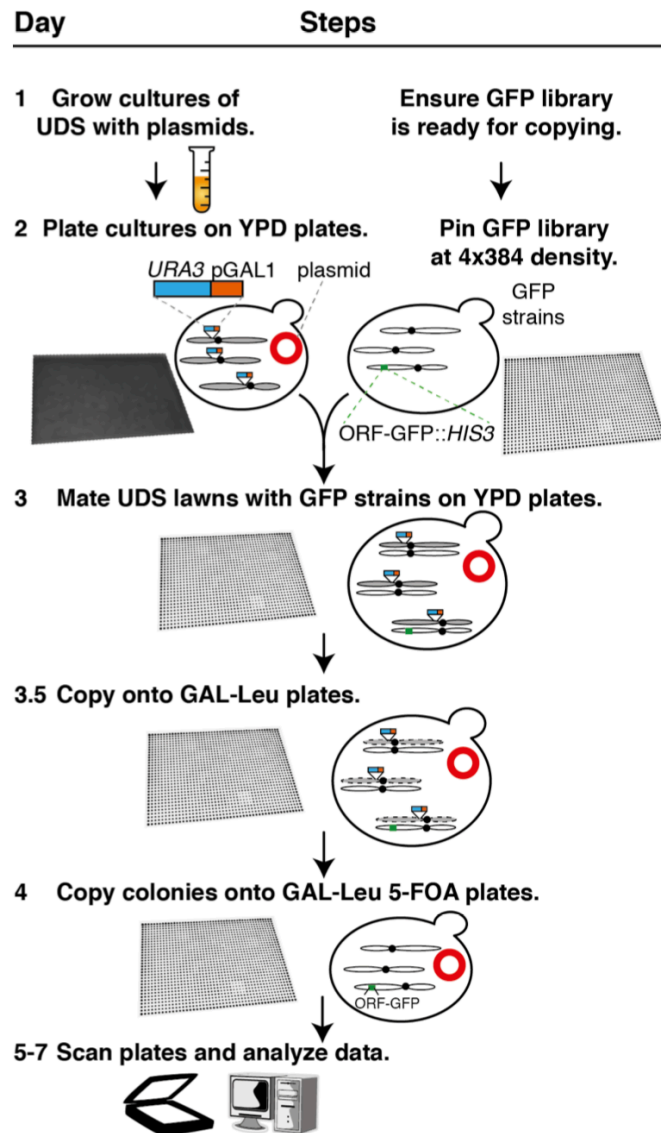


Figure 2-2 The key steps for a typical SPI assay

Please refer to the main text for detailed descriptions of these steps. Adapted from Ref.⁴²⁹ with permission.

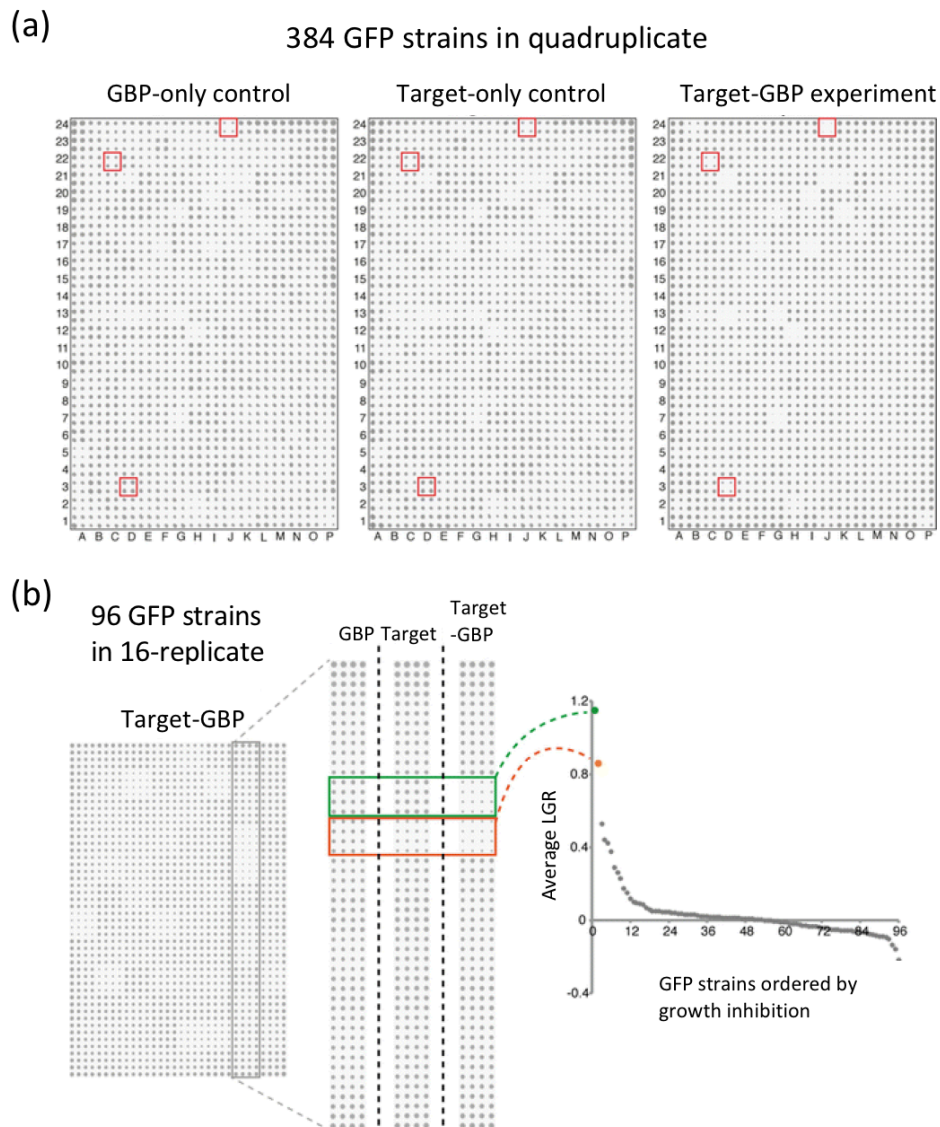


Figure 2-3 Exemplar SPI data

(a) Exemplar images of plates in a proteome-wide SPI screen. (b) Exemplar results for a validation SPI assay with 16 replicates for each GFP strain. Adapted from Ref.⁴²⁹ with permission.

Figure 2-2 illustrates the key steps for a typical SPI assay. The detailed procedures performed were given below:

1. Prepared the rectangular plates containing arrays of GFP strains and, separately, a lawn of each plasmid in the UDS:
 - In the Thorpe lab, a copy of the GFP collection of strains is routinely stored at 4 °C on rectangular YPD agar plates, with 384 strains on each

plate (1 colony for each strain). At the start of the proteome-wide SPI assay, these strains were copied onto fresh rectangular YPD plates by pinning them into the 1536-array format, i.e. with 4 replicates of each strain on each plate (Figure 2-3a). For the validation SPI assays, the selected strains were picked from the GFP collection and pinned also into the 1536-array format, but with 16 replicates for each strain (Figure 2-3b). The new plates were incubated at 30°C overnight for colonies to form.

- 5-10 ml -Leu liquid cultures were set up for the UDSs, each containing a separate plasmid for the SPI assay (e.g. the ones in Figure 2-1), and were incubated with shaking at 30°C overnight. Cells in the overnight cultures were collected by centrifugation at 4000 rpm for 5 min, and were then respectively resuspended in ~400 µl of growth media and plated onto YPD plates by using 4 mm sterile glass beads to evenly spread the cells across each plate. The plates were kept in 30°C overnight for the lawns to grow. Note that each lawn can be used to mate with 4 plates of GFP strains; when there were more GFP strains, more lawns were made.
2. Mixed each GFP strain with the UDS containing one plasmid to allow them to mate:
- The GFP strains from last step were copied onto fresh YPD plates.
 - Cells from each UDS lawns were overlaid respectively onto the GFP strains on the new plates by pinning in the same way.
 - The new plates were incubated at 30°C for 6-7 h to allow the cells to mate.
3. Performed SPA:
- Colonies on the “mating” plates were copied on GAL -Leu plates and incubated at 30°C for 24 h, for counter-selecting the conditional chromosomes from the UDS.
 - The resulting colonies were copied again onto GAL -Leu 5-FOA plates to further eliminate the conditional chromosomes.
 - These final plates were incubated at 30°C for 24-72 h, until colonies of appropriate sizes were formed.
 - **NOTE:** for screening SPIs in diploid cells, instead of performing SPA, the mated colonies were first copied directly onto -His -Leu plates, incubated

at 30°C for 24 h, and then copied again onto -His -Leu plates and further incubated for ~24 h (depending on colony sizes) before plate imaging.

4. Imaged the final plates using the scanner.

2.5.4 Quantitative data analysis

The sizes of the colonies on the plates imaged were quantitatively analysed in order to identify SPIs, i.e. forced protein interactions that appeared to affect cell growth when compared with either or both of the control groups (GBP only and target protein only). The analysis was done following the procedures below:

1. Colony sizes from each plate image were first extracted using the “CM engine” (Colony Measurement engine), which is a freely available ImageJ plug-in and part of the ScreenMill software suite⁴³⁶. The engine was run using standard settings^V. For each assay the output was a “log file”, which contained the information on the size (in pixels), the circularity, and the location coordinates for each colony on all the plates in the assay.
2. The log file was then processed using a PERL script, to extract the information for each pair of plates whose colony sizes were to be compared, i.e. the experiment plate (containing cells with the fusion plasmid) and the control plate (containing cells with either of the control plasmids). The information for each pair was saved as a .txt file.
3. The .txt files from the previous step were loaded respectively into the DR engine (Data Review engine) of the ScreenMill software suite (available online: http://www.rothsteinlab.com/tools/screen_mill/dr_engine_set-up), to compare the colony sizes on the experiment and the control plates. The locations of each strain containing the same GFP-tagged gene were the same on both plates. For the validation assays, there were also some non-GFP control strains added onto certain positions identical on both plates, which were used for normalising the

^V Fine-crop Mode: Automatic; Colony Measurement Method: Standard; Plate Density: 1536; Running Mode: Standard.

colony sizes against inter-plate variations irrelevant of SPIs (for the proteome-wide assay the normalisation was performed using the median colony size for each plate instead). A “key file” containing information on the position of each strain on each plate was loaded into the DR engine, which was then run using the default settings^{VI}. The output was a spreadsheet reporting the relative cell growth for strains containing each GFP-tagged query protein after forcing the interaction between the query protein and the target protein compared with the GBP-only or target protein-only controls. This relative growth was reported as both the z-score and the log growth ratio (LGR).

4. The z-score for each strain was calculated based on the mean and the standard deviation of all relative colony sizes for all the strains across the assay, under the assumption that those colony sizes are normally distributed. Empirically, this assumption is generally true for proteome-wide assays, and therefore, in this thesis, z-scores were used for reporting the results for the Rdh54p proteome-wide SPI assay. By definition, higher z-scores indicate stronger growth defects; a z-score of 2 or greater corresponds to the top 5% of all LGRs.
5. The LGR is the natural log of the ratio of the average colony size of a strain on the control plate(s) and the average colony size of the same strain on the experiment plate. Similar to the z-score, the higher the LGR, the stronger the growth defect. By assuming that yeast colonies are hemispheres, it has been theoretically calculated that a LGR of 0.5 suggests that there are 110% more cells on the control plate(s) than on the experiment using the formula $C/E = e^{1.5LGR}$ (C: number of cells on the control plate(s); E: number of cells on the experiment plate).
6. For the proteome-wide results, a smoothing algorithm was used to adjust the z-scores by normalising the row/column medians towards the plate median, respectively, so as to correct for the spatial anomalies across each plate (e.g. colonies growing larger at one side of the plate due to the agar being uneven). The PERL script can be downloaded on <http://www.source->

^{VI} Statistical Test settings: Normal Distribution; Do not Bonferroni correct *p*-values. Data Normalisation Method: Plate Median (for the genome-wide assay) or Designated Controls (for the validation assays).

[forge.net/projects/zspatialcorrect/files/spatial1_0.plx/download](https://github.com/forge-net/projects/zspatialcorrect/files/spatial1_0.plx/download). For the validation assays this smoothing step was not performed, as most of the strains on the plates exhibited growth defects and therefore could not be normalised using medians.

2.6 Fluorescence microscopy

2.6.1 Wide-field fluorescence microscopy

A Zeiss Axioimager Z2 microscope (Carl Zeiss AG, Germany) was used to perform wide-field fluorescence imaging of fluorescently-labelled yeast cells. In addition to the frame, the microscope system comprises of a Colibri LED light source (Carl Zeiss AG, Germany), several Zeiss filter-sets for imaging in various spectral channels, a DIC prism, a 63x 1.4NA Plan-Apochromat objective lens (Carl Zeiss AG, Germany), and an Orca ER II CCD camera (Hamamatsu, Japan). Fluorescence microscopy was mainly performed in the blue (CFP), green (GFP), and yellow (YFP) channels, and the corresponding filter sets used were Zeiss filter sets 47HE (excitation BP 436/25, dichroic FT 455, emission BP 480/40), 38HE (excitation BP 470/40, dichroic FT 495, emission BP 525/50), and 46 (excitation BP 500/20, dichroic FT 515, emission BP 535/30), respectively. The CCD camera was set for 2x2 binning, and the corresponding effective pixel size in the sample plane was ~205 nm.

Instead of performing quantitative imaging, I mainly used the widefield microscope for checking whether fluorescent kinetochore foci could be seen after tagging a kinetochore protein gene with a fluorescent protein gene by yeast transformation, and for examining the colocalisation of the GFP-tagged query protein and the GBP-RFP-tagged target protein in strains resulting from SPI assays. For both purposes, a 5 ml liquid SC+ADE or -LEU+ADE culture was prepared for each strain by transferring a small colony of cells from an agar plate to the liquid medium and then incubating overnight at 23°C with shaking. On the next day, ~200 µl of the overnight culture was diluted into 5 ml fresh culture medium and further incubated for ~3 h at 23°C with shaking. The cells were then harvested by centrifugation at 4000g for 5 min, discarding most supernatant, and

resuspending the cells in the remaining liquid. Finally, ~3 μ l of the liquid was transferred onto a microscope slide and then covered with a 22 mm coverslip of #1.5 thickness for the subsequent examination using the microscope.

2.6.2 Fluorescence lifetime imaging microscopy (FLIM)

As a major part of my project, I developed a methodology for performing high-content analysis on interactions between endogenously-labelled proteins at budding yeast kinetochore using wide-field time-gated FLIM. Please refer to Chapter 3 for a detailed description of the method.

Chapter 3. *OpenFLIM-HCA* for studying *in vivo* interactions between endogenously labelled proteins at budding yeast kinetochores

This chapter reports the high content analysis (HCA) methodology developed in this project for studying the interactions between proteins endogenously labelled with fluorescent proteins (FPs) at the kinetochores of live budding yeast cells utilising wide-field time-gated FLIM to read out FRET between the fluorophores. The first section reviews the open source platform, “*openFLIM-HCA*”, for automated multiwell plate wide-field time-gated FLIM imaging and the subsequent FLIM data analysis using “*FLIMfit*”, which has been primarily developed by previous members in the Photonics Group at Imperial College London^{330,396,437,438}. The second section discusses the challenges encountered when applying FLIM HCA to fluorescently-labelled endogenous kinetochore-associated proteins expressed in live yeast cells. The subsequent sections describe the modifications I made to the *openFLIM-HCA* platform, in terms of the instrumentation as well as the data acquisition and analysis processes, in order to address the challenges of live cell yeast kinetochore FLIM analysis and optimise the platform for this specific task. Part of the work presented here has been published⁴³⁹.

3.1 Introduction to *openFLIM-HCA*

Assays utilising fluorescence microscopy of biological samples, including fluorescence intensity and lifetime measurements, are inevitably compromised by various types of noise⁴⁴⁰. These include and are not limited to:

- Biological noise:
 - Arising from factors that introduce inhomogeneity in the fluorescence and biological signals of interest, e.g. the expression level of the target construct, the labelling or maturation efficiency of the fluorescent marker, the local physical and biochemical environment, and the spatial and

temporal contexts of the biological signal of interest (e.g. dynamic changes in protein positions and protein interactions);

- Arising from background signals in the regions of interest (ROIs) or from the corresponding out-of-focus regions that can overlap with signals from the ROIs when imaged, e.g. background fluorescence from freely-diffusing fluorophores as opposed to those localised to the ROIs, and autofluorescence from (unlabelled) components in a cell or in the imaging medium. Note that, while the background can be subtracted during analysis, this subtraction can at best remove the mean offset introduced by the background but not the noise.
- Instrumental noise:
 - Arising from noise inherently associated with the instruments in a microscope set-up, e.g. scattered excitation light arriving at the photodetector, background fluorescence generated by components in the optical path, thermal noise and read noise introduced by the photodetector and other electronic components, and the shot noise inevitably arising from the stochastic quantum nature of an incident photon flux.
- Experimental noise:
 - Arising from unwanted variations in experimental conditions, such as short-term and long-term variation of the excitation power, environmental changes (e.g. temperature, ambient light, vibrations) and the sample conditions such as pH, culture density, etc., that can occur within one experiment or between repeated experiments.
- Operator bias
 - Arising from the subjective selection of data for analysis, e.g. rejecting measurements that appear to be wrong or focusing on measurements that appear to have “worked well”.

The presence of significant noise can affect the precision and accuracy of measurements of the fluorescence parameter of interest (e.g. intensity, lifetime, or polarisation), leading to broadening or shifts in the distribution of the measured data. This can limit the ability to distinguish differences between results from different samples via inferential statistics.

Besides specific methods to minimise the impact of the various sources of noise, automated HCA offers a means to improve the statistical robustness of assays, as compared to manual microscopy experiments, through the unsupervised acquisition of many fields of view (FOVs) for each sample in a reasonable time for a single experiment in which the various experimental conditions can be reasonably expected to be constant. A large number of FOVs for each sample can facilitate the averaging of random noise and thus can improve the precision of measured parameters. Also, automated HCA usually entails imaging samples in a multiwell plate format with automatically selected FOVs in each well, and therefore reduces the possibility of introducing human error and operator bias as may happen when performing an imaging assay on samples manually prepared on individual microscopic slides with manually selected FOVs. Furthermore, the measurement of control samples and the inspection of trends in multiwell plate experimental data can help identify and therefore eliminate systematic errors.

As discussed in Section 1.3.3, although FLIM offers a quantitative and robust readout of FRET and therefore serves as a promising method for screening for protein-protein interactions (PPIs), automated FLIM instruments for assaying FRET in an HCA manner are yet to be commercialised and have only been developed in a few research groups^{404–406,408}, likely due to the relative complexity of such instruments and the need for appropriate software for automated data acquisition and FLIM data analysis. From the first demonstration of rapid readouts of FRET in live cells using wide-field time-gated FLIM microscopy in 2007⁴⁴¹ and the first automated multiwell plate FLIM microscope in 2008⁴⁰⁷, the Photonics Group at Imperial College London has developed FLIM HCA, characterising and optimising the system^{399,409} components and applying it to various biological studies^{411,416,439}. More importantly, efforts have made FLIM HCA more accessible to the scientific community, leading to the development of an open source platform, *openFLIM-HCA*^{437,438}. The platform comprises an automated multiwell plate microscope³⁹⁶, an open source data acquisition software tool written as a *μManager*⁴¹³ plug-in for controlling the microscope and performing unsupervised HCA³⁹⁶, and an open source data analysis software tool *FLIMfit*, which allows rapid analysis of large-scale FLIM data with complex fluorescence decay models^{330,442}. This section provides an introduction to this platform, in terms of its instrumentation and the processes for data acquisition and analysis.

3.1.1 Instrumentation

The microscope set-up in the *openFLIM-HCA* platform is shown in Figure 3-1.

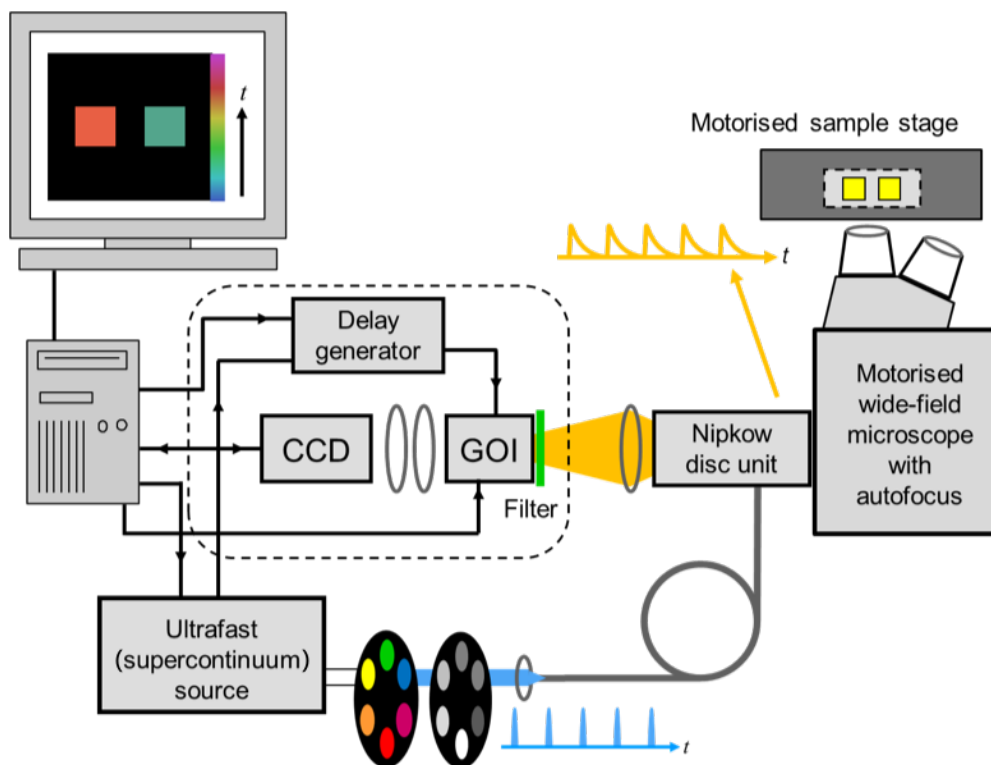


Figure 3-1 Schematic of the automated multiwell plate FLIM microscope in *openFLIM-HCA*

GOI: gated optical intensifier. CCD: charge-coupled device. Adapted from Ref.³⁹⁶.

The microscope is built around an inverted epifluorescence microscope frame (IX81, Olympus, Japan) with a built-in Z-drift compensation (ZDC) module for autofocusing during long-term experiments and with a motorised x-y stage (Märzhäuser Wetzlar GmbH, Germany) where samples, either in a multiwell plate or on a microscopic slide, can be mounted. The pulsed excitation radiation required for wide-field time-gated FLIM is provided by an ultrafast laser source, typically a fibre-laser-pumped supercontinuum source operating at 40 MHz (SC400-4, Fianium, UK) that provides picosecond “white light” pulses covering a spectral range of ~400 - ~2500 nm. Before being coupled into a single-mode polarisation-maintaining optical fibre and delivered to a Nipkow disk unit (CSU-X1, Yokogawa Electric Corporation, Japan) that provides quasi-wide-field optical sectioning, the pulses are spectrally filtered by one of the band-pass filters mounted on a

motorised filter wheel to select the excitation spectrum appropriate to the chosen fluorophore. The excitation intensity can be regulated with neutral density (ND) filters mounted on a second motorised filter wheel. For imaging typical biological samples an average power of $\sim 100\text{--}200\text{ }\mu\text{W}$ at the sample plane is used.

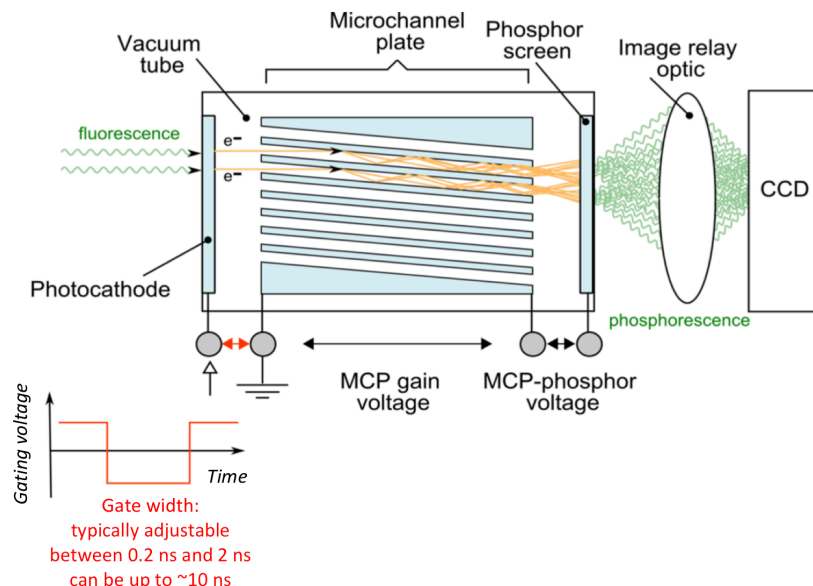


Figure 3-2 Schematic of a GOI for wide-field time-gated FLIM

MCP: microchannel plate. CCD: charge-coupled device. The image relay optic and the CCD camera are outside the GOI. Adapted from Ref.³⁹⁹ with permission.

The key component in the wide-field time-gated FLIM set-up is the gated optical intensifier (GOI), which acts like an ultrafast shutter. A schematic of the GOI used (HRI-HL, Kentech Instruments Ltd., UK) is given in Figure 3-2. The incident fluorescence is converted to photoelectrons at the photocathode of the GOI and thus cannot travel against the positive voltage applied between the photocathode and the front face of a microchannel plate (MCP), until a negative gating voltage is applied (switched in a time scale of <100 ps) and then the photoelectrons are accelerated to the MCP until this negative voltage signal is switched off. The MCP consists of an array of channels, each resembling a photomultiplier tube (PMT). Photoelectrons arriving at the MCP are accelerated by a gain voltage applied across the MCP and excite secondary electrons when colliding with the walls of the channels. The final number of secondary electrons at the exit face of the MCP is dependent on the adjustable gain voltage. These electrons

are then accelerated to a phosphor screen to generate phosphorescence on impact. Thus, the fluorescence signal is effectively amplified for the duration of the negative gating pulse (the “gate width”, typically adjustable between ~ 200 ps to ~ 2 ns). Two camera lenses (Nikon, Japan) are employed to image the phosphor screen onto a cooled CCD camera (Orca ER II, Hamamatsu, Japan) with a demagnification factor of 0.7. Importantly, the GOI gain voltage is triggered by an electronic signal from the excitation laser source at the same repetition rate as the laser pulses, and the gate opening time with respect to each excitation pulse can be controlled with the combination of a coarse delay unit (Precision Programmable Delay Generator, Kentech Instruments Ltd., UK) that enables an offset up to 20 ns with 25 ps precision and a fine delay unit (HDG, Kentech Instruments Ltd., UK) that is computer controlled and offers picosecond increments. By acquiring a stack of time-gated images for a range of delay values, this set-up permits time-gated measurements that can sample fluorescence decays from a whole FOV with the time-gate window set to various temporal positions relative to the excitation pulse, hence enabling wide-field time-gated FLIM.

Each FLIM dataset is a sequence of time-gated fluorescence intensity images of a FOV acquired by the CCD camera at different time-gate delays relative to the excitation pulses. For each time-gated image, the CCD camera integrates over multiple excitation pulses during its exposure time. FLIM datasets for multiple FOVs can be saved as individual OME-TIFF files, i.e. one FOV per file, or as a single OME-TIFF file for all FOVs from a multiwell plate, onto the local hard drive or the OMERO image data management platform⁴⁴³. The local computer controls most aspects of the instrument with a custom-written open source plug-in^{396,437,438} for *μManager*⁴¹³, which enables fully automated FLIM imaging on samples in a multiwell plate for HCA, as described in detail in the following subsection.

3.1.2 Data acquisition

The data acquisition process for a typical FLIM HCA experiment consists of six steps: (i) setting up the instrument, (ii) acquiring a reference dye FLIM dataset, (iii) acquiring a FLIM dataset of the instrument response function (IRF), (iv) acquiring the sample data,

(v) acquiring the time-varying background (TVB) FLIM dataset(s), and (vi) acquiring the constant background datasets.

Setting up the instrument

At the start of an experiment, the instrument must be set up properly in terms of the optical alignment, the selection of the microscope objective lens, the excitation power at the focal plane of the objective (the sample plane), the GOI settings (including the gate width and the gain voltage), and the settings of the CCD camera (hardware binning, etc.). Note that the above settings should remain unchanged throughout an experiment, with the exception of the excitation power, which can be adjusted using different ND filters.

Acquiring a reference dye FLIM dataset

The choice of the reference dye is based on its fluorescence properties, e.g. for an experiment on biological samples labelled with mTurquoise2FP (mTq2FP) or GFP, Coumarin 6 dissolved in ethanol is chosen, as it can be efficiently excited, fluoresces strongly in either channel and its fluorescence decays mono-exponentially with a lifetime in the nanosecond range (~ 2.5 ns)^{414,444}. The acquisition of this dye FLIM dataset enables the instrument set-up to be verified and provides information about the spatially-dependent temporal shift of the IRF across the FOV – a radial shift arising from the “irising” effect of the GOI⁴⁴⁵, which is caused by the temporal delay between the gate voltage being applied to the centre and the rim of the photocathode. For this purpose, the fluorescence decay of the reference dye is finely sampled with a step of 25 ps across the time window between two excitation pulses (e.g. a 12.5 ns window for a laser operating at 80 MHz). Details of how this FLIM dataset can be used for determining the IRF shift is given in Subsection 3.1.3 on data analysis.

Acquiring a FLIM dataset of the IRF

The IRF, $i(t)$, is the temporal response of a system to an impulse input (a Dirac delta function) $\delta(t)$. For a system measuring a fluorescence decay, the measured decay $g(t)$ will be a convolution ($*$) of the IRF $i(t)$ and the actual decay $f(t)$, i.e.:

$$g(t) = f(t) * i(t) \quad (3.1)$$

For our wide-field time-gated FLIM system, the IRF is predominately determined by the shape of the GOI time-gate, as laser pulses $\ll 100$ ps are used for excitation and therefore can be considered as impulse signals compared with the GOI gate width, which is typically adjustable between ~ 200 ps and ~ 2 ns. Some exemplar IRF profiles measured using a new GOI model (HRI-HL, Kentech Instruments Ltd., UK) that provides a larger adjustable range of gate width (from ~ 200 ps to ~ 10 ns) are shown in Figure 3-3. Figure 3-4 presents a simulation of how the IRF can affect the measured profile of a fluorescence decay compared with the actual profile. The IRF of our system is usually measured by finely sampling the excitation light that is back-scattered from the Nipkow disk with 25 ps increments in the same way as is used to measure the reference dye FLIM dataset, and will be referred to in the following text as the “*scatter IRF*”⁴¹¹. Notably, as the response of the GOI may be sensitive to the wavelength of the radiation, in order to accurately measure the IRF for the sample fluorescence, the back-scattered excitation light should pass through the fluorescence emission path (including filter) before reaching the GOI. To achieve this the supercontinuum excitation light should be filtered by an excitation filter whose passband has some overlap with that of the emission filter. The use of this *scatter IRF* dataset in the data analysis process is covered in Subsection 3.1.3.

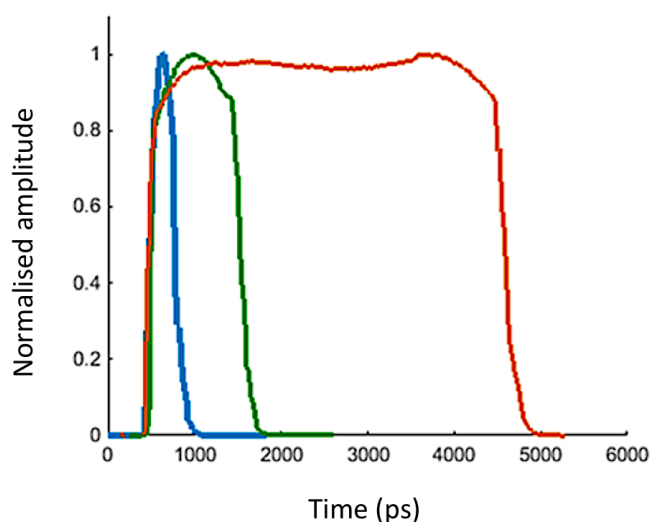


Figure 3-3 Exemplar IRF profiles when using a typical GOI (HRI-HL)

The profiles were acquired with the gate width of the GOI (HRI-HL) set to 200 ps (blue), 1000 ps (green), and 4000 ps (red), respectively. Adapted from Ref.³⁹⁹ with permission.

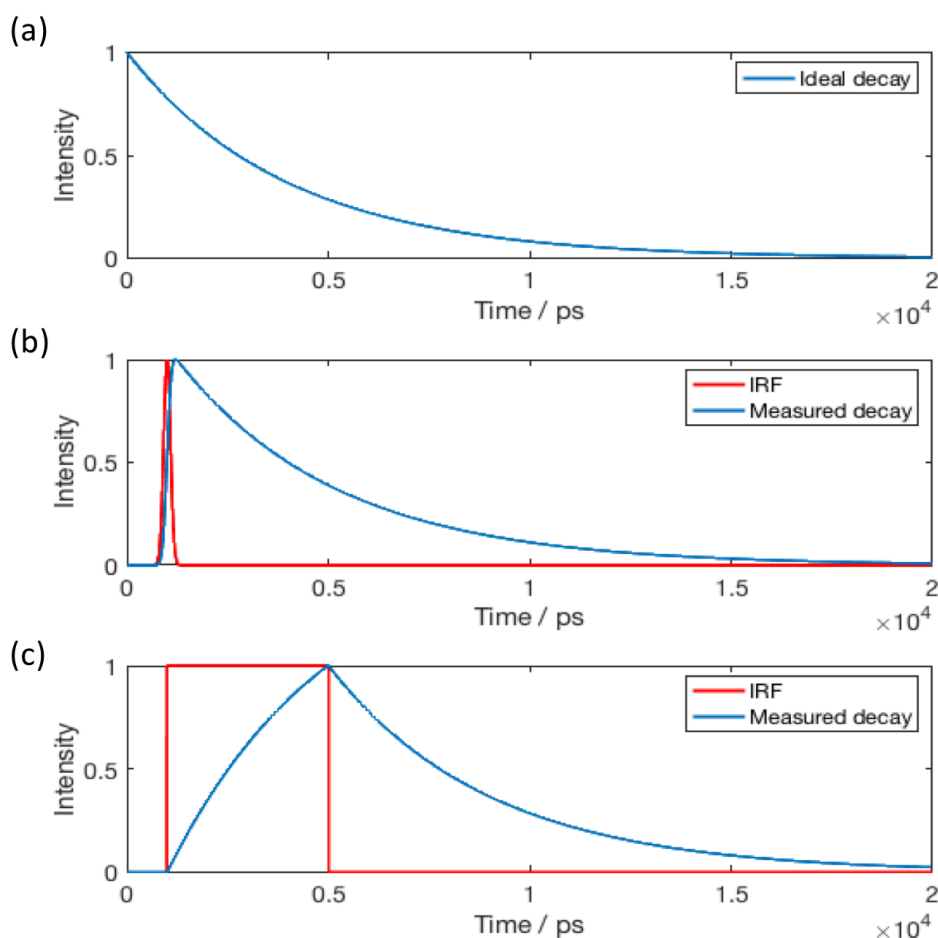


Figure 3-4 Simulation of the impact of the IRF on the measured fluorescence decay

(a) An ideal mono-exponential fluorescence decay with a fluorescence lifetime of 4 ns. (b) The same fluorescence decay measured with an instrument with an IRF that has a Gaussian shape with a 200ps full width at half maximum. (c) The same fluorescence decay measured with an instrument that has a rectangular IRF profile with a width of 4 ns. IRF: instrument response function.

Acquiring the sample FLIM data

For FLIM HCA, the acquisitions of sample data can be fully automated for imaging samples in a multiwell plate using a custom *μManager* plug-in. On the graphic-user interface (GUI) of the plug-in one can first select which wells on a multiwell plate are to be imaged. FOV positions within each well can then be generated automatically, located on a spiral pattern starting from the well centre or on a ring with an adjustable radius to minimise operator bias, or can be selected manually to, for example, ensure optimal cell density in each FOV. Then, the parameters related to the time-gated imaging process that

apply to all FOVs need to be set, including the temporal positions of the time gates and the camera exposure time. The gate positions can be set manually or can be generated across a range with a fixed increment (as in the cases for the reference dye and scatter IRF FLIM measurements), or can be generated automatically through “auto-gating” based on the following equations³⁹⁶:

$$t_1 = t_{peak} - 0.75 \times w_g \quad (3.2)$$

$$t_x = t_{peak} + \tau \times \ln\left(\frac{n-1}{n-x+1}\right) \quad (for\ 2 \leq x \leq n) \quad (3.3)$$

where t_1 is the position of the first time gate, t_x the x -th, t_{peak} the temporal position of the decay’s peak, w_g the GOI gate width, τ the expected mean fluorescence lifetime of the measured decay, and n the total number of time gates (which should be larger than 2). While an optimal gating strategy is yet to be analytically proven, we have empirically found a satisfactory approach for complex fluorescence decay profiles using the above equations to generate 7 time gates across a decay: 1 time-gate before the decay peak, 1 time-gate at the peak ($t_2 = t_{peak}$), and 5 time-gates distributed logarithmically after the peak. In this manner, the changes in the fluorescence intensity between 2 successive gates in the last 6 gates should be roughly equal, assuming that the complex decay approximately matches a mono-exponential decay with the expected lifetime τ . In terms of the camera exposure time, it should be set to a value such that the brightest pixel value is approximately 2/3 of the CCD camera’s dynamic range. When more photons are needed for more accurate lifetime fitting, multiple camera frames can be accumulated at each time-gate delay. The plug-in also enables automated multi-channel FLIM and time-lapse FLIM, but these were not used for the work in this thesis; for details please refer to the *openFLIM-HCA* wiki⁴³⁸.

Acquiring the TVB FLIM dataset

During the automated acquisition of the sample data, a few additional FOVs are typically taken from a well containing only the imaging medium, as the medium itself can introduce an additional fluorescence signal that may overlap with the fluorescence signal of interest when the biological sample is present. This medium background - and any

other background signals introduced by the excitation pulses (e.g. reflection and scattering of the excitation radiation and/or the excited fluorescence from some optical components) - will introduce additional fluorescence lifetime components to the measured fluorescence profile, and these are collectively considered as the TVB. In order to accurately measure the fluorescence lifetime(s) of the sample, the TVB needs to be taken into account, especially for dim samples where the TVB can have a significant contribution to the measured fluorescence, as shown in details in Subsection 3.1.3. Note that the TVB FLIM data should be taken with all settings (e.g. the gate positions and the camera exposure time) being the same as the ones used for acquiring the sample FLIM data.

Acquiring the constant background datasets

The final step for the data acquisition process is taking the datasets for the constant background. The constant background refers to the combination of the background signals in the captured image that are unrelated to the laser excitation pulses and the sample of interest and therefore is distinct from the TVB. Sources of these signals include the ambient lighting, the thermal noise and the read noise from the camera, and the constant offset that is normally added to the pixel values read out by the CCD camera. The overall effect to the background is to elevate the measured signal by a constant level across the whole profile and this should be subtracted properly for accurate lifetime analysis. The measurement of this background should be done for every camera setting used in the experiment, as the background value changes with the camera exposure time and the accumulated frame number. For each measurement, a FLIM dataset with ~100 gates should be taken with the laser shuttered, and the mean pixel value from these time-gated images will be used for background subtraction during data analysis, as shown in the following subsection.

3.1.3 Data analysis

The analysis of the FLIM data is done using the custom-written open source MATLAB-based software tool *FLIMfit*⁴⁴² (Figure 3-5). The whole dataset from an HCA experiment can be loaded in *FLIMfit* and be analysed with high throughput. *FLIMfit* also offers a set

of built-in algorithms for image segmentation, or the ROIs can be segmented by intensity thresholding. Fluorescence lifetimes can be calculated only from the segmented ROIs (or the whole FOV if no segmentation is performed).

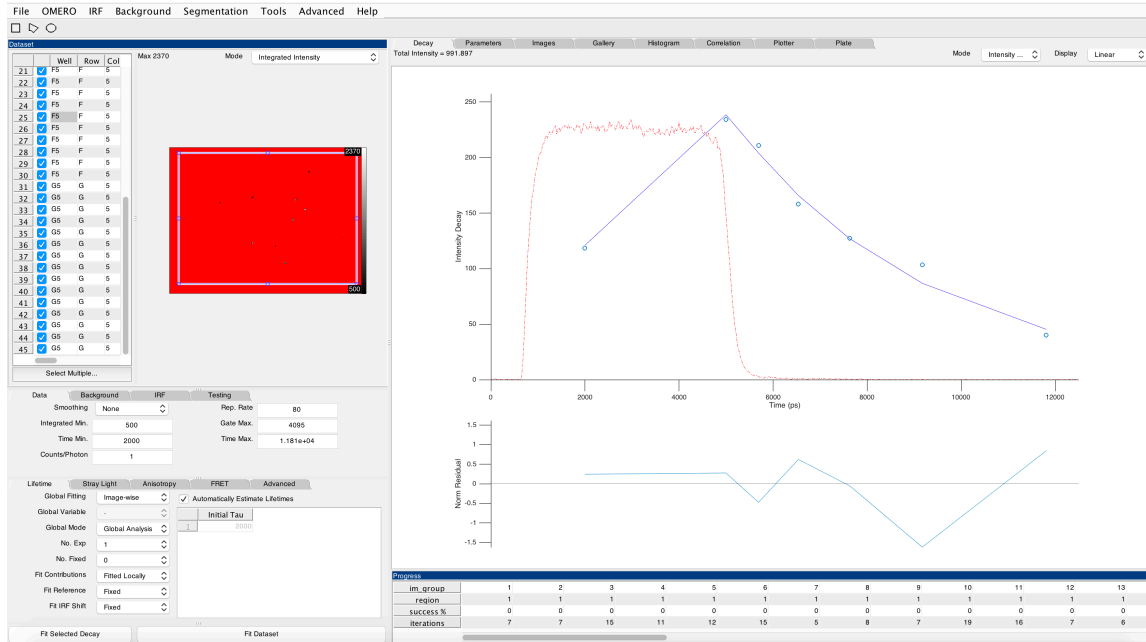


Figure 3-5 The GUI of FLIMfit

The fluorescence decay measured from a FOV with budding yeast cells expressing Ask1p-mTq2FP is shown on the top right panel of this GUI. Red dashed line: IRF. Blue circles: intensity data from different time-gates. Blue line (top right): fitted mono-exponential decay. Blue line (bottom right): normalised fitting residuals.

As discussed earlier, the measured fluorescence decay $g(t)$ differs from the actual decay from the sample $f(t)$ predominately due to the IRF $i(t)$, and is also subject to the impacts of the TVB $b(t)$ and the constant background c . This relationship can be summarised by the following equation:

$$g(t) = [f(t) + b(t)] * i(t) + c \quad (4.1)$$

FLIMfit has the functionality to take account of these background terms. The value for the constant background c is determined by loading in the constant background data taken earlier with the laser shuttered and calculating the mean pixel value contributed by the constant background. This value is then subtracted from the measured signal $g(t)$. The TVB $b(t)$ is more complicated in that it is also spatially varying, mainly due to the

inhomogeneous illumination pattern. Consequently, *FLIMfit* can generate a “TVB intensity map” using the TVB data acquired earlier, i.e. the FLIM dataset of the imaging medium. The temporal profile of the measured TVB (which is actually $b(t) * i(t)$ instead of $b(t)$) is determined using a small region in the centre of the FOV, and then the TVB intensity map is generated by scaling the TVB profile for each pixel across an FOV according to the local pixel value (Figure 3-6). This map is subsequently loaded into *FLIMfit* and provides information about $b(t) * i(t)$ when analysing for $f(t)$ from $g(t)$.

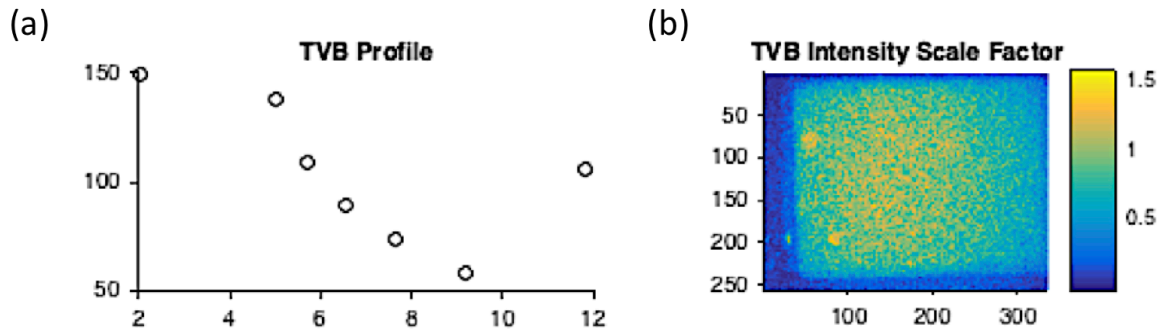


Figure 3-6 An exemplar TVB intensity map generated from a FLIM dataset of the imaging medium SC+ADE

(a) The profile of the TVB signal from a small region in the centre of a FOV containing only the imaging medium SC+ADE. (b) The TVB map containing the intensity scale factor for each pixel across the FOV.

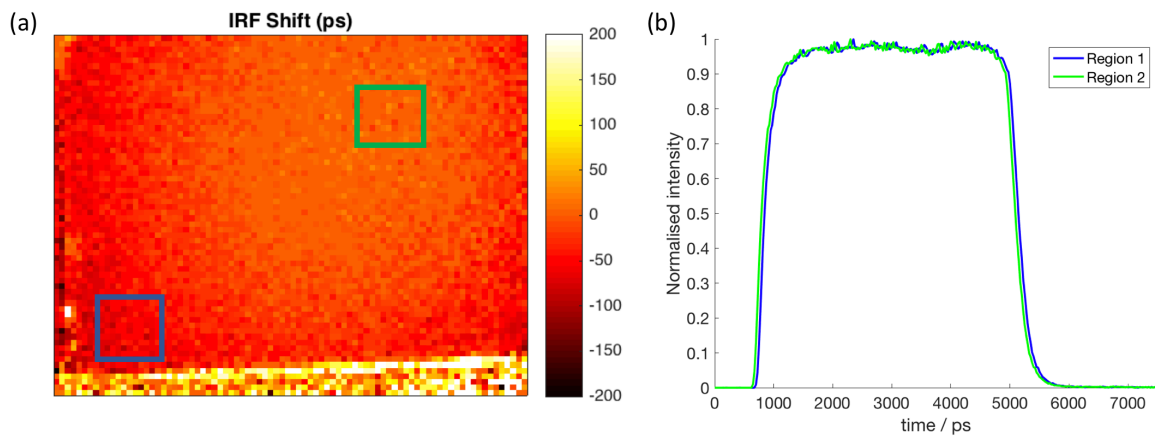


Figure 3-7 Exemplar IRF shift map and two IRF profiles from different positions of a FOV

(a) An exemplar IRF shift map generated by *FLIMfit*, showing the temporal shift of the IRF profile for each pixel relative to the reference IRF profile from a small region in the centre of the

FOV. (b) The IRF profiles from two regions in the FOV in Panel (a). Region 1 is in the blue rectangle in the bottom left. Region 2 is in the green rectangle in the top right. Note the temporal shift between the two IRF profiles.

Owing to the iris effect of the GOI introduced in Subsection 3.1.2, the IRF, $i(t)$, is also spatially varying. While the temporal shape of the IRF (Figure 3-3) is approximately invariant across the FOV, its temporal position is shifted for different pixels. The IRF temporal shape can be obtained from the signal from a small region in the centre of the *scatter IRF* dataset taken earlier (with the constant background subtracted), while an “*IRF shift map*” needs to be generated to correct for the spatially varying temporal shift (Figure 3-7). *FLIMfit* generates the *IRF shift map* by loading in the reference dye FLIM dataset taken earlier, selecting the same small region as the one used for obtaining the IRF shape to get a smooth decay profile of the dye, and cross-correlating that profile with the decay profile from each individual pixel to find the temporal shift for each pixel.

In addition to contributing to the generation of the *IRF shift map*, the reference dye FLIM dataset is used for determining the correct temporal position of the IRF, t_0 . This is necessary as the IRF is measured using back-scattered light from the Nipkow disk, which has a different optical path length (and therefore a different time delay) compared with light from the sample plane. With the reference dye FLIM dataset and the *IRF shift map* loaded in *FLIMfit*, a global value for t_0 can be obtained by globally fitting the fluorescence decay data of the reference dye to a mono-exponential model. When the fitted fluorescence lifetime matches well with the known lifetime of the reference dye (e.g. ~2500 ps for Coumarin 6 dissolved in ethanol), it indicates that the fitted value of t_0 is representative of the true value and can be used for the subsequent lifetime analysis.

After the above procedures have been used to characterise c , $b(t)$, and $i(t)$, the measured fluorescence data from the samples $g(t)$ can be fitted in order to retrieve $f(t)$, which contains the desired fluorescence lifetime information. *FLIMfit* can perform fitting to a mono-exponential decay model that can be described by:

$$f(t) = I_0 \times e^{-\frac{t}{\tau}} \quad (3.5)$$

where τ is the fitted fluorescence lifetime and I_0 is the peak intensity. In cases where the underlying decay profile is more complex, τ represents the effective lifetime. Alternatively, *FLIMfit* can also fit the data to a complex decay model, e.g. a bi-exponential decay:

$$f(t) = I_0 \times \left[\beta \times e^{-\frac{t}{\tau_1}} + (1 - \beta) \times e^{-\frac{t}{\tau_2}} \right] \quad (3.6)$$

where τ_1 is the lifetime of the first decay component, τ_2 the lifetime of the second decay component, and β the fraction of the first decay component. Fitting to a complex decay model may offer more quantitative information, but it requires many more photons for an accurate result. For example, while <200 photons are needed to determine the effective lifetime of a fluorophore with good accuracy, fitting to a bi-exponential model to the same accuracy requires >100000 photons⁴⁰¹. The photon count per pixel in an FLIM experiment, however, is typically limited to a few hundred owing to the photostability of the fluorophores and the need to minimise phototoxicity in biological samples. *FLIMfit* addresses this problem by incorporating a functionality for fitting FLIM data with global analysis^{330,446}, where the photons from all pixels in one FOV or in all FOVs in a large dataset are utilised to estimate the lifetime components, with the assumption that the lifetime components are the same globally and only the pre-exponential factors are variant across the pixels. In particular, the global analysis method fits for both the global lifetime components and the pixel-wise pre-exponential factors simultaneously, i.e. without losing the information of the decay profile at each pixel when determining the lifetime components, and thus is more computationally demanding, in comparison to the global binning method (which is also implemented in *FLIMfit* but is less preferred) where first all the photons are binned together to estimate the lifetime components and then the pre-exponential factors are fitted on a pixel-wise basis⁴⁴⁷. *FLIMfit* is specifically programmed for performing rapid global analysis. For example, it only requires 32 seconds to globally fit a dataset of 394 time-gated datasets each with five time gates and 672×512 pixels, using 2 GB of RAM³³⁰.

3.2 Challenges for *in vivo* FLIM HCA on FP-labelled endogenous proteins at budding yeast kinetochores

The accuracy and the precision of lifetime determination are key considerations for a FLIM experiment: a high accuracy means that the measured lifetime is highly representative of the true value, whereas a high precision means that the variation in the measured lifetimes of duplicated samples is small. While the accuracy and the precision can be affected by the method used for analysing the FLIM data, they are fundamentally limited by the signal-to-noise ratio (S/N) in the acquired data. The signal here is the measured fluorescence of interest, e.g. the time-varying fluorescence from a donor FP, while the noise is the combination of all the biological, instrumental, and experimental factors covered above in Section 3.1.

Previous work from the Photonics Group at Imperial College has shown experimentally that an imaging system employing a GOI typically exhibits a “shot noise-like” behaviour in terms of the S/N of the detected photon number, i.e. the S/N increases almost proportionally with the square root of the detected photon number, $\sqrt{N}$. The S/N should be $\sqrt{N}$ for shot noise-limited performance, but it is slightly higher here due to an excess noise factor imposed by the amplification processes in the GOI (hence the term “shot noise-like”). This result suggests that, with the contributions from all other noise factors being the same, the S/N of our FLIM system, and thus the accuracy and the precision of lifetime determination, should also improve with increasing numbers of detected photons. This has been previously demonstrated with HEK cells expressing an mTurquoiseFP-YFP FRET construct³⁹⁹.

Practically however, the achievable photon number in a FLIM experiment is limited by the properties of the fluorophores used and the number of fluorophores present in the sample. It is also limited by the image acquisition time, which has to be relatively short for practical FLIM HCA, and the excitation power, and therefore the photon flux cannot be increased arbitrarily otherwise significant photobleaching and/or phototoxicity would occur. These considerations limit the achievable S/N for any fluorescence-based HCA approach. This section briefly discusses the specific challenges related to the experiments undertaken for this project - particularly the limited fluorescence signals available from

the FPs labelling endogenous proteins of interest at the kinetochores (which present low copy numbers), the cellular autofluorescence background in live budding yeast cells, and the presence of larger bright dead cells that may contaminate the fluorescence signals of interest.

3.2.1 Limited signal

This project entailed performing *in vivo* FLIM HCA on stably expressed FP-labelled endogenous proteins in live budding yeast kinetochores. Figure 3-8 shows an exemplar fluorescence intensity image of diploid yeast cells expressing Ask1p-mTq2FP acquired with the automated FLIM microscope described later in Section 3.3. The donor fluorophores are typically confined to one or two diffraction-limited spots in each cell (depending on which cell cycle stage the cell is in) due to the clustering of all the kinetochores (32 for a haploid cell during cell division, 16 otherwise; 64 for a diploid cell during cell division, 32 otherwise). The donor fluorescence is relatively weak, as the donors FPs are labelling endogenous proteins of interest with copy numbers in the range of from 1 to ~20 per kinetochore for constitutive KPs (as shown in Table 3-1) and should not be more for motor proteins and regulators that only transiently associate with the kinetochores. In contrast, previous FLIM experiments on fixed or live mammalian cells commonly employ dyes conjugated to antibodies, small molecule dyes staining specific cellular components, or FP constructs overexpressed from plasmids, and thus have much brighter donor fluorescence signals. However, the use of FP-labelled endogenous proteins offer the benefits of studying cellular events such as PPIs under physiological conditions much closer to wild-type and without artefacts resulting from overexpression of proteins or from unnatural heterogeneity in FRET that can be manifest when the donor and the acceptor are separately expressed from transiently transfected plasmids at uncertain levels. FP-labelling of endogenous proteins is straightforward with live budding yeast via yeast transformations (see Section 2.3.3).

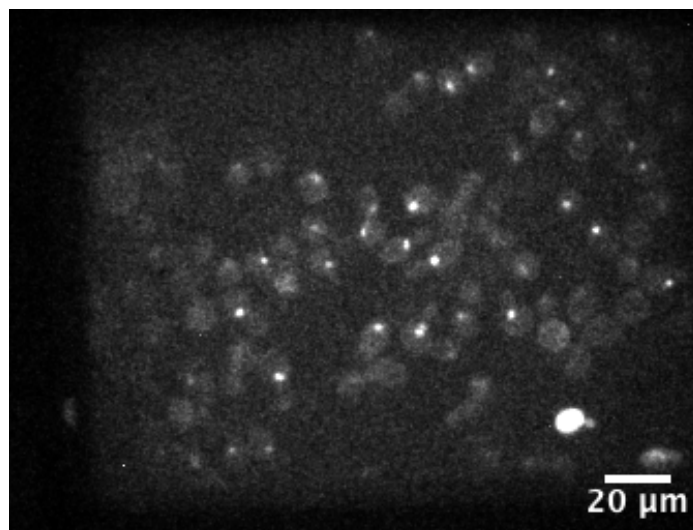


Figure 3-8 Exemplar fluorescence intensity image of diploid budding yeast cells expressing Ask1p-mTq2FP

The image was taken with the automated FLIM microscope. Each bright focus is a collection of Ask1p-mTq2FP molecules in a kinetochore cluster. The dim oval shapes show the autofluorescence from the yeast cells. The bright oval on the bottom right is a dead cell, which typically exhibits strong autofluorescence in both the blue and the green channels.

Table 3-1 Copy numbers per kinetochore for typical kinetochore proteins^{VII}

Kinetochore protein	Copy number per metaphase kinetochore (CNPMK)	Copy number per metaphase focus in diploids
Cse4p	2	64
Ndc10p	4	128
Cep3p	2	64
Ctf19p	3	96
Mtw1p	6 - 7	192 - 224
Nuf2p	8	256
Ask1p	16 - 20	512 - 640

^{VII} Based on the results from Ref.²²².

Overall, the weak signal, together with the strong cellular autofluorescence background that is discussed in the following subsection, make it challenging to achieve adequate S/N in FLIM data of the yeast kinetochores, which leads to lower precision in the lifetime fitting results. These errors may be compounded by poor segmentation of kinetochore foci when their signal is not much higher than the cellular background. To tackle this challenge, efforts should be made to increase the donor fluorescence from the kinetochore, to improve the efficiency of collecting the emitted photons, and to better segment the kinetochores, as discussed in the later subsections in this chapter.

3.2.2 Strong background

Even with the optical sectioning provided by a Nipkow disk unit, the autofluorescence from the imaging media (SC+ADE and -LEU+ADE were used for the experiments presented in this thesis, for cells with endogenous tags and cells with plasmids, respectively; see Chapter 2 for details) and from the yeast cells themselves is still significant compared with the donor fluorescence of interest from the kinetochores, at least when using mTq2FP as the donor, as can be seen in Figure 3-8. Note that in Figure 3-8 the KP labelled by the donor mTq2FP is the outer kinetochore protein Ask1p, which is a higher abundance kinetochore protein compared with the others measured in this study (some examples are shown in Table 3-1Table 3-1); for proteins with lower copy numbers, the contrast between the kinetochore foci and the cellular background was lower, as shown in Figure 3-9.

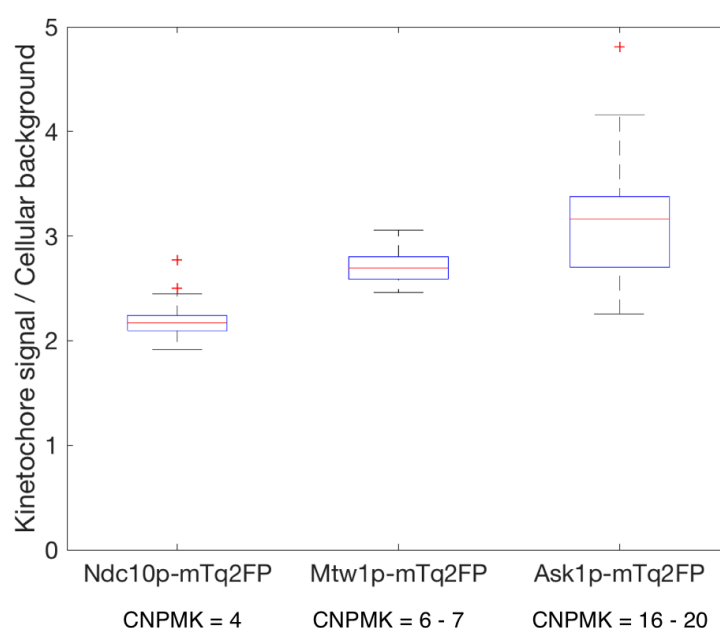


Figure 3-9 Signal-to-background ratios for images of donor-only cells expressing various mTq2FP-labelled KPs respectively

The signal here is defined as the total constant background-subtracted signal levels (i.e. pixel values) from the kinetochore regions integrated over all the time-gated images in the FLIM dataset for each FOV. The background is the total constant background-subtracted signal levels from the cellular region surround each kinetochore region. For the definitions of the kinetochore regions and the cellular regions as well as the algorithm for segmenting them, please see Section 3.5.1. CNPMK: copy number per metaphase kinetochore.

Table 3-2 Exemplar integrated signal levels^{VIII} from the imaging medium SC+ADE, from the cellular regions around the kinetochore foci, and from the kinetochore foci^{IX}), from one FLIM experiment with 43 FOVs of donor-only cells with Ask1p-mTq2FP

Median from SC+ADE (a.u.)	Median from cellular areas (a.u.)	Mean from kinetochore foci (a.u.)
~750	~2500	~8000

^{VIII} Each integrated signal level is the sum of the corresponding signals from the 12 time-gated images of a FLIM dataset with the constant background subtracted.

^{IX} Contributions of the TVB background in these foci were not removed.

Notably, the signal-to-background ratios indicate that a significant amount of the signal measured at the kinetochore foci may be contributed by the cellular autofluorescence and, to a lesser extent, the medium fluorescence (for example see Table 3-2), which can introduce bias to the fitting lifetime results. While the homogeneous medium background can be easily taken account of by generating a TVB intensity map as described in Section 3.1.3, it is not straightforward to exclude the cellular autofluorescence in a similar manner. To make things more complicated, the cellular autofluorescence seems to increase over time (Figure 3-10), which might be due to the accumulation of fluorescent metabolites.

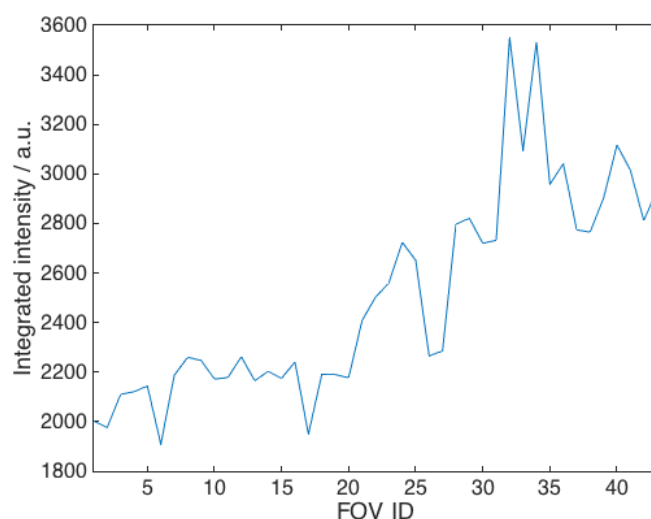


Figure 3-10 Exemplar data showing how cellular autofluorescence increases over time, from one FLIM experiment with 43 FOVs of donor-only cells with Ask1p-mTq2FP

The integrated intensity (i.e. signal level) of the cellular fluorescence for each FOV was calculated as the sum of the constant background-subtracted pixel value from the cellular region surrounding each kinetochore focus in each time-gated image. The FLIM dataset for each FOV contains 12 time-gated images taken at different time relays relative to the excitation pulses.

In order to better deal with this cellular autofluorescence, it would be useful to know the biochemical entities contributing to it and their fluorescence properties, such as the fluorescence lifetimes as well as the excitation and the emission spectra. However, to the best of my knowledge, these have not been well characterised. NADH and FAD can contribute autofluorescence in the detection bands of mTq2FP and GFP⁴⁴⁸, and phosphor-ribosyl-amino-imidazole generated in the adenine synthesis pathway is excited by blue

light and can also contribute autofluorescence in the mTq2FP and GFP channels⁴⁴⁹, especially in the absence of a functional *ADE* gene and/or a limiting quantity of adenine in the imaging medium.

At longer wavelengths the cellular autofluorescence in budding yeast is weaker⁴⁵⁰. As this observation suggests the use of a red FP as the FRET donor, I tried labelling certain KPs with a TagRFP mutant (TagRFP-R67K-S158T), which was reported to be brighter than the commonly used mCherry and also to exhibit superior photostability^{451,452}, but no kinetochore foci were seen, probably because this TagRFP variant was not expressed well in budding yeast cells or it has a long maturation time at the temperature of 23°C used for yeast cell growth and imaging. For the work reported in this thesis, the CFP variant mTq2FP was initially used as the donor for the studies on the kinetochore structure for its high brightness relative to other CFP variants, mono-exponential intrinsic lifetime, and high photostability⁴⁵³. Subsequently GFP (S65T)⁴³³ was used as the donor for the Rdh54p-related studies, since this allowed me to use the GFP-tagged yeast strain collection⁴³² and also exploit the higher relative brightness of GFP compared with mTq2FP. Additionally, all the imaged yeast strains contain an *ADE2* gene, and were grown and imaged in SC+ADE or -LEU+ADE media for suppressing the autofluorescence from phosphor-ribosyl-amino-imidazole.

The cellular autofluorescence was further characterised by fitting the medium background-subtracted cellular autofluorescence signals from regions around the foci from various donor-only kinetochore strains expressing KP-mTq2FP to a mono-exponential decay model. The resulting effective lifetimes were in the range of ~2.7 ns to ~3 ns. The absorption and the emission spectra of the medium background-subtracted cellular autofluorescence were also measured using a spectrofluorometer (RF-5301, Shimadzu Corporation, Japan): the spectra of a dense SC+ADE culture of unlabelled cells was measured and a background was subtracted based on measurements of the spectra of the culture medium SC+ADE. The resulting spectra are shown in Figure 3-11. While these fluorescence lifetime and spectral data are not sufficient to identify the biochemical species responsible for the autofluorescence, they do highlight the need to deal with the cellular autofluorescence in the blue and green channels in order to accurately determine the fluorescence lifetime of the donor FP.

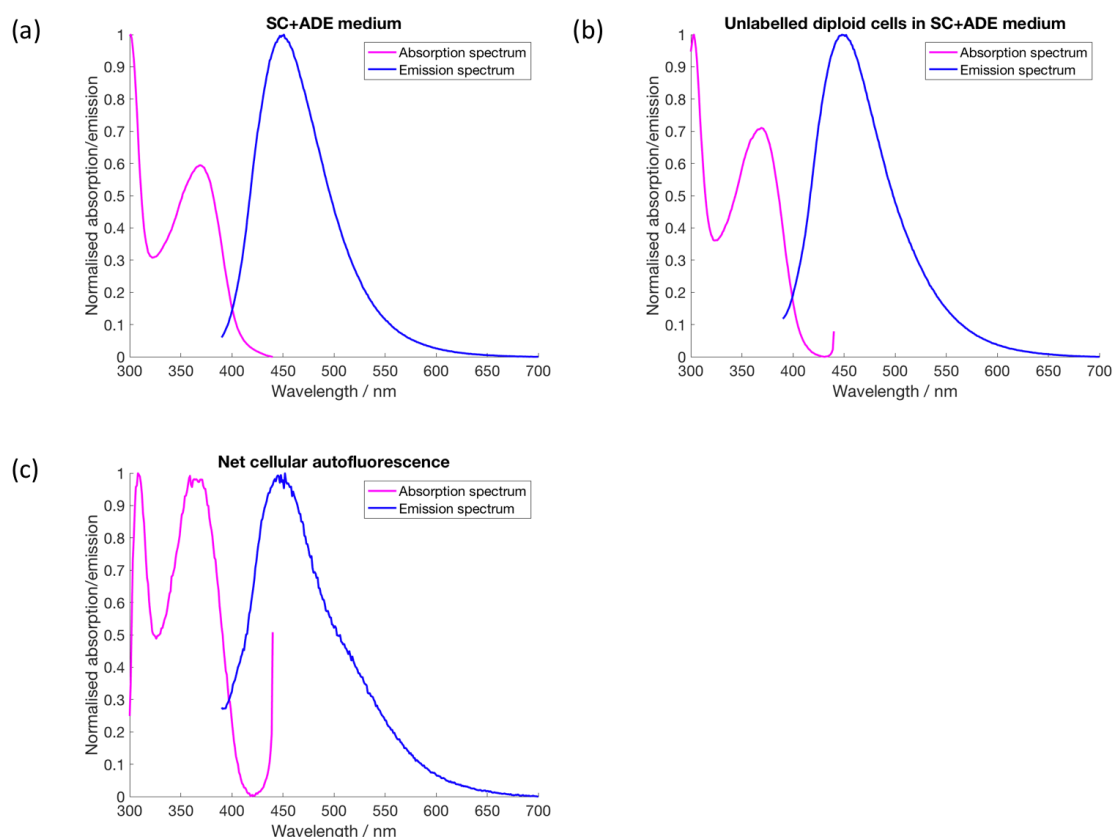


Figure 3-11 The absorption and the emission spectra of (a) the imaging medium SC+ADE, (b) the unlabelled diploid cells in a dense SC+ADE culture, and (c) the net cellular autofluorescence

The absorption spectrum of the net cellular autofluorescence was calculated by subtracting the unnormalised absorption spectrum of the unlabelled diploids cells in a dense SC+ADE culture with the unnormalised absorption spectrum of SC+ADE. The emission spectrum was calculated similarly.

3.2.3 Dead cells

Also apparent in the exemplar fluorescence intensity image of yeast cells (Figure 3-8) is the presence of large bright dead cell(s). It is unclear why dead yeast cells appear to be so bright in the mTq2FP and GFP channels and what fluorescent entities are responsible. When fitted to a mono-exponential decay model, the fluorescence from the dead cells presented an effective lifetime in the range of ~2.7 ns to ~3 ns, similar to that of the cellular autofluorescence, indicating that the dead cell autofluorescence may result from the same biochemical entities. Since the kinetochore foci are segmented mainly based on their brightness before lifetime fitting (see Section 3.5), some regions of the bright dead

cells will inevitably be included and may significantly impact the lifetime results, as the dead cells are so much brighter than the relatively weak kinetochore signals. Hence, the dead cell regions need to be robustly excluded from the segmented kinetochore regions. The method I developed to do this is described below in Section 3.5.1.

3.3 Development of the automated multiwell plate FLIM microscope

During this project I adapted the automated multiwell plate FLIM microscope in the *openFLIM-HCA* platform to address the challenges presented by *in vivo* imaging of FP-labelled endogenous proteins in budding yeast kinetochores imposed by the weak FP signal and the strong autofluorescence background. As illustrated in Figure 3-12, this entailed replacing the supercontinuum laser excitation source with an ultrafast Ti:Sapphire laser to provide higher power and more stable excitation, and implementing an electronically controlled micropump system to maintain the level of immersion liquid for the water-immersion objective lens during automated FLIM HCA.

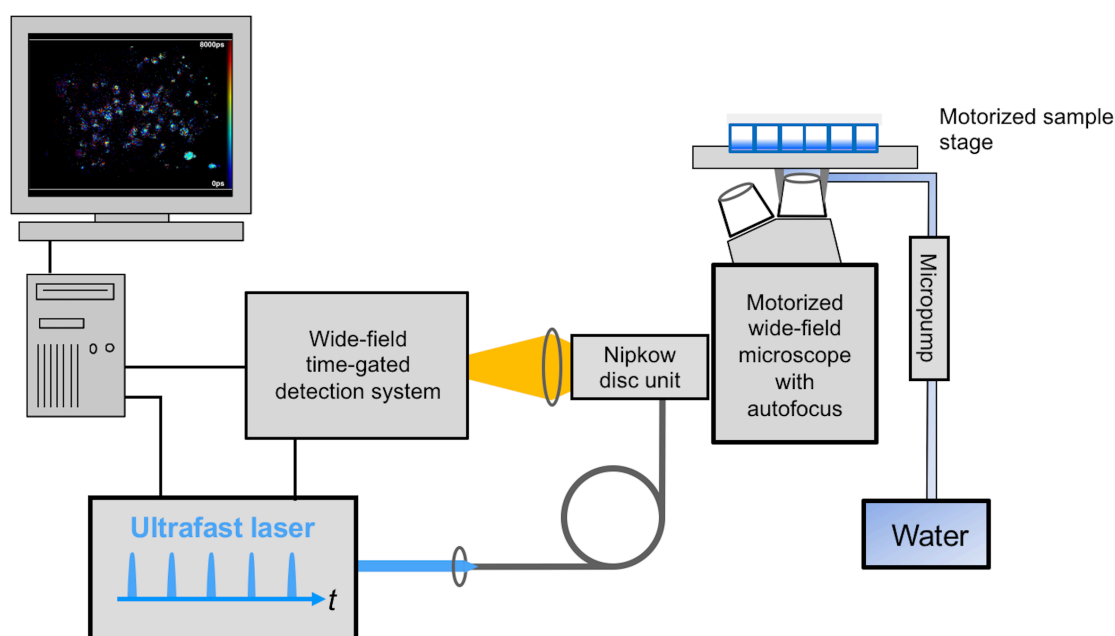


Figure 3-12 Schematic of the modified automated multiwell plate FLIM microscope

Adapted from Ref.⁴³⁹.

3.3.1 The excitation laser source

Although the supercontinuum laser source used in the typical set-up of the *openFLIM-HCA* instrument shown in Figure 3-1 offers the benefit of flexible spectrum selection for multi-colour imaging, it is not the optimal choice for the experiments reported here, which required single-channel excitation in either the mTq2FP (excitation peak at ~434 nm) or GFP (excitation peak at ~488 nm) channels. This is because the spectral power density of the supercontinuum emission is relatively low in the blue and green spectral regions and because the beam quality deteriorated over time, became unstable and stopped being single-mode (Figure 3-13).

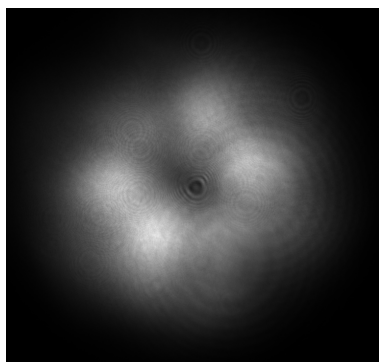


Figure 3-13 Exemplar profile of a beam from a Fianium supercontinuum laser after a 434/17 bandpass filter (for excitation in the mTq2FP channel)

The deterioration of the beam quality from nearly single mode with an approximately Gaussian beam profile to this typical donut-shaped profile prevented efficient coupling of the laser beam into a single-mode optical fibre, limiting the achievable excitation power at the objective lens' focal plane.

With a low output power and low coupling efficiency (<10%) into the single-mode optical fibre that delivered the light to the microscope frame, the supercontinuum laser failed to provide sufficient excitation power to achieve a usable signal from the donor fluorophores at yeast kinetochores. In contrast, a tunable femtosecond Ti:Sapphire laser (Mai Tai HP, Spectra-Physics, UK) and a second-harmonic generation (SHG) unit (#3980 Frequency Doubler / Pulse Selection Unit, Spectra-Physics, UK) provided the pulsed excitation radiation needed for both the mTq2FP and GFP channels with a stable single-mode beam profile and a coupling efficiency of >50%. The schematic of the set-up for generating and coupling the excitation beam into the single-mode fibre is given in Figure 3-14. The

excitation power at the sample plane can be altered using the combination of a half-wave plate and a linear polariser shown in the schematic and was set to $\sim 150 - \sim 200 \mu\text{W}$ at the start of each FLIM experiment performed in this thesis.

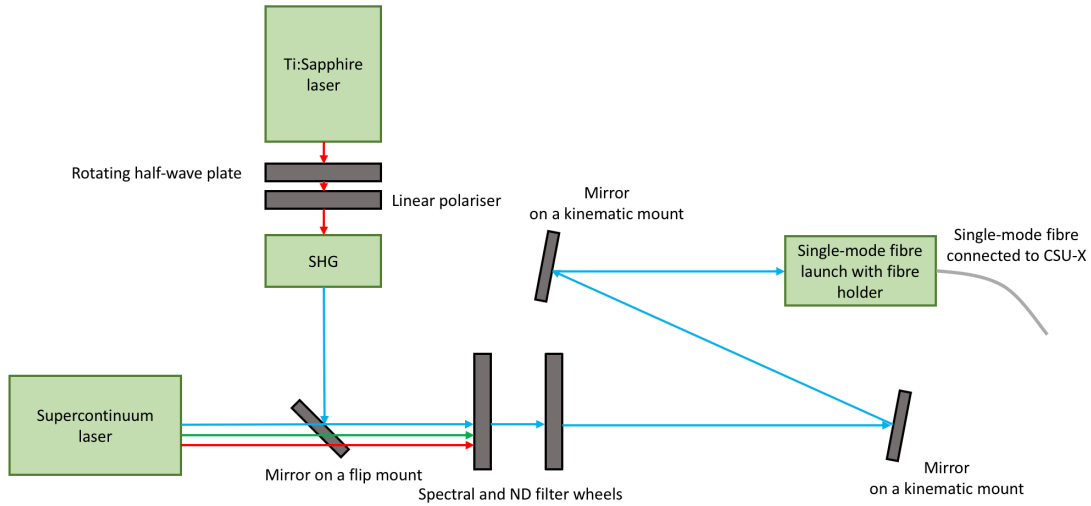


Figure 3-14 Set-up for generating the excitation radiation and coupling it into a single-mode optical fibre

The infra-red output from the Ti:Sapphire laser is frequency-doubled to a blue or green beam by the second harmonic generation (SHG) unit for exciting mTq2FP or GFP. The excitation beam is then reflected from a mirror on a flip mount, which is flipped away from the light pathway when the supercontinuum laser is the excitation source in use. After the flip mirror, the outputs from the two lasers share the light pathway for being coupled into a single-mode fibre that delivers the excitation light to the spinning disk unit (CSU-X). The combination of a rotating half-wave plate and a linear polarisers enables the tuning of the excitaton power from the Ti:Sapphire laser.

3.3.2 The microscope objective lens

In an epifluorescence microscope with a laser beam underfilling the objective lens's aperture exciting a homogeneous sample (or a sample larger than the excitation point spread function (PSF)), the brightness of the detected fluorescence B is dependent on both the numerical aperture NA and the magnification M of the microscope objective following the relation below⁴⁵⁴:

$$B \propto \frac{NA^2}{M^2} \quad (3.7)$$

While live cell imaging of budding yeasts typically uses a 100x, 1.4 NA oil-immersion objective lens for the higher spatial resolution and the higher collection efficiency of the excited fluorescence offered by the high NA⁴⁵⁴, when considering solely the detected brightness it can be beneficial to use a 60x 1.2 NA water-immersion objective lens instead. The two objective lenses that we have available for the FLIM microscope (UPlanSApo 100XO 1.4 NA and UPlanSApo 60XW 1.2 NA, Olympus, Japan) have similar transmittance for excitation in the blue and green channels and so the water-immersion lens should offer a brightness that is ~104% higher than obtained with the oil-immersion lens. Although this improvement in brightness is at the expense of a lower spatial resolution, for our FLIM experiments intended to query protein interactions based on FRET, the spatial resolution is of less importance compared with the brightness that improves the S/N.

More critically for automated HCA, it is easier to maintain the immersion liquid between the objective front lens and the bottom of a multiwell plate for a water-immersion objective lens than for an oil-immersion lens in our inverted microscope set-up. This is because water is much less viscous than immersion oil and so can be easily pumped through a narrow tube. Additionally, water is easier to clean than immersion oil, and excess water is conveniently absorbed by putting tissue paper around the objective lens.

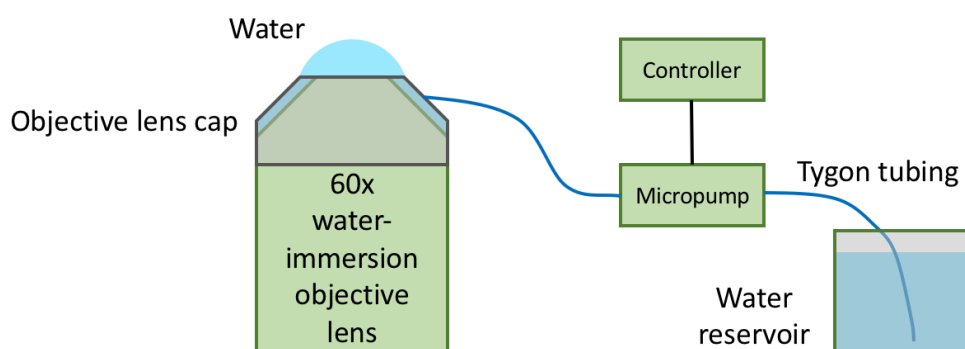


Figure 3-15 Set-up for retaining the immersion liquid on the Olympus water-immersion objective lens

A cap was designed for the objective lens for retaining water at the objective lens' front surface by creating a small reservoir between the cap and the objective lens. Water is pumped into this small reservoir by a micropump to balance the loss through evaporation. The water pump rate is adjustable via the controller for the micropump.

Figure 3-15 shows our set-up for retaining the immersion liquid on our water-immersion objective throughout an HCA experiment that can take a few hours. The key component is a micropump (mp6, Bartels Mikrotechnik GmbH, Germany) that can constantly pump water from a reservoir through standard tygon tubing (mp-t, Bartels Mikrotechnik GmbH, Germany) under the control of an associated controller unit (mp-x, Bartels Mikrotechnik GmbH, Germany). A cap for the water-immersion objective lens was designed so as to create a small reservoir between the cap and the objective lens for retaining water at the objective lens's front surface. The pumping rate was set using the controller to balance water loss through evaporation and changing FOVs while not creating excessive overflow. The electrical signal driving the pump was typically set to “sinusoidal, 30 Hz, 25 V_{peak-peak}”.

3.3.3 The GOI and the CCD camera

The performance of the GOI and the CCD camera strongly impact the quality of FLIM data in terms of the S/N and the spatial resolution³⁹⁹. Compared with a standard GOI that offers a tunable gate width from ~200 ps to 2 ns, the GOI model I used (HRI-HL from Kentech Instruments Ltd.) allows a wider choice of time gates (~200 ps - ~10 ns) (see Figure 3-3). The use of wider time-gates improves the S/N, as more photons can be acquired at each time-gate delay. For the experiments reported in this thesis, the HRI-HL model was consistently used with the gate width set to 4 ns. The CCD camera (Orca ER II, Hamamatsu) was used with 4x4 hardware binning to improve the S/N at the expense of spatial resolution. Considering the 60x magnification from the objective lens and the 0.7x demagnification from the relay lenses between the GOI and the CCD camera, each time-gated image after binning had 336x256 pixels with a pixel size of 615 nm. While this pixel size is insufficient for Nyquist sampling the PSF of the microscope with the 1.2 NA lens, for the intended FLIM experiments this is not a problem.

3.4 Development of the data acquisition workflow

As well as optimising the imaging set-up for my project, I also modified the data acquisition workflow introduced in Section 3.1.3. This section first describes these

modifications along with the rationale behind them, then summarises the workflow used for the experiments reported in this thesis.

3.4.1 Measuring the fluorescence decay of quenched fluorescein instead of the scatter IRF

As mentioned in Sections 3.1.2 & 3.1.3, when using the supercontinuum laser as the excitation source, the excitation light scattered from the Nipkow disk can be measured to provide the *scatter IRF* since the broadband nature of the supercontinuum laser source enables the scattered light to be filtered so that the *scatter IRF* can be recorded in the same spectral region as the sample fluorescence. However, after the laser source was changed to a frequency-doubled Ti:Sapphire one, this was not possible because the narrowband excitation light was efficiently blocked by the emission filter and it would not be optimal to measure the *scatter IRF* at the excitation wavelength due to the wavelength-dependent response of the system. To circumvent this problem, an alternative method to measure the IRF was required. It has been reported that the fluorescence decays of organic dyes with very short lifetimes or dyes quenched to very short lifetimes via collisional quenching can be used to provide IRF measurements for various spectral regions^{455–460}. However, most of these decays still have lifetimes of tens of picoseconds, making them unsuitable as impulse functions for samples with very short lifetimes⁴⁶¹. Another approach that also utilises the fluorescence decay of a dye is the Delta function convolution method (DFCM)^{462,463}. Instead of directly using the decay as the IRF, the DFCM utilises the reference convolution technique⁴⁶⁴, which entails first convolving a modified decay model $\tilde{f}(t)$ with the mono-exponential decay of the reference dye $r(t)$ and then comparing the result to the measured sample decay $g(t)$ so as to recover the decay model $f(t)$, as described by the following equations⁴⁶⁵:

$$g(t) = \tilde{f}(t) * r(t) \quad (3.8)$$

$$\tilde{f}(t) = \sum_i \frac{\alpha_i}{\alpha_r} \left[\delta(t) + \left(\frac{1}{\tau_r} - \frac{1}{\tau_i} \right) e^{-\frac{t}{\tau_i}} \right] \quad (3.9)$$

$$f(t) = \sum_i \alpha_i e^{-\frac{t}{\tau_i}} \quad (3.10)$$

where τ_r is the lifetime of the reference dye, τ_i the i -th lifetime component of the exponential sample decay, and α_r and α_i the corresponding amplitudes. Knowing τ_r and α_r , the sample decay model can be recovered by fitting for τ_i and α_i . This is in contrast to the conventional method shown in Equation 3.1 where the exponential decay model for the sample is convolved with the IRF, and offers the benefit of accounting for the lifetime of the reference dye decay rather than requiring an ultrafast impulse function.

While the DFCM can also work with reference dyes of multi-exponential decays and unknown lifetimes, fewer photons are needed to reach a given fitting accuracy if the reference dye has a mono-exponential decay with a known short lifetime^{465,466}. With that knowledge in mind, I decided to use a mixture of the dye, fluorescein, and the quencher, potassium iodide (KI), as the reference fluorophore for my FLIM experiments utilising the blue (mTq2FP) and the green (GFP) channels.

As the absorption and the fluorescence spectra of fluorescein are known to be pH-dependent^{467,468}, the 1 mM stock solution of fluorescein was made by dissolving the fluorescein (free acid) power (Sigma-Aldrich 46955, Merck, Germany) with MilliQ water and adjusting the pH to neutral (pH7). At this pH, fluorescein should be efficiently excited by both blue and green lights⁴⁶⁸, and thus is ideal for the experiments presented here. Considering that fluorescein's excitation and emission spectra can vary with concentration at high concentrations (due to self-quenching)^{468,469}, for consistency the 1 mM stock solution was always diluted to 250 μ M for imaging. At this concentration, I measured a mono-exponential fluorescence decay with a fluorescence lifetime of ~ 3.6 ns for the aqueous fluorescein solution.

The fluorescence from fluorescein can be quenched by the addition of iodide ions. The quencher iodide ion effectively depopulates the excited fluorophores via collisional quenching and therefore reduces both the intensity and the lifetime of the fluorescein fluorescence^{457,459}. As collisional quenching follows the Stern-Volmer equation⁴⁵⁹, the quenched fluorescence lifetime should decrease with increasing quencher concentration, and with sufficient KI concentration it should be short enough that the quenched fluorescein decay can be used as a reference for the DFCM. To test that, I mixed various amounts of KI (Fluka 60399, Honeywell, US) with 250 μ M fluorescein respectively to

make a series of quenched fluorescein solutions. The results showed that both the fluorescein lifetime and the fluorescence intensity of fluorescein decrease with increasing KI concentration. Therefore, in order to balance the requirements for short lifetime and sufficient photon number, I chose to use the mixture of 250 μM fluorescein and 2 M KI as the reference dye for the DFCM.

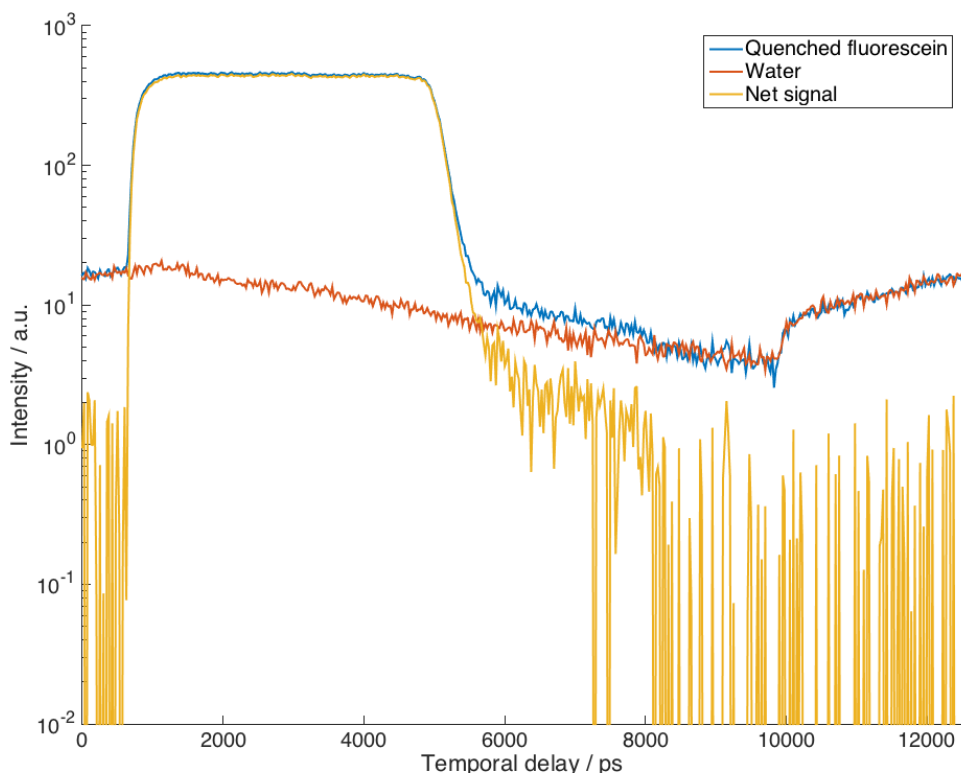


Figure 3-16 Fluorescence decay profiles of the quenched fluorescein solution (250 μM fluorescein with 2 M KI), the water background, and the background-subtracted signal (net signal)

Note the abnormal rises near the end of the decay profiles of both the quenched fluorescein and the water background, which is largely corrected in the background-subtracted net signal.

Figure 3-16 shows an exemplar fluorescence decay profile measured from the quenched fluorescein solution of choice (250 μM fluorescein and 2 M KI). One apparent anomaly is the unexpected rise towards the end of the decay. As a similar rise at the same temporal position was observed for the fluorescence decay of water, the origin of this rise is attributed to some components along the optical pathway that generate background

fluorescence (which would be proportional to the excitation power but not the sample brightness), rather than the scattered or reflected excitation light that reaches the sample plane with a delay and generates further fluorescence from the sample (which would be proportional to the sample brightness). While this background fluorescence should always be present regardless of what is being imaged, it is only relatively significant when the sample being imaged is dim, e.g. the quenched fluorescein, and its presence in the fluorescence signal from yeast kinetochore foci can also be effectively eliminated by the TVB subtraction process discussed in the following section. Therefore, this background in the quenched fluorescein fluorescence should be corrected as well, which was done by subtracting the fluorescence measured from water. As shown in Figure 3-16, after this subtraction, the unwanted background fluorescence can be largely removed, and thus the resulting decay can be used for FLIM data analysis based on the DFCM, which is described in detail in Section 3.5.2.

3.4.2 Pre-photobleaching the cellular autofluorescence

While the introduction of the frequency-doubled Ti:Sapphire laser offered higher excitation power for imaging in both the blue and the green channels, the use of higher excitation power is often accompanied by increased photobleaching. To characterise the photobleaching effect on the FP fluorescence from budding yeast kinetochore foci as well as on the cellular and the imaging medium autofluorescence, I devised a protocol as described below:

1. Prepare the appropriate yeast samples (for details in the yeast sample preparation protocol see Section 3.4.3), and transfer these into separate wells on a 96-well plate:
 - The imaging medium SC+ADE should also be transferred into an individual well, so that its photobleaching behaviour can be characterised separately;
 - Note that when multiple excitation power settings are to be tested, the yeast samples and the media should be transferred into multiple wells, so that different wells will be imaged when the excitation power is changed.

2. Set up the FLIM microscope hardware for yeast kinetochore imaging (for details in the hardware settings see Section 3.4.3).
3. Set the excitation power at the sample plane to a chosen value, which can be measured with a microscope slide photodiode sensor (S170C, Thorlabs Inc., Germany), by rotating the half-wave plate in front of the Ti:Sapphire laser's output (Figure 3-14).
4. Using the custom *μManager* plug-in, set up the microscope for automated FLIM acquisition of 5 FOVs per well in the wells to be imaged for the current power setting, so as to perform averaging on the photobleaching curves measured from each well:
 - For each FLIM acquisition, 121 time-gates should be used, and all delays should be set to the temporal position of the peak FP fluorescence intensity;
 - Each time-gate is imaged with 1 s camera exposure time, so that, in total, a photobleaching curve for 2 min exposure will be acquired for each FOV.
5. If needed, set the excitation power at the sample plane to a different value, and perform the automated FLIM acquisition on a different set of wells.
6. The acquired sample FLIM datasets should then be processed to segment for the kinetochore foci and the corresponding cellular regions for quantifying the cellular autofluorescence:
 - For each acquired dataset, the 121 temporal slices should be summed to generate an “integrated intensity image” for performing the following segmentation steps;
 - Dead cell masks should be generated to exclude the regions with dead cells for following analysis, based on the higher intensities and the larger sizes of the regions compared with the kinetochore foci;
 - Kinetochore foci should be segmented using a modified version of the nonlinear top-hat (NTH) algorithm⁴⁷⁰, the details of which is described in Section 3.5.1. The product of this step is a segmentation mask for each FOV where each kinetochore focus is segmented as a region with 3x3 pixels. Such a region corresponds to an area of 1.8 μm x 1.8 μm in the sample plane, which is much larger than a typical sub-diffraction budding

yeast kinetochore cluster and is chosen to accommodate any movement of the kinetochore cluster during FLIM acquisition;

- A cellular autofluorescence mask can then be generated for each FOV based on the corresponding kinetochore mask, where around each kinetochore region a 7x7 pixel hollow square with 1-pixel thickness is designated as the region for the local cellular autofluorescence background.

7. With the masks generated above, the photobleaching curves of interest can be obtained using a MATLAB script:

- Regions in the kinetochore and the cellular autofluorescence masks that overlap with dead cells are first removed using the dead cell masks;
- The raw FLIM datasets should have the constant background subtracted (using the mean pixel value from the constant background dataset);
- The photobleaching curves for the fluorescence from the kinetochore regions and the cellular autofluorescence can be obtained using the constant background-subtracted FLIM datasets and the corresponding masks. Note that the mean pixel value from all the segmented kinetochore regions in each time-gated image should be used to utilise all the photons from these regions; for the cellular autofluorescence, the mean of all the median values from each region should be used instead to minimise the impact of artefacts due to kinetochore clusters moving into the cellular background regions);
- The photobleaching curve for the net FP fluorescence from each excited yeast sample can be calculated by subtracting the curve for the corresponding cellular autofluorescence from the curve kinetochore regions;
- The photobleaching curves for the imaging medium SC+ADE can be obtained by calculating the mean pixel values from a certain region at the centre of the corresponding SC+ADE-only time-gated images.

Following this protocol, I performed two sets of experiments for the photobleaching behaviours of the two donor FPs used in the subsequent experiments, i.e. mTq2FP and GFP.

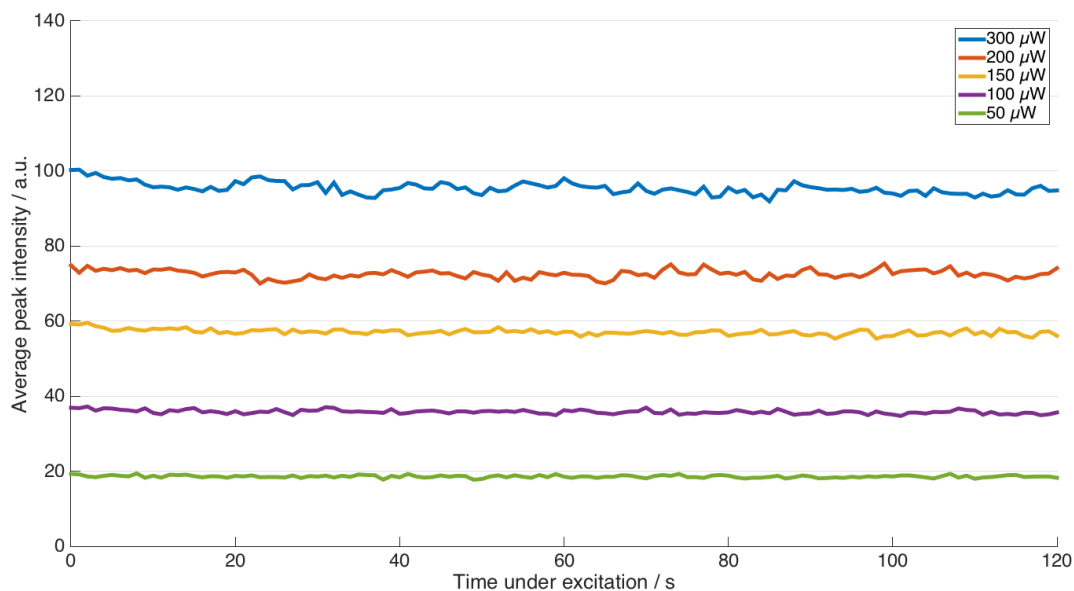


Figure 3-17 Photobleaching curves of SC+ADE averaging over 5 FOVs for various excitation power settings

The excitation power was measured at the objective lens' focal plane before the acquisition of each curve.

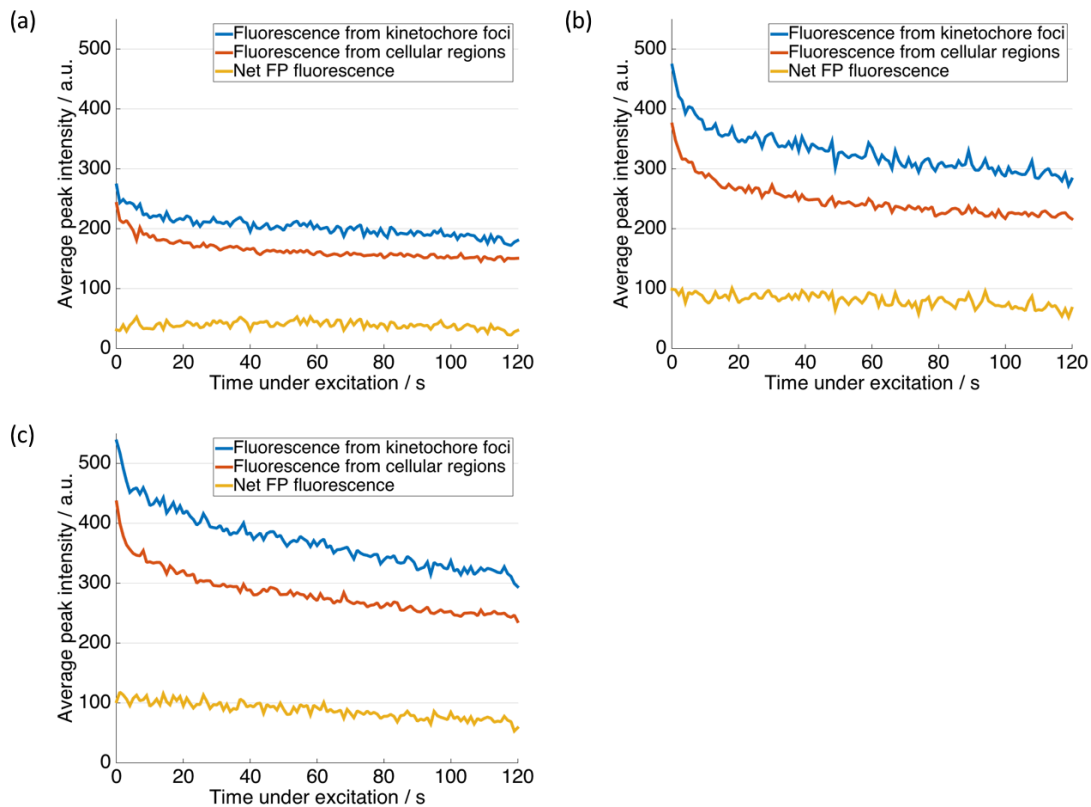


Figure 3-18 Photobleaching curves for the fluorescence signals measured from an endogenously-labelled heterozygous diploid yeast strain containing one *ASK1-mTq2FP* allele under different excitation power settings: (a) 150 μW; (b) 200 μW; (c) 300 μW.

The excitation power was measured at the objective lens' focal plane before each measurement. Note that in all conditions the cellular background-subtracted net fluorescence from Ask1p-mTq2FP (the yellow curve) is much less sensitive to photobleaching than the other two signals. Adapted from Ref.⁴³⁹.

For the experiments on mTq2FP, I used five excitation power settings: 300 μW, 200 μW, 150 μW, 100 μW, and 50 μW (all measured at the water-immersion objective lens's sample plane). The resulting photobleaching curves for the imaging medium SC+ADE plotted in Figure 3-17 show that under these power settings the measured medium fluorescence was barely photobleached throughout the 2 min excitation, and that the average peak intensity increased proportionally with the excitation power. The fluorescence signals measured from the yeast samples, on the other hand, were subject to much stronger photobleaching, as shown in Figure 3-18. The yeast strain in this experiment was an endogenously-labelled heterozygous diploid containing one *ASK1-*

mTq2FP allele and one wild-type *ASK1* allele, and thus ~50% of the Ask1p proteins should be labelled with mTq2FP. Even for this outer kinetochore protein with a relatively high abundance (Table 3-1), the kinetochore foci became hardly distinguishable from the cellular background under 100 μ W or 50 μ W excitation. When excited using the three higher excitation powers, the fluorescence measured from the segmented kinetochore foci exhibited a decrease over time, especially at the start of the excitation. The decrease is consistent with the photobleaching of the cellular autofluorescence, as this autofluorescence showed a similar decrease under prolonged excitation. In fact, the net fluorescence from mTq2FP, calculated by subtracting the fluorescence from the kinetochore foci with the cellular autofluorescence, was relatively stable. Intriguingly, the net FP fluorescence did not scale proportionally with the excitation power, the cause of which is unclear. One possible explanation is that the actual values of the power used were not accurate due to drift in the laser power after calibration at the sample plane; yet this notion is not supported by the results from the SC+ADE datasets (Figure 3-17). It is also possible that the much lower net FP signal in the 150 μ W excitation case was related to being imaged 2-3 hours later than the other two cases, which may result in the cell culture becoming too dense and the cells no longer being in log phase. Nevertheless, from these results I came to the following conclusions:

1. The photobleaching of the cellular autofluorescence is the main cause of the photobleaching of the measured fluorescence from the kinetochore foci;
2. The net FP fluorescence from the kinetochore foci, which is the signal of interest for my experiments, is relatively stable under a reasonable excitation power, and consequently an excitation power of 200 μ W at the sample plane was chosen for the FLIM experiments on imaging mTq2FP;
3. To improve the S/N in a FLIM dataset, before sampling the fluorescence decay of interest the cellular autofluorescence in the FOV should be pre-photobleached; based on the photobleaching curve for the cellular autofluorescence under 200 μ W excitation, a 15-second pre-photobleaching duration was chosen, so that the autofluorescence should be much reduced when sampling the fluorescence decay of interest.

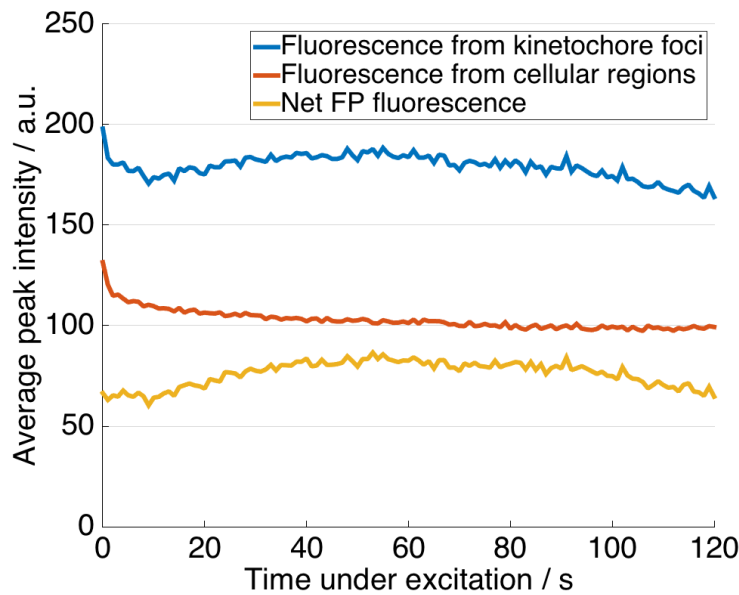


Figure 3-19 Photobleaching curves for the fluorescence signals measured from an endogenously-labelled heterozygous diploid yeast strain containing one *SPC24-GFP* allele under 150 μ W excitation

The excitation power was measured at the objective lens' focal plane. Note the abnormal rises in both the blue and the yellow curves, which suggest that the GFP variant in use (GFP S65T) underwent either photoactivation or reversible photobleaching during the photobleaching process.

Similar results were obtained for photobleaching experiments on GFP. Figure 3-19 shows the results from an endogenously-labelled heterozygous diploid containing one *SPC24-GFP* allele and one wild-type *SPC24* allele under 150 μ W excitation. Compared with the curves shown in Figure 3-18c, where the same excitation power was used for imaging cells with mTq2FP, it is clear that the GFP cells exhibited cellular autofluorescence of a similar photobleaching kinetics but a much lower brightness, possibly because of the different emission filter used or because these cells have a different genetic background (S288C) from the mTq2FP ones (W303)⁴⁷¹. The kinetochore foci with the GFP tags were, on the other hand, brighter than when labelled with mTq2FP, and were still easily distinguishable from the cellular autofluorescence under 150 μ W excitation. This excitation power was subsequently used for all the FLIM experiments on the GFP strains, together with a pre-photobleaching duration of 6 s to suppress cellular autofluorescence. Notably, both the fluorescence from the kinetochore foci and the net FP fluorescence appeared to undergo some photoactivation or reversible photobleaching at the beginning

of the photobleaching curves, which was not seen for mTq2FP but has been reported in other studies^{451,452,472}. While this effect may not strongly impact the FLIM results in terms of revealing FRET, it may introduce additional noise to the data and suggests that the donor for FLIM FRET studies should be chosen with care.

3.4.3 The modified data acquisition workflow

Incorporating the modifications described above, the data acquisition workflow used in my FLIM HCA experiments is summarised below:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		C6	PT208	PT197	PT261	pHT522	PT270	PT244	PT267	pHT449		
C		Water	S+A	PT197	PT261	pHT522	PT270	PT244	PT267	pHT449		
D		QF	-L+A	PT197	PT261	pHT522	PT270	PT244	PT267	pHT449		
E												
F												
G												
H												

Figure 3-20 An exemplar plate map for a FLIM HCA experiment on studying PPIs at the budding yeast kinetochore

This exemplar map shows how the wells in a 96-well plate were typical arranged for the experiments reported in Chapter 4. The grid denotes a 96-well plate. The letters to the left of the grid denote the row numbers, whilst the numbers to the top denote the column numbers. C6: Coumarin 6. QF: quenched fluorescein. S+A: SC+ADE medium. -L+A: -LEU+ADE medium. PT208: unlabelled diploid strain. PT197, PT261, pHT522, PT270, PT244, PT267, pHT449: fluorescently-labelled strains for FLIM imaging. For details of these strains, please refer to Table 4-1. The automated data acquisition for the fluorescently-labelled strains were set to operate in the “horizontal snake” mode, i.e. first horizontally from B10 to B4, then from C4 to C10, and finally from D10 to D4.

1. Prepare the budding yeast samples for the FLIM experiment:
 - For each yeast strain to be imaged, pick a single colony of cells grown on an agar plate and grow them overnight in 5 ml SC+ADE (or -LEU+ADE for plasmid-containing strains) in a 23°C shaking incubator;
 - On the day of the experiment, dilute 100 µl of each overnight culture in 2 ml fresh SC+ADE (or -LEU+ADE), and incubate the new culture at 23°C with shaking for ~3 hr;
 - Seal the Falcon round-bottom polypropylene tubes containing the cultures, and transport them from the yeast lab to the FLIM lab (which takes ~1 hr);
 - Upon arrival, unseal the tubes and incubate them at room temperature (~24°C) with shaking for ~1 hr (while setting up the FLIM instrument);
 - After setting up the instrument (details of which are given below), transfer each culture into 3 wells on a glass-bottom 96-well plate (SensioPlate™, Grenier Bio-One GmbH, Germany), with 200 µl for each well;
 - Transfer the reference dyes (75 µM Coumarin 6, and 250 µM fluorescein quenched by 2 M KI), the imaging medium SC+ADE (and/or -LEU+ADE), the MilliQ water, and some unlabelled cells into additional wells, with 200µl for each well;
 - Figure 3-20 shows an exemplar plate map for a typical experiment. Note that the culture of each strain is transferred into 3 wells that are in different rows but the same column, and the order of the columns containing different strains are randomised for each experiment. The automated image acquisition should be set to operate in the “horizontal snake” mode, with which the acquisition goes from left to right for one row and then from right to left for the following row, so as to partially randomise the timing of imaging for the different strains in the same row.
 - The 96-well plate should then be placed into the microscope chamber at room temperature ~1 hr before imaging the cells to allow the cells to settle, and within this hour the finely sampled FLIM datasets of the reference dyes can be acquired.
2. Set up the FLIM instrument:

- Switch on all the associated parts of the instrument, e.g. the Ti:Sapphire laser, the microscope frame control, the x-y stage control, etc., and start up the custom *μManager* plug-in for controlling the instrument;
- Tune the output wavelength of the laser to the desired value (868 nm for mTq2FP imaging and 975 nm for GFP imaging, which after frequency doubling will become 434 nm and 487.5 nm, respectively), and adjust the direction of the laser beam and the position of the input facet of the optical fibre to optimise the coupling efficiency to >50%;
- Using the *μManager* plug-in, select the appropriate ND filter (ND0), excitation filter (434/17 nm for mTq2FP imaging and none for GFP imaging), dichroic filter (457/514/647 nm for mTq2FP and 488/561 nm for GFP), and emission filter (482/35 nm for mTq2FP and 520/35 nm for GFP) for imaging the yeast samples. Note that due to the relative monochromaticity of the excitation radiation and the lack of a suitable filter at hand, no excitation filter was used for the experiments imaging the GFP donor;
- Select the 60x water-immersion objective using the *μManager* plug-in, and set up the micropump for retaining the immersion liquid as shown in Section 3.3.2 and Figure 3-15. Note that the objective lens should be surrounded by tissue paper to absorb excess water, and the correction collar on the objective lens should be checked to be at the right position (0.17 for matching the #1.5 thickness of the 96-well plate's glass bottom);
- Adjust the excitation power at the sample plane, by putting a microscope slide photodiode sensor (S170C, Thorlabs Inc., Germany) onto the motorised x-y stage, adjusting the z-position of objective so that the immersion water is in contact with the sensor's glass surface, and rotating the half-wave plate in front of the output port of the Ti:Sapphire laser (Figure 3-14) until the power measured by the sensor reaches the desired value (200 μ W for mTq2FP imaging and 150 μ W for GFP imaging);
- Set up the ZDC unit for autofocus using the *μManager* plug-in, by putting the 96-well plate onto the x-y stage, moving to the well containing the unlabelled cells, manually adjusting the z position of the objective lens

until the cells are in focus under bright-field illumination when viewed from the eyepieces, and then setting the autofocus search range to 300 μm and the autofocus offset to 3.5 μm , and lastly manually running the autofocus once so that the current z position is set as a reference point for autofocusing during the later automated FLIM acquisition;

- Set the GOI to operate at 80 MHz (matching the repetition rate of the laser pulses) with 4 ns gate width and 850 V gain voltage and, in the *$\mu\text{Manager}$* plug-in, select the calibration file specific for the laser and the fine delay unit in use. (This ensures the correct delay time between the excitation pulse and the switching of the GOI gate voltage is set when commanded by the *$\mu\text{Manager}$* plug-in. The calibration file takes account of the excitation pulse repetition rate and the specific fine delay unit in use);
- Set the CCD camera to operate with 4x4 hardware binning and 200 ms exposure time and adjust the two camera lenses between the GOI and the CCD camera to ensure that both apertures are fully open and the lenses are focused to infinity so that single photon detection events imaged by the camera are in focus.

3. Take a finely-sampled FLIM dataset of the reference dye Coumarin 6:

- Move to the well containing 200 μl 75 μM Coumarin 6 dissolved in ethanol;
- Perform autofocusing;
- Acquire live images of the dye and use the “Find Max Point” function in the *$\mu\text{Manager}$* plug-in to find the temporal position of the peak of the dye’s fluorescence decay; note that the peak intensity should be set to $\sim 2/3$ of the camera dynamic range to avoid saturation, by selecting an appropriate ND filter in the excitation laser path and setting an appropriate camera exposure time (if it is less than 100 ms, multiple frames should be taken to avoid fixed-pattern noises arising from the spinning disk; typically 300-600 ms was used);
- Adjust the peak position of the decay to 5500 ps using the manual coarse delay unit, so that the rise of the fluorescence is right at the beginning of the temporal window when a 4 ns gate width is used. Note that after this

- step, the setting on the coarse delay unit should not be changed throughout the experiment;
- In the *μManager* plug-in, set up the temporal positions of the time gates for finely sampling the Coumarin 6 fluorescence decay, which is from 12500 ps to 0 ps with a 25 ps step. Note that the decay should be sampled in a reverse order, so as to minimise the impact of phosphorescence persistence from the GOI's phosphor screen on the decay measurement;
 - Acquire the finely-sampled FLIM dataset of the Coumarin 6 dye, which will be used for generating a temporal shift map for the quenched fluorescein decay (equivalent to the *IRF shift map* described in Figure 3-7) and for assessing the performance of the FLIM instrument: when the instrument is performing well, the measured Coumarin 6 decay should appear as a mono-exponential decay with a fitted lifetime of ~ 2.5 ns;
 - When a 600 ms camera exposure time is used, the total acquisition time for this FLIM dataset is <10 min.
4. Take a finely-sampled FLIM dataset of the quenched fluorescein reference dye and the associated water background:
- These two datasets can be acquired in exactly the same way as for the Coumarin 6 FLIM dataset, except that there is no need to adjust the setting on the coarse delay unit;
 - As the fluorescence from the quenched fluorescein is much dimmer than that from Coumarin 6, typically a longer camera exposure time is used, e.g. 2 s per time-gate (which corresponds to a total acquisition time of ~ 30 min per FLIM dataset, i.e. in total this step takes ~ 60 min).
5. Perform automated FLIM acquisition on the budding yeast samples:
- Check that after autofocusing the kinetochore foci can be seen in most cells; if not, slightly adjust the autofocus offset and retry;
 - Adjust the camera exposure time so that the maximum signal from kinetochore foci reaches $\sim 2/3$ of the camera's dynamic range;
 - In the *μManager* plug-in, set the positions for the 7 time gates for sampling each decay using "auto-gating" (see Section 3.1.2), plus several additional gates at the beginning for pre-photobleaching the cellular

autofluorescence (total pre-photobleaching time: 15 s for mTq2FP and 6 s for GFP; for details see Section 3.4.2);

- In the *μManager* plug-in, select the wells to be imaged, and auto-generate 15 non-overlapping FOVs for each well in a spiral pattern starting from the well centre;
 - In the *μManager* plug-in, set the acquisition order of the wells as shown in Figure 3-20, so as to average over any noise related to the timing of imaging, e.g. the status of the cells and the excitation power (in case there is a systematic drift);
 - For the data acquired for this thesis, each of the yeast strains with mTq2FP took ~33 min to image in each experiment (3 s camera exposure per time-gate, 12 time-gates per FOV, 45 FOVs), while each of the GFP ones took ~24 min (2 s camera exposure per time-gate, 10 time-gates per FOV, 45 FOVs).
6. Take the datasets for the constant background:
- Shutter the excitation light;
 - Take a FLIM dataset with ~100 time gates for each of the camera settings used for imaging the dyes and the samples.
7. Switch off and tidy up all the associated parts of the instrument.
8. Additional notes:
- During the experiment the ambient light (e.g. the room light and the microscope chamber light) should be kept to a minimum;
 - There need be enough cells in each FOV to provide sufficient photons for accurate lifetime fitting, while the cell culture should also be in log phase and not be too dense, as otherwise the cellular autofluorescence may increase (Figure 3-10). Therefore, the cell density should be well controlled when transferring the cells into the wells, especially for those to be imaged towards the end of the experiment, by adding less cell cultures to the wells and diluting them with fresh imaging medium to a full volume of 200 μl. Besides, the total length of the automated yeast imaging step should be controlled. For the experiments reported in this

thesis, no more than 10 strains were imaged in each single experiment, i.e. the longest acquisition time was <6 hr.

3.5 Development of the data analysis workflow

This section describes the data analysis workflow specifically developed for the yeast kinetochore FLIM data. This workflow differs from the generic one introduced in Section 3.1.3 in two main aspects: the use of a custom MATLAB-based tool to generate the kinetochore-segmented TVB-subtracted FLIM datasets (where the local TVB from the cellular autofluorescence around each kinetochore focus is subtracted and thus theoretically there is only the FP fluorescence of interest in each dataset), and the use of *FLIMfit* to perform image-wise mono-exponential fitting using the DFCM based on the quenched fluorescein decay instead of the method based on the *scatter IRF*. These two aspects are discussed respectively in the following subsections, together with the statistical test chosen for comparing the image-wise lifetime results.

3.5.1 Image processing using a custom MATLAB-based tool

The raw FLIM datasets for the samples acquired following the workflow above contained not only the fluorescence of interest from the donor FPs at the kinetochores but also unwanted background signals from the cellular and medium autofluorescence and the bright dead cells. For extracting the FP fluorescence of interest in a high-throughput manner for the subsequent lifetime analysis, I wrote a MATLAB-based tool to process the raw datasets. Figure 3-21 shows the GUI of this tool.

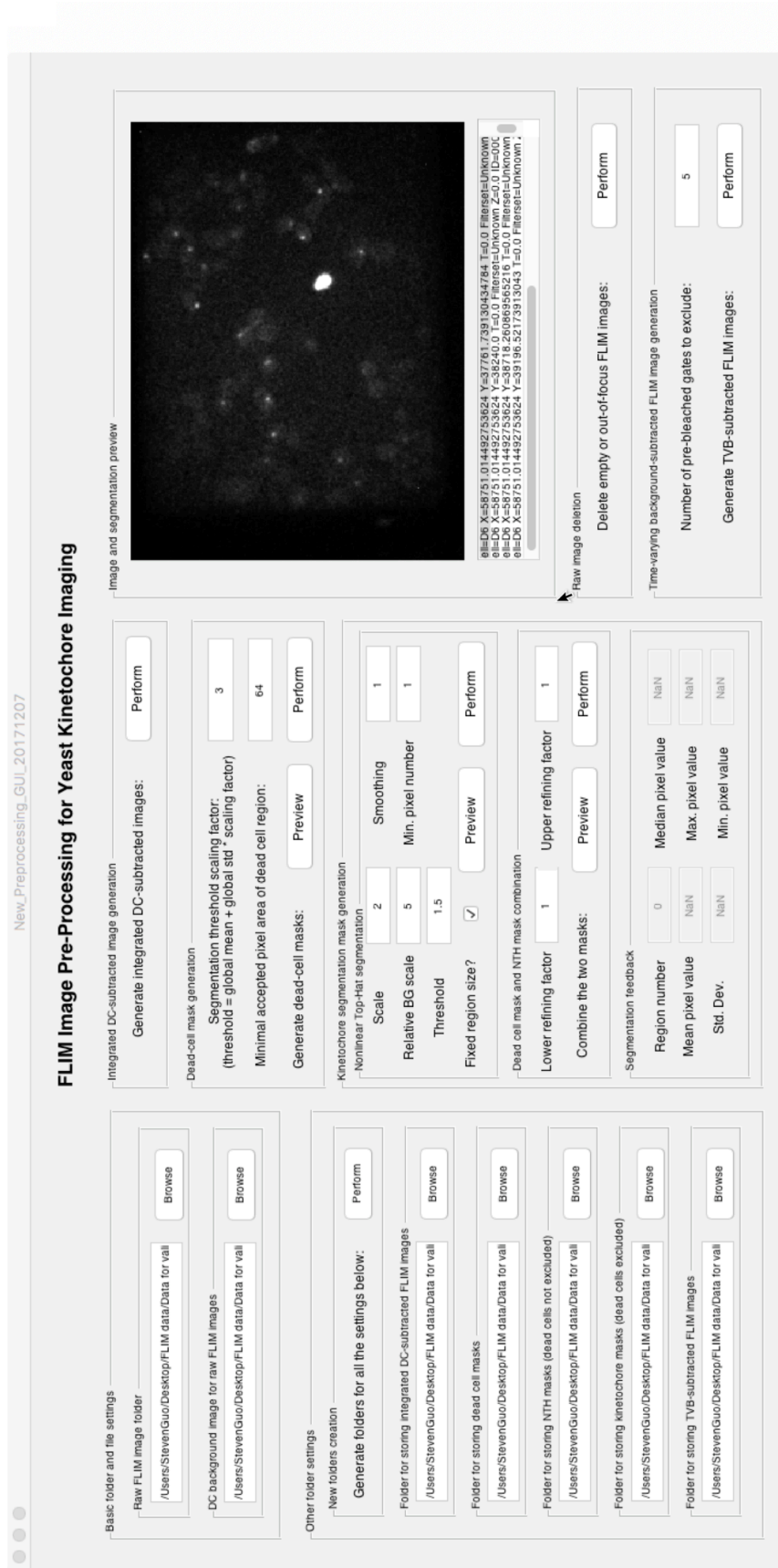


Figure 3-21 The GUI of the MATLAB-based tool for processing the raw FLIM datasets

The functions of this tool include generating a constant background-subtracted integrated intensity image for each raw FLIM dataset, masking out regions with dead cells, segmenting for the kinetochore foci and the corresponding cellular regions, and removing the first few time-gated images in each FLIM dataset that were acquired for pre-photobleaching the sample. All these functions are performed in high-throughput for all raw FLIM datasets loaded in the tool.

The tool requires two inputs: the directory of the folder containing the raw FLIM datasets, and the directory of the constant background dataset. The subfolders for storing the outputs of the tool can be generated automatically within the raw dataset folder.

For processing the raw FLIM datasets, the constant background is first calculated as the mean pixel value from the constant background dataset and removed from the raw FLIM datasets. The multiple time-gated images of each of the constant background-subtracted FLIM datasets are then summed to generate an integrated intensity image. Subsequently, an interactive list for all the generated integrated intensity images is created and, upon selection, the corresponding image is displayed on the GUI as shown in Figure 3-21. As outlined in the protocol for characterising the photobleaching behaviours (Section 3.4.2), these integrated intensity images are used for creating the masks for excluding the dead cells, selecting the kinetochore foci, and evaluating the local TVB around each kinetochore focus.

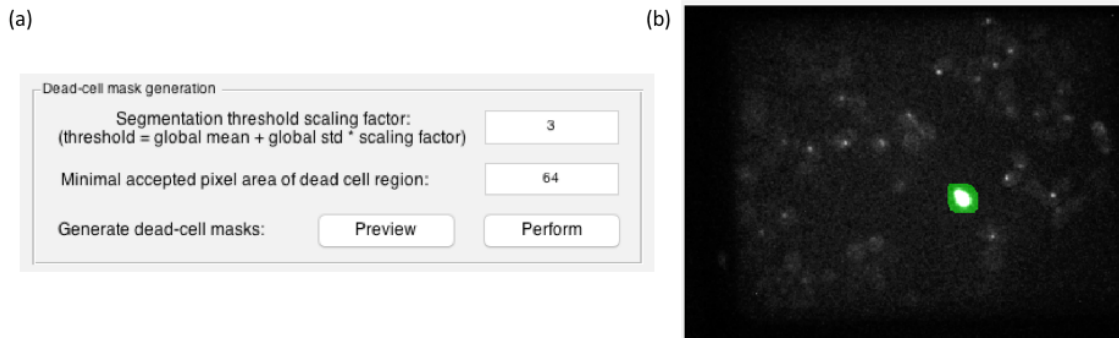


Figure 3-22 The generation of the dead-cell masks

(a) Exemplar settings for generating the dead-cell masks. (b) An exemplar dead-cell mask overlayed on an intensity image, showing the bright dead cell being picked out by the mask in green.

The generation of the dead-cell masks is based on the high brightness and the large size of a dead cell. As shown in Figure 3-22a, the tool requires the input of two parameters to perform this task. The first one is a scaling factor for the global standard deviation of the pixel values, i.e. the standard deviation of the pixel values from all the FLIM datasets for the imaged strain of interest. A threshold in the pixel value is set to the global mean pixel value plus the product of the global standard deviation and the scaling factor, so that only

regions with high pixel values will be selected after the thresholding, which may include both the dead cells and the kinetochore foci. These regions are then filtered by the second parameter, which is the minimal size in pixel number for which a region can be considered as a dead cell, noting that the dead cells are much larger than the kinetochore foci. Lastly, each segmented dead cell region is dilated by 6 pixels to cover a larger area, so that the areas proximal to the dead cells that may present above average noise are also excluded for the following analysis. Figure 3-22b shows an exemplar dead cell mask overlaying with the integrated intensity image, which can be displayed on the GUI as a feedback of the operation so that changes can be made to the two user-defined parameters until the best choice is found.

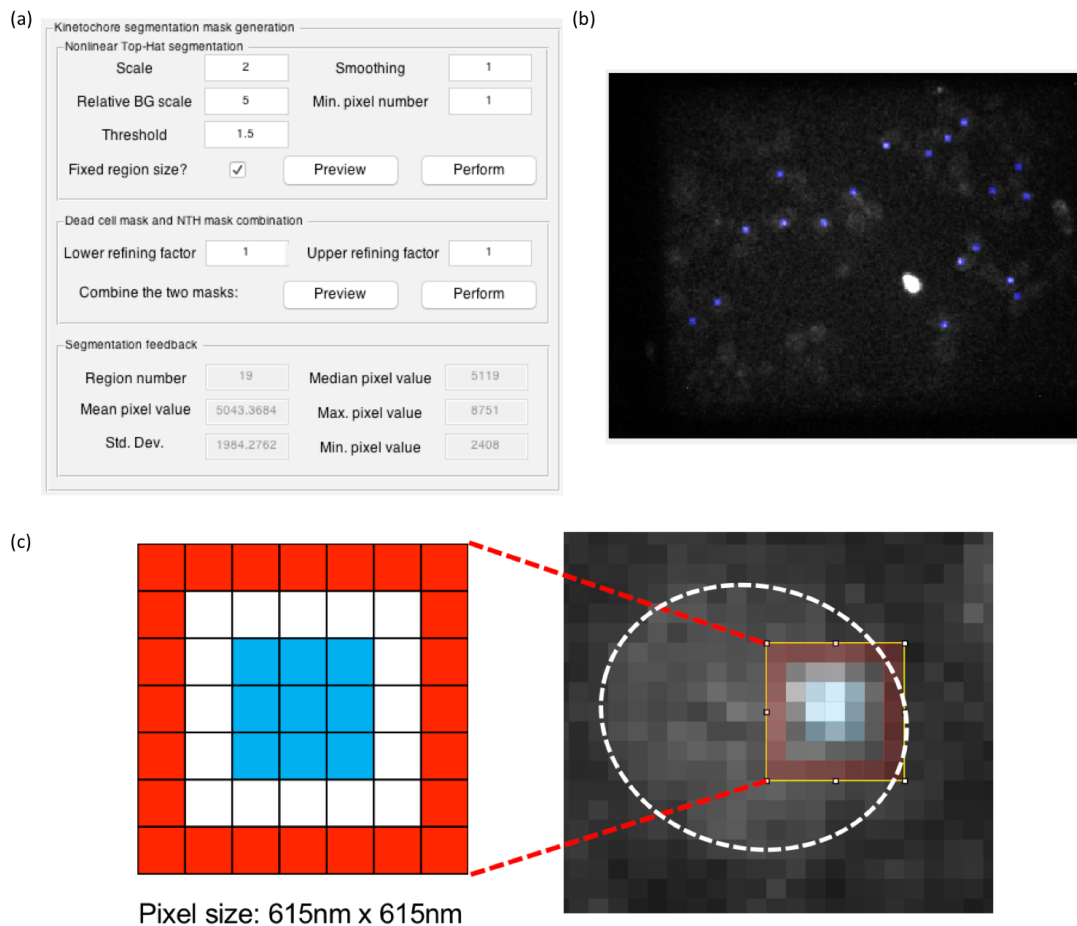


Figure 3-23 The generation of the kinetochore segmentation masks

(a) Exemplar settings for kinetochore segmentation. (b) An exemplar result of kinetochore segmentation, with the segmented kinetochore foci highlighted in blue. (c) The segmented 3x3 region for each kinetochore focus and the corresponding region for quantifying the local TVB background. Adapted from Ref.⁴³⁹.

The segmentation of the kinetochore foci (Figure 3-23) is generally based on the NTH algorithm, which first performs a pixel-wise transformation on the integrated intensity image to enhance the pixels that are bright themselves, have a bright close vicinity (set by the parameter “Scale” in Figure 3-23a), and have a dim far vicinity (set by the parameter “Relative BG scale” in Figure 3-23a), and then thresholds the resulting image by a user-defined value (set by the parameter “Threshold” in Figure 3-23a). This algorithm works well for picking out bright regions of a regular shape, and thus is ideal for segmenting the spot-like kinetochore foci. One issue with the NTH algorithm, however, is that the sizes of the segmented regions are sensitive to the manually selected threshold despite the use of the “Scale” parameter, which may introduce some inconsistencies when processing images from different yeast samples. To cope with this issue, I extended the NTH algorithm to pick out only the brightest pixel in each of the initially segmented regions. Following that, the segmented pixels are filtered by the dead cell mask to remove the pixels in the dead cells, and then further filtered to include only the pixels with values within a window defined by the mean value of the segmented pixels, the corresponding standard deviation, and two factors that scale the standard deviation to set the lower and the upper limits. Finally, the kinetochore segmentation masks are saved to the designated directory and displayed on the GUI with 1-pixel dilation to show clearly the coverage of the kinetochore foci (Figure 3-23b).

The last step is generating the TVB-subtracted FLIM datasets. For this purpose, the constant background-subtracted FLIM datasets and the kinetochore segmentation masks are needed. The first few time-gated images acquired during the pre-photobleaching period should first be removed from each constant background-subtracted FLIM dataset. Next, the resulting FLIM datasets are combined with the kinetochore segmentation masks to include only the fluorescence from the kinetochore foci. Note that, as shown in Figure 3-23c, each of the 1x1 segmented regions in the masks is dilated to a 3x3 region here, which corresponds to an area of $1.8\ \mu\text{m} \times 1.8\ \mu\text{m}$, which is much larger than the size of a kinetochore focus, so that even if the kinetochore focus was not completely stationary, the FP signal of interest can still be extracted. The local TVB for each segmented kinetochore region is subsequently calculated as the median pixel value of the pixels in a

7x7 hollow square with 1-pixel thickness around the kinetochore region in each temporal slice (Figure 3-23c), and this is then subtracted from the mean value of the pixels in the kinetochore region in each corresponding time-gated image. The final products of this step are FLIM datasets where, in each time-gated image, only the pixels in the 3x3 kinetochore regions have non-zero local-TVB-subtracted values (Figure 3-24). These TVB-subtracted FKIM datasets can be loaded into *FLIMfit* for lifetime analysis.

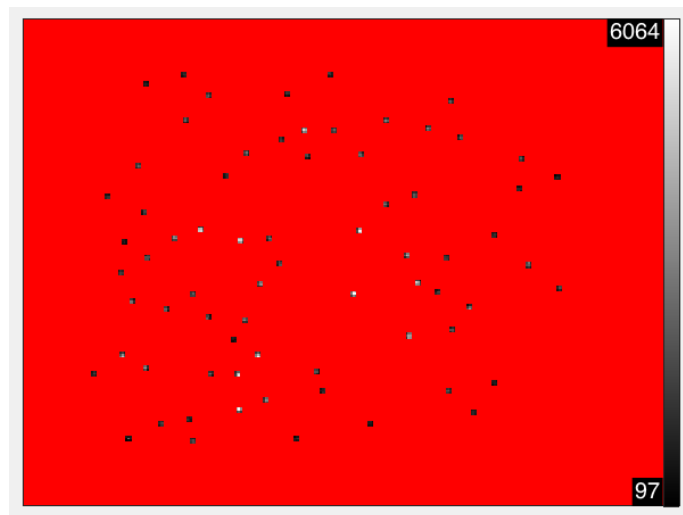


Figure 3-24 Screenshot of an exemplar TVB-subtracted FLIM dataset loaded into *FLIMfit* with cells expressing *Spc24p-GFP* and no *mCherry*

In a TVB-subtracted FLIM dataset, only pixels corresponding to the segmented kinetochore regions have non-zero pixel values. The pixel values for all other pixels are set to zero, so that when the dataset is loaded into *FLIMfit*, no additional step is needed for segmenting the kinetochore foci.

3.5.2 FLIM analysis using *FLIMfit*

As the TVB-subtracted datasets generated via the image processing tool are already segmented for the kinetochore foci and free from the constant background and the TVB, the analysis of these FLIM datasets is more straightforward than the generic workflow introduced in Section 3.1.3. This subsection describes the new workflow that I developed.

Firstly, although the fluorescence decay from the quenched fluorescein dye was measured as an alternative to the *scatter IRF*, a shift map for this fluorescence decay needs to be generated in the same way as for the *IRF shift map* (Section 3.1.3), i.e. utilising the Coumarin 6 FLIM dataset, to account for the iris effect of the GOI.

Next, the fluorescence lifetime of the Coumarin 6 dye should be fitted using the DFCM. To do this, the shift map for the quenched fluorescein decay should be loaded as the IRF, and the IRF type should be set to “Reference” to instruct *FLIMfit* to operate in DFCM mode. A global mono-exponential fit is then performed for the Coumarin 6 decay dataset, with the reference lifetime (of the quenched fluorescein dye) being globally fitted as well. If the fitted lifetime of the Coumarin 6 decay is as expected (~ 2.5 ns), the reference lifetime needed for the DFCM can be fixed to this fitted value and be used for the lifetime fitting of the fluorescence from the yeast cells.

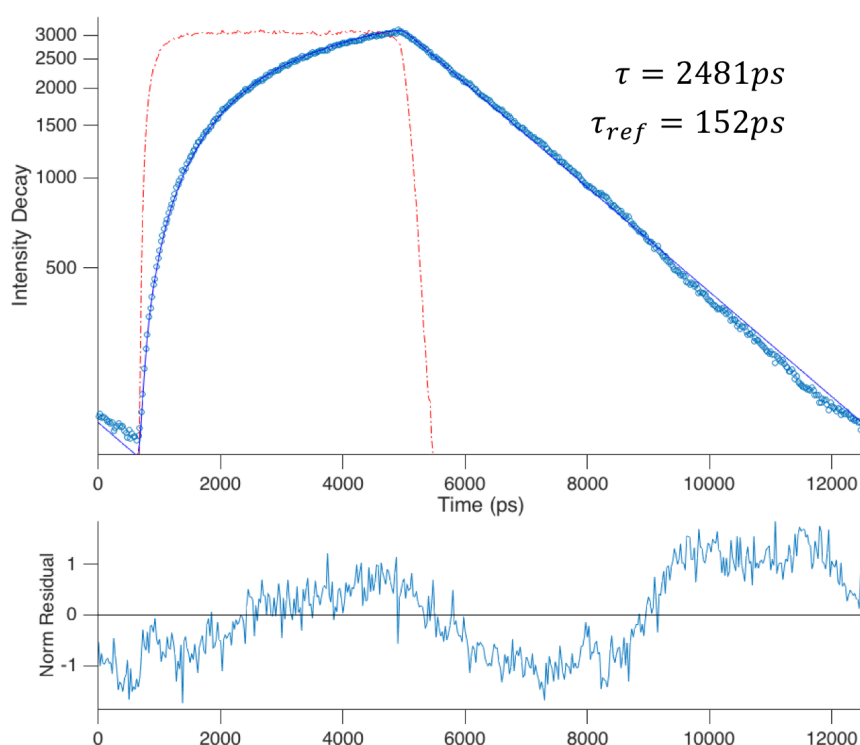


Figure 3-25 Lifetime fitting for the Coumarin 6 fluorescence decay using the DFCM and a fitted reference lifetime for the quenched fluorescein decay

The fluorescence lifetime of Coumarin 6 was globally fitted to be 2481 ps, whilst the reference lifetime of the quenched fluorescein solution was simultaneously fitted to be 152 ps. The fitted lifetime of Coumarin 6 is close to the expected value of ~ 2.5 ns, suggesting that the DFCM approach using the quenched fluorescein decay enables accurate lifetime fitting. The fitted reference lifetime of 152 ps were then applied to analyse the FLIM data from the yeast samples.

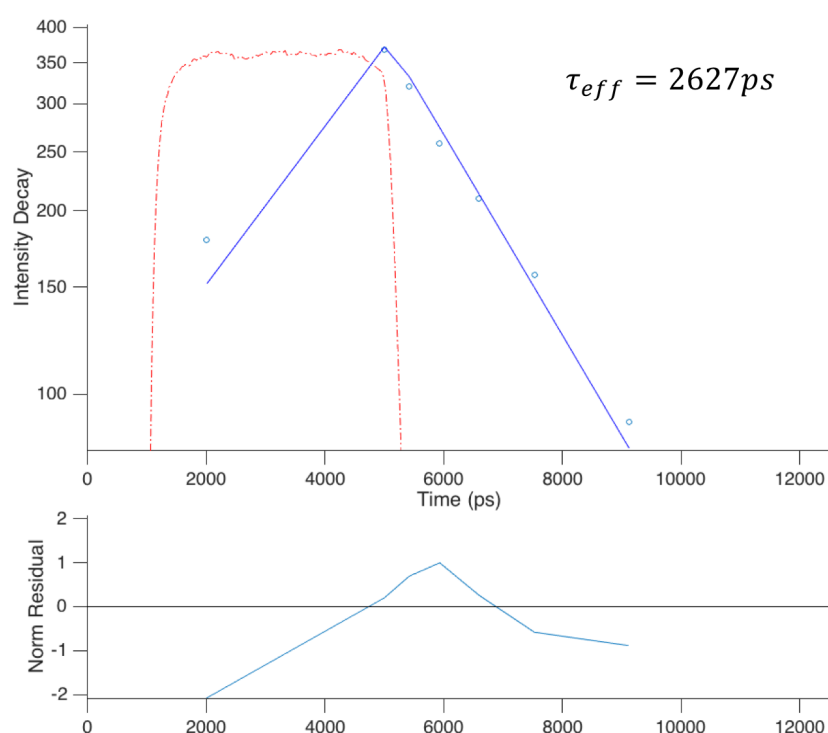


Figure 3-26 Fitting result of the fluorescence decay from the TVB-subtracted dataset shown in Figure 3-24 (with cells expressing Spc24p-GFP and no mCherry)

Using the DFCM and the TVB-subtracted dataset, the image-wise fluorescence lifetime of GFP in cells expressing Spc24p-GFP and no mCherry was fitted to be 2627 ps, close to the expected non-FRETing lifetime of GFP (~2.6 ns).

With the acquired reference lifetime, the TVB-subtracted FLIM datasets of the yeast samples can be analysed using the DFCM. Considering the relatively low number of the FPs at each kinetochore focus, and that the fluorescence from each kinetochore focus should be subject to some significant noise owing to the strong autofluorescence background and other complexities in the biology, etc., I decided to analyse the data using image-wise mono-exponential fitting with global analysis, i.e. to combine all the photons in one FOV for a robustly fitted effective lifetime, instead of performing pixel-wise fitting or fitting the data to a more complex decay model (both of which were tried, but did not improve the results – data not shown). For each strain in one experiment, there should be 45 fitted effective lifetime values, assuming that the FLIM datasets of all FOVs have been acquired with sufficient detected photons. Figure 3-26 shows an example of fitting the fluorescence decay from the TVB-subtracted FLIM dataset represented in Figure 3-24, where a large number of the heterozygous diploid cells expressing the relatively abundant

KP Spc24p labelled with the GFP donor were imaged. Consequently, the average fluorescence decay of all signals from the FOV was not too noisy, and the fitted lifetime was close to the expected value of the GFP donor (~2.6 ns).

3.5.3 The chosen statistical test for comparing the lifetime results

As discussed in the following two chapters, the FLIM HCA experiments performed for this thesis involved comparing the lifetime results from various pairs of yeast strains. In each pair, one strain was the query strain in which the presence of FRET was under investigation, while the other was the negative control for FRET that contained only the donor fluorophore but not the acceptor. The presence of FRET in the query strain would be supported if the query strain exhibited a donor fluorescence lifetime shorter than that of the negative control. For making such a comparison, a statistical test should be performed on two sets of lifetime results from the pair of strains. Based on the nature of the lifetime results, I chose to use the two-sided Wilcoxon rank-sum test⁴⁷³. The two-sided Wilcoxon rank-sum test is a non-parametric counterpart of the t-test for examining whether the two populations where two independent samples were acquired have the same distribution. Being a non-parametric test, it makes no assumption on the normality of the data and is also less sensitive to outliers, and therefore is more suitable for analysing the FLIM data in this project. It tests the null hypothesis that a randomly sampled value from one population is equally likely to be smaller or larger than a randomly sampled value from the other population by mixing all the values in the two independent samples together, ranking them in an ascending order, assigning a rank to each value (starting from 1 for the smallest value), and then summing the ranks of the values belonging to each sample, respectively, resulting in a rank sum for each sample. When performing the test by hand, one can then refer to the Wilcoxon rank-sum table with the rank sum for the first sample and the sizes of the two samples to find the corresponding p -value, i.e. the probability that the null hypothesis is true when such a rank sum is acquired. Alternatively, one can perform the test and acquire a p -value using a software package on a computer; for the tests performed in this project I used the *ranksum* function in MATLAB. If the acquired p -value is smaller than the chosen level of significance α (for this project α was chosen to be 0.05), the null hypothesis should be rejected, and it is supported that the two distributions of the two populations are significantly different. I

also employed the Bonferroni correction to correct the level of significance α when a negative control was compared with multiple query strains⁴⁷⁴. With the Bonferroni correction, when n statistical comparisons are made, the level of significance α would be corrected to $1/n$ of the original value (i.e. $\alpha = \frac{0.05}{n}$ for this project) before being compared with the p -value(s). Correcting α when multiple comparisons are made is necessary for controlling the family-wise error rate, i.e. the probability of getting a Type I error (i.e. incorrectly rejecting the null hypothesis). While there are many other methods for correcting α , the Bonferroni correction was chosen for this project as it is one of the most stringent ones and is straightforward to perform.

In order to examine the reproducibility of the FLIM results, as well as to acquire large sample sizes for more robust and informative statistical comparisons, each yeast strain used for the FLIM experiments in this thesis were imaged in at least two separate experiments. In principle, the results for the same strain from multiple experiments may be directly combined prior to statistical testing; however, the combination process was not trivial and required careful examination. This is described in detail in the following two chapters together with the corresponding lifetime data.

3.6 Summary and discussion

This chapter describes the *openFLIM-HCA* platform developed by the Photonics group at Imperial College London and outlines how I developed it for performing FLIM HCA on the *in vivo* interactions between FP-labelled endogenous proteins at budding yeast kinetochores.

As discussed in the following two chapters, this new FLIM HCA methodology enabled small changes in the donor fluorescence lifetimes between the query strains and the negative controls to be discriminated while imaging multiple yeast strains (up to 10) in a single experiment on a reasonable time scale (~ 33 min per strain for the mTq2FP experiments and ~ 24 min per strain for the GFP experiments). Nevertheless, I believe there is still room for improvements in terms of both the precision of lifetime-measurement and the imaging speed. While these two aspects appear to be contradictory,

as the S/N needs to be higher for improving the precision but tends to be lower when imaging faster, it should be possible to achieve both by using a more suitable donor FP. The donor FP should be chosen based on its brightness and also its photobleaching and spectral properties, so as to withstand a higher excitation power and to better distinguish its fluorescence from the cellular autofluorescence that is mainly in the blue and green part of the visible spectrum. The density of cells to be imaged may also be better controlled, so that each FOV can contain sufficient cells, but not too many to end up with reduced cell fitness and elevated autofluorescence. It may also be advantageous to sample the fluorescence decays with fewer time gates since the FLIM data is fitted to a mono-exponential decay model, hence improving the imaging speed, while retaining the lifetime discrimination. It may also be possible to improve on the segmentation algorithm, e.g. using a machine-learning-based approach to more faithfully extract the signals from the FPs and exclude the dead cells, and thereby improve the accuracy and precision of the fluorescence lifetime measurements. Overall, many aspects of strain design, sample preparation, data acquisition and data analysis may be optimised. However, due to the time constraints of my PhD project, I judged that the methodology described here would be able to validate the use of FLIM FRET for budding yeast kinetochores.

Chapter 4. Application of *openFLIM-HCA* to study *in vivo* interactions between FP-labelled endogenous kinetochore proteins

This chapter reports the demonstration of the capability of the *openFLIM-HCA* platform to study interactions between endogenous kinetochore proteins (KPs) that are labelled by fluorescent proteins (FPs) in live budding yeast cells using FRET, which can provide information about the kinetochore architecture on the nanometre scale. The chapter begins with a brief overview of the importance of the budding yeast kinetochore architecture. This is followed by a description of the FLIM HCA results, including how the raw fluorescence lifetime data from multiple experiments were combined and filtered before being subject to statistical comparisons. The final section then summarises the results and discusses how to improve the experimental design in order to better reveal protein-protein interactions (PPIs) at the budding yeast kinetochore using FLIM HCA. Part of the work presented here has been published⁴³⁹.

4.1 Introduction

The kinetochore of the budding yeast *Saccharomyces cerevisiae* is an excellent example of how the notion “*form follows function*” applies in biology. As reviewed in Section 1.2, the multi-layer architecture of the budding yeast kinetochore, comprising >60 types of constitutively-presented kinetochore proteins (KPs) as well as transiently-associated proteins, elegantly facilitates the kinetochore’s key functions in ensuring faithful chromosome segregation^{475–477}. The current understanding of the functions of both the budding yeast kinetochore and its human homolog has been largely gained from studies on the kinetochore architecture, including those on the structures of the constituent KPs and subcomplexes (e.g. Refs.^{231,243,268,270,313,478}) as well as those on the assembly of these KPs and subcomplexes into an intact kinetochore (e.g. Refs.^{230,240,242,317}). As reviewed in Section 1.2.3, a variety of experimental methods have contributed to these works, ranging

from *in vitro* methods like immunoprecipitation and mass spectroscopy to *in vivo* fluorescence microscopy.

In this chapter I present my work applying the FLIM HCA methodology described in Chapter 3 to identify close ($< \sim 10$ nm) interactions between FP-labelled endogenous KPs in live cells using FRET. The results of this work showed that, despite the challenges described in Section 3.2, the FLIM HCA methodology was able to quantify the donor fluorescence lifetime even when the donor was tagged to an endogenous KP with a copy number of only ~ 2 per kinetochore under an average labelling efficiency of 50%. The measurement of FRET between fluorophores labelling KPs that are known to form dimers validated the capability of the method for identifying PPIs. This work not only demonstrates that the FLIM HCA methodology can serve as a useful tool for *in vivo* studies on the kinetochore architecture, but also indicates that the method may be utilised to identify the interacting partners of regulatory proteins, e.g. Rdh54p, at the kinetochore for better understanding the molecular mechanisms governing the kinetochore's functions (see Chapter 5).

4.2 Results

4.2.1 Strains and plasmids constructed for the experiments

For the FLIM FRET experiments reported in this chapter, I constructed 16 diploid yeast strains expressing KPs labelled with the mTurquoise2 fluorescent protein (mTq2FP) and/or the yellow fluorescent protein (YFP). These strains were created by transformation of wild-type yeast with linear targeting constructs that fuse the sequence encoding the fluorophore in frame with the endogenous KP gene. mTq2FP, a monomeric variant of the cyan fluorescent protein (CFP), was chosen to be the donor fluorescent protein for FRET because of the favourable characteristics it exhibits, e.g. high brightness, high photostability, and a mono-exponential intrinsic fluorescence lifetime (~ 4 ns)⁴⁵³, which are all desirable for FLIM FRET assays. YFP was chosen as the FRET acceptor because it has the appropriate spectral properties for FRET with mTq2FP. In addition, I also constructed 4 yeast plasmids under a constitutively active *CUP1* promoter⁴³⁵, each

expressing a KP tandemly tagged by mTq2FP and YFP, and transformed them into unlabelled diploid cells, respectively. Lists of these strains and plasmids are provided in Table 2-2 and Table 2-3.

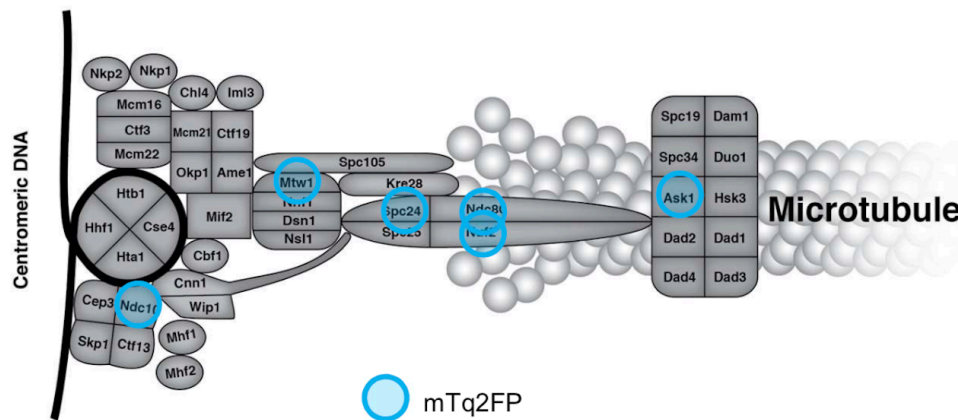


Figure 4-1 Kinetochore proteins selected for being respectively labelled by the FRET donor mTq2FP

The approximate localisations of the 6 selected KPs (Ndc10p, Mtw1p, Spc24p, Ndc80p, Nuf2p, Ask1p) along the kinetochore-microtubule axis are highlighted on a schematic of the budding yeast kinetochore. Adapted from Ref.⁴³⁹.

I categorised the strains and the plasmids above into 6 groups, based on which of the 6 selected KPs (Ndc10p, Mtw1p, Spc24p, Ndc80p, Nuf2p, Ask1p) was tagged with the donor mTq2FP. The 6 KPs were selected to represent different locations along the kinetochore-microtubule axis. In each group there was at least one query strain and one negative control for FRET. The query strain was dual-labelled with both mTq2FP and YFP for FRET measurements, where the genes of the two fluorophores were either fused to the genes of two different KPs (e.g. expressing Ndc10p-mTq2FP and Ask1p-YFP) or fused to the two alleles of the same KP gene (e.g. expressing Ndc10p-mTq2FP and Ndc10p-YFP), while the negative control was a donor-only strain expressing no YFP (and therefore no FRET acceptor for mTq2FP). Additionally, for 4 of the 6 groups there was also a positive control for FRET in each group, which was a diploid strain containing the aforementioned plasmid expressing the chosen KP tandemly tagged by mTq2FP and YFP. As the mTq2FP and the YFP here were linked by an 8-amino-acid flexible linker (four repeats of glycine-serine), they should be close enough for FRET to always occur.

These three types of strains in the Spc24p-mTq2FP group are shown in Figure 4-2 as an example. By comparing the donor fluorescence lifetimes measured from a positive control and from a negative control, it is possible to assess whether the FLIM HCA methodology can detect FRET at the yeast kinetochore. Similarly, comparing the donor fluorescence lifetimes measured from a query strain and a negative control could provide information about the kinetochore structure. Furthermore, the comparison between the FLIM results and the previous knowledge of the kinetochore structure could be used to confirm the FLIM HCA methodology can reveal close proximity (and therefore interactions) between FP-labelled endogenous proteins at the kinetochores of live budding yeast cells. Table 4-1 summarises all these strains involved in the FLIM experiments.

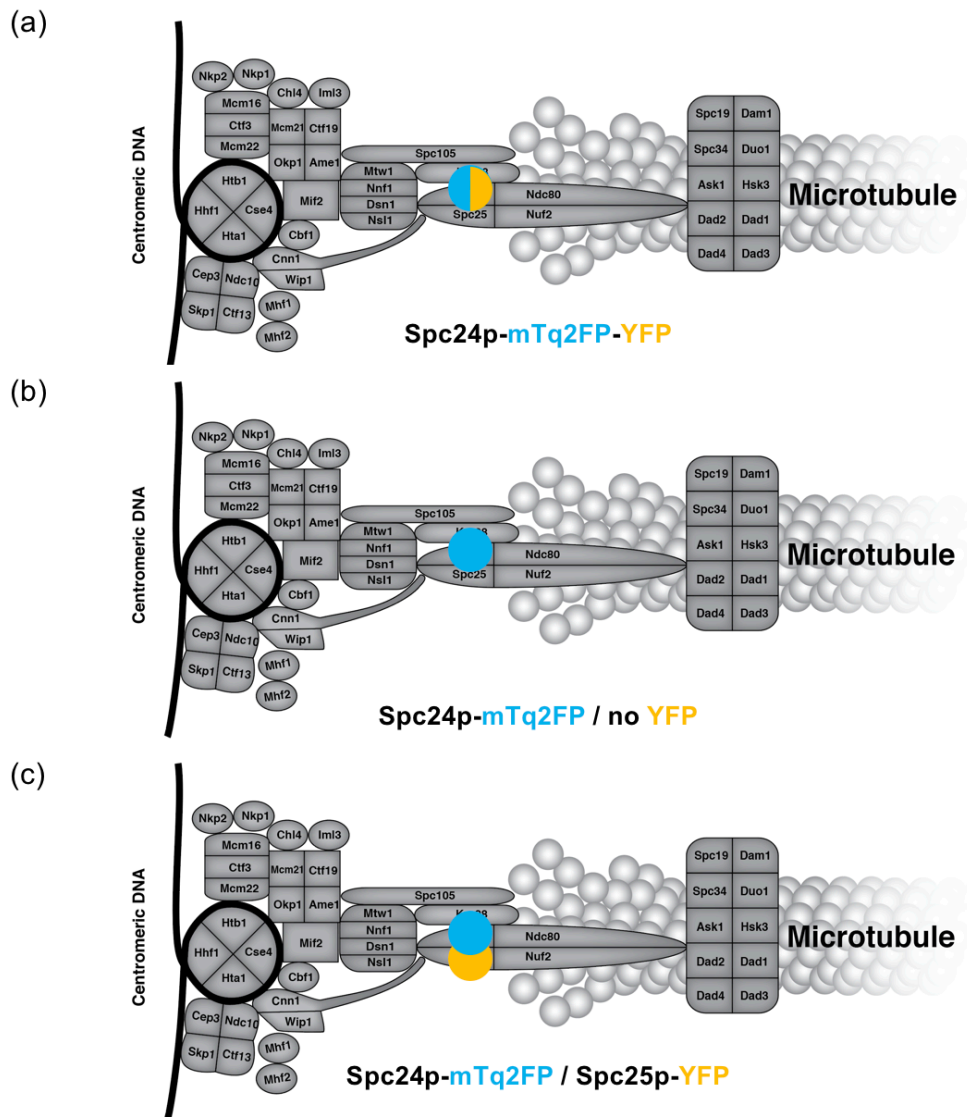


Figure 4-2 The three types of strains in the Spc24p-mTq2FP group

(a) The positive control strain with Spc24p tandemly tagged by the FRET donor mTq2FP and the FRET acceptor YFP. (b) The negative control strain with only Spc24p-mTq2FP and no YFP. (c) The query strain where Spc24p is tagged by mTq2FP whilst Spc25p is tagged by YFP. Adapted from Ref.⁴³⁹.

Table 4-1 Strains imaged in the FLIM experiments reported in this chapter

Group	Name	Strain type	Relevant genotype
Ndc10p-mTq2FP	PT208 + pHT449	Wild-type + positive control plasmid	<i>NDC10-mTq2FP-YFP</i>
	PT197	Query	<i>NDC10-mTq2FP/NDC10-YFP</i>

	PT270	Query	<i>NDC10-mTq2FP/NDC10 ASK1-YFP/ASK1-YFP</i>
	PT261	Negative control	<i>NDC10-mTq2FP/NDC10 No YFP</i>
Mtw1p- mTq2FP	PT208 + pHT448	Wild-type + positive control plasmid	<i>MTW1-mTq2FP-YFP</i>
	PT194	Query	<i>MTW1-mTq2FP/MTW1-YFP</i>
	PT268	Query	<i>MTW1-mTq2FP/MTW1 CTF19-YFP/CTF19-YFP</i>
	PT269	Query	<i>MTW1-mTq2FP/MTW1 NDC10-YFP/NDC10-YFP</i>
	PT260	Negative control	<i>MTW1-mTq2FP/MTW1 No YFP</i>
Spc24p- mTq2FP	PT208 + pHT522	Wild-type + positive control plasmid	<i>SPC24-mTq2FP-YFP</i>
	PT267	Query	<i>SPC24-mTq2FP/SPC24 SPC25-YFP/SPC25-YFP</i>
	PT244	Negative control	<i>SPC24-mTq2FP/SPC24 No YFP</i>
Nuf2p- mTq2FP	PT276	Query	<i>NUF2-mTq2FP/NUF2 ASK1-YFP/ASK1</i>
	PT279	Negative control	<i>NUF2-mTq2FP/NUF2 No YFP</i>
Ndc80p- mTq2FP	PT273	Query	<i>NDC80-mTq2FP/NDC80 NUF2-YFP/NUF2</i>
	PT278	Negative control	<i>NDC80-mTq2FP/NDC80 No YFP</i>
Ask1p- mTq2FP	PT208 + pHT446	Wild-type + positive control plasmid	<i>ASK1-mTq2FP-YFP</i>
	PT192	Query	<i>ASK1-mTq2FP/ASK11-YFP</i>
	PT266	Query	<i>ASK1-mTq2FP/ASK1 NDC10-YFP/NDC10-YFP</i>
	PT258	Negative control	<i>ASK1-mTq2FP/ASK1 No YFP</i>

Notably, as shown later in this chapter, mTq2FP presented slightly different non-FRETing lifetimes in the 6 donor-only negative controls. As mTq2FP was tagged to different KPs in these strains, the lifetime differences could be due to the different local molecular environment. Consequently, donor lifetimes were only compared between two strains in the same group, where the FRET donor mTq2FP was labelling the same KP.

In order to increase the likelihood of the donor fluorophores undergoing FRET, I constructed all but two of the dual-labelled query strains to be heterozygously-tagged for the donor mTq2FP-tagged KP gene (with one wild-type allele and one allele fused to the mTq2FP gene) but homozygously tagged for the acceptor YFP-tagged KP gene. The two exceptions are the query strains in the Ndc80p-mTq2FP group and the Nuf2p-mTq2FP group (highlighted in red in Table 4-1). These two strains failed to produce spores, possibly because the FP-tagged KPs affected meiosis, and therefore could not be engineered by further sporulation and crossing for making them homozygous for the acceptor FP-tagged KP genes. Therefore, these strains were heterozygous for both the FP-tagged KP genes when being imaged in the FLIM experiments. The impact of this on the FLIM results is discussed in Section 4.3. Briefly, the number of the acceptor YFP molecules in these heterozygous/homozygous strains should be approximately twice as many as that in a diploid strain that is heterozygous for both donor and acceptor FP-tagged KP genes; this should increase the number of mTq2FP donors undergoing FRET when the two KPs being studied are in close proximity. The overall effect of this increased number of FRETing mTq2FPs should be an enhanced reduction in the effective donor fluorescence lifetime obtained from a mono-exponential fit.

To create these strains, I followed these steps:

- mating one haploid *MAT α* strain expressing the mTq2FP-tagged KP with another haploid *MAT a* strain expressing the YFP-tagged KP;
- selecting for the resulting dual-labelled diploid strain (which was heterozygous for both the FP-tagged KP genes) using the marker genes linked to the fluorophores (one encoding hygromycin resistance, the other histidine prototrophy);

- sporulating the diploid cells and then selecting for the *MAT α* haploid spore that contained both FP-tagged KP genes;
- mating the selected *MAT α* strain with the earlier YFP-containing *MAT a* strain;
- selecting for the desired diploid strain using auxotrophic markers (*trp1-1* and *lys2 Δ*).

4.2.2 Combining the FLIM data from multiple experiments

I performed the FLIM HCA experiments on the aforementioned yeast strains and the corresponding lifetime analysis for each strain in each experiment following the method described in Chapter 4. In each experiment, 45 FOVs were taken for each strain (from 3 replicate wells with 15 FOVs per well) and no more than 10 strains were imaged. As each FOV required ~45 s for image acquisition, the whole automated acquisition took <6 hr.

In order to acquire a larger sample size for averaging over biological and experimental noise, each strain was imaged in 2 - 4 separate multiwell plate experiments on different days. Before combining the lifetime data for each strain from these multiple experiments, I needed to consider whether there may be any systematic bias impacting data collected on different days since I noted that the lifetimes measured from each negative control strain could vary between experiments. If the variations in the non-FRETing lifetime values for each strain are systematic biases, e.g. due to instrumentation drift, then I would need to correct for this using a normalisation procedure. However, if the variations are random noise, then I can directly combine the data.

To examine whether there were some significant systematic biases among the lifetime values measured from separate experiments, I globally fitted the FLIM datasets for each strain from each experiment to a monoexponential decay model, i.e. using all the photons from the ~45 FOVs to obtain a single lifetime value with the maximum precision, and compared the lifetime values from the strains in one experiment with those from an experiment performed on another day.

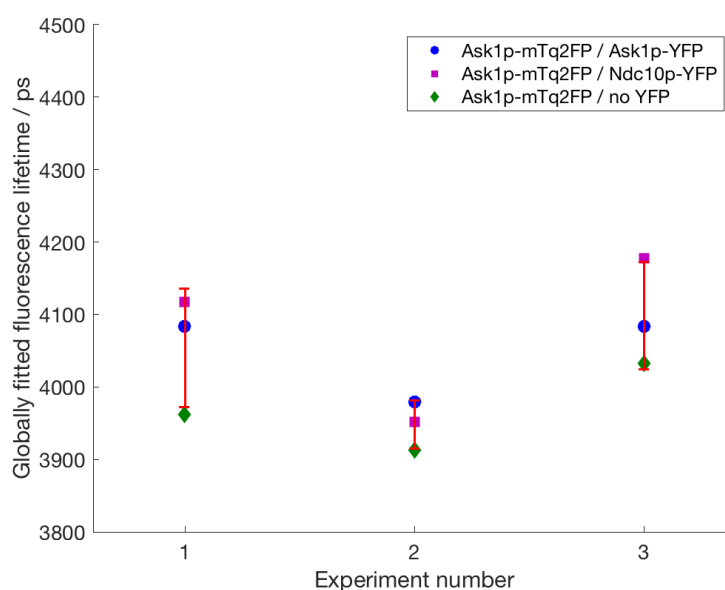


Figure 4-3 Globally fitted donor fluorescence lifetimes for the 3 non-FRETing strains in the Ask1p-mTq2FP group from 3 separate experiments

The variation of the measured fluorescence lifetimes for each strain across 3 separate experiments is comparable to the variation of the non-FRETing lifetimes for 3 strains in each experiment, suggesting that the former variation is more likely caused by random noise instead of any systematic bias.

Figure 4-3 plots the globally fitted lifetime values for 3 strains in the Ask1p-mTq2FP group from 3 separate experiments that did not undergo FRET. Since the donors in all these strains were tagged to the same KP and they were not undergoing FRET with the acceptors, they should exhibit the same non-FRETing fluorescence lifetime. As shown in Figure 4-3, although the lifetime results for each strain exhibited some variations across the 3 experiments within a range of ~200 ps, these variations seemed to indicate the effect of random noise rather than any systematic bias, since similar (~200 ps) levels of variation were seen for the non-FRETing lifetime values within a single multiwell plate experiment (denoted by the error bars that reported the standard deviations). Similar results were seen for strains in the other groups. Therefore, the lifetime results from multiple experiments for each strain were combined directly to make the box plots shown in the later subsection of this chapter (Figure 4-6 and Figure 4-7).

4.2.3 Filtering the image-wise FLIM results based on the total signal level in each FOV

I noted that the numbers of the segmented kinetochore foci in the acquired FLIM images (where each kinetochore focus corresponds to a square region of 9 pixels) exhibited considerable variation, with a mean pixel number of 91 for each FOV and a standard deviation of 61. This variation in the segmented foci is a consequence of the variation in the numbers of cells in different FOVs.

Despite the actions that had been taken to maintain similar cell culture densities for each yeast strain among multiple experiments, e.g. by ensuring that in each experiment the same amount of overnight stationary phase culture was diluted in fresh media and the resulting new culture was incubated for roughly the same amount of time before being transported for FLIM experiments, significant variation in cell density between FOVs was observed. This could be due to several factors, including how long the cell cultures were incubated after the final dilution before imaging (which was affected by the time taken for transporting the samples from the yeast lab to the FLIM lab; the time taken to set up the FLIM instrument, and the positions of the cultures in the 96-well plates - the overall acquisition time for the 96-well plate could be up to 3 hours, which is long enough for the cells to divide twice). The number of visible foci in each FOV could also be affected by how well the optical components in the FLIM instrument were aligned (which would mainly affect the homogeneity of the excitation power across the FOV, hence would cause kinetochore foci towards the edges of the FOV to be dimmer). With the excitation power maintained at the same level ($\sim 200 \mu\text{W}$ measured at the microscope objective lens's focal plane), the variation in the numbers of segmented kinetochore foci in different FOVs led to variation in the total signal, i.e. in the numbers of photons detected from these FOVs. When performing image-wise mono-exponential fitting, all photons from one FOV contribute to the measured fluorescence decay to be fitted. FOVs containing fewer segmented kinetochore regions, and therefore fewer photons, would result in fluorescence decay data with lower signal-to-noise ratio (S/N), and therefore the fitted lifetimes would be less precise (exhibiting a wider distribution). As wide lifetime distributions are undesirable for making statistical comparisons, I decided to investigate the variation in the fitted lifetimes as a function of total signal in more detail.

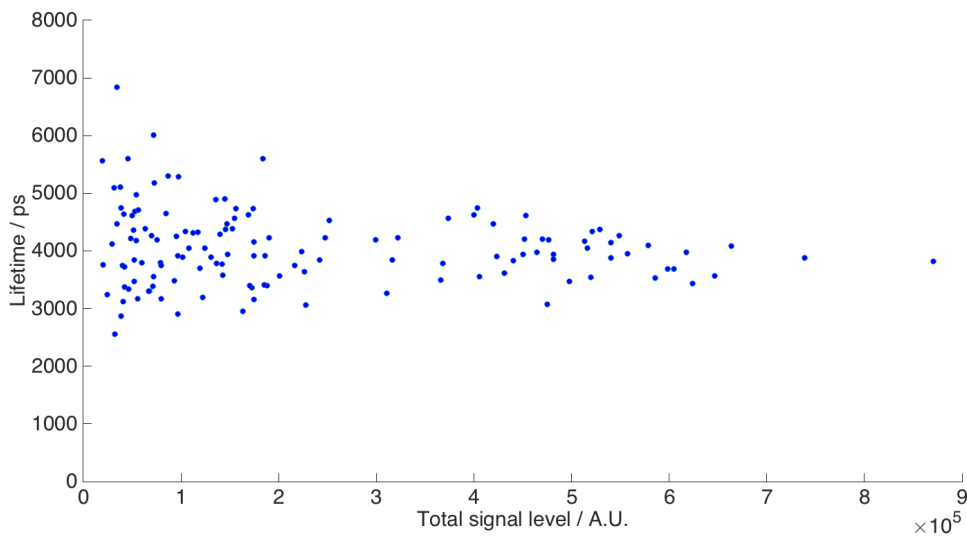


Figure 4-4 Distributions of the fitted image-wise donor fluorescence lifetime against the total signal level in each FOV for the strain expressing Ask1p-mTq2FP and no YFP

The wider lifetime distributions and the seemingly more data points with lifetime values larger than 4 ns at lower total signal levels suggest that there are FOVs with insufficient photon counts for precise and accurate lifetime determination.

I first chose the datasets for the negative control strain in the Ask1p-mTq2FP group (expressing Ask1p-mTq2FP and no YFP) and plotted the fitted image-wise donor fluorescence lifetime against the total signal level for each FOV, as shown in Figure 4-4. Here the total signal level was calculated as the product of the total segmented pixel number and the average digital value of the segmented pixels and was in an arbitrary unit of “digital number”. As the total signal level should be proportional to the product of the detected photon number and the GOI gain, with the GOI gain voltage set to the same value (850 V) for all experiments the total signal level should reflect the detected photon number in each FOV. From the figure, it is apparent that the lifetime values for FOVs with lower total signal levels have wider distributions than those for FOVs with higher total signal levels. It can also be seen that at very low total signal levels, the fitted lifetimes were slightly biased to higher values (compared with the expected non-FRETing lifetime of ~4 ns). Overall, these results suggested that for these particular FLIM datasets there were some FOVs with too few photons for precisely and accurately revealing the true donor fluorescence lifetime.

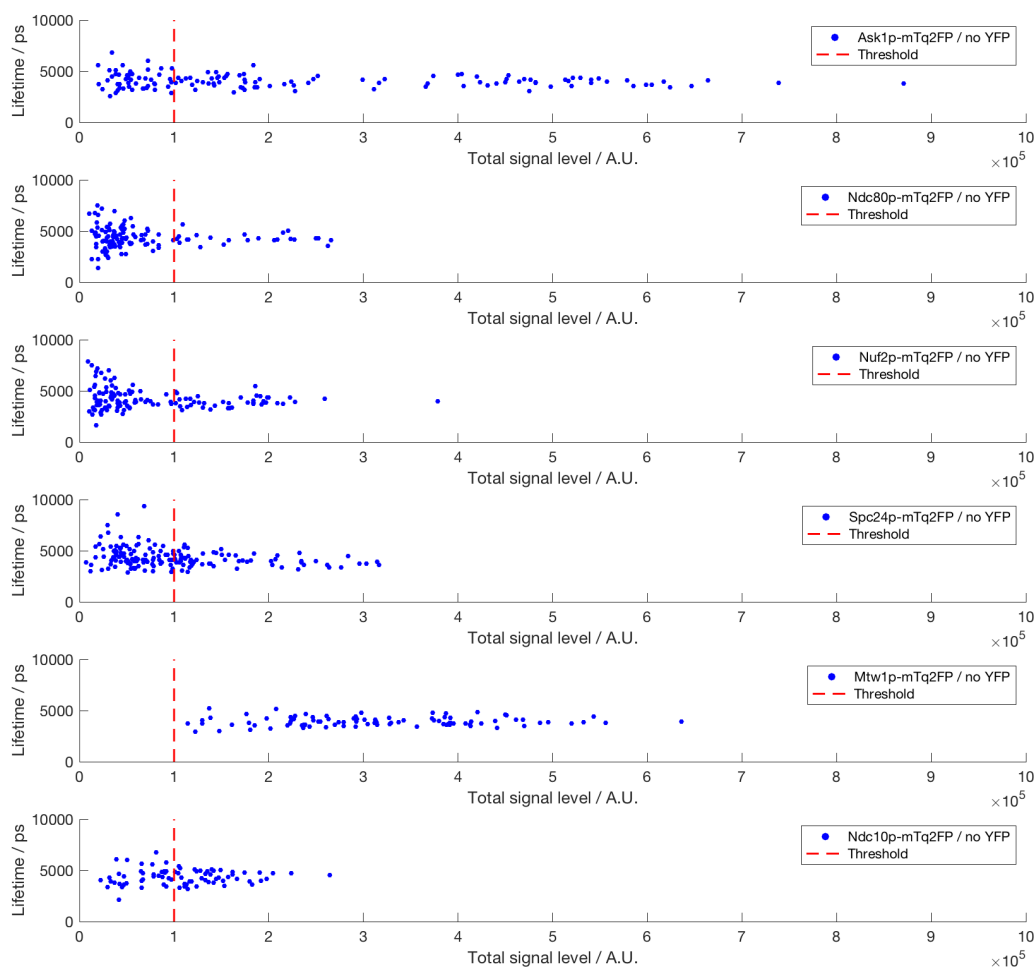


Figure 4-5 Distributions of the fitted image-wise donor fluorescence lifetime against the total signal level in each FOV for all 6 negative control strains

The fitted image-wise donor fluorescence lifetimes for all the 6 negative control strains exhibited a similar total signal level-dependent pattern. A threshold for the total signal level was consequently set to 1×10^5 digital number for excluding FOVs with insufficient photon counts from the following statistical comparisons.

To see if a similar effect was present in the FLIM results for other strains, I made several additional plots of the relationship between the total signal level and the fitted lifetime for all the 6 negative control strains listed in Table 4-1, as shown in Figure 4-5. It is manifest that all the distributions exhibited the same trend, being wider at low signal levels and getting narrower with the signal levels increasing. This trend is expected due to the effect of the shot noise in the FLIM system: the uncertainty of the fitted lifetime should be inversely proportional to the square root of the number of detected photons, hence the higher the total signal level in a FOV the better the precision for the image-

wise lifetime results. It also seems that, similar to the case for the strain with Ask1p-mTq2FP and no YFP (Figure 4-4), for some of the other negative control strains the fitted lifetimes at low signal levels were slightly biased to higher values (compared with the expected value of ~ 4 ns). Establishing the exact cause of these biases needs further investigation; but it is most likely caused by slight over or under compensation for TVB (from the cytoplasm and cell culture medium) by the process outlined in Section 3.5.1, as any errors in TVB subtraction will affect the lifetime measured for dim kinetochores more than for bright kinetochores.

While normally it is desirable to include the results from all the FOVs for making statistical inference, for the FLIM datasets presented above this may not be true, owing to the higher uncertainties and the potential biases in the fitted fluorescence lifetimes for the FOVs with lower signal levels. Consequently, I decided to filter the datasets by setting a global threshold for the total signal level, which was chosen to be 1×10^5 digital number as shown in Figure 4-5 (denoted as the dashed red line in each plot), and excluding all FOVs whose total signals levels were below that threshold. After filtering out those data of high-variability, statistical comparisons were then performed for the remaining lifetime results, as reported in the following subsections.

4.2.4 All positive controls exhibited significantly reduced donor fluorescence lifetimes

For each of the 4 positive control strains (for the Spc24p-mTq2FP, the Ndc10p-mTq2FP, the Mtw1p-mTq2FP, and the Ask1p-mTq2FP groups, respectively), I compared the measured donor fluorescence lifetime with that measured from the corresponding donor-only negative control strain. As shown in the box plots in Figure 4-6a, Figure 4-7a, Figure 4-7b, and Figure 4-7d, in each case the donor fluorescence lifetime measured from the positive control was significantly shorter than that from the negative control under the chosen statistical test (the two-sided Wilcoxon rank-sum test with Bonferroni correction for multiple comparisons; see Section 3.5.3 for details). The results demonstrate that, by measuring the donor fluorescence lifetime, the FLIM HCA method is capable of detecting FRET between closely located donor and acceptor fluorophores clustered at the compact budding yeast kinetochores.

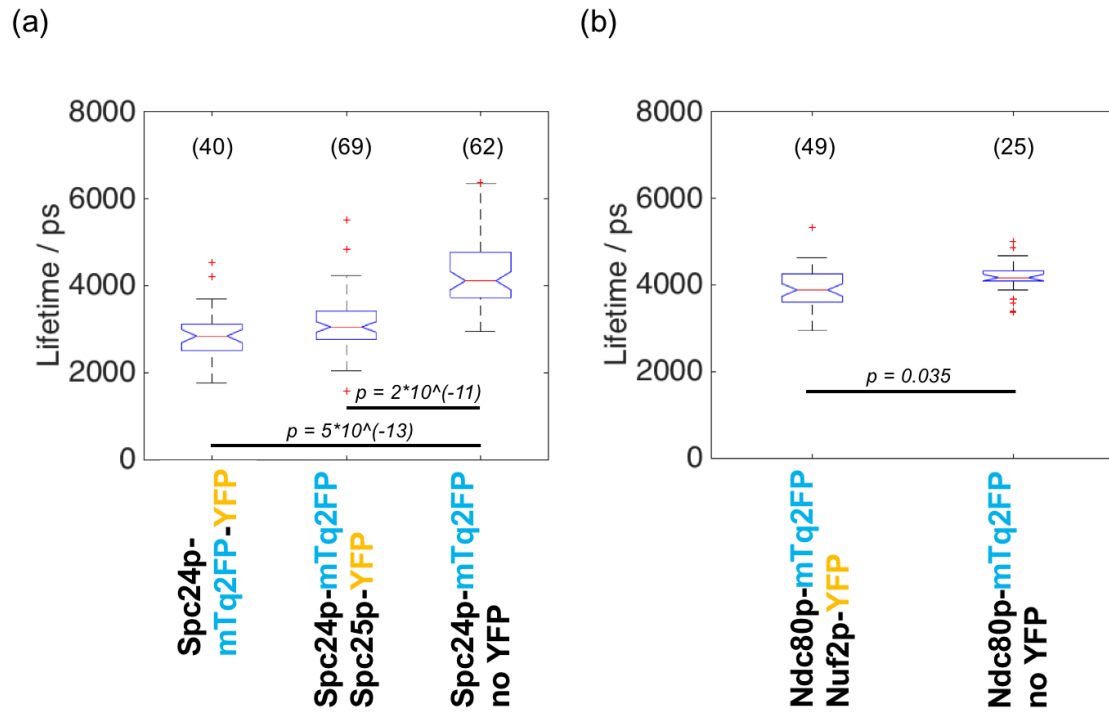


Figure 4-6 FLIM results for the strains in the (a) Spc24p-mTq2FP and (b) Ndc80p-mTq2FP groups

Each box plot shows the median value (bar) and the quartiles (box) of the fitted image-wise donor fluorescence lifetime values. The notches indicate the 95% confidence interval of the median. The whiskers correspond to the minimum of the data range or $1.5 \times$ the interquartile range. Extreme data points are marked as outliers. The number of FOVs analyzed for each strain is shown in parentheses above the corresponding box. The statistical test performed was the two-sided Wilcoxon rank-sum test. A Bonferroni correction was performed to correct the α value for multiple comparisons in each group. P-values smaller than the corrected α values are shown in the box plots. Adapted from Ref.⁴³⁹.

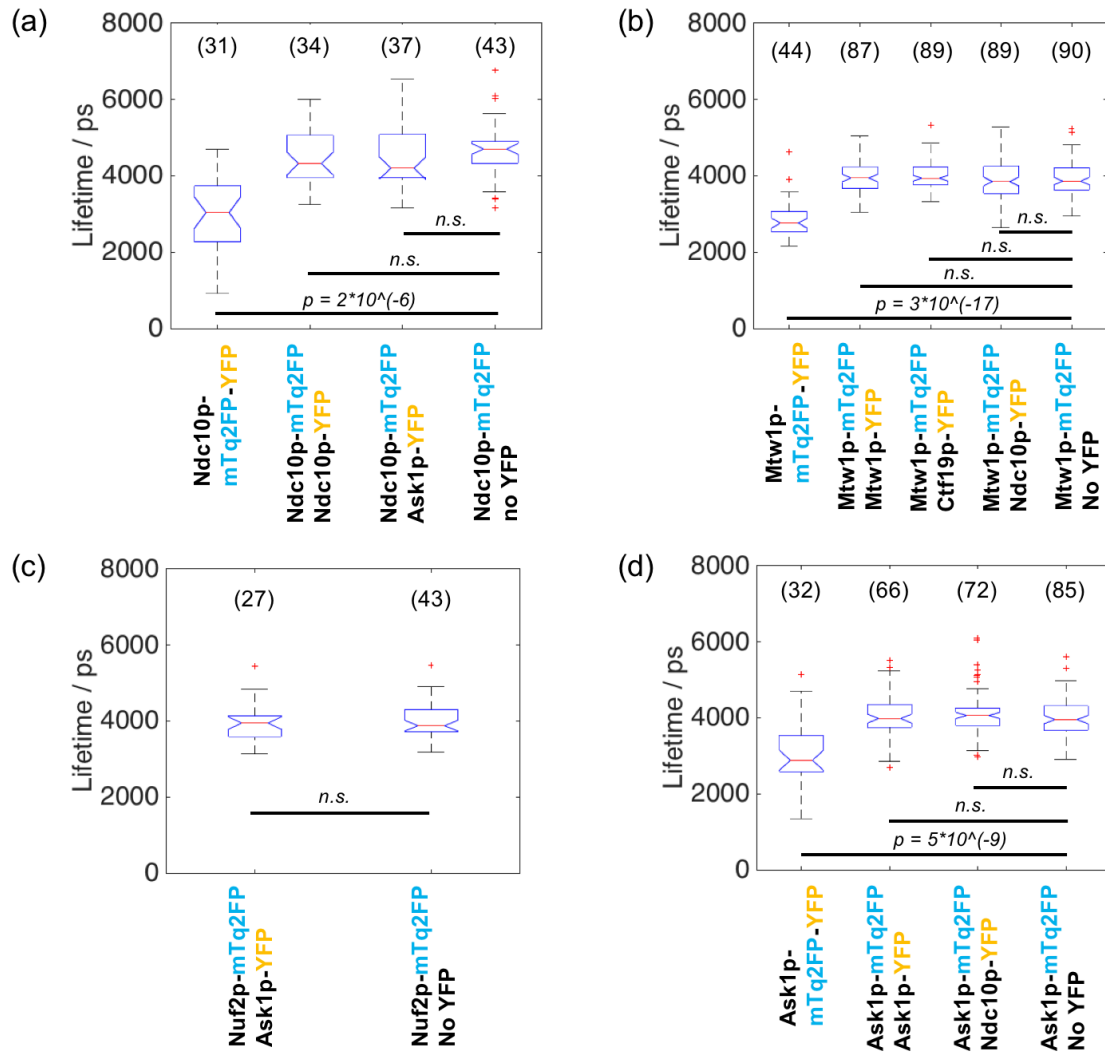


Figure 4-7 FLIM results for the strains in the (a) Ndc10p-mTq2FP, (b) Mtw1p-mTq2FP, (c) Nuf2p-mTq2FP, and (d) Ask1p-mTq2FP groups

None of the query strains in these groups showed a significantly different lifetime value compared with their corresponding donor-only negative controls under the two-sided Wilcoxon rank-sum test with the Bonferroni correction for correcting for multiple comparisons within each group. n.s.: non-significant. Each box plot shows the median value (bar) and the quartiles (box) of the fitted image-wise donor fluorescence lifetime values. The notches indicate the 95% confidence interval of the median. The whiskers correspond to the minimum of the data range or $1.5 \times$ the interquartile range. Extreme data points are marked as outliers. The number of FOVs analyzed for each strain is shown in parentheses above the corresponding box. Adapted from Ref.⁴³⁹.

4.2.5 Two query strains in the Spc24p-mTq2FP and the Ndc80p-mTq2FP groups exhibited significantly reduced donor fluorescence lifetimes

Among the 10 query strains, 2 of them, one each in the Spc24p-mTq2FP and the Ndc80p-mTq2FP groups, showed significantly shorter donor fluorescence lifetimes than that from

the corresponding negative controls, as shown in Figure 4-6, when tested individually with a significance level of $\alpha=0.5$ and with the Bonferroni correction applied for multiple comparisons within each group of strains. The median lifetime reduction seen in the strain expressing Spc24p-mTq2FP and Spc25p-YFP was 1064 ps (Figure 4-6a), while for the strain expressing Ndc80p-mTq2FP and Nuf2p-YFP the median reduction was 284 ps (Figure 4-6b). These data acquired with the FLIM HCA method are consistent with the previous notions that Spc24p dimerises with Spc25p while Ndc80p dimerises with Nuf2p^{248,252,479}, and therefore support that this methodology is capable of identifying endogenously-tagged KPs that are in close proximity, which provides information about the kinetochore structure in live budding yeast cells.

4.2.6 All other query strains exhibited no difference in the donor fluorescence lifetime between the corresponding negative controls

For the remaining 8 query strains in the Ndc10p-mTq2FP, the Mtw1p-mTq2FP, the Nuf2p-mTq2FP, and the Ask1p-mTq2FP groups, none of them showed significantly different fluorescence lifetime when compared with the corresponding negative controls (Figure 4-7). While most of these results matched the expectations based on previous knowledge of the kinetochore structure, FRET was expected in the strain where Ndc10p was stochastically tagged by mTq2FP and YFP, as it was reported previously that in each kinetochore there should be two copies of Ndc10p that forms a homodimer near the centromere^{213,480}. The absence of a fluorescence lifetime reduction in this strain (Figure 4-7a) may be due to the C-termini of the two Ndc10p molecules in the homodimer being sufficiently far apart to prevent FRET, or it could be a false-negative result owing to the low copy number of this inner KP (with only ~2-4 copies in each kinetochore²²⁰⁻²²²) and/or the stochastic labelling strategy. Overall, these results are satisfying in that they suggest that the FLIM HCA methodology is largely free of false-positive results and can consistently read out the non-FRETing lifetimes, even for KPs with very low copy numbers per kinetochore.

4.3 Summary and discussion

In this chapter, I have presented results for FLIM HCA experiments on the budding yeast kinetochore structure that explored the interactions between several pairs of FP-labelled endogenous KPs in asynchronised live yeast cells using FRET. The results were compared with prior knowledge of the kinetochore structure, so as to validate the capability of the FLIM HCA methodology described in Chapter 3 for identifying PPIs at the budding yeast kinetochore.

For these experiments, I constructed 16 endogenously labelled diploid strains to serve as query strains or negative controls for FRET (Table 4-1). In addition, I constructed 4 plasmid-containing diploid strains, which were positive controls for FRET with each plasmid expressing a KP tandemly tagged by mTq2FP and YFP, with a 8-amino-acid flexible linker in between the two FPs (Table 4-1). I chose to express these tandemly-tagged KPs from plasmids as I was unable to label the endogenous KPs using the two-FP tag, which could be due to this large two-FP tag compromising the kinetochore function. Under a *CUP1* promoter, the FP-tagged KP expressed from a plasmid should contribute typically half of the total number of that specific KP at the kinetochore in a diploid strain⁴⁸¹.

The 20 strains above were categorised into 6 groups, based on which of the 6 KPs (Ndc10p, Mtw1p, Ndc80p, Nuf2p, Spc24p, Ask1p) was labelled with the donor mTq2FP, and there was at least one query strain and one negative control in each group. The lifetime results from the FLIM HCA experiments were compared only between strains in the same group, as I noted that the median non-FRETing fluorescence lifetimes of mTq2FP measured for the negative control strains of the 6 groups (Ndc10p-mTq2FP, Mtw1p-mTq2FP, Ndc80p-mTq2FP, Nuf2p-mTq2FP, Spc24p-mTq2FP, Ask1p-mTq2FP) were different, being 4705 ps, 3863 ps, 4170 ps, 3878 ps, 3863 ps, and 3955 ps, respectively (with the corresponding 95% confidence intervals being 140 ps, 96 ps, 75 ps, 140 ps, 209 ps, and 108 ps, respectively). Similar variations in fluorescence lifetime were also seen for green fluorescent protein (GFP) when tagged to different proteins at the budding yeast plasma membrane⁴¹⁹, and it is likely to be due to that the different FP-tagged proteins were in slightly different microenvironments. It is also possible that the

strategy I employed for subtracting the local TVB around each kinetochore (see Section 3.5.1) was not optimal, which resulted in either under- or over-subtraction of the TVB and therefore introduced biases to the fitted lifetime values.

In order to average over the potential biological and experimental noise, multiple FLIM HCA experiments were performed on each strain. As the donor fluorescence lifetimes of the strains in each group did not exhibit systematic shifts between different multiwell plate experiments, the small variations in the measured lifetimes were attributed to random noise instead of systematic bias, and therefore the lifetime results for from different experiments were directly combined together for each strain.

The combined FLIM results for each strain were then subjected to a filtering process, where the lifetime values from FOVs with low total signal levels were discarded owing to the lower precision and accuracy they exhibited (Figure 4-4 and Figure 4-5), so that only the data of higher quality were used for the subsequent statistical comparisons using the two-sided Wilcoxon rank-sum test with the Bonferroni correction for multiple comparisons within each group of strains.

In addition to the 4 positive control strains, I detected significantly reduced lifetimes in two query strains, in both of which KPs in the well-characterised NDC80 complex were fluorescently tagged. The NDC80 complex, localised at the outer kinetochore, comprises four components that form two heterodimers (Ndc80p-Nuf2p and Spc24p-Spc25p), and the C-termini of the proteins in each dimer have been shown to be in close proximity^{248,252,479}. Previous ratiometric FRET measurements also indicated that the C-termini of Spc24p and Spc25p are closer than that of the other two proteins²²⁰⁻²²². My FLIM HCA results are consistent with these data. However, the ratiometric FRET data^{316,317} suggest that the FRET efficiency for the Ndc80p-mTq2FP/Nuf2p-YFP pair should be closer to that for the Spc24p-mTq2FP/Spc25p-YFP pair than I observed. This discrepancy may be partly explained by the Ndc80p-mTq2FP/Nuf2p-YFP strain I used for the FLIM assays was heterozygous in the Nuf2p-YFP gene, so that ~50% of the Nuf2p proteins were not tagged by YFP and therefore led to a lower measured “effective FRET efficiency”. It should also be noted that, while I applied the Bonferroni correction to correct for the chosen significance level of $\alpha=0.5$ for multiple comparisons within each group of strains, I did not apply it for correcting the total of 10 comparisons between

query strains and negative control strains in the 6 groups. Without correction for multiple comparisons, a significance level of $\alpha=0.5$ means that for every 20 comparisons 1 may appear to be a false-positive by chance. Nevertheless, the fact that two positive results were identified out of the total 10 comparisons and that they are supported by previous studies suggest that these two hits are unlikely to be false-positives. If time allowed a more rigorous statistical analysis could be performed to confirm these findings; alternately, further experiments with improved capability in discriminating small differences in fluorescence lifetimes could be performed.

For the other query strains where the FRET donor/acceptor fluorophore pair were respectively tagged to different KPs, no significant change in the donor fluorescence lifetime was seen relative to that from the negative controls. These results are expected, as each pair of the fluorescently-tagged KPs should be well separated along the kinetochore-microtubule axis within each kinetochore (e.g. for Ask1p-mTq2FP/Ndc10p-YFP, see the review by Cieřliński and Ries and references therein³¹⁴). These data also rule out interkinetochore FRET, despite that in budding yeast all the kinetochores colocalise into one or two diffraction-limited foci within the nucleus. These data further suggest that the FLIM HCA methodology can consistently reveal the non-FRETing donor fluorescence lifetimes without introducing false-positive results, even when the donor-tagged KP has a copy number as low as ~2-4 per kinetochore (e.g. Ndc10p).

The two query strains where the FRET pair were stochastically tagged to the same type of KP (expressing Mtw1p-mTq2FP/Mtw1p-YFP and Ndc10p-mTq2FP/Ndc10p-YFP, respectively) also exhibited no significantly changed donor fluorescence lifetime. For the strain with fluorescently-tagged Mtw1p, the non-FRETing result is supported by a previous ratiometric FRET study that showed that the N-termini of Mtw1p molecules in one kinetochore are in close proximity, but the C-termini are not³¹⁷. For the other strain, however, a reduction in the fluorescence lifetime of the Ndc10p-tagged mTq2FP was expected, as Ndc10p was expected to form a homodimer proximal to the centromere. The absence of FRET could be explained by the dimerisation domain of the Ndc10p being far away from the C terminus, and therefore the C-termini of the two Ndc10p molecules in one dimer are >10 nm away. This is supported by one study that reported the structure of Ndc10p for another yeast species, *Kluyveromyces lactis*, whose dimerisation domain was

found to be at the centre of the protein sequence²¹³, but conflicts with another study in *S.cerevisiae* that suggested that the C-terminus of Ndc10p (residues 551-957) is responsible for dimerisation⁴⁸². Alternatively, this could be a false-negative result caused by the low S/N owing to the low copy number of the Ndc10p dimer (currently thought to be one at each centromere), which increased the variability of the measured lifetimes (see the wide inter-quartile ranges in Figure 4-7a). Furthermore, since the Ndc10p molecules were stochastically labelled with the FRET pair, on average only ~50% of the imaged donors would undergo FRET. If indeed only a fraction of the Ndc10p dimers are undergoing FRET, the FRET signal could be too small a fraction of the measured donor signal to be distinguished from the non-FRETing signal. Therefore, this potential false-negative result could be indicating the limit of the FLIM HCA methodology for studying proteins with extremely low copy numbers.

For the FRET pair (mTq2FP and YFP) used in this study, the interquartile ranges of the donor fluorescence lifetimes measured from all the strains were 240-1450 ps. The corresponding 95% confidence intervals of the median lifetimes, calculated using the numbers of FOVs reported in Figure 4-6 and Figure 4-7 and under the assumption of normal distribution⁴⁸³, were in the range of 75-410 ps. Although the statistical power for revealing differences in the donor fluorescence lifetimes could be improved by increasing the samples sizes, as the uncertainty would decrease with the square root of the sample size, it would require imaging many more FOVs for a large improvement, which may not be practical for live cell experiments, since such a prolonged experiment may compromise the fitness of the cells. Consequently, to enhance the power of the FLIM HCA methodology for identifying PPIs, one should seek to maximize the S/N of the donor fluorescence signal, e.g. by judicious choice of fluorophores or careful matching of the instrument's spectral properties to the donor fluorescence, in order to reduce the uncertainty in the measured donor lifetime. For example, these assays could be improved by employing bright donor FPs that emit in a spectral window with less overlap with the cellular autofluorescence (such as enhanced GFP [EGFP]). The potential lifetime change could also be increased through selecting a more suitable acceptor for the chosen donor. The FPs selected for yeast kinetochore imaging should ideally have optimised maturation rates for yeast cultures at 23 °C, and they should be linked to the KPs of interest with flexible linkers for more random fluorophore orientations. Alternatively, the SNAP-tag

methodology is an attractive option for labelling of endogenous KPs, as it is smaller in size than FPs and can be covalently tagged by small molecule dyes that can be both brighter and more photostable than FPs⁴⁸⁴. However, although an electroporation-based protocol has been published for transporting small molecule dyes into intact budding yeast cells⁴⁸⁵, after following the protocol I was not able to observe any specific binding of the dye SNAP-Cell 430 to the SNAP-tag endogenously labelling various KPs in either live or fixed cells. The cause of this remains to be explored.

Overall, the work presented in this chapter has validated that the FLIM HCA methodology for identifying interactions between FP-labelled endogenous KP at the budding yeast kinetochore in live cells using FRET, despite the relatively low copy numbers of the KPs. To the best of my knowledge, this is the first demonstration of FLIM HCA of PPIs at the compact budding yeast kinetochore. This provides a high-resolution approach to study, not just the kinetochore structure, but also interactions between KPs and regulatory proteins for cell cycle progression in live budding yeast cells. This may provide a better understanding of the kinetochore functions in ensuring faithful chromosome segregation.

Chapter 5. Investigation of the role of Rdh54p in the adaptation to the spindle assembly checkpoint-dependent cell cycle arrest

Rdh54p is an ATP-dependent DNA translocase functioning in DNA damage repair. However, it has another less well-studied function in promoting the adaptation to cell cycle arrest in response to DNA damage, which has been shown to be dependent on the spindle assembly checkpoint (SAC). This chapter reports the experiments performed to investigate this intriguing function of Rdh54p, focusing on Rdh54p's potential interacting partners at the kinetochore, which is the regulatory hub of the SAC and is also where Rdh54p constitutively localises. The first section provides an introduction to Rdh54p in terms of its known characteristics and functions. The second section presents the results of the synthetic physical interaction (SPI) assays performed to screen for the SPI partners of Rdh54p, first across the budding yeast proteome and then focusing on the kinetochore-related proteins. The third section presents the results of the FLIM experiments, which were performed in search for Rdh54p's interacting partners at the kinetochore following the procedures described in Chapter 3. The fourth section at the end summaries my current progress on this topic and discusses potential future work.

5.1 Introduction

The budding yeast protein Rdh54p is an ATP-dependent DNA translocase that belongs to the SWI2/SNF2 family of helicase-like chromatin-remodelling proteins^{486,487}. Its structure contains the 7 helicase-like motifs conserved among most DNA helicases. This Swi2/Snf2 helicase domain is essential for Rdh54p's DNA-dependent ATPase activity that provides the energy through ATP hydrolysis for the protein to translocate along double-stranded DNA (dsDNA) and to remodel chromatin and DNA-protein complexes⁴⁸⁸ (Figure 5-1).

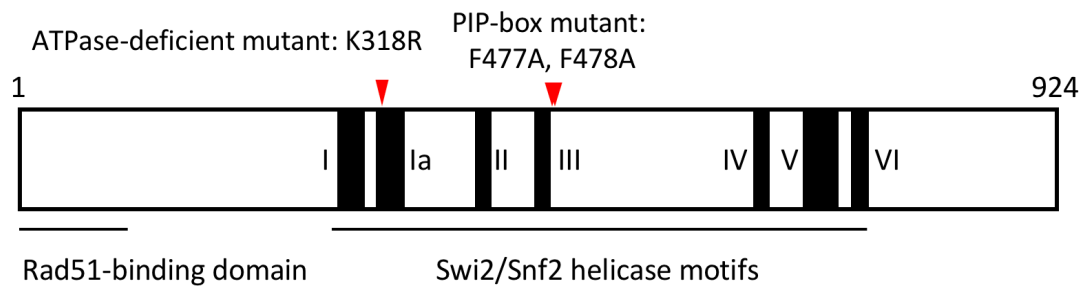


Figure 5-1 Schematic of *Rdh54p* and its known domains

The positions of the mutated amino acids in the ATPase-deficient mutant *rdh54p-K318R* and in the PIP-box mutant *rdh54p-pip* are indicated by the red triangles.

Rdh54p was first identified as a homolog of *Rad54p*^{489–491} (hence the name Rad homolog 54). *Rad54p* is also a member of the SWI2/SNF2 family and functions in homologous recombination (HR) for DNA damage repair. In the presence of a DNA double-strand break (DSB), *Rad54p* interacts *Rad51p*, a paralog of bacterial recombinase *RecA* that binds to 3' single-stranded DNA (ssDNA) resulting from the processing of the primary lesion, and promotes the *Rad51p*-bound ssDNA's invasion into the homologous dsDNA to form a displacement loop (D-loop) by unwinding the naked dsDNA and generating unconstrained negative and positive supercoils upon ATP hydrolysis^{492–494}. It is also capable of displacing *Rad51p* from dsDNA, likely to facilitate DNA synthesis from the invaded ssDNA following the formation of the nascent D-loop^{494,495}. Similarly, *Rdh54p* has been shown to physically interact with *Dmclp*, a meiosis-specific *RecA* paralog, in an yeast two-hybrid assay⁴⁹⁶ (and therefore was also named as Two-hybrid interaction with Dmclp, *Tid1p*), to function together with *Rad51p* and/or *Dmclp* during HR^{493,497}, to translocate along and supercoil DNA and reposition nucleosomes through its ATP-dependent translocase and chromatin remodelling activities^{493,498–500}, and to remove *Rad51p* and *Dmclp* from dsDNA^{497,498,501}. Moreover, both *Rad54p* and *Rdh54p* have a conserved PCNA-interacting protein box motif, which may facilitate their interactions with the PCNA (proliferating cell nuclear antigen) clamp that is involved in various cellular processes including DNA replication and repair⁵⁰², but the exact functions of this motif in the two proteins are yet to be elucidated^{503,504}.

Despite the aforementioned similarities, Rad54p and Rdh54p have distinct roles in HR as revealed by both *in vitro* and *in vivo* studies: (1) Rad54p is primarily involved in the intrahomolog recombination process through its interaction with Rad51p, whereas Rdh54p specialises in promoting interhomolog recombination through interactions with Rad51p in mitosis and interactions with both Rad51p and Dmc1p in meiosis^{490,491,505,506}; (2) Rad54p preferentially removes Rad51p from DNA damage foci after D-loop formation for promoting the subsequent DNA synthesis, whereas Rdh54p preferentially removes Rad51p as well as Dmc1p from undamaged dsDNA to maintain a sufficient pool of free Rad51p/Dmc1p and prevent the toxic nonspecific binding of the recombinases to dsDNA^{497,501,507,508}. The Rdh54p-mediated interhomolog recombination pathway is more active in meiosis prophase than the Rad54p-mediated intrahomolog pathway for repairing the meiosis-specific programmed DSBs and generating crossovers between homologous chromosomes. Rdh54p may facilitate this preference by regulating the cohesion between sister chromatids, presumably through its chromatin remodelling activity that remodels the mitotic cohesin protein Mcd1p at DNA DSB sites^{509,510} and/or through its interaction with Dmc1p⁵¹⁰. It is also speculated that Rdh54p plays a role in D-loop stabilisation and extension during meiotic recombination⁵¹¹. Other reported recombination-related roles of Rdh54p include facilitating interchromosomal template switching⁵¹², regulating chromatin through its increased associations with chromatin-associated factors and its elevated distribution to centromeric and pericentromeric regions in the absence of two histone chaperones, Asf1p and Cac1p⁵¹³, and stabilising bridge-induced translocated chromosomes^{514,515}. Notably, Rdh54p's ATPase activity appears to be vital for a proper response to DNA damage induced by methyl methanesulfonate (MMS), as both the *rdh54Δ* and the ATPase-deficient *rdh54-K318R* mutants are more sensitive to MMS, while its N-terminal Rad51p-interacting domain (Figure 5-1) is dispensable in this regard but is required for the D-loop formation and the removal of excess Rad51p from dsDNA^{498,516}. This suggests that the Rdh54p-mediated response to MMS-induced DNA damage is different from its canonical roles in HR.

Interestingly, Rdh54p is indeed required for the adaptation to prolonged G2/M arrest induced by the DNA damage checkpoint (DDC; for a review, see Section 1.1.2). It has been shown that a *rdh54Δ* mutant is permanently arrested (for >24 hours) prior to anaphase in the presence of an DNA DSB induced by the site-specific HO

endonuclease¹³³, in contrast to wild-type cells that normally adapt to this checkpoint-mediated arrest and resume the cell cycle within 8-12 hours^{126,128,133}. The Rdh54p protein is also needed for the division of cells devoid of Cdc13p-mediated chromosome capping⁵¹⁷. The permanent arrest phenotype can be rescued by mutating Rfalp, a subunit of the RPA complex, but not by the deletion of some other recombination factors (e.g. Yku70p, Rad52p, Rad51p, and Rad54p) or the overexpression of the Rdh54p's homolog Rad54p¹³³. The ATPase activity of Rdh54p is essential for adapting the arrest, as expressing *rdh54p*-K318R does not rescue the adaptation phenotype seen in *rdh54Δ*¹³³. Phosphorylation of Rdh54p by Mec1p has been shown to occur in the presence of damaged DNA, and its dephosphorylation (as well as the inactivation of Rad53p) also requires Rdh54p's ATPase activity, the lack of which leads to Rad53p-dependent Rdh54p hyper-phosphorylation⁵¹⁸.

Remarkably, deleting Mad2p, a component of the SAC, partly suppresses the DNA damage-induced permanent arrest in *rdh54Δ*. Considering that Rdh54p is constitutively localised to the kinetochore⁷⁰, a regulatory hub for the SAC (see Section 1.1.3), it is possible that Rdh54p plays some role in SAC control. Rdh54 is also recruited to DNA repair sites when DNA damage occurs⁷⁰, and since the SAC has been shown to be activated in response to prolonged DNA damage (see Section 1.1.4), it seems possible that Rdh54p is the component that connects the DDC and the SAC signals. It would therefore be interesting to identify the interacting partners of Rdh54p in the yeast proteome and particularly at the kinetochore, so as to help elucidate the molecular mechanism of how Rdh54p achieves its functions in checkpoint adaptation. Although a functional human homolog of Rdh54p is yet to be identified^{519–521}, a better understanding of the checkpoint adaptation process could still contribute to our knowledge of tumorigenesis, during which checkpoint signals are often impaired¹⁴².

5.2 SPI screens for Rdh54p's interacting partners

To search for the potential interacting partners of Rdh54p that may contribute to Rdh54p's role in checkpoint adaptation, I employed the Synthetic Physical Interaction (SPI) assay described in Section 2.5. I hoped that the SPI assays, which should identify

proteins that lead to growth defect phenotypes when artificially associated with Rdh54p or Rdh54p's mutants, could highlight a few proteins or protein complexes that would be worth following up with further experiments. Based on previous SPI studies, it has been shown that SPIs are often found between a target protein and a query protein that normally function together but require timely regulation for their interaction, and among query proteins that share common functions^{428,522,523}.

5.2.1 Proteome-wide screens identified Rdh54p SPIs related to the DNA damage response and the kinetochore

I initially undertook two proteome-wide screens for Rdh54p's potential interacting partners, one in haploid cells and the other in diploid cells. Procedures for these screens are described in detail in Section 2.5. Briefly, for each screen, three plasmids based on pWJ1512⁴²⁴ were transformed into the universal donor strain (UDS) W8161-2B⁴²⁴: one “fusion” plasmid, expressing Rdh54p fused to a GBP and an RFP, and two “control” plasmids, expressing either Rdh54p alone without GBP or the GBP-RFP construct without Rdh54p (Figure 5-2a). Each transformed UDS was then mated respectively with the ~4300 strains in a budding yeast GFP collection⁴²⁵ on rectangular agar plates in a high-throughput manner using a pinning robot, resulting in 12 plates of mated strains, with 384 strains on each plate and 4 replicates of colonies for each strain. The resulting diploid strains were either brought back to haploids through Selected Ploidy Ablation (SPA)⁴²⁴ for the haploid screen (Figure 5-2b), or directly analysed for the diploid screen results following two more steps of replica plating onto -His -Leu selection plates using the pinning robot (Figure 5-2c). The growth of colonies was quantitative assessed by scanning the plates, then using the ScreenMill software⁴³⁶ to analyse colony sizes, which allowed me to calculate log growth ratios (LGRs, the natural logarithm of the ratio of control divided by experimental colony size). For the proteome-wide screens, the LGRs were converted to z-scores by fitting the data to a normal distribution. Some haploid strains resulting from SPA were examined using fluorescence microscopy to confirm the colocalisation of the GBP-RFP-tagged Rdh54p and the GFP-tagged query protein, an example of which is shown in Figure 5-2d (where Rdh54p colocalised with Dad3p, a component of the microtubule-binding DAM1 complex).

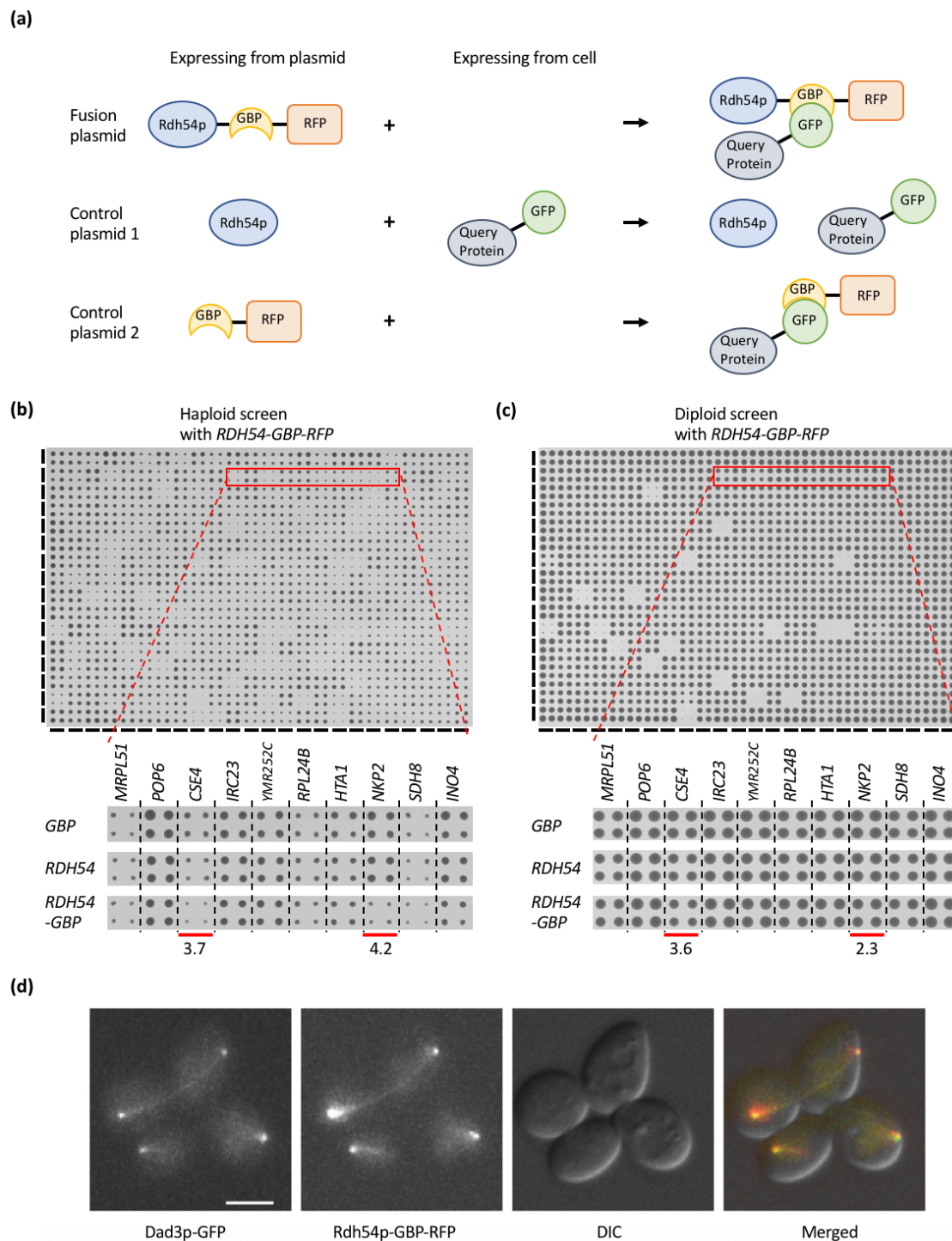


Figure 5-2 Proteome-wide screens for SPIs of Rdh54p

(a) Schematic of the forced association of Rdh54p to a GFP-tagged query protein by the GBP and the associations of the constructs expressed from the two controls to the query protein. (b) Exemplar image of a plate showing the haploid screen results for Rdh54p SPIs, with four colonies of each GFP strain containing a plasmid expressing Rdh54p-GBP-RFP arrayed in a 2x2 format on the agar plate. The colony sizes of the GFP strains on this plate were compared with those of the same GFP strains on the two control plates, and the ones with high z-scores are highlighted

with red bars and the corresponding z-scores. (c) Exemplar image of a plate showing the diploid screen results for Rdh54p SPIs. (d) Images taken by a fluorescence microscope, showing the colocalisation of Rdh54p-GBP-RFP with a GFP-tagged kinetochore protein Dad3p.

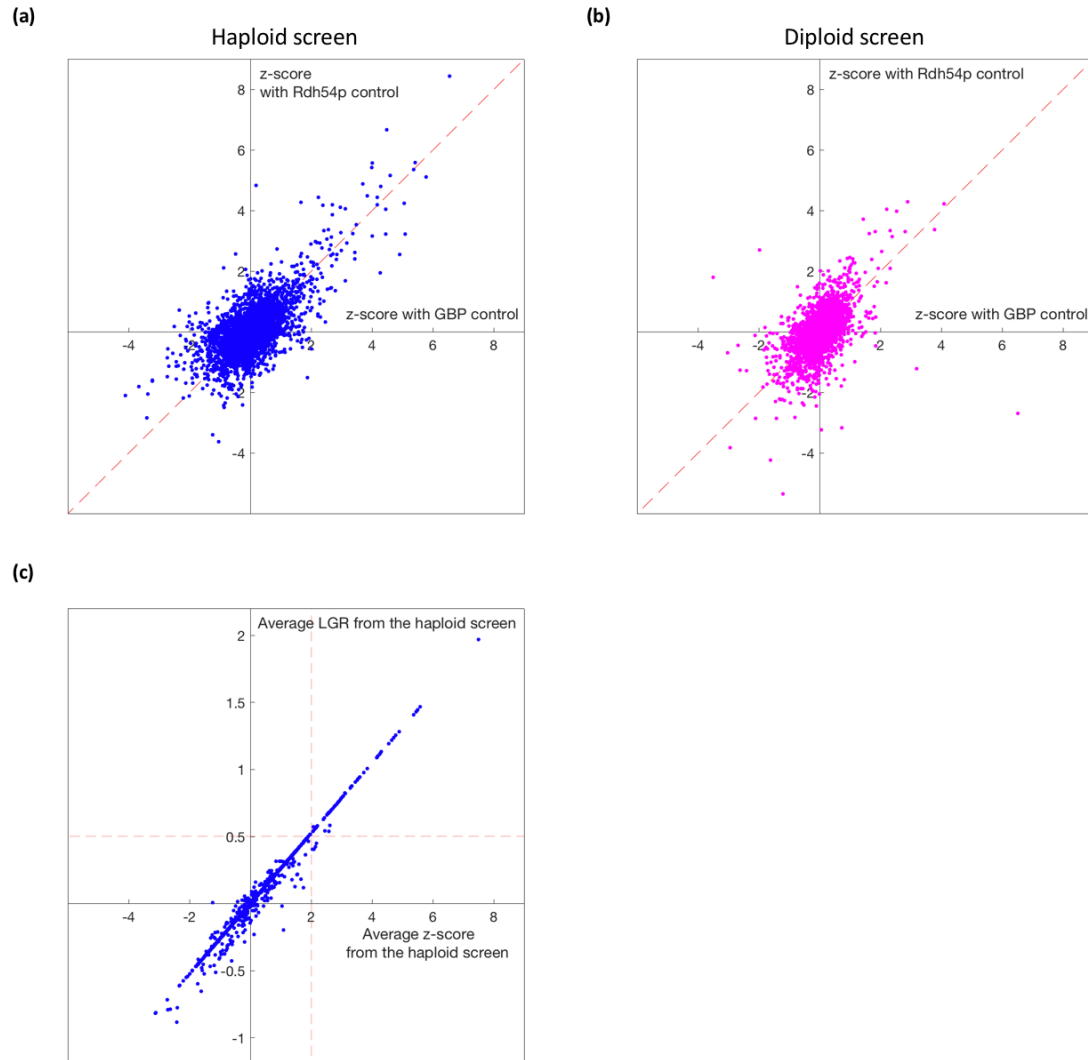


Figure 5-3 Correlations in the results of the proteome-wide screens for Rdh54p SPIs in haploid and diploid strains

(a) Correlation between the z-scores for the haploid screen calculated respectively with the two controls. (b) Correlation between the z-scores for the diploid screen calculated respectively with the two controls. (c) Correlation between the average z-scores and the average LGRs for the haploid screen.

I compared Rdh54p-GBP separately with both of the controls to obtain two related sets of z-score data (Rdh54p-GBP vs. Rdh54p and Rdh54p-GBP vs. GBP). I then calculated

the average z-score of these two, as a measure of how the forced association of the two proteins affects the cell growth. The use of average z-scores was justified by the observation that elevated z-scores obtained from the two controls were highly correlated for both the haploid and the diploid screens, as shown in Figure 5-3a and Figure 5-3b. Z-scores and LGRs also correlated well as shown in Figure 5-3c. Note that a z-score or LGR value of ~ 0 corresponds to a strain that grew similarly on the experimental plate and the control plates. In contrast, positive z-scores or LGRs indicate forced associations that caused slow growth.

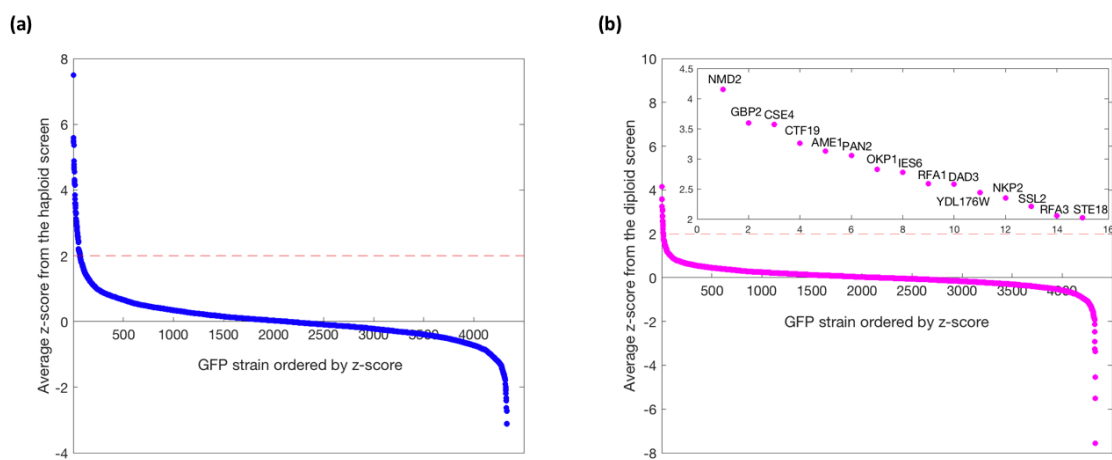


Figure 5-4 Results of the initial proteome-wide screens for *Rdh54p* SPIs in haploid and diploid strains

(a) Results for the haploid screen plotted with descending z-scores. (b) Results for the diploid screen with descending z-scores. The diploid *Rdh54p* SPIs (with z-scores higher than 2) were shown in the inset with the gene names annotated.

Figure 5-4a plots the z-scores for the haploid screen in descending order from left to right. I decided to set a threshold for the z-score at $z=2$; the strains whose z-scores are above this threshold were subsequently regarded as showing SPI-induced growth defects. For the haploid screen, 61 SPIs were identified for *Rdh54p*. For the diploid screen, which was performed in case the GBP-tagged *Rdh54p* expressed in a haploid strain could lead to the mislocalisation of an essential GFP-tagged protein and consequently a false position result, I expected that some of the SPIs seen in the haploid screen would be suppressed in the heterozygous diploid strains that contain an untagged version of the GFP-tagged protein. Indeed, only 6 of the 61 haploid SPIs appeared in the diploid results, besides 9

newly identified diploid SPIs. The results for the diploid screen are shown in Figure 5-4b, with the SPIs highlighted in the inset. Notably, among the 6 SPIs common for both screens, apart from the centromeric histone Cse4p that frequently appears as a SPI regardless of what the target protein is⁵²², 3 of them (Nkp2p, Ame1p, Okp1p) belong to the large CTF19 complex at the kinetochore, and the other 2 (Rfa1p, Rfa2p) are units of the trimeric RPA complex. This is interesting, considering that Rdh54p has been shown to constitutively localise at the kinetochore⁷⁰ and that both RPA and Rdh54p are involved in DNA damage repair and checkpoint function^{128,133}.

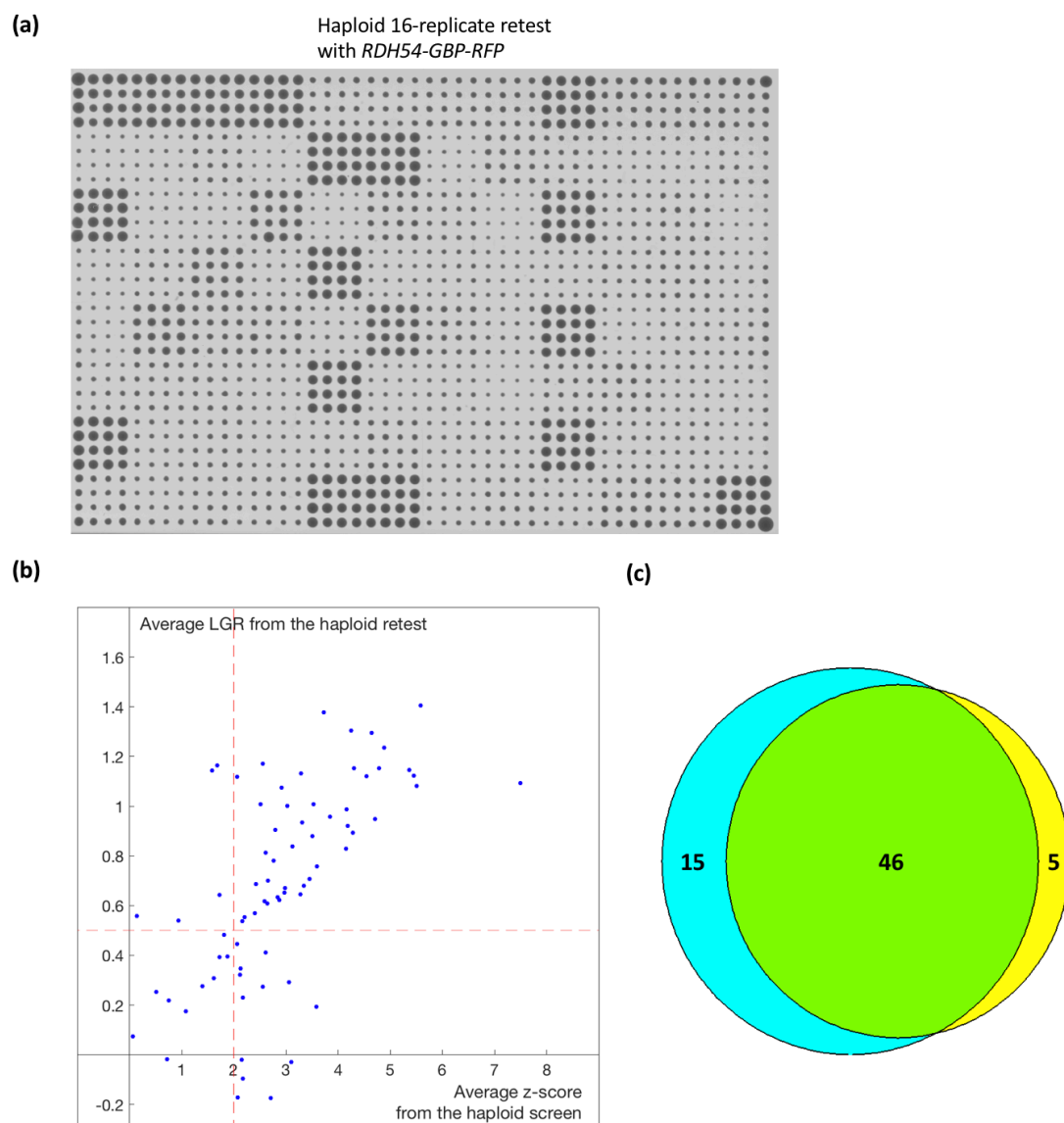


Figure 5-5 Results from the 16-replicate retest for haploid *Rdh54p* SPIs

(a) Exemplar image of a plate showing the results of the haploid 16-replicate retest for *Rdh54p* SPIs, with 16 colonies of each strain containing a plasmid expressing *Rdh54p*-GBP-RFP arrayed in a 4x4 format on the agar plate. (b) Correlation between the average z-scores from the initial haploid screen and the average LGRs from the retest. (c) Venn diagram showing that among all the 61 haploid *Rdh54p* SPIs identified in the initial screen, 46 of them were validated by the retest whilst 15 were not. The retest also identified 5 new *Rdh54p* SPIs.

Table 5-1 Genes related to the *Rdh54p* haploid SPIs from the 16-replicate retest

	Genes (ranked based on their z-scores in descending order, with “non-frequent flyers” marked in red)
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Validated haploid SPIs	<i>SPC29, CSE4, DDC2, SEC24, BBP1, AME1, MTR10, NOP9, SEC10, MEX67, YRA2, DOA4, FRQ1, NUP192, BRL1, KAP120, SPC42, NOP8, OKP1, RFA1, RFA2, ACL4, KIP3, NKP2, RTT105, CSE1, LEU3, TSR2, CDC12, SLY1, RPA49, BDF1, PRP5, TPD3, RFA3, KRI1, MTR2, DID4, VPS4, UBP3, VPS28, NEO1, SPC24, BUD23, ENP1, ARC35</i>
Newly-identified SPIs from the retest	<i>MOB2, STS1, SPT15, DAD1, CEP3</i>

Although 55 out of the 61 SPIs for the haploid screen above were absent in the diploid screen, at least 62% (34/55) of them could still be interesting, since they are not “frequent flyers” seen in many other SPI screens⁵²² but are specific to Rdh54p. This suggests that these interactions, although suppressed in diploid cells, are unlikely to be caused by mislocalisation of the query proteins and could have functional significance. I therefore decided to retest the haploid SPIs with more colonies for each strain (16 replicates per strain) for validation (Figure 5-5a). I also included a few additional GFP strains, which had been identified as being sensitive to association with mutant versions of Rdh54p (see Section 5.2.2). Figure 5-5b and Figure 5-5c illustrate the comparisons between the results from the initial haploid screen and the retest. For the retest, I used the LGR to report cell growth as the z-score was inapplicable in this case where the distribution of LGRs was not normal. I set the threshold for the LGR at 0.5 (Figure 5-5a), which corresponds to a z-score of 2 (Figure 5-4c) and represents a control colony that is 110% larger than the experimental colony. Among the 61 SPIs identified in the initial screen, 46 of them were validated in the retest (including the 6 SPIs common to the initial haploid and diploid screens), while there were also 5 new SPIs that were not identified in the original screen (false negatives). In general, the “16-replicate” retest indicates the degree of robustness of the SPI methodology. A list of the 51 Rdh54p haploid SPI genes found in the retest is provided in Table 5-1, including the 46 genes validated for the initial haploid screen and the 5 additional genes identified in the 16-replicate retest. The genes are ranked by their corresponding LGRs from the retest (from high to low). Note that, as shown in previous SPI studies⁵²², certain proteins are particularly sensitive to being associated with other proteins, some of which were found in the Rdh54p SPIs; I term these “frequent flyers”, and have marked the “non-frequent flyers” in Table 1-1 in red.

Table 5-2 Gene Ontology analysis for the “non-frequent flyer” SPI genes identified by the 16-replicate retest

Process	Gene name
Nuclear transport ($p = 7.15 \times 10^{-4}$)	<i>NOP9, BUD23, MTR2, YRA2, NUP192, ENP1</i>
Function	Gene name
Damaged DNA binding ($p = 9.62 \times 10^{-4}$)	<i>RFA1, RFA2, DDC2</i>
Component	Gene name
Condensed chromosome kinetochore ($p = 8.48 \times 10^{-4}$)	<i>NKP2, OKP1, AME1, DAD1</i>
DNA replication factor A complex ($p = 3.18 \times 10^{-4}$)	<i>RFA1, RFA2</i>

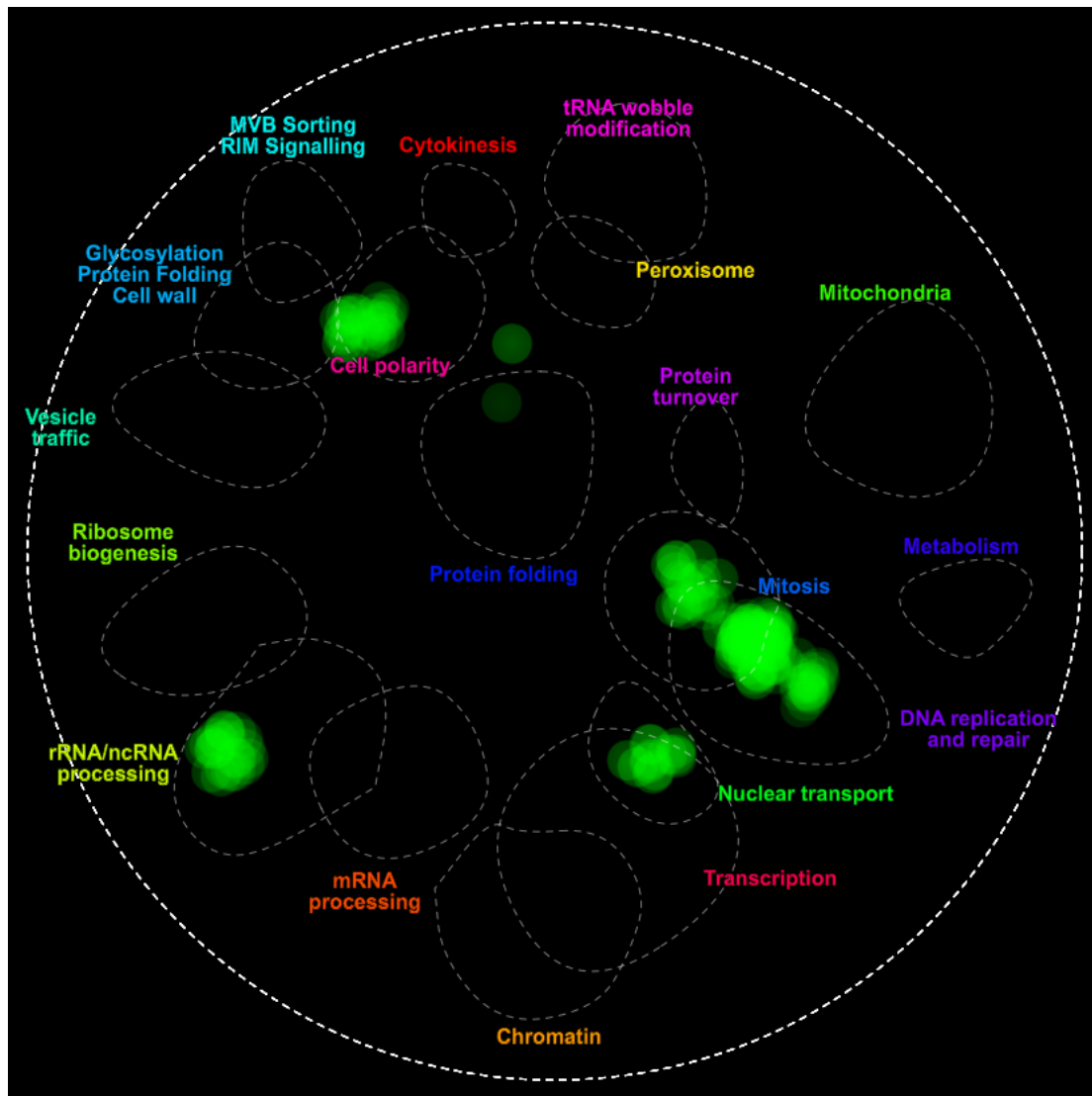


Figure 5-6 Functional similarities of the “non-frequent flyer” genes of the *Rdh54p* haploid SPIs as revealed by TheCellMap.org

These genes were found to be enriched for several biological processes/functions, including “DNA replication and repair” and “mitosis”.

To examine whether the *Rdh54p* SPIs share similar functions, I performed a gene ontology (GO) analysis on the “non-frequent flyer” genes listed in Table 5-1 using GOrilla (<http://cbl-gorilla.cs.technion.ac.il>)⁵²⁴. The GO analysis indeed revealed several highly enriched biological processes, molecular functions, and cellular components within the SPI genes, which include the genes related to *Rdh54p*’s known function in the DNA damage response (*RFA1*, *RFA2*, *DDC2*) and the genes of some kinetochore components (*NKP2*, *OKP1*, *AME1*, *DAD1*). The functional similarities of the SPI genes

were further confirmed with TheCellMap.org, an online database containing genetic interaction data obtained with genome-scale synthetic genetic array (SGA) assays^{525,526}, as the SPIs genes are shown to be enriched for genetic interactions for several biological processes, including “DNA replication and repair” and “mitosis” (Figure 5-6).

5.2.2 Rdh54p SPIs related to the CTF19 complex at the kinetochore were suppressed in the proteome-wide rdh54p-pip SPI screens but not in the ones for rdh54p-K318R SPIs

Besides the wild-type Rdh54p, I also conducted SPI screens for two mutants of Rdh54p: rdh54p-pip, which contains two amino acid changes in Rdh54p’s PIP-box motif (F477A,F478A), and rdh54p-K318R, which is deficient in the ATPase activity owing to the mutation in the Walker A motif. The screens were performed in haploid cells, with only the plasmid expressing GBP used as the control. The strongest hits from the initial proteome-wide screens were validated with 16-replicate retests. The results for the PIP-box mutant rdh54p-pip and the ATPase mutant rdh54p-K318R are shown in Figure 5-7 and Figure 5-8, respectively.

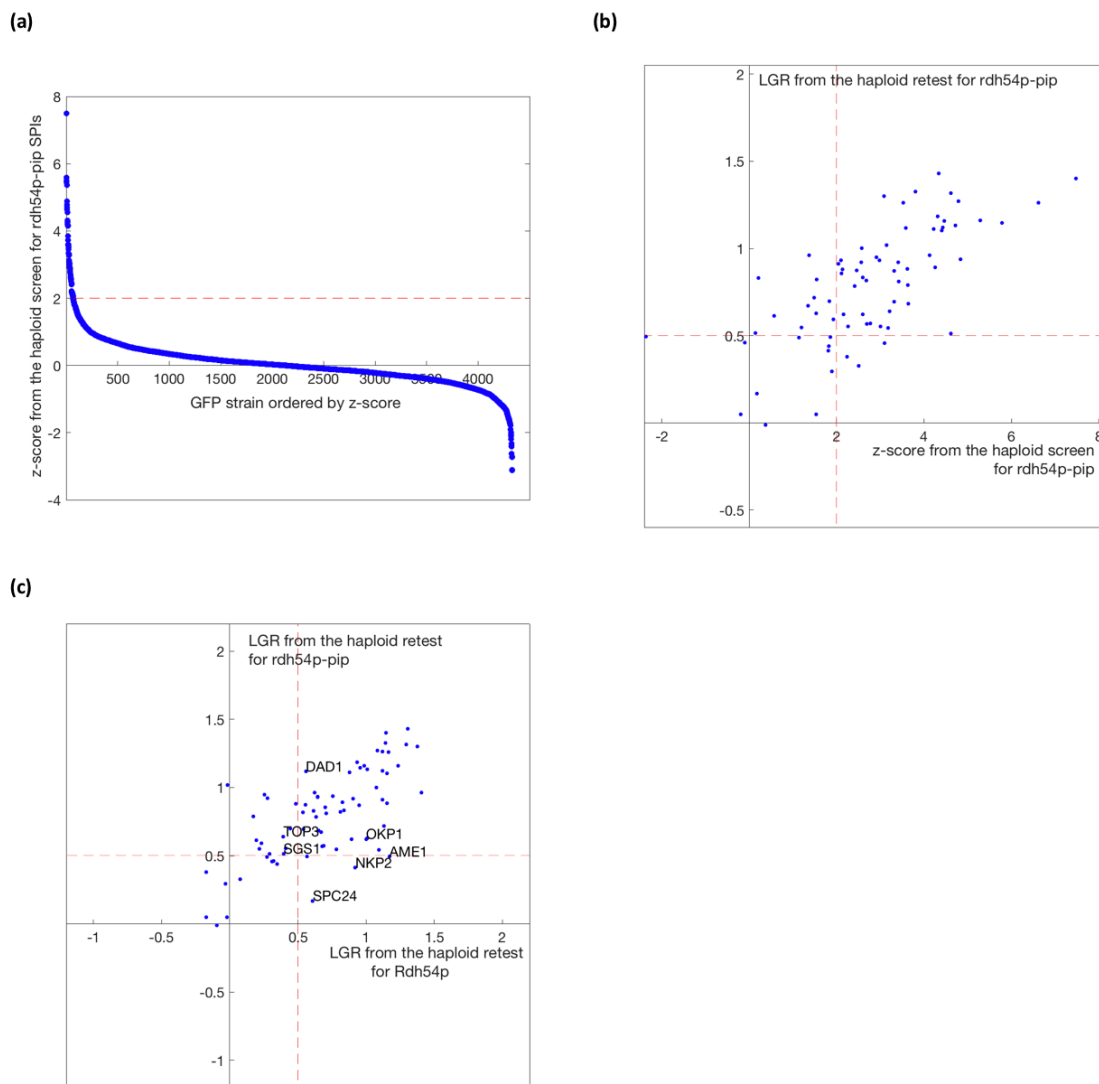


Figure 5-7 Results of the initial proteome-wide screen and the 16-replicate retest for haploid *rdh54p*-pip SPIs

(a) Results for the haploid screen plotted with descending z-scores. (b) Correlation between the z-scores from the initial screen and the LGRs from the retest. (c) Comparisons between the results of the haploid retest for *Rdh54p* SPIs and the results of the haploid retest for *rdh54p*-pip SPIs. Strains that exhibited notable differences between the two screens are annotated.

Table 5-3 Genes related to the haploid *rdh54p*-pip SPIs from the 16-replicate retest

	Genes (ranked based on their z-scores in descending order, with “non-frequent flyers” marked in red)
Validated haploid SPIs	<i>DDC2</i> , <i>SEC10</i> , <i>STS1</i> , <i>SEC24</i> , <i>CSE4</i> , <i>BRL1</i> , <i>YRA2</i> , <i>MOB2</i> , <i>KIP3</i> , <i>RFA1</i> , <i>BBP1</i> , <i>RFA2</i> , <i>SPC42</i> , <i>DOA4</i> , <i>DAD1</i> , <i>LEU3</i> , <i>MTR10</i> , <i>MET30</i> , <i>KAP120</i> , <i>SPC29</i> , <i>INO4</i> , <i>BDF1</i> , <i>SPT15</i> , <i>VPS4</i> , <i>TAF10</i> , <i>RTT105</i> , <i>FRQ1</i> , <i>CDC12</i> , <i>NOP9</i> , <i>CBK1</i> , <i>ENP1</i> , <i>ACL1</i> , <i>TPD3</i> , <i>TSR2</i> , <i>ARC35</i> , <i>PRP5</i> , <i>APL4</i> , <i>UBP3</i> ,

	<i>CEP3</i> , <i>DID4</i> , <i>TOP3</i> , <i>CSE1</i> , <i>OKP1</i> , <i>RFA3</i> , <i>KRI1</i> , <i>CDC40</i> , <i>KIN28</i> , <i>NUP192</i> , <i>SGS1</i>
Newly-identified SPIs from the retest	<i>VPS28</i> , <i>NEO1</i> , <i>SLY1</i> , <i>MEX67</i> , <i>SIN4</i> , <i>MTR2</i> , <i>NOP8</i> , <i>ARC18</i> , <i>RMD1</i> , <i>RPA49</i> , <i>SNF7</i>

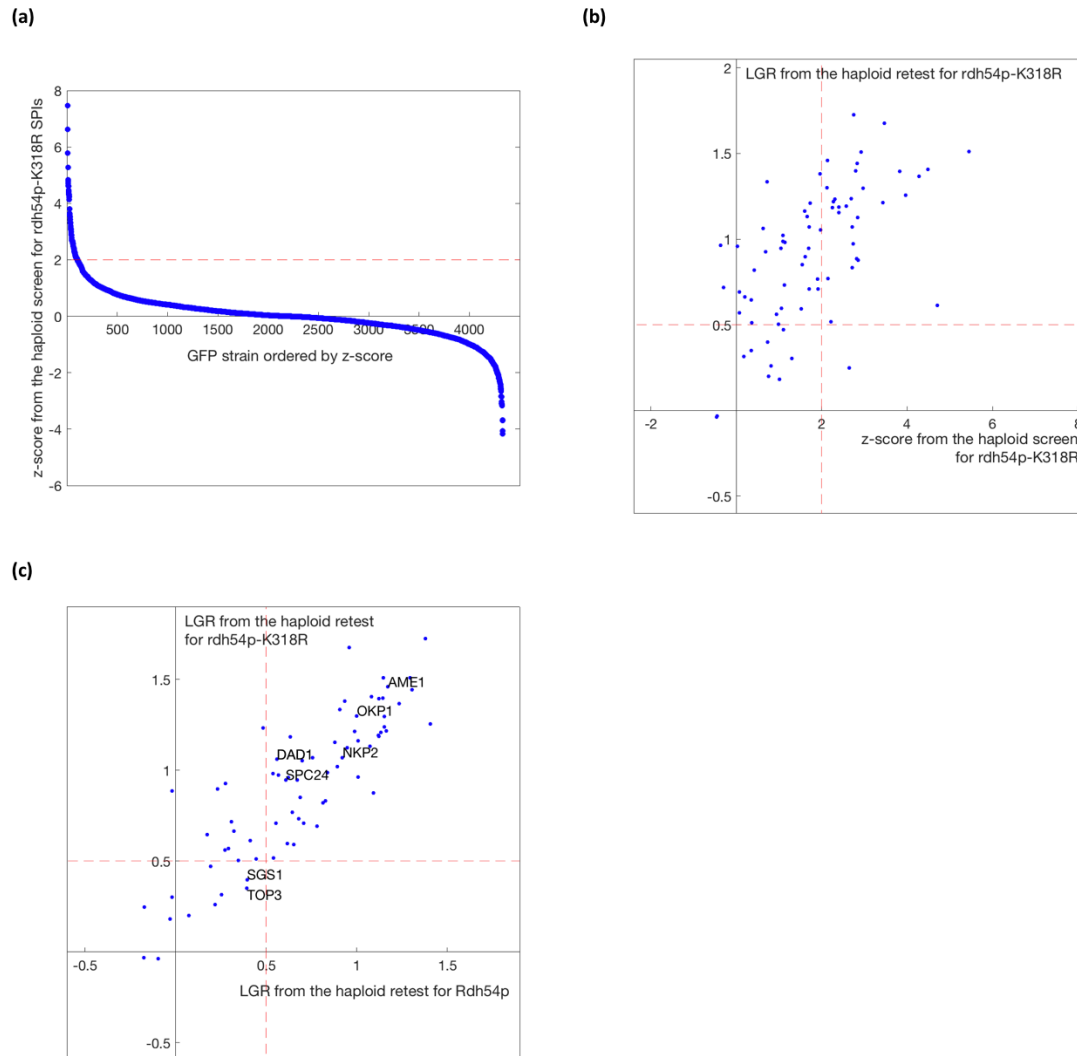


Figure 5-8 Results of the initial screen and the 16-replicate retest for haploid *rdh54p-K318R* SPIs

(a) Results for the haploid screen plotted with descending z-scores. (b) Correlation between the z-scores from the initial screen and the LGRs from the retest. (c) Comparisons between the results of the haploid retest for *Rdh54p* SPIs and the results of the haploid retest for *rdh54p-K318R* SPIs. Strains that exhibited notable differences between the two screens are annotated.

Table 5-4 Genes related to the haploid *rdh54p*-K318R SPIs from the 16-replicate retest

	Genes (ranked based on their z-scores in descending order, with “non-frequent flyers” marked in red)
Validated haploid SPIs	<i>CSE4</i> , <i>RFA2</i> , <i>SEC10</i> , <i>SEC24</i> , <i>AME1</i> , <i>DDC2</i> , <i>BRL1</i> , <i>STS1</i> , <i>YRA2</i> , <i>BBP1</i> , <i>OKP1</i> , <i>MTR10</i> , <i>SPC29</i> , <i>NOP9</i> , <i>CBK1</i> , <i>MOB2</i> , <i>RFA1</i> , <i>FRQ1</i> , <i>DOA4</i> , <i>UBP3</i> , <i>LEU3</i> , <i>ACL4</i> , <i>BDF1</i> , <i>BUD23</i> , <i>MET30</i> , <i>NUP192</i> , <i>CDC12</i> , <i>VPS4</i> , <i>CDC40</i> , <i>CEP3</i>
Newly-identified SPIs from the retest	<i>KIP3</i> , <i>RTT105</i> , <i>MEX67</i> , <i>SPC42</i> , <i>KAP120</i> , <i>NKP2</i> , <i>DAD1</i> , <i>TPD3</i> , <i>CSE1</i> , <i>TSR2</i> , <i>ARC35</i> , <i>NOP8</i> , <i>VPS28</i> , <i>SPC24</i> , <i>MTR2</i> , <i>TAF10</i> , <i>RMD1</i> , <i>RFA3</i> , <i>SLY1</i> , <i>SPT15</i> , <i>KRI1</i> , <i>YTM1</i> , <i>ENP1</i> , <i>PRP5</i> , <i>RPA49</i> , <i>VMA3</i> , <i>APL4</i> , <i>NEO1</i> , <i>DID4</i> , <i>SNF7</i> , <i>ABF1</i> , <i>SIN4</i> , <i>RCL1</i>

For *rdh54p*-pip, 92 SPIs were identified in the initial screen, ~57% (52/92) of which were selected for the retest. In the retest, ~94% (49/52) of the selected SPIs were confirmed and 11 more SPIs were identified (Table 5-3). To examine if there was any difference between the SPIs for the wild-type *Rdh54p* and those for the mutant *rdh54p*-pip, I compared the results for the two proteins from the 16-replicate retests. As illustrated in Figure 5-7c, while many SPIs were common for both proteins, there were also some interesting discrepancies. Of particular interest are the kinetochore-related SPIs: the LGRs for most of them (*Nkp2p*, *Okp1p*, *Amel1p*, and *Spc24p*) were suppressed by the presence of the PIP-box mutation. In contrast, the *rdh54p*-pip SPI with *Dad1p* appeared stronger than for that with wild-type *Rdh54p*. Two other SPIs, *Sgs1p* and *Top3p*, which are two of the three components of the *Sgs1p*-*Top3p*-*Rmi3p* complex (a strain with *Rmi1p*-GFP was not in the GFP library used for the SPI assays reported here) that is involved in DNA damage repair⁵²⁷, were also specific for *rdh54p*-pip but not for wild-type *Rdh54p*. These differences show that the PIP-box motif of *Rdh54p* is important for its SPI phenotype with the kinetochore and the DNA damage site.

For the ATPase-deficient mutant *rdh54p*-K318R, however, no such differences were seen. 38 SPIs were initially discovered for *rdh54p*-K318R, while the retest validated ~97% (31/32) of the selected initial SPIs and identified 35 new SPIs (Table 5-4). As shown in Figure 5-8c, the 4 kinetochore-related SPIs (*Nkp2p*, *Okp1p*, *Amel1p* and *Spc24p*) for *Rdh54p* remained as SPIs for *rdh54p*-K318R, whereas both *Sgs1p* and *Top3p* were still not SPIs. Overall, the SPI results from the two *Rdh54p* mutants indicate that only the

rdh54-PIP mutation appears to change the SPI phenotypes, specifically suppressing SPIs with the kinetochore and enhancing SPIs with the Sgs1p-Top3p-Rmi3p complex.

5.2.3 Results from the screens focusing on kinetochore-related query proteins were highly consistent with those from the proteome-wide screens under normal growth condition

As the proteome-wide screens for Rdh54p SPIs had identified several hits at the kinetochore and some of these hits were affected by the PIP-box mutant *rdh54p-pip* but not the ATPase-deficient mutant *rdh54p-K318R*, I wished to more closely examine the effects for forced associations of Rdh54p or *rdh54p-pip* with just kinetochore proteins (KPs) and some other proteins related to the kinetochore, not just under the normal growth condition (see this section) but also upon drug treatments that induce microtubule instability and DNA damage (see Section 5.2.4). For this purpose, I selected a subset of the GFP-tagged strains from the GFP library used for the proteome-wide screens and arrayed them onto one rectangular agar plate together with a non-GFP control strain in the 1536-array format with 16 replicate colonies for each strain using the ROTOR robot. Table 5-5 summarizes the GFP strains used for the kinetochore screens, with the gene names of the GFP-tagged proteins grouped by the subcomplexes and/or functional categories to which they belong.

Table 5-5 GFP strains used for the kinetochore screens (denoted by the gene names)

Subcomplex/Category	Gene name
Centromeric histones	<i>CSE4, HTA1, HTA2, HTB1, HTB2, HHT1, HHT2, HHF1</i>
CBF3	<i>NDC10, CEP3, SKP1</i>
COMA	<i>CTF19, OKP1, MCM21, AME1</i>
CTF19 (excluding COMA)	<i>NKP2, CTF3, MCM16, MCM22, CHL4, IML3, CNN1</i>
Other inner kinetochore proteins	<i>CBF1, MIF2, YBP2</i>
MIND	<i>MTW1, NNF1, NSL1, DSN1</i>
SPC105	<i>SPC105, KRE28</i>
NDC80	<i>NDC80, NUF2, SPC24, SPC25</i>
DAM1	<i>DAM1, ASK1, DAD1, DAD2, DAD3, DAD4, SPC19, SPC34</i>

Spindle pole body	<i>SPC42, SPC97, SPC98, TUB4</i>
Nuclear pore complex (NPC)	<i>NUP53, NUP59, NUP157, NUP170</i>
MT-associated proteins (MAPs) and related proteins	<i>STU1, STU2, BIK1, BIM1, KAR9</i>
Motors and related proteins	<i>KIP1, KIP2, KIP3, CIN8, DYN1, DYN2, KAR3, SLK19, PAC1</i>
CPC	<i>IPL1, BIR1, SLI15</i>
SAC	<i>MPS1, MAD1, MAD2, MAD3, BUB1, BUB3, CDC20</i>
Other regulators	<i>CDC5, KIC1, GLC7, TPD3, PPH21, PPH22, CDC14, SGO1, SGT1, BFA1, MOB1, LRS4, CSM1, ULP2</i>

I first performed SPI screens for both Rdh54p and rdh54p-pip in haploid cells under normal growth conditions. Both screens were then repeated for validation. Similar to the proteome-wide screens, two types of control plasmids were used for the Rdh54p screens (expressing GBP-RFP or Rdh54p alone) while only one type was used for the rdh54p-pip screens (expressing GBP-RFP). LGRs were used to quantify colony growth, and the threshold was set to LGR=0.5 as before.

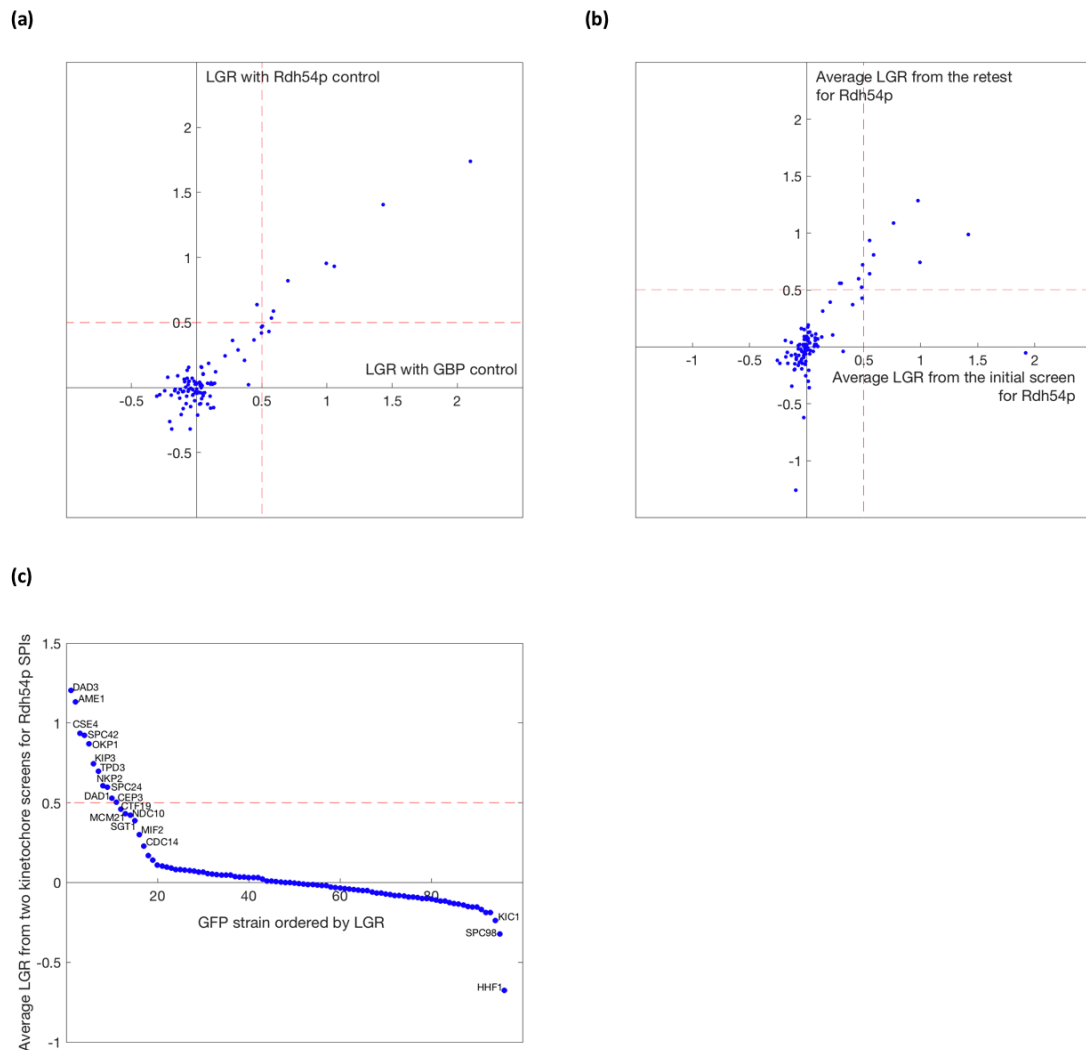


Figure 5-9 Results of the initial kinetochore screen and the retest for haploid *Rdh54p* SPIs

(a) Correlation between the LGRs for the initial screen calculated respectively with the two controls. (b) Correlation between the average LGRs from the initial screen and the average LGRs from the retest. (c) The LGRs averaged from the initial screen and the retest plotted in descending order. Strains with notable LGRs are annotated.

For the initial *Rdh54p* kinetochore screen, the two sets of LGRs determined using the two controls were well correlated (Figure 5-9a), similar to the proteome-wide screen results (Figure 5-3a). Hence, the average LGR was used as before to report the growth of each strain. A good correlation was also seen between the results from the initial screen and the retest (Figure 5-9b), and therefore, for each strain, the two LGRs averaged for the two controls from the two screens were further averaged to combine the results from the two

screens. Figure 5-9c illustrates these average LGRs from the two screens in descending order. All the kinetochore-related Rdh54p SPIs discovered in the proteome-wide screens (namely Ame1p, Cse4p, Spc42p, Okp1p, Kip3p, Tpd3p, Nkp2p, Spc24p, Dad1p and Cep3p) were again found as SPIs; in fact, of all the SPIs identified in this kinetochore-specific screen, only Dad3p was not a haploid SPI in the previous proteome-wide screen, yet it was found as a SPI in the diploid proteome-wide screen (Figure 5-4b). This high level of consistency among the results from different screens further demonstrates the robustness of the SPI methodology. In addition, a few other strains exhibited LGRs close to the threshold, which interestingly were all inner kinetochore-related ones (Ctf19p, Mcm21p, Ndc10p, Sgt1p, and Mif2p). Cdc14p also showed a slightly elevated LGR compared with the majority of strains, although the LGR was well below the threshold.

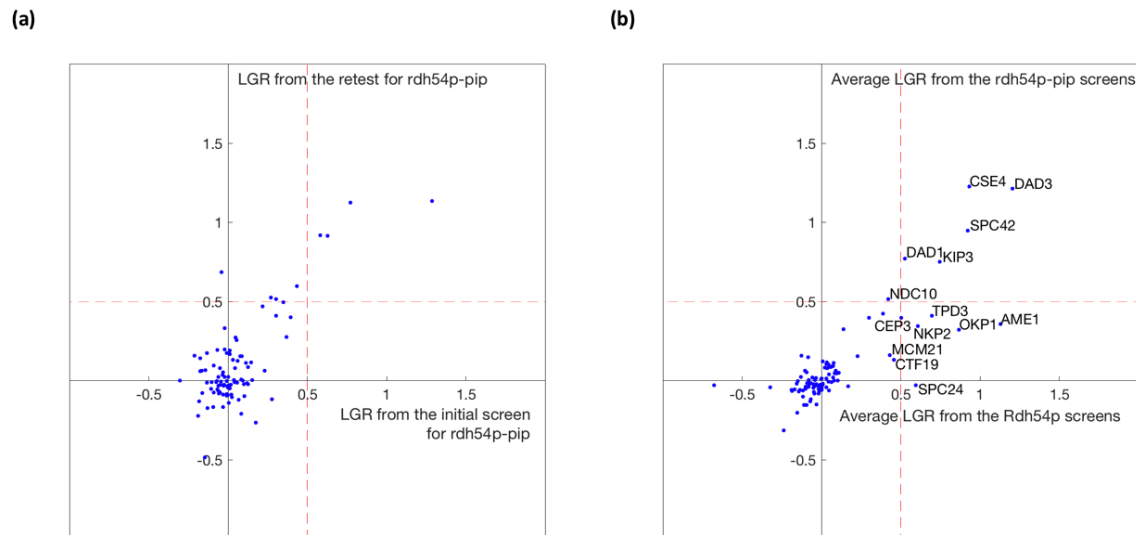


Figure 5-10 Results of the initial kinetochore screen and the retest for haploid rdh54p-pip SPIs

(a) Correlation between the LGRs from the initial screen and the LGRs from the retest. (b) Comparison between the average LGRs from the kinetochore screens for Rdh54p SPIs and the average LRGs from the kinetochore screens for rdh54p-pip SPIs.

For the rdh54p-pip kinetochore-specific screen and the retest, the two resulting sets of elevated LGRs again exhibited a good correlation (Figure 5-10a). The average LGR for each strain was then taken and compared with the corresponding average LGR from the Rdh54p screens, to see how the mutations in the PIP-box motif affected the SPI results.

The result of this comparison, as shown in Figure 5-10b, agrees with the one for the proteome-wide screen results shown in Figure 5-7c: while some GFP-tagged strains exhibited similarly high LGRs that were above or close to the threshold (Dad3p, Cse4p, Spc42p, Dad1p, Kip3p, Ndc10p, and to a lesser extent Cep3p and Tpd3p) in both the Rdh54p screens and the rdh54p-pip screen, some other strains were much more sensitive to Rdh54p than to rdh54p-pip. Consistent with the previous results, the latter strains included the ones from the COMA or the large CTF19 complexes (Ctf19p, Okp1p, Mcm21p, Ame1p, and Nkp2p) and the NDC80 complex member Spc24p (which was initially thought to be a frequent flyer in the proteome-wide screens for Rdh54p SPIs but was later found to be insensitive to the forced association with rdh54p-pip).

5.2.4 Additional SPIs were identified for Rdh54p and rdh54p-pip in the kinetochore screens in the presence of checkpoint-inducing drugs

The previous section showed that under normal growth conditions a number of kinetochore-related proteins when forced to associate with Rdh54p caused defects in cell growth, some of which were dependent on Rdh54p's PIP-box motif. It is possible that some of these associations could occur naturally at certain stages of a cell cycle to facilitate Rdh54p's functions, e.g. to promote the adaptation to the SAC-dependent DDC after a prolonged cell cycle arrest^{133,138}, and only cause growth defects when forced in an untimely manner. Therefore, I speculated that, in the presence of an activated checkpoint, some forced associations might make the cells grow differently than others. To test this notion, I used the GFP strains in Table 5-5 and performed screens for Rdh54p SPIs and rdh54p-pip SPIs with the strains grown on agar plates with the addition of benomyl (15 µg/ml) or MMS (0.02%). Benomyl is a microtubule depolymerising drug, which disrupts the kinetochore-microtubule attachments and therefore triggers the SAC, whereas MMS introduces DNA damage that leads to the activation of the DDC.

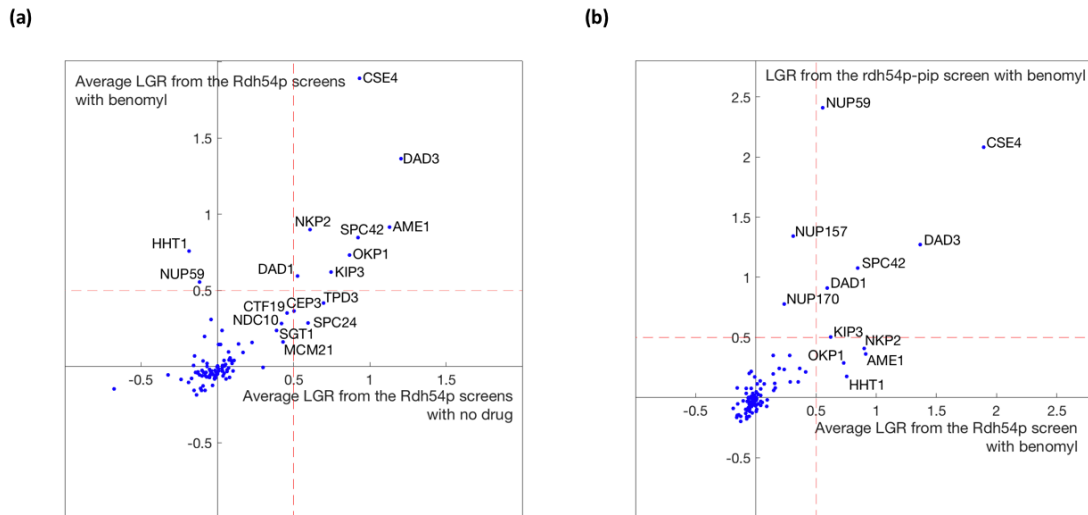


Figure 5-11 Results of the *Rdh54p* and *rdh54p-pip* SPI screens with benomyl

(a) Comparison between the average LGRs from the kinetochore screens for *Rdh54p* SPIs with no added drug and the average LGRs from the kinetochore screens for *Rdh54p* SPIs with benomyl. (b) Comparison between the average LGRs from the kinetochore screens for *Rdh54p* SPIs with benomyl and the average LGRs from the kinetochore screens for *rdh54p-pip* SPIs with benomyl.

Figure 5-11a compares the results of *Rdh54p* SPI screens with and without the addition of benomyl. In the screens with benomyl, the LGRs for *Tpd3p*, *Spc24p* and *Cep3p* dropped below the threshold, while a NPC component *Nup59p* and a histone H3 protein *Hht1p* exhibited higher LGRs (Figure 5-11a). When comparing the screen results for *Rdh54p* SPIs and *rdh54p-pip* SPIs in the presence of benomyl, I found that besides *Nup59p* two more NPC components (*Nup157p* and *Nup170p*) became SPIs for *rdh54p-pip* in the presence of benomyl (Figure 5-11b). For the KPs, on the other hand, the addition of benomyl had little effect on the SPI phenotypes: as seen with no drug addition, several components of the CTF19 complex (*Nkp2p*, *Okp1p*, *Ame1p*) became insensitive to the forced association with *Rdh54p* when the latter's PIP-box motif was mutated (Figure 5-11b). A similar change in sensitivity was also seen for the histone *Hht1p*.

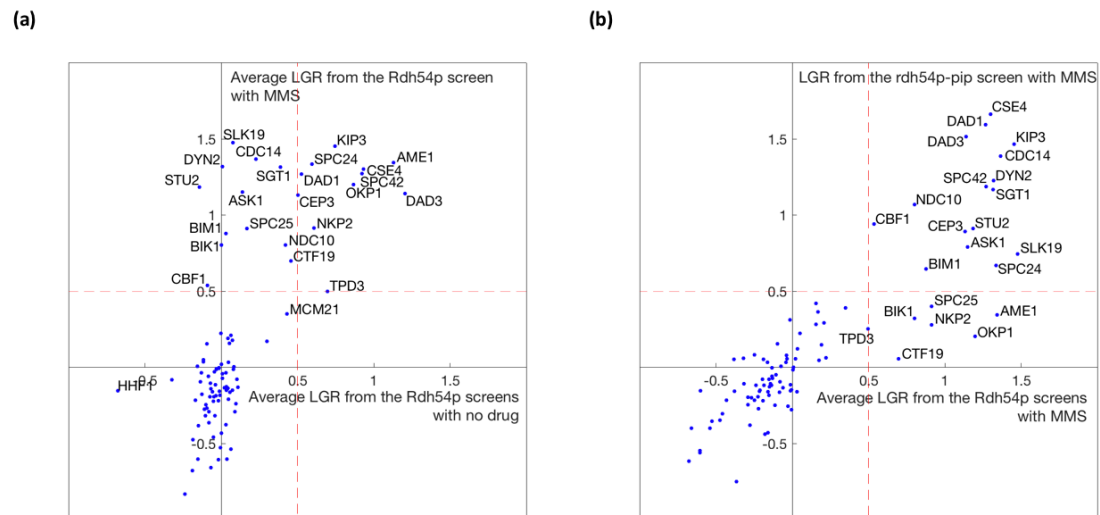


Figure 5-12 Results of the *Rdh54p* and *rdh54p-pip* SPI screens with MMS

(a) Comparison between the average LGRs from the kinetochore screens for *Rdh54p* SPIs with no added drug and the average LGRs from the kinetochore screens for *Rdh54p* SPIs with MMS. (b) Comparison between the average LGRs from the kinetochore screens for *Rdh54p* SPIs with MMS and the average LGRs from the kinetochore screens for *rdh54p-pip* SPIs with MMS.

In the presence of MMS, in addition to the SPIs identified under unperturbed growth condition, many more hits were found for both *Rdh54p* (Figure 5-12A) and *rdh54p-pip* (Figure 5-12B). These included two mitotic exit-related proteins (*Cdc14p* and *Slk19p*), an outer kinetochore protein (*Ask1p*), and three inner kinetochore proteins (*Cbf2p*, *Ndc10p*, and *Sgt1p*). Curiously, many MAPs and motor proteins (*Dyn2p*, *Stu2p*, *Bim1p*, and *Bik1p*) were also found to be SPIs for both *Rdh54p* and *rdh54p-pip* in these screens, in addition to *Kip3p* that appeared as hits for all other screens. It is worth noting that in MMS the *Rdh54p* SPIs belonging to the CTF19 complex (*Ctf19p*, *Okp1p*, *Ame1p*, and *Nkp2p*) were again suppressed with the *rdh54p-pip* mutant, and similar behaviours were seen for *Spc25p*, *Bik1p*, and *Tpd3p*.

5.2.5 Summary and discussion

In this section, I reported the results of various screens for the SPIs of the wild-type *Rdh54p* and two of its mutants, *rdh54p-pip* and *rdh54p-K318R*.

In the search of the SPIs of Rdh54p and its mutants across the yeast proteome, I found that several hits related to the early stage of the DNA damage response. All the three subunits of the RPA complex (Rfa1p, Rfa2p, Rfa3p) were identified as Rdh54p SPIs (although Rfa3p is a “frequent flyer” in other SPI screens as well), together with Ddc2p. The RPA complex plays a critical role in the DNA damage response by promoting DNA end resection, binding to the ssDNA generated from the resection, and subsequently promoting the recruitments of various proteins required for DNA damage repair and DDC signalling⁸². These proteins include Ddc2p, which is the interacting partner of Mec1p that initiates the DDC at ssDNA^{70,528}. Both the RPA subunits and Ddc2p also appeared as SPIs for the two mutants of Rdh54p. In contrast, Sgs1p and Top3p, the subunits of the STR complex (Sgs1p-Top3p-Rmi1p; a strain with Rmi1p-GFP was not in the GFP library used for the SPI screens) that works together with RPA in both DNA end resection and DNA decatenation^{80,527}, were only found as SPIs for the PIP-box mutant *rdh54p-pip*. It is intriguing that the forced associations of Rdh54p or mutants to these proteins functioning at the early stage of the DNA damage response can cause growth defects, even in the absence of DNA damage-inducing drugs. It would be informative to monitor the cell cycle progression of single cells using live cell microscopy to examine if inducing such forced associations lead to premature DDC silencing in the presence of DNA damage, as a positive result would imply that Rdh54p promotes the DDC adaptation by disrupting the upstream checkpoint signals. It would also be worth further dissecting the functional differences between the PIP-box mutant *rdh54p-pip* and the ATPase mutant *rdh54p-K318R*. Two studies on the PIP-box motif of the Rdh54p homolog Rad54p suggest that the functional defects caused by mutating residues in this motif are more related to the abolishment of the protein’s ATPase activity than its reduced interaction with the PCNA clamp^{503,504}. However, my finding that Sgs1p and Top3p were SPIs for *rdh54p-pip* but not for *rdh54p-K318R* (as well as the differential reactions of several KPs to the mutants; discussed below) suggests that the two mutants have distinct functions. It might be that both activities of Rdh54p are required for it to function together with the PCNA, with the PIP-box enabling the Rdh54p-PCNA interaction and the ATPase activity facilitating Rdh54p’s canonical functions as a translocase and a chromatin remodeller. In fact, the budding yeast PCNA Pol30p was almost a SPI for Rdh54p with a z-score of 1.97 in the initial proteome-wide screen, while the z-scores for the two mutants were only ~1.

The proteome-wide screens also identified Rdh54p SPIs enriched at the kinetochore, which were further examined with screens focusing on the kinetochore-related proteins. The results from the proteome-wide screens and from the kinetochore screens were highly consistent and included proteins belonging to various subcomplexes/categories:

- centromeric histone: Cse4p*;
- CBF3: Cep3p*;
- CTF19: Okp1p, Ame1p, Nkp2p;
- NDC80: Spc24p*;
- DAM1: Dad1p, Dad3p;
- spindle pole body: Spc42p*;
- motor: Kip3p
- PP2A phosphatase: Tpd3p

Note that the proteins marked by asterisks are “frequent flyers” in other SPI screens, and therefore may not bear meaningful information specific to Rdh54p’s function. The enrichment of the “non-frequent flyers” in the CTF19 complex is particularly interesting: two other CTF19 components, Ctf19p and Mcm21p, also exhibited LGRs close to the threshold in the kinetochore screens for Rdh54p SPIs, and all these five proteins (Okp1p, Ame1p, Nkp2p, Ctf19p, Mcm21p) exhibited considerably lower LGRs in the screens for rdh54p-pip SPIs. Similar trends were also seen for Spc24p and Tpd3p. The differential reactions of these proteins to the forced associations with Rdh54p/rdh54p-K318R and rdh54p-pip again highlight the functional importance of Rdh54p’s PIP-box motif. These data demonstrate a strong dominant growth effect of constitutive Rdh54p association with the CTF19 complex, dependent upon the PIP box; however, this does not explain Rdh54p’s normal function at the kinetochore. Previous SPI screens show that proteins often show SPIs when constitutively associated with their normal interacting proteins. Thus, one possibility is that Rdh54p constitutively localises at the kinetochore by interacting with some CTF19 components, and its action there depends upon the PIP box, perhaps via interacting with RPA. It may be more likely that the forced association of Rdh54p with the CTF19 components may affect its function in promoting the adaptation to the SAC-dependent DDC. If time allowed, I would be interested to test if the SPIs are dependent upon the SAC.

I also performed kinetochore screens for Rdh54p or rdh54p-pip SPIs with the addition of benomyl or MMS to the agar plates for triggering the SAC or the DDC, respectively.

For the screens with benomyl, I noted that several components of the NPC behaved differently compared with the results of the screens performed under normal growth condition: Nup59p became a SPI for both Rdh54p and rdh54p-pip, with the SPI phenotype for rdh54p-pip being much stronger, whereas Nup157p and Nup170p were rdh54p-pip-specific SPIs. The NPCs are evolutionarily conserved large protein complexes embedded in the nuclear envelope that act as channels for nucleocytoplasmic exchange of macromolecules and also have important roles in spindle assembly, kinetochore function, and the SAC (reviewed in Ref.⁵²⁹). Interestingly, the three NPC components identified as rdh54p-pip SPIs (together with the Nup59p paralog Nup53p as well as two other NPC components Mlp1p and Mlp2p) have been found to be associated with the SAC components Mad1p and Mad2p^{200,201} in budding yeast, and this association is conserved across distinct organisms⁵³⁰. The NPCs seem to be docking sites for Mad1p and Mad2p, only releasing them for their relocalisation to unattached kinetochore(s) when the SAC is activated and relying the cycling of Mad1p between the NPCs and the kinetochores for regulating nuclear transport in the presence of the SAC^{531,532}. It needs to be investigated further why Rdh54p, which has a putative role in promoting SAC adaptation, causes a growth defect when forced to the NPC. More experiments are also needed to examine whether rdh54p-pip retains Rdh54p's function in checkpoint adaptation and to explain why the three NPC components are more sensitive to rdh54p-pip association.

For the screens with MMS, many proteins exhibited increased sensitivity to the associations with both Rdh54p and rdh54p-pip (Figure 5-12). This increased number of kinetochore-related SPIs induced by MMS might be a result of the linkage between the DDC and the SAC (see Section 1.1.4), but it is unclear whether this phenotype indeed bears meaningful information about Rdh54p's putative role in the DDC or it merely reflects the cell's increased sensitivity to forced associations in general in the presence of DNA damage. The former possibility may be supported since Cep3p, a CBF3 component whose phosphorylation by the DDC kinase Mec1p promotes a Mad2p-dependent checkpoint arrest¹⁹⁴, exhibited much higher z-scores in the screens with MMS than under

normal growth condition. To my surprise, quite a few MAPs and motors were found as Rdh54p or rdh54p-pip SPIs when MMS was added, but they were largely unaffected by the addition of benomyl that should more directly disrupt microtubules. This might be explained by that the kinetochore-microtubule attachments were affected by the presence of global MMS-induced DNA damage as well, whereas the amount of benomyl used in the screens might be sufficient to trigger the SAC by disrupting a few microtubules but were not high enough to cause a profound impact on the activities of those MAPs and motors.

Overall, the SPI screens have identified a number of proteins that might be related to Rdh54p's function in checkpoint adaptation and have also revealed that the PIP-box motif of Rdh54p has functional roles distinct from the protein's ATPase activity. Nevertheless, it is difficult to interpret the SPI results since the exact cause of the observed growth defect is not clear, especially in the presence of checkpoint signals, as both prolonged checkpoint arrest and premature checkpoint adaptation could lead to reduced colony size. Therefore, these SPI results need to be followed with further experiments, for example by using time-lapse live-cell imaging to examine how the forced associations affect a cell's behaviour in the presence of checkpoint-inducing reagents.

5.3 FLIM screens for Rdh54p's interacting partners

Considering that Rdh54p is known to constitutively localise at the kinetochore and that several KPs presented hits for the Rdh54p SPI screens described above, it would be interesting to more precisely explore the location at the kinetochore where Rdh54p normally resides. To this end, I utilised the FLIM HCA methodology described in Chapter 3 and validated by the experiments discussed in Chapter 4 to screen for interacting partner(s) of Rdh54p at the kinetochore. This section reports the results of FLIM experiments that explored the proximity of Rdh54p's C-terminal end to the C-terminal ends of various selected KPs using FRET.

5.3.1 Strains constructed for the Rdh54p FLIM screens

I utilised the budding yeast GFP library used in the previous SPI screens to construct the diploid strains to be imaged in the FLIM screens. I selected 16 KPs representing various locations along the kinetochore-microtubule axis, many of which were found as SPIs for Rdh54p, as candidates of the FLIM screens for Rdh54's interacting partners (Figure 5-13), and I picked out the corresponding 16 *MATa* strains from the GFP library. I also constructed a *MATα* strain expressing Rdh54p-mCherry, by inserting a DNA fragment encoding mCherry and a marker for hygromycin resistance at the end of the *RDH54* gene via yeast transformation for genomic integration. These selected *MATa* GFP strains were then mated respectively with the *MATα* Rdh54p-mCherry strain to construct the dual-labelled diploid strains that were heterozygous for the genes of both the FRET donor GFP and the acceptor mCherry (note that the GFP protein expressed from the yeast GFP library is in fact the variant GFP-S65T^{432,433,533}, but for simplicity I denoted it here as GFP). For each of these dual-labelled strains, I also constructed a donor-only diploid strain by mating the corresponding *MATa* GFP strain with a wild-type *MATα* strain to serve as a negative control for FRET. Constructing one negative control for each dual-labelled strain was necessary, as I expected that the donor GFP would exhibit different non-FRETing lifetimes when it was tagged to different KPs (as was found for mTq2FP), the FRET donor I used for the previous FLIM experiments for validating the methodology reported in Chapter 4. In principle, it would be more efficient to label Rdh54p with GFP and the KPs with mCherry, as then only one donor-only negative control would need to be made for all the dual-labelled query strains. However, Rdh54p-GFP did not exhibit kinetochore foci discernible by either a wide-field fluorescence microscope or the FLIM microscope and so this approach did not work. Further, kinetochore foci were also not observed in a strain expressing Rdh54p-mTq2FP; for reasons not yet clear, kinetochore foci were only seen in a strain with Rdh54p-YFP (using the variant YFP-10C⁵³⁴). One possibility is that owing to the tendency of the YFP molecules to dimerise with each other, there are more Rdh54p molecules at the kinetochore in the Rdh54p-YFP strain than in a wild-type strain. I was also unable to make the query strains homozygous for the acceptor gene to increase the opportunity of FRET (as I did for most of the strains reported in Chapter 4). This is because the strains used in this chapter are of the S288C background,

which tend to sporulate poorly compared with the W303-derivative strains used in Chapter 4.

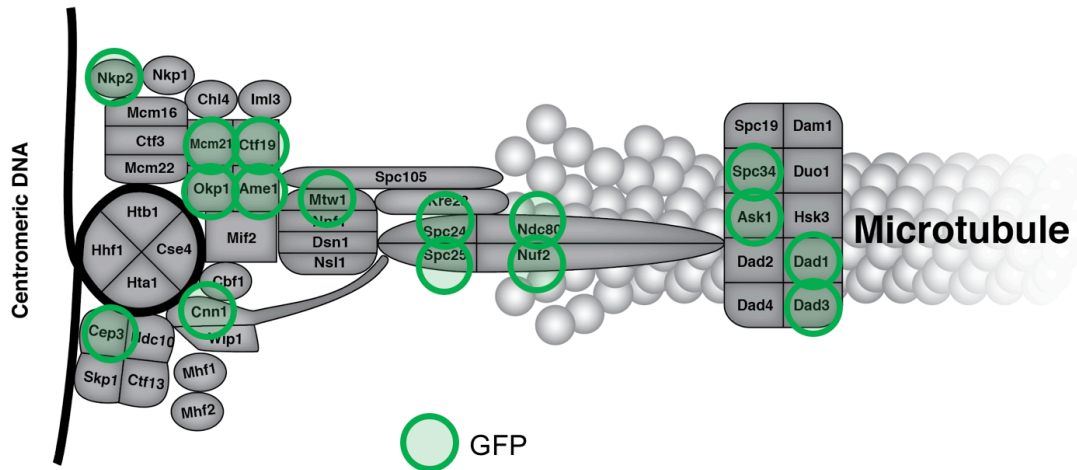


Figure 5-13 Kinetochore proteins selected for being respectively labelled by the FRET donor GFP

The approximate localisations of the 16 selected KPs (Cep3p, Cnn1p, Nkp2p, Ctf19p, Okp1p, Mcm21p, Ame1p, Mtw1p, Spc24p, Spc25p, Ndc80p, Nuf2p, Spc34p, Ask1p, Dad1p, Dad3p) along the kinetochore-microtubule axis are highlighted on a schematic of the budding yeast kinetochore.

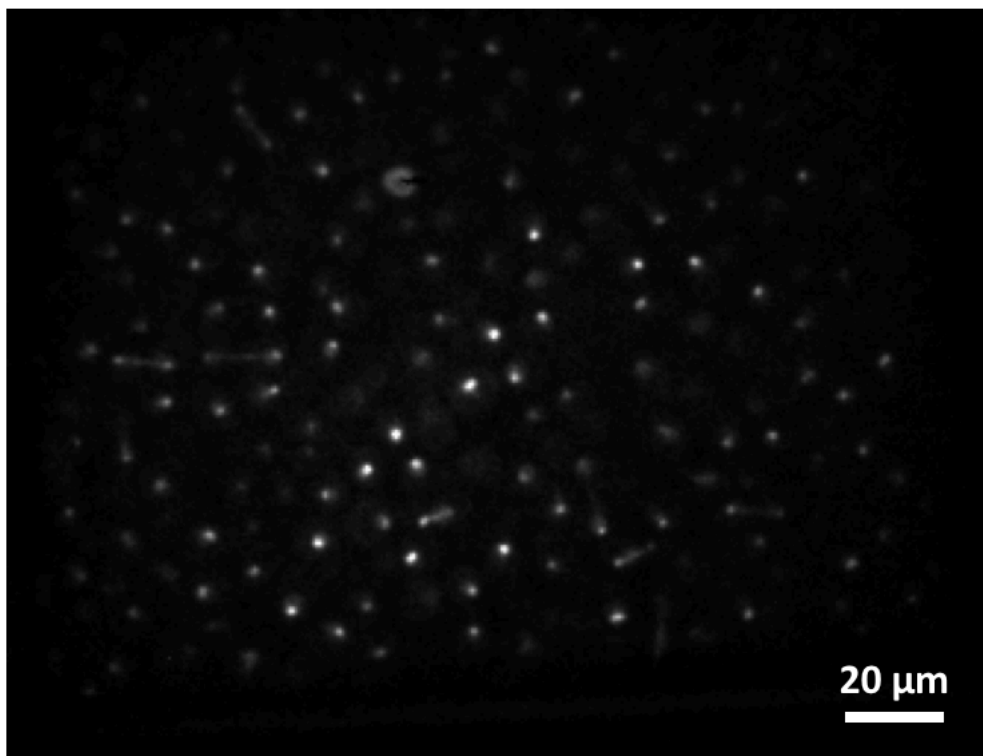


Figure 5-14 Exemplar fluorescence intensity image of diploid budding yeast cells expressing Ask1p-GFP

The image was taken with the automated FLIM microscope. Each bright focus is a collection of Ask1p-GFP molecules in a kinetochore cluster.

5.3.2 Combining the image-wise fitted lifetime results from multiple experiments and filtering the data based on the total signal level in each FOV

I performed the FLIM experiments and the subsequent FLIM data analysis following the method described in Chapter 3. Similar to the experiments reported in Chapter 4, in each of these experiments I transferred the cell culture of each strain into 3 replicate wells and took 45 FOVs in total for each strain, i.e. 15 FOVs per well. Each FOV was imaged with 3 time-gates for pre-photobleaching and 7 for sampling the fluorescence decay, with the image for each time-gate integrated for 2 s on the CCD camera. With the time needed for stage movement and auto-focusing added, each FOV required ~30 s for image acquisition. This was ~33% faster than imaging mTq2FP in the previous experiments, mainly due to the higher brightness of GFP compared with mTq2FP and the weaker autofluorescence background in the GFP channel that required less time for pre-photobleaching, as shown

in the exemplar fluorescence intensity image of cells expressing Ask1p-GFP in Figure 5-13 (in comparison to the exemplar image shown in Figure 3-8 where Ask1p was labelled with mTq2FP). In total, it took ~23 min to image each strain and thus <6 hr for an experiment involving 14 strains. Only 14 pairs out of the 16 pairs of constructed strains were imaged, as no GFP foci were seen in 2 of the strains (Cnn1p-GFP and Spc34p-GFP) under the FLIM microscope.

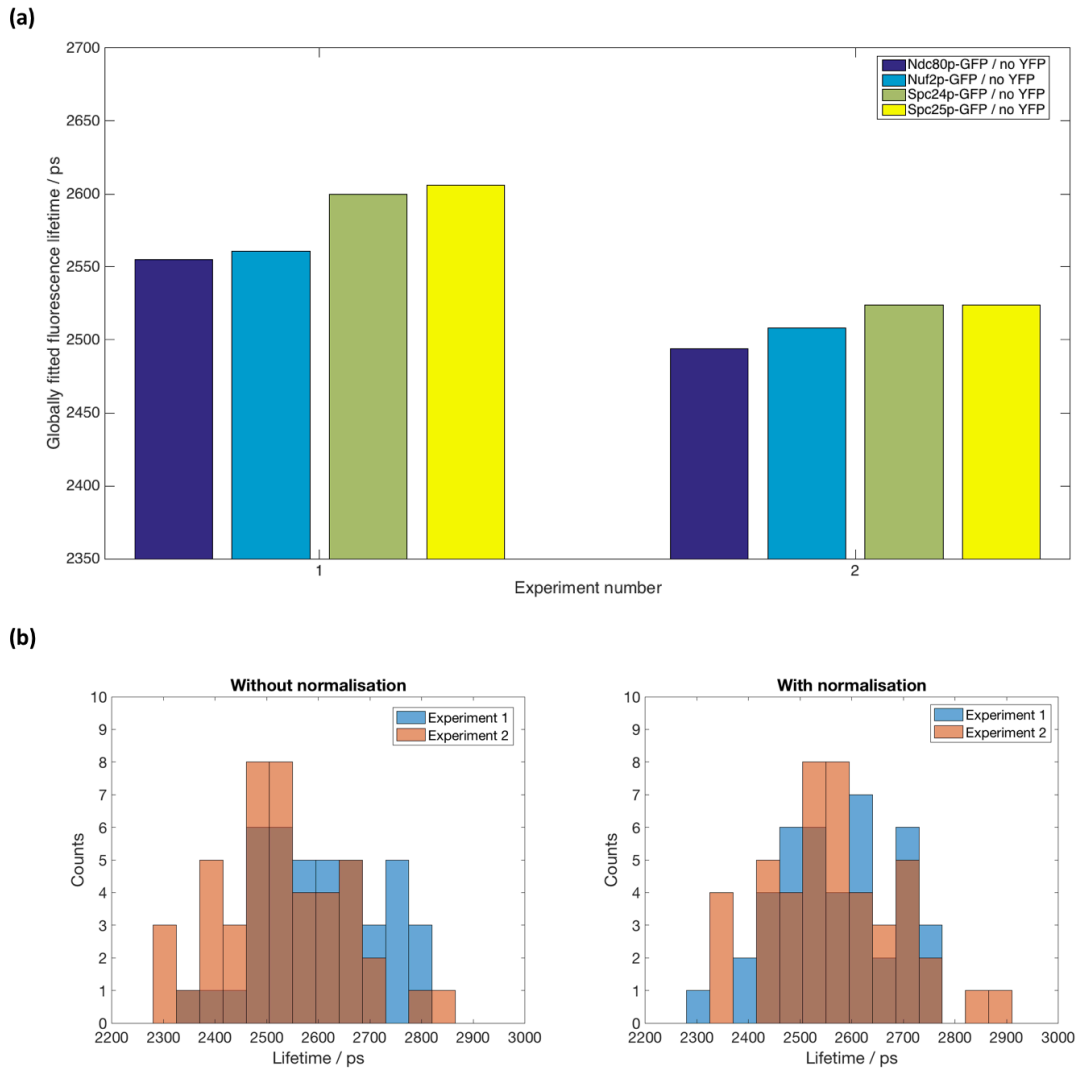


Figure 5-15 Lifetime results from multiple experiments for the same strain needed to be normalised before being combined

(a) Globally fitted donor fluorescence lifetimes for the 4 donor-only strains related to the NDC80 complex from 2 separate experiments.(b) The effect of the normalisation operation on the image-wise lifetimes results for the donor-only strain expressing Spc24p-GFP.

To minimise potential biases introduced to the results during the prolonged experiments owing to the deterioration of cell fitness of time, the positions of the wells of all strains on a 96-well plate were semi-randomised in the same manner as in Figure 3-20. I also conducted at least 2 separate experiments on different days for each strain to further average over biological and experimental noise. To determine whether the results for a strain from separate experiments can be directly combined together for further statistical comparisons, as done for the experiments in Chapter 4, I examined the globally fitted mono-exponential donor fluorescence lifetimes for all the donor-only control strains. Unlike what was seen in Chapter 4, these measurements did seem to present systematic shifts in the measured lifetime values from different multiwell plate experiments compared with the impact of noise within a multiwell plate experiment. Figure 5-15a provides an example of these lifetime shifts using the results from the 4 donor-only strains related to the NDC80 complex at the kinetochore. The presence of these shifts, which could be due to some systematic biases as discussed in Section 4.2.2, could skew the true underlying distributions of the lifetimes if the lifetime results are combined directly. To reduce the impact of these shifts, I decided to perform a normalisation operation on the lifetime results for each strain from multiple experiments when combining them. The normalisation operation was performed using a custom-written MATLAB script following the algorithm below:

1. Load in the image-wise lifetime results for a pair of strains, i.e. a dual-labelled query strain and the corresponding donor-only control, from one experiment;
2. Get the median lifetime from the control strain's results, m_{c1} , and then normalise the image-wise lifetime results for both strains towards that median lifetime by dividing them with m_{c1} , resulting in two normalised unitless lifetime datasets, τ_{qnu1} (for the query strain) and τ_{cnu1} (for the control strain);
3. Repeat the above two steps for the results for the same pair of strains from a second experiment, i.e. to get another median lifetime from the control strain, m_{c2} , and normalise all the lifetime results, and keep repeating if there are results from more experiments for the same pair of strains;
4. Use the median lifetime from each experiment ($m_{c1}, m_{c1}, \dots, m_{cn}$) to calculate a mean median lifetime (m_c) weighted by the total signal count in the control

strain's segmented kinetochore images from each experiment ($s_{c1}, s_{c1}, \dots, s_{cn}$),

$$\text{i.e. } m_c = \frac{\sum_{i=1}^n (m_{ci} \times s_{ci})}{\sum_{i=1}^n s_{ci}}.$$

5. Combine the normalised unitless lifetime datasets into one single dataset for each strain, and then multiply the unitless lifetime values with m_c to get the final normalised lifetime dataset for each strain.

Figure 5-15b shows an example of how the normalisation operation reduced the effect of lifetime shifts between separate experiments on the distribution of the image-wise lifetimes for the donor-only strain expressing Spc24p-GFP.

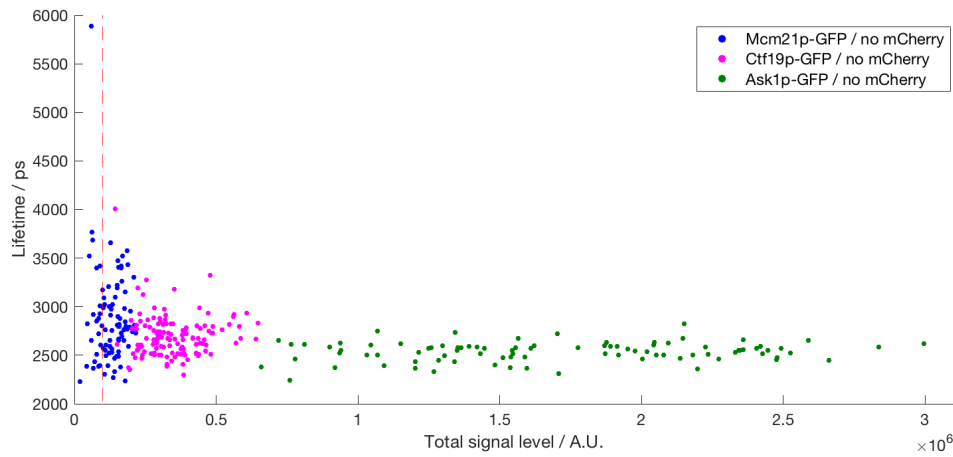


Figure 5-16 Distributions of the fitted image-wise donor fluorescence lifetimes against the total signal level of each FOV for 3 different donor-only strains

A threshold for the total signal level was consequently set to 1×10^5 digital number (red dashed line) for excluding FOVs with insufficient photon counts from the following statistical comparisons.

After combining the image-wise lifetime results, I decided to filter them based on the total signal level in each FOV, as was done with the data in Chapter 4. As shown by the exemplar results for 3 strains in Figure 5-16, similar to what was found in Figure 4-4 and Figure 4-5 the distribution of the image-wise lifetimes still exhibited a dependency on the total signal level of each FOV; the higher the total signal level, the narrower the lifetime distribution. The brightness of GFP relative to the autofluorescence background was much higher than that of mTq2FP, and therefore it allowed the acquisition of more signal using lower excitation power, fewer time gates, and shorter exposure time compared with

the experiments using mTq2FP as the donor. Nevertheless, for the KPs at the inner kinetochore, e.g. Mcm21p and Ctf19p as shown in Figure 5-16, the acquired total signal levels for some images could still be too low for precise estimation of the underlying donor fluorescence lifetime. I note that, while Mcm21p was expected to have the same protein copy number as Ctf19p, as both of them belong to the COMA complex and each contributes one copy to one complex, the measured average pixel intensity, as well as the total signal level per FOV, were much lower for Mcm21p-GFP than the Ctf19p-GFP. The cause of this is unclear but was not investigated further due to lack of time. I decided to set a threshold for the total signal level for each FOV at 1×10^5 digital number and to only use the lifetime results from the FOVs that satisfied this criterion for the subsequent statistical analyses. I expected that restricting the analysis to these high-quality data would increase the likelihood of revealing an underlying donor fluorescence lifetime difference between a query strain and a control strain that would indicate the presence of FRET.

5.3.3 A reduction in the donor fluorescence lifetime seen in the positive control strain validates the use of GFP/mCherry as the FRET pair

After processing the lifetime results as described in the previous two subsections, I compared the donor fluorescence lifetime result from a dual-labelled diploid strain expressing Spc24p-GFP and Spc25p-mCherry (heterozygous for both the FP genes) with that from the corresponding donor-only control. The dual-labelled strain was constructed to be a positive control for FRET, nothing that the C-termini of Spc24p and Spc25p were shown to be in close proximity in Chapter 4. As demonstrated in Figure 5-17, this strain indeed exhibited a significantly reduced donor fluorescence lifetime compared with the donor-only control when the results were statistically compared using the two-sided Wilcoxon rank-sum test. While the observed reduction in the median lifetime was only 141 ps, which is much lower than that found in Chapter 4 (1064 ps) when mTq2FP and YFP were used as the FRET pair, this result nevertheless validated GFP and mCherry as a working, although not ideal, FRET fluorophore pair in budding yeast cells. The differences in the measured lifetime reduction and the effective FRET efficiency could be partly explained by the fact that there were more acceptors for the donors to FRET

with in the strain used in Chapter 4, as it was homozygous for the acceptor gene (see the discussion in Section 4.3).

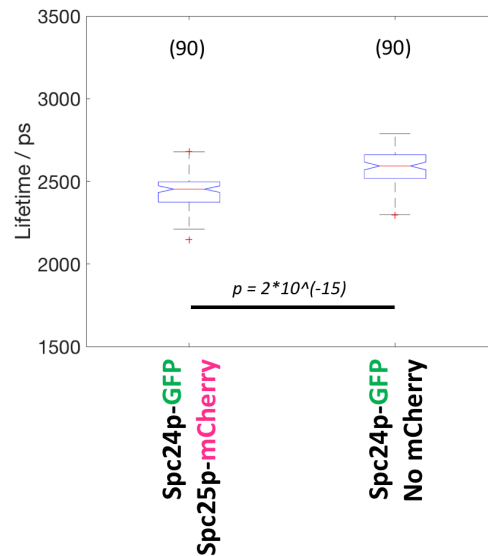


Figure 5-17 FLIM results of the strain expressing Spc24p-GFP/Spc25p-mCherry and the corresponding donor-only strain

Each box plot shows the median value (bar) and the quartiles (box) of the fitted image-wise donor fluorescence lifetime values. The notches indicate the 95% confidence interval of the median. The whiskers correspond to the minimum of the data range or $1.5 \times$ the interquartile range. Extreme data points are marked as outliers. The number of FOVs analyzed for each strain is shown in parentheses above the corresponding box. The statistical test performed was the two-sided Wilcoxon rank-sum test.

5.3.4 FLIM results suggest that Rdh54p may be localised in proximity with Ctf19p and Nkp2p in the CTF19 complex at the kinetochore

Similar to Section 5.3.3, the lifetime results for each pair of the strains in the Rdh54p FLIM screens were compared using the two-sided Wilcoxon rank-sum test. Among the 14 analysed query strains, 2 of them exhibited statistically significant lifetime reductions when tested individually with a significance level of $\alpha=0.05$, which were the Ctf19p-GFP and the Nkp2p-GFP strains (Figure 5-18a and Figure 5-18b). My methodology was unable to detect a statistically significant lifetime change between any other pair of query strain and donor-only strain (Figure 5-19).

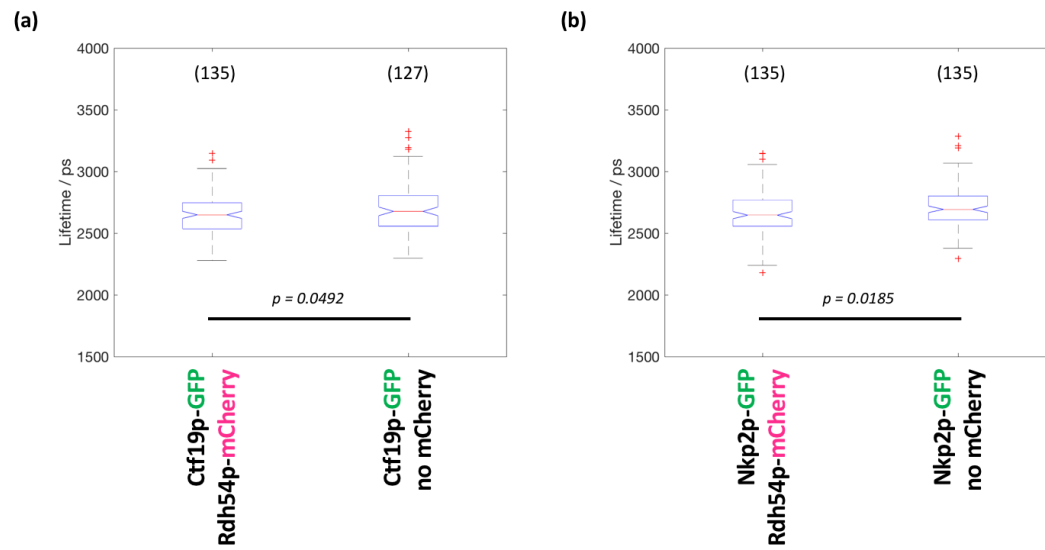


Figure 5-18 FLIM results for the two pairs of strains where significant reductions in the donor fluorescence lifetimes of the query strains were seen

(a) The query strain expressing Ctf19p-GFP and Rdh54p-mCherry exhibited a significantly reduced donor fluorescence lifetime as compared with the donor-only negative control using the two-sided Wilcoxon rank-sum test. (b) The query strain expressing Nkp2p-GFP and Rdh54p-mCherry exhibited a significantly reduced donor fluorescence lifetime as compared with the donor-only negative control using the two-sided Wilcoxon rank-sum test. Each box plot shows the median value (bar) and the quartiles (box) of the fitted image-wise donor fluorescence lifetime values. The notches indicate the 95% confidence interval of the median. The whiskers correspond to the minimum of the data range or $1.5 \times$ the interquartile range. Extreme data points are marked as outliers. The number of FOVs analyzed for each strain is shown in parentheses above the corresponding box.

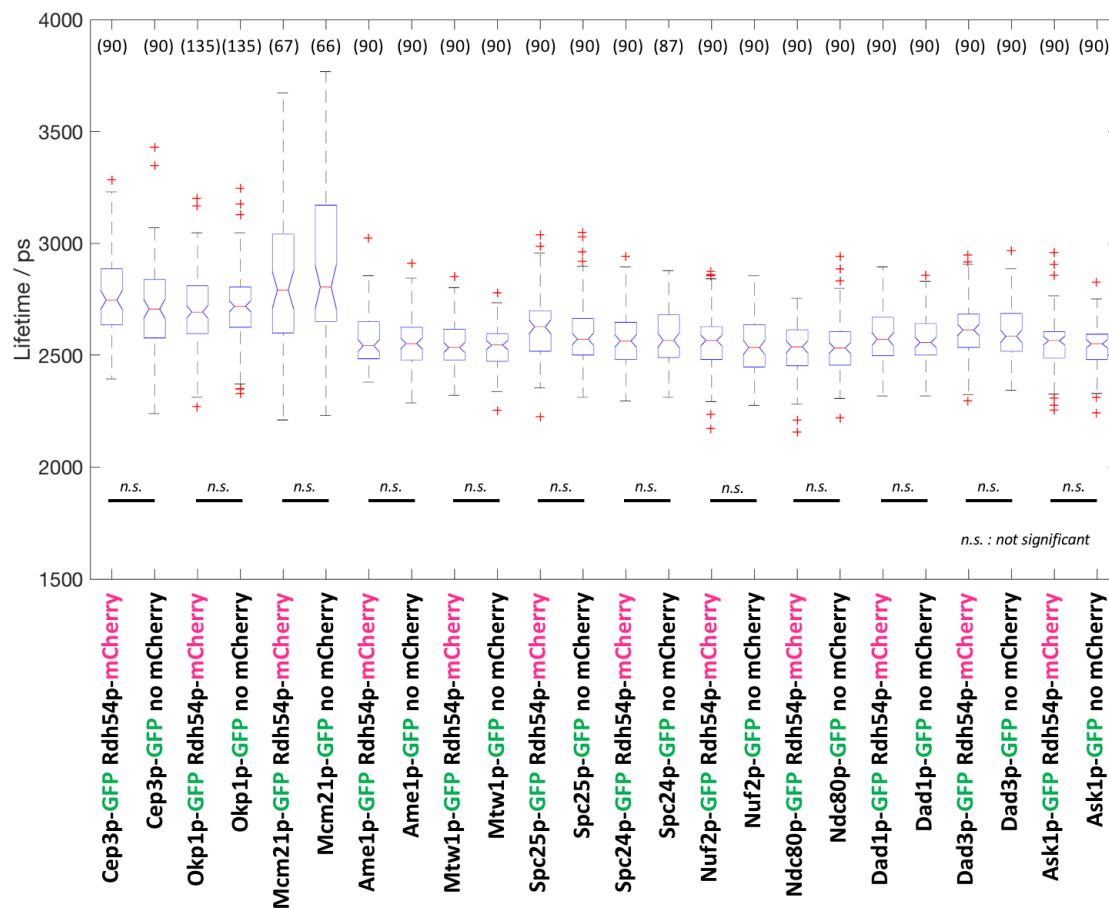


Figure 5-19 FLIM results for all the other strains where no significant donor fluorescence lifetime change was seen

None of the query strains in these groups showed a significantly different lifetime value compared with their corresponding donor-only negative controls under the two-sided Wilcoxon rank-sum test. n.s.: non-significant. Each box plot shows the median value (bar) and the quartiles (box) of the fitted image-wise donor fluorescence lifetime values. The notches indicate the 95% confidence interval of the median. The whiskers correspond to the minimum of the data range or $1.5 \times$ the interquartile range. Extreme data points are marked as outliers. The number of FOVs analyzed for each strain is shown in parentheses above the corresponding box.

Based on the FLIM results, it seems that Ctf19p and Nkp2, both of which belong the CTF19 complex at the inner kinetochore, are in close proximity with Rdh54p. However, it has to be noted that, among the 3 separate FLIM experiments performed for each pair of strains that constituted the final combined results shown in Figure 5-18, only in one experiment was a lifetime reduction seen for each pair of strains, respectively; in the other two experiments there was no significant lifetime difference for each pair of strains. The reductions in the median lifetimes in the combined results were also quite small compared

with what was seen in the positive control strain in the above subsection, being 28 ps for the Ctf19p-GFP pair and 47 ps for the Nkp2p-GFP pair, which was reflected by the relatively large *p*-values.

5.3.5 Summary and discussion

In this section, I reported the application of the FLIM HCA platform to undertake FRET-based screens to identify interacting partners of Rdh54p at the kinetochore.

For these screens, I constructed 16 query strains where the donor GFP and the acceptor mCherry were endogenously tagged to a KP and Rdh54p, respectively, as well as 16 corresponding donor-only strains to provide negative controls for FRET. Since 2 out of these 16 pairs of strains did not exhibit discernible kinetochore foci (Cnn1p-GFP and Spc34p-GFP), only 14 pairs of strains were imaged. Due to the use of fewer time-gates for each FLIM image (10 vs. 12) and a shorter exposure time (2 s vs. 3 s) for each time-gate (made possible by the relatively higher brightness of GFP compared with mTq2FP and the lower cellular autofluorescence in the green channel), only ~30 s was required to image each FOV, thereby increasing the imaging speed by ~33% compared to the imaging of the strains expressing mTq2FP-labelled KPs as reported in Chapter 4. As there seemed to be a more significant systematic shift between the donor fluorescence lifetimes measured for the same strain but from different experiments, I employed a normalisation-based algorithm to correct for this shift when combining FLIM results from multiple experiments. The combined FLIM results for each strain were then filtered based on the total signal level in each FOV, and only FOVs with sufficient photon counts were retained for the subsequent statistical comparisons.

The validity of using GFP and mCherry as a FRET pair was confirmed by a positive control strain for FRET, where Spc24p and Spc25p were labelled by the FRET pair respectively: a reduction in the donor GFP fluorescence lifetime compared with the corresponding donor-only negative control was observed as expected. Two query strains, one with Ctf19p-GFP/Rdh54p-mCherry and the other with Nkp2p-GFP/Rdh54p-mCherry, also exhibited reductions in the donor fluorescence lifetime compared with their negative controls, suggesting that FRET occurred between the fluorophores and that

Rdh54p's constitutive localisation at the kinetochore is near the CTF19 complex. However, it should be noted that these reductions in the median lifetimes were small (28 ps and 47 ps respectively) compared to the non-FRETing donor lifetimes of ~2600 ps measured from the two negative controls, and they were not observed in every experiment. It is likely that these small lifetime reductions were close to the noise level, and therefore in some experiments they were buried by the noise; this could be improved as discussed below. Another possible reason for the low donor lifetime reductions upon FRET - and the lack of them for some of these strains - could be that the relative orientation of the FRETing fluorophore dipoles was constrained to be close to orthogonal, which would have reduced the FRET efficiency and led to false negative results concerning the proximity of the fluorophores⁵³⁵. In addition, the statistical tests were applied for each query strain in isolation. Given the significance level chosen of $\alpha=0.5$, if 20 query strains are tested then we would expect one strain to show a positive interaction through chance alone. The fact that we obtain two query strains that exceed our chosen significance level out of 14 tested indicates that at least one of these is likely to be a real one. If time allowed then a more rigorous statistical analysis could be performed, but further experimental repeats could also be performed in order to allow these small changes in fluorescence lifetime to be detected more reliably.

Overall, the results of the FLIM screens are encouraging and they have identified the CTF19 complex as a potential localisation of Rdh54p at the kinetochore - where the SPI screens also found hits. The results of these two sets of screens support the idea that Rdh54p's localisation near the CTF19 complex is regulated by the cell cycle and has a functional role that can negatively affect cell growth when Rdh54p is forced to stay at that location throughout the cell cycle. One possibility is that, considering the role of the CTF19 complex in recruiting pericentromeric cohesin²³⁷ and the translocase activity of Rdh54p, Rdh54p might promote checkpoint adaptation by affecting sister-chromatid cohesin. These ideas should be tested by further experiments.

Similar to the discussions in Chapter 3 and Chapter 4, a number of further developments could improve the capability of the FLIM method for identifying PPIs. For example, instead of using strains of the S288C background that are poor in sporulation, W303-derived strains should be chosen instead, so as to allow the construction of dual-labelled

query strains that are homozygous for the *RDH54-mCherry* gene via further sporulation and crossing (see Section 4.2.1). This would increase the number of mCherry acceptors in the cell and potentially increase the FRET efficiency. It may also be worthwhile to choose a different FRET pair: the effective FRET efficiency of only 5.4% measured from the positive control strain with Spc24p-GFP/Spc25p-mCherry is much lower than the 25.9% efficiency measured from the strain with mTq2FP and YFP labelling the same pair of KPs. This cannot be fully accounted for by the 50% fewer acceptor molecules in the former strain that is heterozygous for the *SPC25-mCherry* gene and therefore indicates that GFP and mCherry are not FRETing optimally. An optimal donor fluorophore may still fluoresce in the green channel, where the cellular autofluorescence has been found to be much weaker than in the blue channel; alternatively, a red or far-red donor and corresponding acceptor may be found. This could facilitate multiplexed FLIM/FRET of two different FRET pairs in the same budding yeast cell, e.g. if used with the mTq2FP/YFP FRET pair. Ideally, a new donor should be even brighter than GFP to enable the use of a shorter exposure time for imaging at each time-gate. Combined with the use of fewer time-gates to sample the fluorescence decay, this could further reduce the time for imaging each FOV and enable the screening of more strains or replicates in each multiwell plate experiment.

After identifying a better FRET pair, the PPIs between Rdh54p and KPs could also be further examined using the FLIM HCA method. In the experiments reported in this section, the fluorophores were always tagged to the C-termini of proteins; changing the labelling to the N-termini of some proteins may lead to additional information about Rdh54p's localisation. It should also be checked whether the PIP-box mutant *rdh54p-pip*, which abolished the sensitivity of the CTF19 components to forced associations with Rdh54p, also localises near the CTF19 complex; my current assumption is that the PIP-box motif is required for both the localisation and the function of Rdh54p near the CTF19 complex. Furthermore, it would be interesting to explore whether the kinetochore localisation of Rdh54p or *rdh54p-pip* changes in the presence of activated DDC or SAC and after prolonged cell cycle arrest.

5.4 Summary and future work

In this chapter, I reported my work to begin exploring how Rdh54p achieves its uncharacterised function in promoting the adaptation to the SAC-dependent DDC. To this end, I used the SPI screens to systematically query for the proteins in the yeast proteome, or related to the kinetochore, that are sensitive to the forced association with Rdh54p or its mutants *rdh54p-pip* and *rdh54p-K318R*, and I identified hits enriched for the DNA damage response and the kinetochore (especially the CTF19 complex). Under checkpoint-inducing growth conditions, some other hits were found. All these results imply that Rdh54p indeed has a role in both the DDC and the SAC, and its PIP-box motif may be important for its kinetochore-related functions. I also employed the FLIM FRET screens to examine the constitutive localisation of Rdh54p at the kinetochore and found that Rdh54p may reside near the CTF19 components Ctf19p and Nkp2p. Both the SPI and FLIM FRET results are worth following up, as discussed in detail in Section 5.2.5 and Section 5.3.5.

Besides the follow-up experiments proposed in Section 5.2.5 and Section 5.3.5, work can be done to investigate Rdh54p's checkpoint adaptation function from other angles. For example, as it has not yet been reported whether Rdh54p promotes the checkpoint adaptation when only the SAC is activated (e.g. by using a microtubule-depolymerising drug or forcing the association of Mps1p and Spc105p⁵²³) in the absence of a persistent DNA DSB, this should be tested to confirm that Rdh54p has a role outside the DNA damage response. Another example is that, as Rdh54p has been shown to compensate for the functions of the chromatin assembly factors Asf1p and Cac1p and to redistribute to pericentromeric chromatin in their absence⁵¹³, it would be interesting to explore whether the Rdh54p-dependent checkpoint adaptation pathway is partially overlapped with the Asf1p- and Cac1p-dependent DDC recovery pathway⁵³⁶. While it is not yet possible to propose a working model for the exact mechanism of how Rdh54p functions in promoting the adaptation to the SAC-dependent DDC, the data reported in this chapter and the proposed experiments should bring us closer to the answer.

Chapter 6. Conclusion and outlook

In this thesis I have described my work to establish and apply a FLIM HCA methodology to study *in vivo* PPIs at the budding yeast kinetochore utilising FRET. Specifically, I have:

- Established FLIM FRET as a tool for HCA of *in vivo* PPIs at the budding yeast kinetochore for the first time, addressing the challenges imposed by the limited copy numbers of FP-labelled endogenous proteins and the relatively strong cellular autofluorescence in the blue and green channels (Chapter 3).
- Validated the FLIM HCA methodology by showing that the method can reliably identify known PPIs between specific KPs at the kinetochore (Chapter 4).
- Used SPI screens to show that the DNA repair protein Rdh54p causes growth defects when forced to associate with a number of KPs, most of which belong to the CTF19/COMA complex, supporting the idea that Rdh54p's constitutive localisation at the CTF19/COMA complex has functional significance. Several components related to the DDC and the SAC also exhibited sensitivity to the forced association of Rdh54p, which could be related to Rdh54p's function in promoting the adaptation to the DDC-induced SAC-dependent cell cycle arrest (Chapter 5).
- Applied the FLIM HCA method to assay interactions between KPs and the DNA repair protein Rdh54p, observing that Rdh54p seems to constitutively localise adjacent to the Nkp2p and Ctf19p KPs, which belong to the CTF19/COMA complex of the kinetochore (Chapter 5).

These findings have been discussed in Chapters 3-5, together with suggestions to advance these studies.

One priority for future studies would be to improve the FLIM HCA methodology, specifically to make it more sensitive to interactions between low abundance proteins at the kinetochore. The key challenges are the low number of the photons to be detected from kinetochore foci as well as background autofluorescence from the cells and their medium. The following work could help address these challenges:

- To change the donor fluorophore to a different FP, or other fluorescent marker, that provides higher brightness and which fluoresces in a spectral channel that presents less autofluorescence background. This would increase the sensitivity of the assay and/or the speed of image acquisition. An appropriate acceptor fluorophore would then need to be identified to complement the new donor fluorophore. Both the donor and the acceptor fluorophores should be photostable and compatible with live budding yeast imaging at 23°C.
- To improve the algorithm for segmenting kinetochore foci. This could better identify weak kinetochore signals and distinguish them from the background. A machine learning-based approach may be able to distinguish different types of foci that represent different phases of the cell cycle (such as the two closely aligned foci that represent metaphase kinetochores).
- To improve the lifetime fitting strategy. Instead of performing image-wise mono-exponential fitting, global fitting to a suitable complex decay profile may yield more quantitative data about the population fraction of the FRETing donor and may more effectively suppress the impact of background in the detected signal. Alternatively, for bright kinetochore foci, it might be possible to perform lifetime fitting to the fluorescence signals from individual cells. Moreover, with a machine learning-based segmentation algorithm that could discriminate different types of kinetochore foci, it would be interesting to acquire lifetime values for each subpopulation of cells that have the same type of kinetochore foci in a FOV.
- To improve the statistical analysis of the lifetime results. The currently employed method that relies on the p -value for reporting statistical significance can be susceptible to false positive results: for a chosen significance level of $\alpha=0.5$, comparisons between 20 pairs of data with identical distributions could present one pair with an apparent statistical difference by chance. Hence, the significance level should be adjusted accordingly when multiple comparisons are made. For the work reported here I used the stringent Bonferroni correction to correct for only the multiple comparisons made within a group of strains that have the same donor-tagged KP, which are presented in Chapter 4, but not for the comparisons presented in Chapter 5 where different pairs of strains have different donor-tagged

KPs. For future work, a more suitable statistical analysis to correct for multiple comparisons should be explored.

In terms of future biology studies, the work described in this thesis suggests that Rdh54p functions close to the CTF19/COMA complex in the kinetochore and this could be further explored. The function of the CTF19/COMA complex is not entirely clear; however, it plays roles in establishing the kinetochore structure and in influencing the chromatin around the centromere (for example, affecting chromatin condensation and sister-chromatid cohesion). The observation of Rdh54p's localisation near this complex is therefore intriguing, as the CTF19/COMA complex provides an interface between the kinetochore structure and surrounding DNA, and as Rdh54p is canonically described as a DNA repair protein with helicase motifs. Rdh54p also promotes the adaptation to the cell cycle arrest that is induced by persistent DNA damage and is dependent on the SAC. My data implicates CTF19/COMA as the likely location within the kinetochore that Rdh54p may function. However, the data does not tell us whether this function is related to the DDC/SAC adaptation. The following possibilities could be explored:

- Rdh54p may affect cohesin adjacent to the kinetochore. There is evidence in meiosis that Rdh54p is involved in remodelling cohesin and the pericentromeric region is a key site for cohesin loading.
- Rdh54p may downregulate the DDC, although exactly how this function would be achieved is unclear. It may be through interaction with RPA or Ddc2p-Mec1p (all of which were sensitive to forced Rdh54p association).
- Rdh54p may act directly upon the kinetochore to inhibit the SAC, for example, by inhibiting some component(s) of the SAC. However, this is unlikely since there is no documented activity of Rdh54p in SAC regulation.

For future work it would be possible to test these ideas in several ways:

- By mapping cohesin distribution using ChIP-Seq (chromatin immunoprecipitation followed by sequencing) around the centromere in wild type or *rdh54* mutants, both in undamaged cells and those containing a DSB. This could determine whether Rdh54p affects cohesin adjacent to centromeres.

- By examining whether the SPIs between Rdh54p and RPA/Ddc2p inhibit the DDC. This would define whether Rdh54 is primarily inhibiting or shutting down the cells normal response to DNA damage. For example, epistasis analysis between RDH54 over-expression and DDC mutants may be informative.
- By testing whether Rdh54p can inhibit SAC activation using an artificial SAC activator (e.g. a Mps1p-Spc105p fusion). Although this is unlikely, it is important to ascertain whether Rdh54 can disrupt normal SAC function.

Finally, my work to demonstrate FLIM HCA as a novel method to study *in vivo* PPIs at the budding yeast kinetochore could be adapted to study other foci in budding yeast cells (e.g. DNA repair foci and SPBs), or even other cellular structures (nuclear pores, cell membrane, mitochondria, etc.).

Publication and Presentations

Wenjun Guo, Sunil Kumar, Frederick Görlitz, Edwin Garcia, Yuriy Alexandrov, Ian Munro, Douglas Kelly, Sean Warren, Peter Thorpe, Christopher Dunsby, Paul French, “Automated fluorescence lifetime imaging high-content analysis of Förster resonance energy transfer between endogenously labeled kinetochore proteins in live budding yeast cells”, SLAS Tech, 2019, doi: 10.1177/2472630318819240, Publication

Wenjun Guo, Frederick Görlitz, Sunil Kumar, Douglas Kelly, Sean Warren, Yuriy Alexandrov, Edwin Garcia, Ian Munro, Peter Thorpe, Christopher Dunsby, Paul French, “Automated time-gated FLIM multi-well plate microscope for identifying interactions between endogenously labelled kinetochore proteins in live yeast cells based on FRET”, Focus on Microscopy, Singapore, 2018, Oral presentation by Wenjun Guo

Peter Thorpe, Paul French, Christopher Dunsby, **Wenjun Guo**, “Rdh54, a key regulator of the mitotic checkpoint, associates with the COMA complex of the kinetochore”, FASEB Yeast Chromosome Biology, Steamboat Springs, US, 2018, Poster presentation by Peter Thorpe

Sunil Kumar, Yuriy Alexandrov, Edwin Garcia, **Wenjun Guo**, Anca Margineanu, George Chennell, Frederick Görlitz, Douglas Kelly, Sean Warren, Gordon Kennedy, Jia Chan, Delphine Flatters, Hiroshi Kondo, Steven Hooper, Ian Munro, Mark Neil, Matilda Katan, Alexander Sardini, James McGinty, David Carling, Peter Thorpe, Erik Sahai, Christopher Dunsby, Paul French, “Fluorescence lifetime imaging for high content analysis and preclinical applications”, GRC on Lasers in Medicine and Biology, Lewiston, US, 2018, Poster presentation by Paul French

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Institution name	Imperial College London
Expected presentation date	May 2019
Portions	Figures 1(a) and 1(c)
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Figure 1-8

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References

1. Botstein, D.; Fink, G. Yeast: An Experimental Organism for Modern Biology. *Science* **1988**, *240*, 1439–1443.
2. Goffeau, A.; Barrell, B. G.; Bussey, H.; et al. Life with 6000 Genes. *Science* **1996**, *274*, 546–547.
3. Herskowitz, I. Life Cycle of the Budding Yeast *Saccharomyces Cerevisiae*. *Microbiol. Rev.* **1988**, *52*, 536–553.
4. Botstein, D.; Fink, G. R. Yeast: An Experimental Organism for 21st Century Biology. *Genetics* **2011**, *189*, 695–704.
5. Hartwell, L. H.; Culotti, J.; Reid, B. Genetic Control of the Cell-Division Cycle in Yeast. I. Detection of Mutants. *Proc. Natl. Acad. Sci.* **1970**, *66*, 352–359.
6. Hartwell, L. H.; Culotti, J.; Pringle, J. R.; et al. Genetic Control of the Cell Division Cycle in Yeast. *Science* **1974**, *183*, 46–51.
7. Hartwell, L. H.; Weinert, T. A. Checkpoints: Controls That Ensure the Order of Cell Cycle Events. *Science* **1989**, *246*, 629–634.
8. Nurse, P. Genetic Control of Cell Size at Cell Division in Yeast. *Nature* **1975**, *256*, 547–551.
9. Beach, D.; Durkacz, B.; Nurse, P. Functionally Homologous Cell Cycle Control Genes in Budding and Fission Yeast. *Nature* **1982**, *300*, 706–709.
10. Evans, T.; Rosenthal, E. T.; Youngblom, J.; et al. Cyclin: A Protein Specified by Maternal mRNA in Sea Urchin Eggs That Is Destroyed at Each Cleavage Division. *Cell* **1983**, *33*, 389–396.
11. Bloom, J.; Cross, F. R. Multiple Levels of Cyclin Specificity in Cell-Cycle Control. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 149–160.
12. de Bruin, R. A. M.; McDonald, W. H.; Kalashnikova, T. I.; et al. Cln3 Activates G1-Specific Transcription via Phosphorylation of the SBF Bound Repressor Whi5. *Cell* **2004**, *117*, 887–898.
13. Costanzo, M.; Nishikawa, J. L.; Tang, X.; et al. CDK Activity Antagonizes Whi5, an Inhibitor of G1/S Transcription in Yeast. *Cell* **2004**, *117*, 899–913.
14. Cross, F. R.; Tinkelenberg, A. H. A Potential Positive Feedback Loop Controlling CLN1 and CLN2 Gene Expression at the Start of the Yeast Cell Cycle. *Cell* **1991**, *65*, 875–883.
15. Dirick, L.; Nasmyth, K. Positive Feedback in the Activation of G1 Cyclins in Yeast. *Nature* **1991**, *351*, 754–757.
16. Skotheim, J. M.; Di Talia, S.; Siggia, E. D.; et al. Positive Feedback of G1 Cyclins

- Ensures Coherent Cell Cycle Entry. *Nature* **2008**, *454*, 291–296.
17. Henchoz, S.; Chi, Y.; Catarin, B.; et al. Phosphorylation- and Ubiquitin-Dependent Degradation of the Cyclin-Dependent Kinase Inhibitor Far1p in Budding Yeast. *Genes Dev.* **1997**, *11*, 3046–3060.
 18. Schwob, E.; Böhm, T.; Mendenhall, M. D.; et al. The B-Type Cyclin Kinase Inhibitor P40SIC1 Controls the G1 to S Transition in *S. Cerevisiae*. *Cell* **1994**, *79*, 233–244.
 19. Charvin, G.; Oikonomou, C.; Siggia, E. D.; et al. Origin of Irreversibility of Cell Cycle Start in Budding Yeast. *PLoS Biol.* **2010**, *8*, e1000284.
 20. Nasmyth, K. At the Heart of the Budding Yeast Cell Cycle. *Trends Genet.* **1996**, *12*, 405–412.
 21. Epstein, C. B.; Cross, F. R. CLB5: A Novel B Cyclin from Budding Yeast with a Role in S Phase. *Genes Dev.* **1992**, *6*, 1695–1706.
 22. Kühne, C.; Linder, P. A New Pair of B-Type Cyclins from *Saccharomyces Cerevisiae* That Function Early in the Cell Cycle. *EMBO J.* **1993**, *12*, 3437–3447.
 23. Schwob, E.; Nasmyth, K. CLB5 and CLB6, a New Pair of B Cyclins Involved in DNA Replication in *Saccharomyces Cerevisiae*. *Genes Dev.* **1993**, *7*, 1160–1175.
 24. Donaldson, A. D.; Raghuraman, M. K.; Friedman, K. L.; et al. CLB5-Dependent Activation of Late Replication Origins in *S. Cerevisiae*. *Mol. Cell* **1998**, *2*, 173–182.
 25. Dahmann, C.; Diffley, J. F.; Nasmyth, K. A. S-Phase-Promoting Cyclin-Dependent Kinases Prevent Re-Replication by Inhibiting the Transition of Replication Origins to a Pre-Replicative State. *Curr. Biol.* **1995**, *5*, 1257–1269.
 26. Lanker, S.; Valdivieso, M. H.; Wittenberg, C. Rapid Degradation of the G1 Cyclin Cln2 Induced by CDK-Dependent Phosphorylation. *Science* **1996**, *271*, 1597–1601.
 27. Richardson, H.; Lew, D. J.; Henze, M.; et al. Cyclin-B Homologs in *Saccharomyces Cerevisiae* Function in S Phase and in G2. *Genes Dev.* **1992**, *6*, 2021–2034.
 28. Fitch, I.; Dahmann, C.; Surana, U.; et al. Characterization of Four B-Type Cyclin Genes of the Budding Yeast *Saccharomyces Cerevisiae*. *Mol. Biol. Cell* **1992**, *3*, 805–818.
 29. Rahal, R.; Amon, A. Mitotic CDKs Control the Metaphase-Anaphase Transition and Trigger Spindle Elongation. *Genes Dev.* **2008**, *22*, 1534–1548.
 30. Stegmeier, F.; Visintin, R.; Amon, A. Separase, Polo Kinase, the Kinetochore Protein Slk19, and Spo12 Function in a Network That Controls Cdc14 Localization during Early Anaphase. *Cell* **2002**, *108*, 207–220.
 31. Shou, W.; Seol, J. H.; Baskerville, C.; et al. Exit from Mitosis Is Triggered by

- Tem1-Dependent Release of the Protein Phosphatase Cdc14 from Nucleolar RENT Complex. *Cell* **1999**, *97*, 233–244.
32. Visintin, R.; Hwang, E. S.; Amon, A. Cfi1 Prevents Premature Exit from Mitosis by Anchoring Cdc14 Phosphatase in the Nucleolus. *Nature* **1999**, *398*, 818–823.
 33. Azzam, R.; Chen, S. L.; Shou, W.; et al. Phosphorylation by Cyclin B-Cdk Underlies Release of Mitotic Exit Activator Cdc14 from the Nucleolus. *Science* **2004**, *305*, 516–519.
 34. Jaspersen, S. L.; Morgan, D. O. Cdc14 Activates Cdc15 to Promote Mitotic Exit in Budding Yeast. *Curr. Biol.* **2000**, *10*, 615–618.
 35. Bardin, A. J.; Visintin, R.; Amon, A. A Mechanism for Coupling Exit from Mitosis to Partitioning of the Nucleus. *Cell* **2000**, *102*, 21–31.
 36. Visintin, R.; Craig, K.; Hwang, E. S.; et al. The Phosphatase Cdc14 Triggers Mitotic Exit by Reversal of Cdk-Dependent Phosphorylation. *Mol. Cell* **1998**, *2*, 709–718.
 37. Jaspersen, S. L.; Charles, J. F.; Tinker-Kulberg, R. L.; et al. A Late Mitotic Regulatory Network Controlling Cyclin Destruction in *Saccharomyces Cerevisiae*. *Mol. Biol. Cell* **1998**, *9*, 2803–2817.
 38. Grallert, A.; Boke, E.; Hagting, A.; et al. A PP1-PP2A Phosphatase Relay Controls Mitotic Progression. *Nature* **2015**, *517*, 94–98.
 39. Johnson, A.; Skotheim, J. M. Start and the Restriction Point. *Curr. Opin. Cell Biol.* **2013**, *25*, 717–723.
 40. Weinert, T. A.; Hartwell, L. H. The RAD9 Gene Controls the Cell Cycle Response to DNA Damage in *Saccharomyces Cerevisiae*. *Science* **1988**, *241*, 317–322.
 41. Sandell, L. L.; Zakian, V. A. Loss of a Yeast Telomere: Arrest, Recovery, and Chromosome Loss. *Cell* **1993**, *75*, 729–739.
 42. Siede, W.; Friedberg, A. S.; Dianova, I.; et al. Characterization of G1 Checkpoint Control in the Yeast *Saccharomyces Cerevisiae* Following Exposure to DNA-Damaging Agents. *Genetics* **1994**, *138*, 271–281.
 43. Burhans, W. Apoptosis-like Yeast Cell Death in Response to DNA Damage and Replication Defects. *Mutat. Res. Mol. Mech. Mutagen.* **2003**, *532*, 227–243.
 44. Clémenson, C.; Marsolier-Kergoat, M.-C. DNA Damage Checkpoint Inactivation: Adaptation and Recovery. *DNA Repair (Amst)*. **2009**, *8*, 1101–1109.
 45. Ciccia, A.; Elledge, S. J. The DNA Damage Response: Making It Safe to Play with Knives. *Mol. Cell* **2010**, *40*, 179–204.
 46. Baldo, V.; Liang, J.; Wang, G.; et al. Preserving Yeast Genetic Heritage through DNA Damage Checkpoint Regulation and Telomere Maintenance. *Biomolecules* **2012**, *2*, 505–523.
 47. Lopes, M.; Cotta-Ramusino, C.; Pellicioli, A.; et al. The DNA Replication

- Checkpoint Response Stabilizes Stalled Replication Forks. *Nature* **2001**, *412*, 557–561.
48. Pardo, B.; Crabbé, L.; Pasero, P. Signaling Pathways of Replication Stress in Yeast. *FEMS Yeast Res.* **2017**, *17*.
 49. Lew, D. J. The Morphogenesis Checkpoint: How Yeast Cells Watch Their Figures. *Curr. Opin. Cell Biol.* **2003**, *15*, 648–653.
 50. Howell, A. S.; Lew, D. J. Morphogenesis and the Cell Cycle. *Genetics* **2012**, *190*, 51–77.
 51. Hoyt, M. A.; Totis, L.; Roberts, B. T. S. *Cerevisiae* Genes Required for Cell Cycle Arrest in Response to Loss of Microtubule Function. *Cell* **1991**, *66*, 507–517.
 52. Li, R.; Murray, A. W. Feedback Control of Mitosis in Budding Yeast. *Cell* **1991**, *66*, 519–531.
 53. London, N.; Biggins, S. Signalling Dynamics in the Spindle Checkpoint Response. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 736–748.
 54. Vernieri, C.; Chirolì, E.; Francia, V.; et al. Adaptation to the Spindle Checkpoint Is Regulated by the Interplay between Cdc28/Clbs and PP2A^{Cdc55}. *J. Cell Biol.* **2013**, *202*, 765–778.
 55. Piatti, S.; Venturetti, M.; Chirolì, E.; et al. The Spindle Position Checkpoint in Budding Yeast: The Motherly Care of MEN. *Cell Div.* **2006**, *1*, 2.
 56. Mendenhall, M. D.; Hodge, A. E. Regulation of Cdc28 Cyclin-Dependent Protein Kinase Activity during the Cell Cycle of the Yeast *Saccharomyces Cerevisiae*. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 1191–1243.
 57. Lindahl, T. Instability and Decay of the Primary Structure of DNA. *Nature* **1993**, *362*, 709–715.
 58. Hakem, R. DNA-Damage Repair; the Good, the Bad, and the Ugly. *EMBO J.* **2008**, *27*, 589–605.
 59. Mathiasen, D. P.; Lisby, M. Cell Cycle Regulation of Homologous Recombination in *Saccharomyces Cerevisiae*. *FEMS Microbiol. Rev.* **2014**, *38*, 172–184.
 60. Bauer, N. C.; Corbett, A. H.; Doetsch, P. W. The Current State of Eukaryotic DNA Base Damage and Repair. *Nucleic Acids Res.* **2015**, *14*, gkv1136.
 61. Aguilera, A.; Gómez-González, B. Genome Instability: A Mechanistic View of Its Causes and Consequences. *Nat. Rev. Genet.* **2008**, *9*, 204–217.
 62. Hanahan, D.; Weinberg, R. A. Hallmarks of Cancer: The next Generation. *Cell* **2011**, *144*, 646–674.
 63. Lisby, M.; Rothstein, R. Choreography of Recombination Proteins during the DNA Damage Response. *DNA Repair (Amst.)*. **2009**, *8*, 1068–1076.
 64. Symington, L. S.; Gautier, J. Double-Strand Break End Resection and Repair

- Pathway Choice. *Annu. Rev. Genet.* **2011**, *45*, 247–271.
65. Yamamoto, A.; Guacci, V.; Koshland, D. Pds1p, an Inhibitor of Anaphase in Budding Yeast, Plays a Critical Role in the APC and Checkpoint Pathway(S). *J. Cell Biol.* **1996**, *133*, 99–110.
 66. Cohen-Fix, O.; Koshland, D. The Metaphase-to-Anaphase Transition: Avoiding a Mid-Life Crisis. *Curr. Opin. Cell Biol.* **1997**, *9*, 800–806.
 67. Harrison, J. C.; Haber, J. E. Surviving the Breakup: The DNA Damage Checkpoint. *Annu. Rev. Genet.* **2006**, *40*, 209–235.
 68. Finn, K.; Lowndes, N. F.; Grenon, M. Eukaryotic DNA Damage Checkpoint Activation in Response to Double-Strand Breaks . *Cell. Mol. Life Sci.* **2012**, *69*, 1447–1473.
 69. D’Amours, D.; Jackson, S. P. The Mre11 Complex: At the Crossroads of Dna Repair and Checkpoint Signalling. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 317–327.
 70. Lisby, M.; Barlow, J. H.; Burgess, R. C.; et al. Choreography of the DNA Damage Response: Spatiotemporal Relationships among Checkpoint and Repair Proteins. *Cell* **2004**, *118*, 699–713.
 71. Wu, D.; Topper, L. M.; Wilson, T. E. Recruitment and Dissociation of Nonhomologous End Joining Proteins at a DNA Double-Strand Break in *Saccharomyces Cerevisiae*. *Genetics* **2008**, *178*, 1237–1249.
 72. Clerici, M.; Mantiero, D.; Guerini, I.; et al. The Yku70-Yku80 Complex Contributes to Regulate Double-Strand Break Processing and Checkpoint Activation during the Cell Cycle. *EMBO Rep.* **2008**, *9*, 810–818.
 73. Nakada, D.; Matsumoto, K.; Sugimoto, K. ATM-Related Tel1 Associates with Double-Strand Breaks through an Xrs2-Dependent Mechanism. *Genes Dev.* **2003**, *17*, 1957–1962.
 74. Limbo, O.; Yamada, Y.; Russell, P. Mre11-Rad50-Dependent Activity of ATM/Tel1 at DNA Breaks and Telomeres in the Absence of Nbs1. *Mol. Biol. Cell* **2018**, *29*, 1389–1399.
 75. Cassani, C.; Vertemara, J.; Bassani, M.; et al. The ATP-Bound Conformation of the Mre11-Rad50 Complex Is Essential for Tel1/ATM Activation. *Nucleic Acids Res.* **2019**, *47*, 3550–3567.
 76. Cannavo, E.; Cejka, P. Sae2 Promotes DsDNA Endonuclease Activity within Mre11-Rad50-Xrs2 to Resect DNA Breaks. *Nature* **2014**, *514*, 122–125.
 77. Mimitou, E. P.; Symington, L. S. Sae2, Exo1 and Sgs1 Collaborate in DNA Double-Strand Break Processing. *Nature* **2008**, *455*, 770–774.
 78. Zhu, Z.; Chung, W.-H.; Shim, E. Y.; et al. Sgs1 Helicase and Two Nucleases Dna2 and Exo1 Resect DNA Double-Strand Break Ends. *Cell* **2008**, *134*, 981–994.
 79. Mimitou, E. P.; Symington, L. S. DNA End Resection--Unraveling the Tail. *DNA*

- Repair (Amst)*. **2011**, *10*, 344–348.
80. Cejka, P.; Cannavo, E.; Polaczek, P.; et al. DNA End Resection by Dna2-Sgs1-RPA and Its Stimulation by Top3-Rmi1 and Mre11-Rad50-Xrs2. *Nature* **2010**, *467*, 112–116.
 81. Chen, H.; Lisby, M.; Symington, L. S. RPA Coordinates DNA End Resection and Prevents Formation of DNA Hairpins. *Mol. Cell* **2013**, *50*, 589–600.
 82. Symington, L. S.; Rothstein, R.; Lisby, M. Mechanisms and Regulation of Mitotic Recombination in *Saccharomyces Cerevisiae*. *Genetics* **2014**, *198*, 795–835.
 83. Zou, L.; Elledge, S. J. Sensing DNA Damage through ATRIP Recognition of RPA-SsDNA Complexes. *Science* **2003**, *300*, 1542–1548.
 84. Mantiero, D.; Clerici, M.; Lucchini, G.; et al. Dual Role for *Saccharomyces Cerevisiae* Tel1 in the Checkpoint Response to Double-Strand Breaks. *EMBO Rep.* **2007**, *8*, 380–387.
 85. Shiotani, B.; Zou, L. Single-Stranded DNA Orchestrates an ATM-to-ATR Switch at DNA Breaks. *Mol. Cell* **2009**, *33*, 547–558.
 86. Melo, J. A.; Cohen, J.; Toczyski, D. P. Two Checkpoint Complexes Are Independently Recruited to Sites of DNA Damage in Vivo. *Genes Dev.* **2001**, *15*, 2809–2821.
 87. Majka, J.; Burgers, P. M. J. Yeast Rad17/Mec3/Ddc1: A Sliding Clamp for the DNA Damage Checkpoint. *Proc. Natl. Acad. Sci.* **2003**, *100*, 2249–2254.
 88. Majka, J.; Binz, S. K.; Wold, M. S.; et al. Replication Protein A Directs Loading of the DNA Damage Checkpoint Clamp to 5'-DNA Junctions. *J. Biol. Chem.* **2006**, *281*, 27855–27861.
 89. Majka, J.; Niedziela-Majka, A.; Burgers, P. M. J. The Checkpoint Clamp Activates Mec1 Kinase during Initiation of the DNA Damage Checkpoint. *Mol. Cell* **2006**, *24*, 891–901.
 90. Navadgi-Patil, V. M.; Burgers, P. M. Yeast DNA Replication Protein Dpb11 Activates the Mec1/ATR Checkpoint Kinase. *J. Biol. Chem.* **2008**, *283*, 35853–35859.
 91. Navadgi-Patil, V. M.; Burgers, P. M. The Unstructured C-Terminal Tail of the 9-1-1 Clamp Subunit Ddc1 Activates Mec1/ATR via Two Distinct Mechanisms. *Mol. Cell* **2009**, *36*, 743–753.
 92. Ngo, G. H. P.; Balakrishnan, L.; Dubarry, M.; et al. The 9-1-1 Checkpoint Clamp Stimulates DNA Resection by Dna2-Sgs1 and Exo1. *Nucleic Acids Res.* **2014**, *42*, 10516–10528.
 93. Ngo, G. H. P.; Lydall, D. The 9-1-1 Checkpoint Clamp Coordinates Resection at DNA Double Strand Breaks. *Nucleic Acids Res.* **2015**, *43*, 5017–5032.
 94. Downs, J. A.; Lowndes, N. F.; Jackson, S. P. A Role for *Saccharomyces Cerevisiae*

- Histone H2A in DNA Repair. *Nature* **2000**, *408*, 1001–1004.
95. Shroff, R.; Arbel-Eden, A.; Pilch, D.; et al. Distribution and Dynamics of Chromatin Modification Induced by a Defined DNA Double-Strand Break. *Curr. Biol.* **2004**, *14*, 1703–1711.
 96. Kim, J.-A.; Kruhlak, M.; Dotiwala, F.; et al. Heterochromatin Is Refractory to Gamma-H2AX Modification in Yeast and Mammals. *J. Cell Biol.* **2007**, *178*, 209–218.
 97. Unal, E.; Arbel-Eden, A.; Sattler, U.; et al. DNA Damage Response Pathway Uses Histone Modification to Assemble a Double-Strand Break-Specific Cohesin Domain. *Mol. Cell* **2004**, *16*, 991–1002.
 98. Litwin, I.; Pilarczyk, E.; Wysocki, R. The Emerging Role of Cohesin in the DNA Damage Response. *Genes (Basel)*. **2018**, *9*, 581.
 99. Toh, G. W.-L.; O'Shaughnessy, A. M.; Jimeno, S.; et al. Histone H2A Phosphorylation and H3 Methylation Are Required for a Novel Rad9 DSB Repair Function Following Checkpoint Activation. *DNA Repair (Amst)*. **2006**, *5*, 693–703.
 100. Grenon, M.; Costelloe, T.; Jimeno, S.; et al. Docking onto Chromatin via the *Saccharomyces Cerevisiae* Rad9 Tudor Domain. *Yeast* **2007**, *24*, 105–119.
 101. Vialard, J. E.; Gilbert, C. S.; Green, C. M.; et al. The Budding Yeast Rad9 Checkpoint Protein Is Subjected to Mec1/Tel1-Dependent Hyperphosphorylation and Interacts with Rad53 after DNA Damage. *EMBO J.* **1998**, *17*, 5679–5688.
 102. Emili, A. MEC1-Dependent Phosphorylation of Rad9p in Response to DNA Damage. *Mol. Cell* **1998**, *2*, 183–189.
 103. Sweeney, F. D.; Yang, F.; Chi, A.; et al. *Saccharomyces Cerevisiae* Rad9 Acts as a Mec1 Adaptor to Allow Rad53 Activation. *Curr. Biol.* **2005**, *15*, 1364–1375.
 104. Gilbert, C. S.; Green, C. M.; Lowndes, N. F. Budding Yeast Rad9 Is an ATP-Dependent Rad53 Activating Machine. *Mol. Cell* **2001**, *8*, 129–136.
 105. Sanchez, Y.; Bachant, J.; Wang, H.; et al. Control of the DNA Damage Checkpoint by Chk1 and Rad53 Protein Kinases through Distinct Mechanisms. *Science* **1999**, *286*, 1166–1171.
 106. Blankley, R. T.; Lydall, D. A Domain of Rad9 Specifically Required for Activation of Chk1 in Budding Yeast. *J. Cell Sci.* **2004**, *117*, 601–608.
 107. Chen, Y.; Caldwell, J. M.; Pereira, E.; et al. ATRMec1 Phosphorylation-Independent Activation of Chk1 in Vivo. *J. Biol. Chem.* **2009**, *284*, 182–190.
 108. Usui, T.; Foster, S. S.; Petrini, J. H. J. Maintenance of the DNA-Damage Checkpoint Requires DNA-Damage-Induced Mediator Protein Oligomerization. *Mol. Cell* **2009**, *33*, 147–159.
 109. Liang, F.; Wang, Y. DNA Damage Checkpoints Inhibit Mitotic Exit by Two Different Mechanisms. *Mol. Cell. Biol.* **2007**, *27*, 5067–5078.

110. Ciosk, R.; Zachariae, W.; Michaelis, C.; et al. An ESP1/PDS1 Complex Regulates Loss of Sister Chromatid Cohesion at the Metaphase to Anaphase Transition in Yeast. *Cell* **1998**, *93*, 1067–1076.
111. Wang, H.; Liu, D.; Wang, Y.; et al. Pds1 Phosphorylation in Response to DNA Damage Is Essential for Its DNA Damage Checkpoint Function. *Genes Dev.* **2001**, *15*, 1361–1372.
112. Agarwal, R.; Tang, Z.; Yu, H.; et al. Two Distinct Pathways for Inhibiting Pds1 Ubiquitination in Response to DNA Damage. *J. Biol. Chem.* **2003**, *278*, 45027–45033.
113. Queralt, E.; Lehane, C.; Novák, B.; et al. Downregulation of PP2A(Cdc55) Phosphatase by Separase Initiates Mitotic Exit in Budding Yeast. *Cell* **2006**, *125*, 719–732.
114. Baro, B.; Rodriguez-Rodriguez, J.-A.; Calabria, I.; et al. Dual Regulation of the Mitotic Exit Network (MEN) by PP2A-Cdc55 Phosphatase. *PLoS Genet.* **2013**, *9*, e1003966.
115. Cheng, L.; Hunke, L.; Hardy, C. F. Cell Cycle Regulation of the *Saccharomyces Cerevisiae* Polo-like Kinase Cdc5p. *Mol. Cell. Biol.* **1998**, *18*, 7360–7370.
116. Chen, H.; Donnianni, R. A.; Handa, N.; et al. Sae2 Promotes DNA Damage Resistance by Removing the Mre11-Rad50-Xrs2 Complex from DNA and Attenuating Rad53 Signaling. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, E1880-7.
117. Colombo, C. V.; Menin, L.; Ranieri, R.; et al. Uncoupling Sae2 Functions in Downregulation of Tel1 and Rad53 Signaling Activities. *Genetics* **2019**, *211*, 515–530.
118. Vaze, M. B.; Pellicioli, A.; Lee, S. E.; et al. Recovery from Checkpoint-Mediated Arrest after Repair of a Double-Strand Break Requires Srs2 Helicase. *Mol. Cell* **2002**, *10*, 373–385.
119. Yeung, M.; Durocher, D. Srs2 Enables Checkpoint Recovery by Promoting Disassembly of DNA Damage Foci from Chromatin. *DNA Repair (Amst.)*. **2011**, *10*, 1213–1222.
120. Kim, J.-A.; Haber, J. E. Chromatin Assembly Factors Asf1 and CAF-1 Have Overlapping Roles in Deactivating the DNA Damage Checkpoint When DNA Repair Is Complete. *Proc. Natl. Acad. Sci.* **2009**, *106*, 1151–1156.
121. Tsabar, M.; Waterman, D. P.; Aguilar, F.; et al. Asf1 Facilitates Dephosphorylation of Rad53 after DNA Double-Strand Break Repair. *Genes Dev.* **2016**, *30*, 1211–1224.
122. Leroy, C.; Lee, S. E.; Vaze, M. B.; et al. PP2C Phosphatases Ptc2 and Ptc3 Are Required for DNA Checkpoint Inactivation after a Double-Strand Break. *Mol. Cell* **2003**, *11*, 827–835.
123. Guillemain, G.; Ma, E.; Mauger, S.; et al. Mechanisms of Checkpoint Kinase Rad53 Inactivation after a Double-Strand Break in *Saccharomyces Cerevisiae*. *Mol.*

Cell. Biol. **2007**, *27*, 3378–3389.

124. O'Neill, B. M.; Szyjka, S. J.; Lis, E. T.; et al. Pph3–Psy2 Is a Phosphatase Complex Required for Rad53 Dephosphorylation and Replication Fork Restart during Recovery from DNA Damage. *Proc. Natl. Acad. Sci.* **2007**, *104*, 9290–9295.
125. Keogh, M.-C.; Kim, J.-A.; Downey, M.; et al. A Phosphatase Complex That Dephosphorylates Γ H2AX Regulates DNA Damage Checkpoint Recovery. *Nature* **2006**, *439*, 497–501.
126. Toczyski, D. P.; Galgoczy, D. J.; Hartwell, L. H. CDC5 and CKII Control Adaptation to the Yeast DNA Damage Checkpoint. *Cell* **1997**, *90*, 1097–1106.
127. Bartek, J.; Lukas, J. DNA Damage Checkpoints: From Initiation to Recovery or Adaptation. *Curr. Opin. Cell Biol.* **2007**, *19*, 238–245.
128. Lee, S. E.; Moore, J. K.; Holmes, A.; et al. Saccharomyces Ku70, Mre11/Rad50 and RPA Proteins Regulate Adaptation to G2/M Arrest after DNA Damage. *Cell* **1998**, *94*, 399–409.
129. Pellicioli, A.; Lee, S. E.; Lucca, C.; et al. Regulation of Saccharomyces Rad53 Checkpoint Kinase during Adaptation from DNA Damage-Induced G2/M Arrest. *Mol. Cell* **2001**, *7*, 293–300.
130. Clerici, M.; Mantiero, D.; Lucchini, G.; et al. The Saccharomyces Cerevisiae Sae2 Protein Negatively Regulates DNA Damage Checkpoint Signalling. *EMBO Rep.* **2006**, *7*, 212–218.
131. Eapen, V. V.; Sugawara, N.; Tsabar, M.; et al. The Saccharomyces Cerevisiae Chromatin Remodeler Fun30 Regulates DNA End Resection and Checkpoint Deactivation. *Mol. Cell. Biol.* **2012**, *32*, 4727–4740.
132. Lee, S. E.; Pellicioli, A.; Vaze, M. B.; et al. Yeast Rad52 and Rad51 Recombination Proteins Define a Second Pathway of DNA Damage Assessment in Response to a Single Double-Strand Break. *Mol. Cell. Biol.* **2003**, *23*, 8913–8923.
133. Lee, S. E.; Pellicioli, A.; Malkova, A.; et al. The Saccharomyces Recombination Protein Tid1p Is Required for Adaptation from G2/M Arrest Induced by a Double-Strand Break. *Curr. Biol.* **2001**, *11*, 1053–1057.
134. Dibitetto, D.; Ferrari, M.; Rawal, C. C.; et al. Slx4 and Rtt107 Control Checkpoint Signalling and DNA Resection at Double-Strand Breaks. *Nucleic Acids Res.* **2016**, *44*, 669–682.
135. Vidanes, G. M.; Sweeney, F. D.; Galicia, S.; et al. CDC5 Inhibits the Hyperphosphorylation of the Checkpoint Kinase Rad53, Leading to Checkpoint Adaptation. *PLoS Biol.* **2010**, *8*, e1000286.
136. Ratsima, H.; Serrano, D.; Pascariu, M.; et al. Centrosome-Dependent Bypass of the DNA Damage Checkpoint by the Polo Kinase Cdc5. *Cell Rep.* **2016**, *14*, 1422–1434.

137. Dotiwala, F.; Eapen, V. V.; Harrison, J. C.; et al. DNA Damage Checkpoint Triggers Autophagy to Regulate the Initiation of Anaphase. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, E41-9.
138. Dotiwala, F.; Harrison, J. C.; Jain, S.; et al. Mad2 Prolongs DNA Damage Checkpoint Arrest Caused by a Double-Strand Break via a Centromere-Dependent Mechanism. *Curr. Biol.* **2010**, *20*, 328–332.
139. Gillett, E. S.; Espelin, C. W.; Sorger, P. K. Spindle Checkpoint Proteins and Chromosome-Microtubule Attachment in Budding Yeast. *J. Cell Biol.* **2004**, *164*, 535–546.
140. Pinsky, B. A.; Kung, C.; Shokat, K. M.; et al. The Ipl1-Aurora Protein Kinase Activates the Spindle Checkpoint by Creating Unattached Kinetochore. *Nat. Cell Biol.* **2006**, *8*, 78–83.
141. Kops, G. J. P. L.; Weaver, B. A. A.; Cleveland, D. W. On the Road to Cancer: Aneuploidy and the Mitotic Checkpoint. *Nat. Rev. Cancer* **2005**, *5*, 773–785.
142. Pfau, S. J.; Amon, A. Chromosomal Instability and Aneuploidy in Cancer: From Yeast to Man. *EMBO Rep.* **2012**, *13*, 515–527.
143. Funk, L. C.; Zasadil, L. M.; Weaver, B. A. Living in CIN: Mitotic Infidelity and Its Consequences for Tumor Promotion and Suppression. *Dev. Cell* **2016**, *39*, 638–652.
144. Biggins, S.; Murray, A. W. The Budding Yeast Protein Kinase Ipl1/Aurora Allows the Absence of Tension to Activate the Spindle Checkpoint. *Genes Dev.* **2001**, *15*, 3118–3129.
145. Stern, B. M.; Murray, A. W. Lack of Tension at Kinetochore Activates the Spindle Checkpoint in Budding Yeast. *Curr. Biol.* **2001**, *11*, 1462–1467.
146. Hardwick, K. G.; Weiss, E.; Luca, F. C.; et al. Activation of the Budding Yeast Spindle Assembly Checkpoint without Mitotic Spindle Disruption. *Science* **1996**, *273*, 953–956.
147. London, N.; Ceto, S.; Ranish, J. A.; et al. Phosphoregulation of Spc105 by Mps1 and PP1 Regulates Bub1 Localization to Kinetochore. *Curr. Biol.* **2012**, *22*, 900–906.
148. Aravamudhan, P.; Chen, R.; Roy, B.; et al. Dual Mechanisms Regulate the Recruitment of Spindle Assembly Checkpoint Proteins to the Budding Yeast Kinetochore. *Mol. Biol. Cell* **2016**, *27*, 3405–3417.
149. Kemmler, S.; Stach, M.; Knapp, M.; et al. Mimicking Ndc80 Phosphorylation Triggers Spindle Assembly Checkpoint Signalling. *EMBO J.* **2009**, *28*, 1099–1110.
150. Aravamudhan, P.; Goldfarb, A. A.; Joglekar, A. P. The Kinetochore Encodes a Mechanical Switch to Disrupt Spindle Assembly Checkpoint Signalling. *Nat. Cell Biol.* **2015**, *17*, 868–879.
151. Primorac, I.; Weir, J. R.; Chiroli, E.; et al. Bub3 Reads Phosphorylated MELT

- Repeats to Promote Spindle Assembly Checkpoint Signaling. *Elife* **2013**, *2*, e01030.
152. Chen, R. H.; Brady, D. M.; Smith, D.; et al. The Spindle Checkpoint of Budding Yeast Depends on a Tight Complex between the Mad1 and Mad2 Proteins. *Mol. Biol. Cell* **1999**, *10*, 2607–2618.
 153. London, N.; Biggins, S. Mad1 Kinetochores Recruitment by Mps1-Mediated Phosphorylation of Bub1 Signals the Spindle Checkpoint. *Genes Dev.* **2014**, *28*, 140–152.
 154. De Antoni, A.; Pearson, C. G.; Cimini, D.; et al. The Mad1/Mad2 Complex as a Template for Mad2 Activation in the Spindle Assembly Checkpoint. **2005**, *15*, 214–225.
 155. Sudakin, V.; Chan, G. K.; Yen, T. J. Checkpoint Inhibition of the APC/C in HeLa Cells Is Mediated by a Complex of BUBR1, BUB3, CDC20, and MAD2. *J. Cell Biol.* **2001**, *154*, 925–936.
 156. Luo, X.; Tang, Z.; Rizo, J.; et al. The Mad2 Spindle Checkpoint Protein Undergoes Similar Major Conformational Changes upon Binding to Either Mad1 or Cdc20. *Mol. Cell* **2002**, *9*, 59–71.
 157. Chao, W. C. H.; Kulkarni, K.; Zhang, Z.; et al. Structure of the Mitotic Checkpoint Complex. *Nature* **2012**, *484*, 208–213.
 158. Lim, H. H.; Goh, P. Y.; Surana, U. Cdc20 Is Essential for the Cyclosome-Mediated Proteolysis of Both Pds1 and Clb2 during M Phase in Budding Yeast. *Curr. Biol.* **1998**, *8*, 231–234.
 159. Burton, J. L.; Solomon, M. J. Mad3p, a Pseudosubstrate Inhibitor of APCCdc20 in the Spindle Assembly Checkpoint. *Genes Dev.* **2007**, *21*, 655–667.
 160. Izawa, D.; Pines, J. Mad2 and the APC/C Compete for the Same Site on Cdc20 to Ensure Proper Chromosome Segregation. *J. Cell Biol.* **2012**, *199*, 27–37.
 161. Han, J. S.; Holland, A. J.; Fachinetti, D.; et al. Catalytic Assembly of the Mitotic Checkpoint Inhibitor BubR1-Cdc20 by a Mad2-Induced Functional Switch in Cdc20. *Mol. Cell* **2013**, *51*, 92–104.
 162. Lau, D. T. C.; Murray, A. W. Mad2 and Mad3 Cooperate to Arrest Budding Yeast in Mitosis. *Curr. Biol.* **2012**, *22*, 180–190.
 163. Izawa, D.; Pines, J. The Mitotic Checkpoint Complex Binds a Second CDC20 to Inhibit Active APC/C. *Nature* **2015**, *517*, 631–634.
 164. Gross, F.; Bonaiuti, P.; Hauf, S.; et al. Implications of Alternative Routes to APC/C Inhibition by the Mitotic Checkpoint Complex. *PLoS Comput. Biol.* **2018**, *14*, e1006449.
 165. Sewart, K.; Hauf, S. Different Functionality of Cdc20 Binding Sites within the Mitotic Checkpoint Complex. *Curr. Biol.* **2017**, *27*, 1213–1220.

166. Foster, S. A.; Morgan, D. O. The APC/C Subunit Mnd2/Apc15 Promotes Cdc20 Autoubiquitination and Spindle Assembly Checkpoint Inactivation. *Mol. Cell* **2012**, *47*, 921–932.
167. Wang, R.; Burton, J. L.; Solomon, M. J. Transcriptional and Post-Transcriptional Regulation of Cdc20 during the Spindle Assembly Checkpoint in *S. Cerevisiae*. *Cell. Signal.* **2017**, *33*, 41–48.
168. Ji, Z.; Gao, H.; Yu, H. Kinetochore Attachment Sensed by Competitive Mps1 and Microtubule Binding to Ndc80C. *Science* **2015**, *348*, 1260–1264.
169. Hiruma, Y.; Sacristan, C.; Pachis, S. T.; et al. Competition between MPS1 and Microtubules at Kinetochores Regulates Spindle Checkpoint Signaling. *Science* **2015**, *348*, 1264–1267.
170. Palframan, W. J.; Meehl, J. B.; Jaspersen, S. L.; et al. Anaphase Inactivation of the Spindle Checkpoint. *Science* **2006**, *313*, 680–684.
171. Pinsky, B. A.; Nelson, C. R.; Biggins, S. Protein Phosphatase 1 Regulates Exit from the Spindle Checkpoint in Budding Yeast. **2009**, *19*, 1182–1187.
172. Bokros, M.; Gravenmier, C.; Jin, F.; et al. Fin1-PP1 Helps Clear Spindle Assembly Checkpoint Protein Bub1 from Kinetochores in Anaphase. *Cell Rep.* **2016**.
173. Moura, M.; Osswald, M.; Leça, N.; et al. Protein Phosphatase 1 Inactivates Mps1 to Ensure Efficient Spindle Assembly Checkpoint Silencing. *Elife* **2017**, *6*, 576.
174. Rosenberg, J. S.; Cross, F. R.; Funabiki, H. KNL1/Spc105 Recruits PP1 to Silence the Spindle Assembly Checkpoint. *Curr. Biol.* **2011**, *21*, 942–947.
175. Jin, F.; Wang, Y. The Signaling Network That Silences the Spindle Assembly Checkpoint upon the Establishment of Chromosome Bipolar Attachment. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 21036–21041.
176. Sitry-Shevah, D.; Kaisari, S.; Teichner, A.; et al. Role of Ubiquitylation of Components of Mitotic Checkpoint Complex in Their Dissociation from Anaphase-Promoting Complex/Cyclosome. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 1777–1782.
177. Yang, Y.; Tsuchiya, D.; Lacefield, S. Bub3 Promotes Cdc20-Dependent Activation of the APC/C in *S. Cerevisiae*. *J. Cell Biol.* **2015**, *209*, 519–527.
178. Minshull, J.; Straight, A.; Rudner, A. D.; et al. Protein Phosphatase 2A Regulates MPF Activity and Sister Chromatid Cohesion in Budding Yeast. *Curr. Biol.* **1996**, *6*, 1609–1620.
179. Rossio, V.; Galati, E.; Ferrari, M.; et al. The RSC Chromatin-Remodeling Complex Influences Mitotic Exit and Adaptation to the Spindle Assembly Checkpoint by Controlling the Cdc14 Phosphatase. *J. Cell Biol.* **2010**, *191*, 981–997.
180. Bonaiuti, P.; Chirolì, E.; Gross, F.; et al. Cells Escape an Operational Mitotic Checkpoint through a Stochastic Process. *Curr. Biol.* **2018**, *28*, 28-37.e7.

181. Rudner, A. D.; Hardwick, K. G.; Murray, A. W. Cdc28 Activates Exit from Mitosis in Budding Yeast. *J. Cell Biol.* **2000**, *149*, 1361–1376.
182. Goto, G. H.; Mishra, A.; Abdulle, R.; et al. Bub1-Mediated Adaptation of the Spindle Checkpoint. *PLoS Genet.* **2011**, *7*, e1001282.
183. Toda, K.; Naito, K.; Mase, S.; et al. APC/C-Cdh1-Dependent Anaphase and Telophase Progression during Mitotic Slippage. *Cell Div.* **2012**, *7*, 4.
184. Nagai, M.; Ushimaru, T. Cdh1 Is an Antagonist of the Spindle Assembly Checkpoint. *Cell. Signal.* **2014**, *26*, 2217–2222.
185. Rieder, C. L.; Maiato, H. Stuck in Division or Passing through: What Happens When Cells Cannot Satisfy the Spindle Assembly Checkpoint. *Dev. Cell* **2004**, *7*, 637–651.
186. Rossio, V.; Galati, E.; Piatti, S. Adapt or Die: How Eukaryotic Cells Respond to Prolonged Activation of the Spindle Assembly Checkpoint. *Biochem. Soc. Trans.* **2010**, *38*, 1645–1649.
187. Sinha, D.; Duijf, P. H. G.; Khanna, K. K. Mitotic Slippage: An Old Tale with a New Twist. *Cell Cycle* **2019**, *18*, 7–15.
188. Brito, D. A.; Rieder, C. L. Mitotic Checkpoint Slippage in Humans Occurs via Cyclin B Destruction in the Presence of an Active Checkpoint. *Curr. Biol.* **2006**, *16*, 1194–1200.
189. Lawrence, K. S.; Engebrecht, J. The Spindle Assembly Checkpoint: More than Just Keeping Track of the Spindle. *Trends cell Mol. Biol.* **2015**, *10*, 141–150.
190. Garber, P. M.; Rine, J. Overlapping Roles of the Spindle Assembly and DNA Damage Checkpoints in the Cell-Cycle Response to Altered Chromosomes in *Saccharomyces Cerevisiae*. *Genetics* **2002**, *161*, 521–534.
191. Clerici, M.; Baldo, V.; Mantiero, D.; et al. A Tel1/MRX-Dependent Checkpoint Inhibits the Metaphase-to-Anaphase Transition after UV Irradiation in the Absence of Mec1. *Mol. Cell. Biol.* **2004**, *24*, 10126–10144.
192. Gibson, D. G.; Bell, S. P.; Aparicio, O. M. Cell Cycle Execution Point Analysis of ORC Function and Characterization of the Checkpoint Response to ORC Inactivation in *Saccharomyces Cerevisiae*. *Genes Cells* **2006**, *11*, 557–573.
193. Feng, W.; Bachant, J.; Collingwood, D.; et al. Centromere Replication Timing Determines Different Forms of Genomic Instability in *Saccharomyces Cerevisiae* Checkpoint Mutants during Replication Stress. *Genetics* **2009**, *183*, 1249–1260.
194. Strecker, J.; Gupta, G. D.; Zhang, W.; et al. DNA Damage Signalling Targets the Kinetochore to Promote Chromatin Mobility. *Nat. Cell Biol.* **2016**, *18*, 281–290.
195. Kim, E. M.; Burke, D. J. DNA Damage Activates the SAC in an ATM/ATR-Dependent Manner, Independently of the Kinetochore. **2008**, *4*, e1000015.
196. Palou, R.; Palou, G.; Quintana, D. G. A Role for the Spindle Assembly Checkpoint

- in the DNA Damage Response. *Curr. Genet.* **2017**, *63*, 275–280.
197. Clémenson, C.; Marsolier-Kergoat, M.-C. The Spindle Assembly Checkpoint Regulates the Phosphorylation State of a Subset of DNA Checkpoint Proteins in *Saccharomyces Cerevisiae*. *Mol. Cell. Biol.* **2006**, *26*, 9149–9161.
 198. Géli, V.; Lisby, M. Recombinational DNA Repair Is Regulated by Compartmentalization of DNA Lesions at the Nuclear Pore Complex. *BioEssays* **2015**, *37*, 1287–1292.
 199. Freudenreich, C. H.; Su, X. A. Relocalization of DNA Lesions to the Nuclear Pore Complex. *FEMS Yeast Res.* **2016**, *16*, fow095.
 200. Iouk, T.; Kerscher, O.; Scott, R. J.; et al. The Yeast Nuclear Pore Complex Functionally Interacts with Components of the Spindle Assembly Checkpoint. *J. Cell Biol.* **2002**, *159*, 807–819.
 201. Scott, R. J.; Lusk, C. P.; Dilworth, D. J.; et al. Interactions between Mad1p and the Nuclear Transport Machinery in the Yeast *Saccharomyces Cerevisiae*. *Mol. Biol. Cell* **2005**, *16*, 4362–4374.
 202. Rodriguez-Bravo, V.; Maciejowski, J.; Corona, J.; et al. Nuclear Pores Protect Genome Integrity by Assembling a Premitotic and Mad1-Dependent Anaphase Inhibitor. *Cell* **2014**, *156*, 1017–1031.
 203. Jessulat, M.; Maltby, R. H.; Nguyen-Tran, D.-H.; et al. Spindle Checkpoint Factors Bub1 and Bub2 Promote DNA Double-Strand Break Repair by Nonhomologous End Joining. *Mol. Cell. Biol.* **2015**, *35*, 2448–2463.
 204. Biggins, S. The Composition, Functions, and Regulation of the Budding Yeast Kinetochore. *Genetics* **2013**, *194*, 817–846.
 205. McDonald, K. L.; O'Toole, E. T.; Mastronarde, D. N.; et al. Kinetochore Microtubules in PTK Cells. *J. Cell Biol.* **1992**, *118*, 369–383.
 206. Winey, M.; Mamay, C. L.; O'Toole, E. T.; et al. Three-Dimensional Ultrastructural Analysis of the *Saccharomyces Cerevisiae* Mitotic Spindle. *J. Cell Biol.* **1995**, *129*, 1601–1615.
 207. Burrack, L. S.; Berman, J. Flexibility of Centromere and Kinetochore Structures. *Trends Genet.* **2012**, *28*, 204–212.
 208. Clarke, L. Centromeres: Proteins, Protein Complexes, and Repeated Domains at Centromeres of Simple Eukaryotes. *Curr. Opin. Genet. Dev.* **1998**, *8*, 212–218.
 209. Cai, M. J.; Davis, R. W. Purification of a Yeast Centromere-Binding Protein That Is Able to Distinguish Single Base-Pair Mutations in Its Recognition Site. *Mol. Cell. Biol.* **1989**, *9*, 2544–2550.
 210. Cai, M.; Davis, R. W. Yeast Centromere Binding Protein CBF1, of the Helix-Loop-Helix Protein Family, Is Required for Chromosome Stability and Methionine Prototrophy. *Cell* **1990**, *61*, 437–446.

211. Lechner, J.; Carbon, J. A 240 Kd Multisubunit Protein Complex, CBF3, Is a Major Component of the Budding Yeast Centromere. *Cell* **1991**, *64*, 717–725.
212. Jehn, B.; Niedenthal, R.; Hegemann, J. H. In Vivo Analysis of the *Saccharomyces Cerevisiae* Centromere CDEIII Sequence: Requirements for Mitotic Chromosome Segregation. *Mol. Cell. Biol.* **1991**, *11*, 5212–5221.
213. Cho, U.-S.; Harrison, S. C. Ndc10 Is a Platform for Inner Kinetochore Assembly in Budding Yeast. *Nat. Struct. Mol. Biol.* **2011**, *19*, 48–55.
214. Stoler, S.; Keith, K. C.; Curnick, K. E.; et al. A Mutation in CSE4, an Essential Gene Encoding a Novel Chromatin-Associated Protein in Yeast, Causes Chromosome Nondisjunction and Cell Cycle Arrest at Mitosis. *Genes Dev.* **1995**, *9*, 573–586.
215. Meluh, P. B.; Yang, P.; Glowczewski, L.; et al. Cse4p Is a Component of the Core Centromere of *Saccharomyces Cerevisiae*. *Cell* **1998**, *94*, 607–613.
216. Furuyama, S.; Biggins, S. Centromere Identity Is Specified by a Single Centromeric Nucleosome in Budding Yeast. *Proc. Natl. Acad. Sci.* **2007**, *104*, 14706–14711.
217. Camahort, R.; Li, B.; Florens, L.; et al. Scm3 Is Essential to Recruit the Histone H3 Variant Cse4 to Centromeres and to Maintain a Functional Kinetochore. *Mol. Cell* **2007**, *26*, 853–865.
218. Stoler, S.; Rogers, K.; Weitze, S.; et al. Scm3, an Essential *Saccharomyces Cerevisiae* Centromere Protein Required for G2/M Progression and Cse4 Localization. *Proc. Natl. Acad. Sci.* **2007**, *104*, 10571–10576.
219. Mizuguchi, G.; Xiao, H.; Wisniewski, J.; et al. Nonhistone Scm3 and Histones CenH3-H4 Assemble the Core of Centromere-Specific Nucleosomes. *Cell* **2007**, *129*, 1153–1164.
220. Aravamudhan, P.; Felzer-Kim, I.; Joglekar, A. P. The Budding Yeast Point Centromere Associates with Two Cse4 Molecules during Mitosis. *Curr. Biol.* **2013**, *23*, 770–774.
221. Wisniewski, J.; Hajj, B.; Chen, J.; et al. Imaging the Fate of Histone Cse4 Reveals de Novo Replacement in S Phase and Subsequent Stable Residence at Centromeres. *Elife* **2014**, *3*, e02203.
222. Joglekar, A. P.; Bouck, D. C.; Molk, J. N.; et al. Molecular Architecture of a Kinetochore–Microtubule Attachment Site. *Nat. Cell Biol.* **2006**, *8*, 581–585.
223. Kitamura, E.; Tanaka, K.; Kitamura, Y.; et al. Kinetochore Microtubule Interaction during S Phase in *Saccharomyces Cerevisiae*. *Genes Dev.* **2007**, *21*, 3319–3330.
224. Musacchio, A.; Desai, A. A Molecular View of Kinetochore Assembly and Function. *Biology (Basel)*. **2017**, *6*, 5.
225. Meluh, P. B.; Koshland, D. Budding Yeast Centromere Composition and Assembly as Revealed by in Vivo Cross-Linking. *Genes Dev.* **1997**, *11*, 3401–

3412.

226. Ortiz, J.; Stemmann, O.; Rank, S.; et al. A Putative Protein Complex Consisting of Ctf19, Mcm21, and Okp1 Represents a Missing Link in the Budding Yeast Kinetochore. *Genes Dev.* **1999**, *13*, 1140–1155.
227. Cohen, R. L.; Espelin, C. W.; De Wulf, P.; et al. Structural and Functional Dissection of Mif2p, a Conserved DNA-Binding Kinetochore Protein. *Mol. Biol. Cell* **2008**, *19*, 4480–4491.
228. Kato, H.; Jiang, J.; Zhou, B.-R.; et al. A Conserved Mechanism for Centromeric Nucleosome Recognition by Centromere Protein CENP-C. *Science* **2013**, *340*, 1110–1113.
229. Hornung, P.; Troc, P.; Malvezzi, F.; et al. A Cooperative Mechanism Drives Budding Yeast Kinetochore Assembly Downstream of CENP-A. *J. Cell Biol.* **2014**, *206*, 509–524.
230. Pekgöz Altunkaya, G.; Malvezzi, F.; Demianova, Z.; et al. CCAN Assembly Configures Composite Binding Interfaces to Promote Cross-Linking of Ndc80 Complexes at the Kinetochore. *Curr. Biol.* **2016**, *26*, 2370–2378.
231. Hinshaw, S. M.; Harrison, S. C. The Structure of the Ctf19c/CCAN from Budding Yeast. *Elife* **2019**, *8*.
232. Schmitzberger, F.; Richter, M. M.; Gordiyenko, Y.; et al. Molecular Basis for Inner Kinetochore Configuration through RWD Domain-Peptide Interactions. *EMBO J.* **2017**, *36*, 3458–3482.
233. Pot, I.; Measday, V.; Snyderman, B.; et al. Chl4p and Iml3p Are Two New Members of the Budding Yeast Outer Kinetochore. *Mol. Biol. Cell* **2003**, *14*, 460–476.
234. Hinshaw, S. M.; Harrison, S. C. An Iml3-Chl4 Heterodimer Links the Core Centromere to Factors Required for Accurate Chromosome Segregation. *Cell Rep.* **2013**, *5*, 29–36.
235. Natsume, T.; Müller, C. A.; Katou, Y.; et al. Kinetochores Coordinate Pericentromeric Cohesion and Early DNA Replication by Cdc7-Dbf4 Kinase Recruitment. *Mol. Cell* **2013**, *50*, 661–674.
236. Fernius, J.; Nerusheva, O. O.; Galander, S.; et al. Cohesin-Dependent Association of Scc2/4 with the Centromere Initiates Pericentromeric Cohesion Establishment. *Curr. Biol.* **2013**, *23*, 599–606.
237. Hinshaw, S. M.; Makrantonis, V.; Harrison, S. C.; et al. The Kinetochore Receptor for the Cohesin Loading Complex. *Cell* **2017**, *171*, 72–84.e13.
238. Schleiffer, A.; Maier, M.; Litos, G.; et al. CENP-T Proteins Are Conserved Centromere Receptors of the Ndc80 Complex. *Nat. Cell Biol.* **2012**, *14*, 604–613.
239. Bock, L. J.; Pagliuca, C.; Kobayashi, N.; et al. Cnn1 Inhibits the Interactions between the KMN Complexes of the Yeast Kinetochore. *Nat. Cell Biol.* **2012**, *14*,

614–624.

- 240. Malvezzi, F.; Litos, G.; Schleiffer, A.; et al. A Structural Basis for Kinetochore Recruitment of the Ndc80 Complex via Two Distinct Centromere Receptors. *EMBO J.* **2013**, *32*, 409–423.
- 241. Thapa, K. S.; Oldani, A.; Pagliuca, C.; et al. The Mps1 Kinase Modulates the Recruitment and Activity of Cnn1(CENP-T) at *Saccharomyces Cerevisiae* Kinetochores. *Genetics* **2015**, *200*, 79–90.
- 242. Lang, J.; Barber, A.; Biggins, S. An Assay for de Novo Kinetochore Assembly Reveals a Key Role for the CENP-T Pathway in Budding Yeast. *Elife* **2018**, *7*, 785.
- 243. Dimitrova, Y. N.; Jenni, S.; Valverde, R.; et al. Structure of the MIND Complex Defines a Regulatory Focus for Yeast Kinetochore Assembly. *Cell* **2016**, *167*, 1014–1027.e12.
- 244. Petrovic, A.; Keller, J.; Liu, Y.; et al. Structure of the MIS12 Complex and Molecular Basis of Its Interaction with CENP-C at Human Kinetochores. *Cell* **2016**, *167*, 1028–1040.e15.
- 245. Akiyoshi, B.; Nelson, C. R.; Biggins, S. The Aurora B Kinase Promotes Inner and Outer Kinetochore Interactions in Budding Yeast. *Genetics* **2013**, *194*, 785–789.
- 246. Maskell, D. P.; Hu, X.-W.; Singleton, M. R. Molecular Architecture and Assembly of the Yeast Kinetochore MIND Complex. *J. Cell Biol.* **2010**, *190*, 823–834.
- 247. Pagliuca, C.; Draviam, V. M.; Marco, E.; et al. Roles for the Conserved Spc105p/Kre28p Complex in Kinetochore-Microtubule Binding and the Spindle Assembly Checkpoint. *PLoS One* **2009**, *4*, e7640.
- 248. Wei, R. R.; Sorger, P. K.; Harrison, S. C. Molecular Organization of the Ndc80 Complex, an Essential Kinetochore Component. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 5363–5367.
- 249. Ciferri, C.; De Luca, J.; Monzani, S.; et al. Architecture of the Human Ndc80-Hec1 Complex, a Critical Constituent of the Outer Kinetochore. *J. Biol. Chem.* **2005**, *280*, 29088–29095.
- 250. Wang, H.-W.; Long, S.; Ciferri, C.; et al. Architecture and Flexibility of the Yeast Ndc80 Kinetochore Complex. *J. Mol. Biol.* **2008**, *383*, 894–903.
- 251. Valverde, R.; Ingram, J.; Harrison, S. C. Conserved Tetramer Junction in the Kinetochore Ndc80 Complex. *Cell Rep.* **2016**, *17*, 1915–1922.
- 252. Ciferri, C.; Pasqualato, S.; Screpanti, E.; et al. Implications for Kinetochore-Microtubule Attachment from the Structure of an Engineered Ndc80 Complex. *Cell* **2008**, *133*, 427–439.
- 253. Alushin, G. M.; Musinipally, V.; Matson, D.; et al. Multimodal Microtubule Binding by the Ndc80 Kinetochore Complex. *Nat. Struct. Mol. Biol.* **2012**, *19*, 1161–1167.

254. Lampert, F.; Mieck, C.; Alushin, G. M.; et al. Molecular Requirements for the Formation of a Kinetochore-Microtubule Interface by Dam1 and Ndc80 Complexes. *J. Cell Biol.* **2013**, *200*, 21–30.
255. Maure, J.-F.; Komoto, S.; Oku, Y.; et al. The Ndc80 Loop Region Facilitates Formation of Kinetochore Attachment to the Dynamic Microtubule plus End. *Curr. Biol.* **2011**, *21*, 207–213.
256. Kim, J. ook; Zelter, A.; Umbreit, N. T.; et al. The Ndc80 Complex Bridges Two Dam1 Complex Rings. *Elife* **2017**, *6*, 805.
257. Cheeseman, I. M.; Brew, C.; Wolyniak, M.; et al. Implication of a Novel Multiprotein Dam1p Complex in Outer Kinetochore Function. *J. Cell Biol.* **2001**, *155*, 1137–1145.
258. Cheeseman, I. M.; Anderson, S.; Jwa, M.; et al. Phospho-Regulation of Kinetochore-Microtubule Attachments by the Aurora Kinase Ipl1p. *Cell* **2002**, *111*, 163–172.
259. Li, Y.; Bachant, J.; Alcasabas, A. A.; et al. The Mitotic Spindle Is Required for Loading of the DASH Complex onto the Kinetochore. *Genes Dev.* **2002**, *16*, 183–197.
260. Miranda, J. J. L.; De Wulf, P.; Sorger, P. K.; et al. The Yeast DASH Complex Forms Closed Rings on Microtubules. *Nat. Struct. Mol. Biol.* **2005**, *12*, 138–143.
261. Westermann, S.; Avila-Sakar, A.; Wang, H.-W.; et al. Formation of a Dynamic Kinetochore-Microtubule Interface through Assembly of the Dam1 Ring Complex. *Mol. Cell* **2005**, *17*, 277–290.
262. Welburn, J. P. I.; Grishchuk, E. L.; Backer, C. B.; et al. The Human Kinetochore Ska1 Complex Facilitates Microtubule Depolymerization-Coupled Motility. *Dev. Cell* **2009**, *16*, 374–385.
263. Jeyaprakash, A. A.; Santamaria, A.; Jayachandran, U.; et al. Structural and Functional Organization of the Ska Complex, a Key Component of the Kinetochore-Microtubule Interface. *Mol. Cell* **2012**, *46*, 274–286.
264. van Hooff, J. J. E.; Snel, B.; Kops, G. J. P. L. Unique Phylogenetic Distributions of the Ska and Dam1 Complexes Support Functional Analogy and Suggest Multiple Parallel Displacements of Ska by Dam1. *Genome Biol. Evol.* **2017**, *9*, 1295–1303.
265. Helgeson, L. A.; Zelter, A.; Riffle, M.; et al. Human Ska Complex and Ndc80 Complex Interact to Form a Load-Bearing Assembly That Strengthens Kinetochore-Microtubule Attachments. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 2740–2745.
266. Wang, H.-W.; Ramey, V. H.; Westermann, S.; et al. Architecture of the Dam1 Kinetochore Ring Complex and Implications for Microtubule-Driven Assembly and Force-Coupling Mechanisms. *Nat. Struct. Mol. Biol.* **2007**, *14*, 721–726.
267. Ramey, V. H.; Wang, H.-W.; Nakajima, Y.; et al. The Dam1 Ring Binds to the E-

- Hook of Tubulin and Diffuses along the Microtubule. *Mol. Biol. Cell* **2011**, *22*, 457–466.
268. Jenni, S.; Harrison, S. C. Structure of the DASH/Dam1 Complex Shows Its Role at the Yeast Kinetochores-Microtubule Interface. *Science* **2018**, *360*, 552–558.
 269. Lawrimore, J.; Bloom, K. S.; Salmon, E. D. Point Centromeres Contain More than a Single Centromere-Specific Cse4 (CENP-A) Nucleosome. *J. Cell Biol.* **2011**, *195*, 573–582.
 270. Ng, C. T.; Deng, L.; Chen, C.; et al. Electron Cryotomography Analysis of Dam1C/DASH at the Kinetochores-Spindle Interface in Situ. *J. Cell Biol.* **2019**, *218*, 455–473.
 271. Tien, J. F.; Umbreit, N. T.; Gestaut, D. R.; et al. Cooperation of the Dam1 and Ndc80 Kinetochores Complexes Enhances Microtubule Coupling and Is Regulated by Aurora B. *J. Cell Biol.* **2010**, *189*, 713–723.
 272. Lampert, F.; Hornung, P.; Westermann, S. The Dam1 Complex Confers Microtubule plus End-Tracking Activity to the Ndc80 Kinetochores Complex. *J. Cell Biol.* **2010**, *189*, 641–649.
 273. Umbreit, N. T.; Miller, M. P.; Tien, J. F.; et al. Kinetochores Require Oligomerization of Dam1 Complex to Maintain Microtubule Attachments against Tension and Promote Biorientation. *Nat. Commun.* **2014**, *5*, 4951.
 274. Ortiz, J.; Funk, C.; Schäfer, A.; et al. Stu1 Inversely Regulates Kinetochores Capture and Spindle Stability. *Genes Dev.* **2009**, *23*, 2778–2791.
 275. Funk, C.; Schmeiser, V.; Ortiz, J.; et al. A TOGL Domain Specifically Targets Yeast CLASP to Kinetochores to Stabilize Kinetochores Microtubules. *J. Cell Biol.* **2014**, *205*, 555–571.
 276. Vasileva, V.; Gierliński, M.; Yue, Z.; et al. Molecular Mechanisms Facilitating the Initial Kinetochores Encounter with Spindle Microtubules. *J. Cell Biol.* **2017**, *216*, 1609–1622.
 277. Kitamura, E.; Tanaka, K.; Komoto, S.; et al. Kinetochores Generate Microtubules with Distal plus Ends: Their Roles and Limited Lifetime in Mitosis. *Dev. Cell* **2010**, *18*, 248–259.
 278. Miller, M. P.; Asbury, C. L.; Biggins, S. A TOG Protein Confers Tension Sensitivity to Kinetochores-Microtubule Attachments. *Cell* **2016**, *165*, 1428–1439.
 279. Humphrey, L.; Felzer-Kim, I.; Joglekar, A. P. Stu2 Acts as a Microtubule Destabilizer in Metaphase Budding Yeast Spindles. *Mol. Biol. Cell* **2018**, *29*, 247–255.
 280. Tanaka, T. U.; Stark, M. J. R.; Tanaka, K. Kinetochores Capture and Bi-Oriented on the Mitotic Spindle. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 929–942.
 281. Tanaka, K.; Mukae, N.; Dewar, H.; et al. Molecular Mechanisms of Kinetochores Capture by Spindle Microtubules. *Nature* **2005**, *434*, 987–994.

282. Tanaka, K.; Kitamura, E.; Kitamura, Y.; et al. Molecular Mechanisms of Microtubule-Dependent Kinetochore Transport toward Spindle Poles. *J. Cell Biol.* **2007**, *178*, 269–281.
283. Tanaka, T. U.; Rachidi, N.; Janke, C.; et al. Evidence That the Ipl1-Sli15 (Aurora Kinase-INCENP) Complex Promotes Chromosome Bi-Orientation by Altering Kinetochore-Spindle Pole Connections. *Cell* **2002**, *108*, 317–329.
284. Tanaka, T. U. Kinetochore-Microtubule Interactions: Steps towards Bi-Orientation. *EMBO J.* **2010**, *29*, 4070–4082.
285. Lampson, M. A.; Cheeseman, I. M. Sensing Centromere Tension: Aurora B and the Regulation of Kinetochore Function. *Trends Cell Biol.* **2011**, *21*, 133–140.
286. Carmena, M.; Wheelock, M.; Funabiki, H.; et al. The Chromosomal Passenger Complex (CPC): From Easy Rider to the Godfather of Mitosis. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 789–803.
287. Kalantzaki, M.; Kitamura, E.; Zhang, T.; et al. Kinetochore-Microtubule Error Correction Is Driven by Differentially Regulated Interaction Modes. *Nat. Cell Biol.* **2015**, *17*, 421–433.
288. Akiyoshi, B.; Nelson, C. R.; Ranish, J. A.; et al. Analysis of Ipl1-Mediated Phosphorylation of the Ndc80 Kinetochore Protein in *Saccharomyces Cerevisiae*. *Genetics* **2009**, *183*, 1591–1595.
289. Sarangapani, K. K.; Akiyoshi, B.; Duggan, N. M.; et al. Phosphoregulation Promotes Release of Kinetochores from Dynamic Microtubules via Multiple Mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 7282–7287.
290. García-Rodríguez, L. J.; Kasciukovic, T.; Denninger, V.; et al. Aurora B-INCENP Localization at Centromeres/Inner Kinetochores Is Required for Chromosome Bi-Orientation in Budding Yeast. *Curr. Biol.* **2019**, *29*, 1536-1544.e4.
291. Knockleby, J.; Vogel, J. The COMA Complex Is Required for Sli15/INCENP-Mediated Correction of Defective Kinetochore Attachments. *Cell Cycle* **2009**, *8*, 2570–2577.
292. Sandall, S.; Severin, F.; McLeod, I. X.; et al. A Bir1-Sli15 Complex Connects Centromeres to Microtubules and Is Required to Sense Kinetochore Tension. *Cell* **2006**, *127*, 1179–1191.
293. Yoon, H. J.; Carbon, J. Participation of Bir1p, a Member of the Inhibitor of Apoptosis Family, in Yeast Chromosome Segregation Events. *Proc. Natl. Acad. Sci.* **1999**, *96*, 13208–13213.
294. Shimogawa, M. M.; Widlund, P. O.; Riffle, M.; et al. Bir1 Is Required for the Tension Checkpoint. *Mol. Biol. Cell* **2009**, *20*, 915–923.
295. Fernius, J.; Hardwick, K. G. Bub1 Kinase Targets Sgo1 to Ensure Efficient Chromosome Biorientation in Budding Yeast Mitosis. *PLoS Genet.* **2007**, *3*, e213.
296. Kawashima, S. A.; Yamagishi, Y.; Honda, T.; et al. Phosphorylation of H2A by

- Bub1 Prevents Chromosomal Instability through Localizing Shugoshin. *Science* **2010**, *327*, 172–177.
297. Jin, F.; Bokros, M.; Wang, Y. Premature Silencing of the Spindle Assembly Checkpoint Is Prevented by the Bub1-H2A-Sgo1-PP2A Axis in *Saccharomyces Cerevisiae*. *Genetics* **2017**, *205*, 1169–1178.
 298. Stephens, A. D.; Haase, J.; Vicci, L.; et al. Cohesin, Condensin, and the Intramolecular Centromere Loop Together Generate the Mitotic Chromatin Spring. *J. Cell Biol.* **2011**, *193*, 1167–1180.
 299. Eshleman, H. D.; Morgan, D. O. Sgo1 Recruits PP2A to Chromosomes to Ensure Sister Chromatid Bi-Oriented during Mitosis. *J. Cell Sci.* **2014**, *127*, 4974–4983.
 300. Peplowska, K.; Wallek, A. U.; Storchova, Z. Sgo1 Regulates Both Condensin and Ipl1/Aurora B to Promote Chromosome Biorientation. *PLoS Genet.* **2014**, *10*, e1004411.
 301. Hsu, J. Y.; Sun, Z. W.; Li, X.; et al. Mitotic Phosphorylation of Histone H3 Is Governed by Ipl1/Aurora Kinase and Glc7/PP1 Phosphatase in Budding Yeast and Nematodes. *Cell* **2000**, *102*, 279–291.
 302. Pinsky, B. A.; Kotwaliwale, C. V.; Tatsutani, S. Y.; et al. Glc7/Protein Phosphatase 1 Regulatory Subunits Can Oppose the Ipl1/Aurora Protein Kinase by Redistributing Glc7. *Mol. Cell. Biol.* **2006**, *26*, 2648–2660.
 303. Maure, J.-F.; Kitamura, E.; Tanaka, T. U. Mps1 Kinase Promotes Sister-Kinetochore Bi-Oriented by a Tension-Dependent Mechanism. *Curr. Biol.* **2007**, *17*, 2175–2182.
 304. Storchová, Z.; Becker, J. S.; Talarek, N.; et al. Bub1, Sgo1, and Mps1 Mediate a Distinct Pathway for Chromosome Biorientation in Budding Yeast. *Mol. Biol. Cell* **2011**, *22*, 1473–1485.
 305. Pereira, G.; Schiebel, E. Separase Regulates INCENP-Aurora B Anaphase Spindle Function through Cdc14. *Science* **2003**, *302*, 2120–2124.
 306. Mirchenko, L.; Uhlmann, F. Sli15(INCENP) Dephosphorylation Prevents Mitotic Checkpoint Reengagement Due to Loss of Tension at Anaphase Onset. *Curr. Biol.* **2010**, *20*, 1396–1401.
 307. Dhatchinamoorthy, K.; Shivaraju, M.; Lange, J. J.; et al. Structural Plasticity of the Living Kinetochore. *J. Cell Biol.* **2017**, *216*, 3551–3570.
 308. De Wulf, P.; McAinsh, A. D.; Sorger, P. K. Hierarchical Assembly of the Budding Yeast Kinetochore from Multiple Subcomplexes. *Genes Dev.* **2003**, *17*, 2902–2921.
 309. Driver, J. W.; Geyer, E. A.; Bailey, M. E.; et al. Direct Measurement of Conformational Strain Energy in Protofilaments Curling Outward from Disassembling Microtubule Tips. *Elife* **2017**, *6*, 576.
 310. Asbury, C. L.; Gestaut, D. R.; Powers, A. F.; et al. The Dam1 Kinetochore Complex Harnesses Microtubule Dynamics to Produce Force and Movement. *Proc.*

- Natl. Acad. Sci. U. S. A.* **2006**, *103*, 9873–9878.
311. Akiyoshi, B.; Sarangapani, K. K.; Powers, A. F.; et al. Tension Directly Stabilizes Reconstituted Kinetochore-Microtubule Attachments. *Nature* **2010**, *468*, 576–579.
 312. Gonen, S.; Akiyoshi, B.; Iadanza, M. G.; et al. The Structure of Purified Kinetochores Reveals Multiple Microtubule-Attachment Sites. *Nat. Struct. Mol. Biol.* **2012**, *19*, 925–929.
 313. Leber, V.; Nans, A.; Singleton, M. R. Structural Basis for Assembly of the CBF3 Kinetochore Complex. *EMBO J.* **2018**, *37*, 269–281.
 314. Cieśliński, K.; Ries, J. The Yeast Kinetochore - Structural Insights from Optical Microscopy. *Curr. Opin. Chem. Biol.* **2014**, *20*, 1–8.
 315. Joglekar, A. P.; Bloom, K.; Salmon, E. D. In Vivo Protein Architecture of the Eukaryotic Kinetochore with Nanometer Scale Accuracy. *Curr. Biol.* **2009**, *19*, 694–699.
 316. Joglekar, A.; Chen, R.; Lawrimore, J. A Sensitized Emission Based Calibration of FRET Efficiency for Probing the Architecture of Macromolecular Machines. *Cell. Mol. Bioeng.* **2013**, *6*, 369–382.
 317. Aravamudhan, P.; Felzer-Kim, I.; Gurunathan, K.; et al. Assembling the Protein Architecture of the Budding Yeast Kinetochore-Microtubule Attachment Using FRET. *Curr. Biol.* **2014**, *24*, 1437–1446.
 318. Berezin, M. Y.; Achilefu, S. Fluorescence Lifetime Measurements and Biological Imaging. *Chem. Rev.* **2010**, *110*, 2641–2684.
 319. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Lakowicz, J. R., Ed.; Springer Science & Business Media: Boston, MA, 2007.
 320. Valeur, B.; Berberan-Santos, M. N. *Molecular Fluorescence*; John Wiley & Sons, 2013.
 321. Sugden, J. K. Photochemistry of Dyes and Fluorochromes Used in Biology and Medicine: Some Physicochemical Background and Current Applications. *Biotech. Histochem.* **2004**, *79*, 71–90.
 322. Icha, J.; Weber, M.; Waters, J. C.; et al. Phototoxicity in Live Fluorescence Microscopy, and How to Avoid It. *BioEssays* **2017**, *39*.
 323. Dixon, J. M.; Taniguchi, M.; Lindsey, J. S. PhotochemCAD 2: A Refined Program with Accompanying Spectral Databases for Photochemical Calculations. *Photochem. Photobiol.* **2005**, *81*, 212–213.
 324. Kasha, M. Characterization of Electronic Transitions in Complex Molecules. *Discuss. Faraday Soc.* **1950**, *9*, 14–19.
 325. Harmany, Z. T.; Fereidouni, F.; Levenson, R. M. Spectral Unmixing Methods and Tools for the Detection and Quantitation of Collagen and Other Macromolecules in Tissue Specimens. *Methods Mol. Biol.* **2017**, *1627*, 491–509.

326. Baruah, M.; Qin, W.; Flors, C.; et al. Solvent and PH Dependent Fluorescent Properties of a Dimethylaminostyryl Borondipyrromethene Dye in Solution. *J. Phys. Chem. A* **2006**, *110*, 5998–6009.
327. Wan, Q.-S.; Wang, T.; Zhang, K.-H. Biomedical Optical Spectroscopy for the Early Diagnosis of Gastrointestinal Neoplasms. *Tumour Biol.* **2017**, *39*, 1010428317717984.
328. Kneen, M.; Farinas, J.; Li, Y.; et al. Green Fluorescent Protein as a Noninvasive Intracellular PH Indicator. *Biophys. J.* **1998**, *74*, 1591–1599.
329. Oliver, A. E.; Baker, G. A.; Fugate, R. D.; et al. Effects of Temperature on Calcium-Sensitive Fluorescent Probes. *Biophys. J.* **2000**, *78*, 2116–2126.
330. Warren, S. C.; Margineanu, A.; Alibhai, D.; et al. Rapid Global Fitting of Large Fluorescence Lifetime Imaging Microscopy Datasets. *PLoS One* **2013**, *8*, e70687.
331. Ranjit, S.; Malacrida, L.; Jameson, D. M.; et al. Fit-Free Analysis of Fluorescence Lifetime Imaging Data Using the Phasor Approach. *Nat. Protoc.* **2018**, *13*, 1979–2004.
332. Jameson, D. M.; Ross, J. A. Fluorescence Polarization/Anisotropy in Diagnostics and Imaging. *Chem. Rev.* **2010**, *110*, 2685–2708.
333. Zhang, H.; Wu, Q.; Berezin, M. Y. Fluorescence Anisotropy (Polarization): From Drug Screening to Precision Medicine. *Expert Opin. Drug Discov.* **2015**, *10*, 1145–1161.
334. Chan, F. T. S.; Kaminski, C. F.; Schierle, G. S. K. HomoFRET Fluorescence Anisotropy Imaging as a Tool to Study Molecular Self-Assembly in Live Cells. *ChemPhysChem* **2011**, *12*, 500–509.
335. Köhler Illumination - Köhler Illumination in Transmitted Light. *olympus-lifescience.com*.
336. Schermelleh, L.; Ferrand, A.; Huser, T.; et al. Super-Resolution Microscopy Demystified. *Nat. Cell Biol.* **2019**, *21*, 72–84.
337. Petráň, M.; Hadravský, M.; Egger, M. D.; et al. Tandem-Scanning Reflected-Light Microscope*. *J. Opt. Soc. Am.* **1968**.
338. Tanaami, T.; Otsuki, S.; Tomosada, N.; et al. High-Speed 1-Frame/Ms Scanning Confocal Microscope with a Microlens and Nipkow Disks. *Appl. Opt.* **2002**.
339. Oreopoulos, J.; Berman, R.; Browne, M. Spinning-Disk Confocal Microscopy: Present Technology and Future Trends. *Methods Cell Biol.* **2014**, *123*, 153–175.
340. Pawley, J. B. *Handbook of Biological Confocal Microscopy: Third Edition*; 2006.
341. Conchello, J. A.; Lichtman, J. W. Optical Sectioning Microscopy. *Nature Methods*, 2005.
342. Denk, W.; Strickler, J. H.; Webb, W. W. Two-Photon Laser Scanning Fluorescence Microscopy. *Science (80-.)*. **1990**.

343. Huisken, J.; Swoger, J.; Del Bene, F.; et al. Optical Sectioning Deep inside Live Embryos by Selective Plane Illumination Microscopy. *Science* (80-.). **2004**.
344. Maioli, V.; Chennell, G.; Sparks, H.; et al. Time-Lapse 3-D Measurements of a Glucose Biosensor in Multicellular Spheroids by Light Sheet Fluorescence Microscopy in Commercial 96-Well Plates. *Sci. Rep.* **2016**.
345. Axelrod, D. Total Internal Reflection Fluorescence Microscopy in Cell Biology. *Traffic*, 2001.
346. Förster, T. Zwischenmolekulare Energiewanderung Und Fluoreszenz. *Ann. Phys.* **1948**, 437, 55–75.
347. Förster, T. Energy Migration and Fluorescence. 1946. *J. Biomed. Opt.* **2012**, 17, 11002.
348. Van Der Meer, B. W. Kappa-Squared: From Nuisance to New Sense. *Rev. Mol. Biotechnol.* **2002**.
349. Patterson, G. H.; Piston, D. W.; Barisas, B. G. Förster Distances between Green Fluorescent Protein Pairs. *Anal. Biochem.* **2000**, 284, 438–440.
350. Okamoto, K.; Sako, Y. Recent Advances in FRET for the Study of Protein Interactions and Dynamics. *Curr. Opin. Struct. Biol.* **2017**, 46, 16–23.
351. Arai, Y.; Nagai, T. Extensive Use of FRET in Biological Imaging. *Reprod. Syst. Sex. Disord.* **2013**, 62, 419–428.
352. Day, R. N.; Davidson, M. W. Fluorescent Proteins for FRET Microscopy: Monitoring Protein Interactions in Living Cells. *BioEssays* **2012**, 34, 341–350.
353. Hochreiter, B.; Pardo-Garcia, A.; Schmid, J. A. Fluorescent Proteins as Genetically Encoded FRET Biosensors in Life Sciences. *Sensors* **2015**, 15, 26281–26314.
354. Jares-Erijman, E. A.; Jovin, T. M. FRET Imaging. *Nat. Biotechnol.* **2003**, 21, 1387–1395.
355. Piston, D. W.; Kremers, G.-J. Fluorescent Protein FRET: The Good, the Bad and the Ugly. *Trends Biochem. Sci.* **2007**, 32, 407–414.
356. Shrestha, D.; Jenei, A.; Nagy, P.; et al. Understanding FRET as a Research Tool for Cellular Studies. *Int. J. Mol. Sci.* **2015**, 16, 6718–6756.
357. Vereb, G.; Nagy, P.; Szöllösi, J. Flow Cytometric FRET Analysis of Protein Interaction. *Methods Mol. Biol.* **2011**, 699, 371–392.
358. Karpova, T. S.; Baumann, C. T.; He, L.; et al. Fluorescence Resonance Energy Transfer from Cyan to Yellow Fluorescent Protein Detected by Acceptor Photobleaching Using Confocal Microscopy and a Single Laser. *J. Microsc.* **2003**, 209, 56–70.
359. Van Munster, E. B.; Kremers, G. J.; Adjobo-Hermans, M. J. W.; et al. Fluorescence Resonance Energy Transfer (FRET) Measurement by Gradual Acceptor Photobleaching. *J. Microsc.* **2005**, 218, 253–262.

360. Zeug, A.; Woehler, A.; Neher, E.; et al. Quantitative Intensity-Based FRET Approaches--a Comparative Snapshot. *Biophys. J.* **2012**, *103*, 1821–1827.
361. Leavesley, S. J.; Britain, A. L.; Cichon, L. K.; et al. Assessing FRET Using Spectral Techniques. *Cytometry. A* **2013**, *83*, 898–912.
362. Padilla-Parra, S.; Auduge, N.; Coppey-Moisand, M.; et al. Non Fitting Based FRET-FLIM Analysis Approaches Applied to Quantify Protein-Protein Interactions in Live Cells. *Biophys. Rev.* **2011**, *3*, 63–70.
363. Rizzo, M. A.; Piston, D. W. High-Contrast Imaging of Fluorescent Protein FRET by Fluorescence Polarization Microscopy. *Biophys. J.* **2005**, *88*, L14-6.
364. Bader, A. N.; Hofman, E. G.; Voortman, J.; et al. Homo-FRET Imaging Enables Quantification of Protein Cluster Sizes with Subcellular Resolution. *Biophys. J.* **2009**, *97*, 2613–2622.
365. Levitt, J. A.; Morton, P. E.; Fruhwirth, G. O.; et al. Simultaneous FRAP, FLIM and FAIM for Measurements of Protein Mobility and Interaction in Living Cells. *Biomed. Opt. Express* **2015**, *6*, 3842–3854.
366. Becker, W. Fluorescence Lifetime Imaging--Techniques and Applications. *J. Microsc.* **2012**, *247*, 119–136.
367. Marcu, L. Fluorescence Lifetime Techniques in Medical Applications. *Ann. Biomed. Eng.* **2012**, *40*, 304–331.
368. Squire, A.; Verveer, P. J.; Bastiaens, P. I. Multiple Frequency Fluorescence Lifetime Imaging Microscopy. *J. Microsc.* **2000**, *197*, 136–149.
369. Verveer, P. J.; Squire, A.; Bastiaens, P. I. Global Analysis of Fluorescence Lifetime Imaging Microscopy Data. *Biophys. J.* **2000**, *78*, 2127–2137.
370. Verveer, P. J.; Bastiaens, P. I. H. Evaluation of Global Analysis Algorithms for Single Frequency Fluorescence Lifetime Imaging Microscopy Data. *J. Microsc.* **2003**, *209*, 1–7.
371. Kremers, G.-J.; van Munster, E. B.; Goedhart, J.; et al. Quantitative Lifetime Unmixing of Multiexponentially Decaying Fluorophores Using Single-Frequency Fluorescence Lifetime Imaging Microscopy. *Biophys. J.* **2008**, *95*, 378–389.
372. Clayton, A. H. A.; Hanley, Q. S.; Verveer, P. J. Graphical Representation and Multicomponent Analysis of Single-Frequency Fluorescence Lifetime Imaging Microscopy Data. *J. Microsc.* **2004**, *213*, 1–5.
373. Digman, M. A.; Caiolfa, V. R.; Zamai, M.; et al. The Phasor Approach to Fluorescence Lifetime Imaging Analysis. *Biophys. J.* **2008**, *94*, L14-6.
374. Gadella Jr, T. W. J.; Jovin, T. M.; Clegg, R. M. Fluorescence Lifetime Imaging Microscopy (FLIM): Spatial Resolution of Microstructures on the Nanosecond Time Scale. *Biophys. Chem.* **1993**, *48*, 221–239.
375. Lakowicz, J. R.; Berndt, K. W. Lifetime-selective Fluorescence Imaging Using an

- Rf Phase-sensitive Camera. *Rev. Sci. Instrum.* **1998**, *62*, 1727–1734.
376. Esposito, A.; Oggier, T.; Gerritsen, H.; et al. All-Solid-State Lock-in Imaging for Wide-Field Fluorescence Lifetime Sensing. *Opt. Express* **2005**, *13*, 9812–9821.
 377. Zhao, Q.; Schelen, B.; Schouten, R.; et al. Modulated Electron-Multiplied Fluorescence Lifetime Imaging Microscope: All-Solid-State Camera for Fluorescence Lifetime Imaging. *J. Biomed. Opt.* **2012**, *17*, 126020.
 378. Chen, H.; Holst, G.; Gratton, E. Modulated CMOS Camera for Fluorescence Lifetime Microscopy. *Microsc. Res. Tech.* **2015**, *78*, 1075–1081.
 379. Carlsson, K.; Liljeborg, A. Simultaneous Confocal Lifetime Imaging of Multiple Fluorophores Using the Intensity-Modulated Multiple-Wavelength Scanning (IMS) Technique. *J. Microsc.* **1998**.
 380. Booth, M. J.; Wilson, T. Low-cost, Frequency-domain, Fluorescence Lifetime Confocal Microscopy. *J. Microsc.* **2004**, *214*, 36–42.
 381. Hanson, K. M.; Behne, M. J.; Barry, N. P.; et al. Two-Photon Fluorescence Lifetime Imaging of the Skin Stratum Corneum PH Gradient. *Biophys. J.* **2002**, *83*, 1682–1690.
 382. Behne, M. J.; Meyer, J. W.; Hanson, K. M.; et al. NHE1 Regulates the Stratum Corneum Permeability Barrier Homeostasis MICROENVIRONMENT ACIDIFICATION ASSESSED WITH FLUORESCENCE LIFETIME IMAGING. *J. Biol. Chem.* **2002**, *277*, 47399–47406.
 383. Krishnan, R. V.; Saitoh, H.; Terada, H.; et al. Development of a Multiphoton Fluorescence Lifetime Imaging Microscopy System Using a Streak Camera. *Rev. Sci. Instrum.* **2003**, *74*, 2714–2721.
 384. Biskup, C.; Zimmer, T.; Kelbauskas, L.; et al. Multi-Dimensional Fluorescence Lifetime and FRET Measurements. *Microsc. Res. Tech.* **2007**, *70*, 442–451.
 385. Sun, Y.; Chaudhari, A. J.; Lam, M.; et al. Multimodal Characterization of Compositional, Structural and Functional Features of Human Atherosclerotic Plaques. *Biomed. Opt. Express* **2011**, *2*, 2288–2298.
 386. Buurman, E. P.; Sanders, R.; Draaijer, A.; et al. Fluorescence Lifetime Imaging Using a Confocal Laser Scanning Microscope. *Scanning* **1992**, *14*, 155–159.
 387. De Grauw, C. J.; Gerritsen, H. C. Multiple Time-Gate Module for Fluorescence Lifetime Imaging. *Appl. Spectrosc.* **2001**, *55*, 670–678.
 388. Gerritsen, H. C.; Asselbergs, M. A. H.; Agronskaia, A. V.; et al. Fluorescence Lifetime Imaging in Scanning Microscopes: Acquisition Speed, Photon Economy and Lifetime Resolution. *J. Microsc.* **2002**, *206*, 218–224.
 389. Salthammer, T. Numerical Simulation of Pile-up Distorted Time-Correlated Single Photon Counting (TCSPC) Data. *J. Fluoresc.* **1992**, *2*, 23–27.
 390. Turgeman, L.; Fixler, D. The Influence of Dead Time Related Distortions on Live

- Cell Fluorescence Lifetime Imaging (FLIM) Experiments. *J. Biophotonics* **2014**, 7, 442–452.
391. Chizhik, A.; Ruhlandt, D.; Gregor, I.; et al. Dead-Time Correction of Fluorescence Lifetime Measurements and Fluorescence Lifetime Imaging. *Opt. Express* **2016**, 24, 9429–9445.
 392. Patting, M.; Reisch, P.; Sackrow, M.; et al. Fluorescence Decay Data Analysis Correcting for Detector Pulse Pile-up at Very High Count Rates. *Opt. Eng.* **2018**, 57, 31305.
 393. Wahl, M.; Röhlicke, T.; Rahn, H.-J.; et al. Integrated Multichannel Photon Timing Instrument with Very Short Dead Time and High Throughput. *Rev. Sci. Instrum.* **2013**, 84, 43102.
 394. Kumar, S.; Dunsby, C.; De Beule, P. A. A.; et al. Multifocal Multiphoton Excitation and Time Correlated Single Photon Counting Detection for 3-D Fluorescence Lifetime Imaging. *Opt. Express* **2007**, 15, 12548–12561.
 395. Rinnenthal, J. L.; Börnchen, C.; Radbruch, H.; et al. Parallelized TCSPC for Dynamic Intravital Fluorescence Lifetime Imaging: Quantifying Neuronal Dysfunction in Neuroinflammation. *PLoS One* **2013**, 8, e60100.
 396. Görlitz, F.; Kelly, D. J.; Warren, S. C.; et al. Open Source High Content Analysis Utilizing Automated Fluorescence Lifetime Imaging Microscopy. *J. Vis. Exp.* **2017**, e55119–e55119.
 397. McGinty, J.; Dunsby, C. W.; French, P. M. W. Fluorescence Lifetime Spectroscopy and Imaging: Principles and Applications in Biomedical Diagnostics BT - Fluorescence Lifetime Spectroscopy and Imaging. In *Fluorescence Lifetime Spectroscopy and Imaging*; Marcu, L.; French, P. M. W.; Elson, D. S., Eds.; CRC Press, 2014; pp. 235–248.
 398. Coleman, D. M.; Minami, S.; Uchida, T.; et al. A Two-Dimensional Fluorescence Lifetime Imaging System Using a Gated Image Intensifier. *Appl. Spectrosc.* **1991**, 45, 360–366.
 399. Sparks, H.; Görlitz, F.; Kelly, D. J.; et al. Characterisation of New Gated Optical Image Intensifiers for Fluorescence Lifetime Imaging. *Rev. Sci. Instrum.* **2017**, 88, 13707.
 400. Dowling, K.; Dayel, M. J.; Lever, M. J.; et al. Fluorescence Lifetime Imaging with Picosecond Resolution for Biomedical Applications. *Opt. Lett.* **1998**.
 401. Köllner, M.; Wolfrum, J. How Many Photons Are Necessary for Fluorescence-Lifetime Measurements? *Chem. Phys. Lett.* **1992**, 200, 199–204.
 402. Rowley, M. I.; Barber, P. R.; Coolen, A. C. C.; et al. Bayesian Analysis of Fluorescence Lifetime Imaging Data BT - Multiphoton Microscopy in the Biomedical Sciences XI. In *Multiphoton Microscopy in the Biomedical Sciences XI*; Periasamy, A.; König, K.; So, P. T. C., Eds.; International Society for Optics and Photonics, 2011; Vol. 7903, p. 790325.

403. Rowley, M. I.; Coolen, A. C. C.; Vojnovic, B.; et al. Robust Bayesian Fluorescence Lifetime Estimation, Decay Model Selection and Instrument Response Determination for Low-Intensity FLIM Imaging. *PLoS One* **2016**, *11*, e0158404.
404. Esposito, A.; Dohm, C. P.; Bähr, M.; et al. Unsupervised Fluorescence Lifetime Imaging Microscopy for High Content and High Throughput Screening. *Mol. Cell. Proteomics* **2007**, *6*, 1446–1454.
405. Grecco, H. E.; Roda-Navarro, P.; Girod, A.; et al. *In Situ* Analysis of Tyrosine Phosphorylation Networks by FLIM on Cell Arrays. *Nat. Methods* **2010**, *7*, 467–472.
406. Guzmán, C.; Oetken-Lindholm, C.; Abankwa, D. Automated High-Throughput Fluorescence Lifetime Imaging Microscopy to Detect Protein–Protein Interactions: *J. Lab. Autom.* **2015**, *21*, 238–245.
407. Talbot, C. B.; McGinty, J.; Grant, D. M.; et al. High Speed Unsupervised Fluorescence Lifetime Imaging Confocal Multiwell Plate Reader for High Content Analysis. *J. Biophotonics* **2008**, *1*, 514–521.
408. Matthews, D. R.; Fruhwirth, G. O.; Weitsman, G.; et al. A Multi-Functional Imaging Approach to High-Content Protein Interaction Screening. *PLoS One* **2012**, *7*, e33231.
409. McGinty, J.; Requejo-Isidro, J.; Munro, I.; et al. Signal-to-Noise Characterization of Time-Gated Intensifiers Used for Wide-Field Time-Domain FLIM. *J. Phys. D. Appl. Phys.* **2009**, *42*, 135103.
410. Alibhai, D.; Kelly, D. J.; Warren, S.; et al. Automated Fluorescence Lifetime Imaging Plate Reader and Its Application to Förster Resonant Energy Transfer Readout of Gag Protein Aggregation. *J. Biophotonics* **2013**, *6*, 398–408.
411. Kelly, D. J.; Warren, S. C.; Kumar, S.; et al. An Automated Multiwell Plate Reading Flim Microscope for Live Cell Autofluorescence Lifetime Assays. *J. Innov. Opt. Health Sci.* **2014**, *07*, 1450025.
412. Kelly, D. J.; Warren, S. C.; Alibhai, D.; et al. Automated Multiwell Fluorescence Lifetime Imaging for Förster Resonance Energy Transfer Assays and High Content Analysis. *Anal. Methods* **2015**, *7*, 4071–4089.
413. Edelstein, A. D.; Tsuchida, M. A.; Amodaj, N.; et al. Advanced Methods of Microscope Control Using MManager Software. *J. Biol. methods* **2014**, *1*, 10.
414. Sun, Y.; Day, R. N.; Periasamy, A. Investigating Protein-Protein Interactions in Living Cells Using Fluorescence Lifetime Imaging Microscopy. *Nat. Protoc.* **2011**, *6*, 1324–1340.
415. Day, R. N. Measuring Protein Interactions Using Förster Resonance Energy Transfer and Fluorescence Lifetime Imaging Microscopy. *Methods* **2014**, *66*, 200–207.
416. Margineanu, A.; Chan, J. J.; Kelly, D. J.; et al. Screening for Protein-Protein Interactions Using Förster Resonance Energy Transfer (FRET) and Fluorescence

- Lifetime Imaging Microscopy (FLIM). *Sci. Rep.* **2016**, *6*, 28186.
417. Ernst, S.; Batisse, C.; Zarrabi, N.; et al. Regulatory Assembly of the Vacuolar Proton Pump VoV1-ATPase in Yeast Cells by FLIM-FRET BT - Multiphoton Microscopy in the Biomedical Sciences X. In *Multiphoton Microscopy in the Biomedical Sciences X*; Periasamy, A.; So, P. T. C.; König, K., Eds.; International Society for Optics and Photonics, 2010; Vol. 7569, p. 75690W.
 418. Schreiber, G.; Barberis, M.; Scolari, S.; et al. Unraveling Interactions of Cell Cycle-Regulating Proteins Sic1 and B-Type Cyclins in Living Yeast Cells: A FLIM-FRET Approach. *FASEB J.* **2012**, *26*, 546–554.
 419. Strachotová, D.; Holoubek, A.; Kučerová, H.; et al. Ato Protein Interactions in Yeast Plasma Membrane Revealed by Fluorescence Lifetime Imaging (FLIM). *Biochim. Biophys. Acta* **2012**, *1818*, 2126–2134.
 420. Chen, J.; Miller, A.; Kirchmaier, A. L.; et al. Single-Molecule Tools Elucidate H2A.Z Nucleosome Composition. *J. Cell Sci.* **2012**, *125*, 2954–2964.
 421. Suzuki, A.; Badger, B. L.; Haase, J.; et al. How the Kinetochore Couples Microtubule Force and Centromere Stretch to Move Chromosomes. *Nat. Cell Biol.* **2016**, *18*, 382–392.
 422. Zou, H.; Rothstein, R. Holliday Junctions Accumulate in Replication Mutants via a RecA Homolog-Independent Mechanism. *Cell* **1997**, *90*, 87–96.
 423. Brachmann, C. B.; Davies, A.; Cost, G. J.; et al. Designer Deletion Strains Derived from *Saccharomyces Cerevisiae* S288C: A Useful Set of Strains and Plasmids for PCR-Mediated Gene Disruption and Other Applications. *Yeast* **1998**, *14*, 115–132.
 424. Reid, R. J. D.; González-Barrera, S.; Sunjevaric, I.; et al. Selective Ploidy Ablation, a High-Throughput Plasmid Transfer Protocol, Identifies New Genes Affecting Topoisomerase I-Induced DNA Damage. *Genome Res.* **2011**, *21*, 477–486.
 425. Tkach, J. M.; Yimit, A.; Lee, A. Y.; et al. Dissecting DNA Damage Response Pathways by Analysing Protein Localization and Abundance Changes during DNA Replication Stress. *Nat. Cell Biol.* **2012**, *14*, 966–976.
 426. Gietz, R. D.; Schiestl, R. H. High-Efficiency Yeast Transformation Using the LiAc/SS Carrier DNA/PEG Method. *Nat. Protoc.* **2007**, *2*, 31–34.
 427. Hoffman, C. S.; Winston, F. A Ten-Minute DNA Preparation from Yeast Efficiently Releases Autonomous Plasmids for Transformation of *Escherichia Coli*. *Gene* **1987**, *57*, 267–272.
 428. Ólafsson, G.; Thorpe, P. H. Synthetic Physical Interactions Map Kinetochore Regulators and Regions Sensitive to Constitutive Cdc14 Localization. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 10413–10418.
 429. Ólafsson, G.; Thorpe, P. H. Rewiring the Budding Yeast Proteome Using Synthetic Physical Interactions. *Methods Mol. Biol.* **2018**, *1672*, 599–612.
 430. Rothbauer, U.; Zolghadr, K.; Tillib, S.; et al. Targeting and Tracing Antigens in

- Live Cells with Fluorescent Nanobodies. *Nat. Methods* **2006**, *3*, 887–889.
431. Rothbauer, U.; Zolghadr, K.; Muyldermans, S.; et al. A Versatile Nanotrap for Biochemical and Functional Studies with Fluorescent Fusion Proteins. *Mol. Cell. Proteomics* **2008**, *7*, 282–289.
 432. Huh, W.-K.; Falvo, J. V.; Gerke, L. C.; et al. Global Analysis of Protein Localization in Budding Yeast. *Nature* **2003**, *425*, 686–691.
 433. Heim, R.; Cubitt, A. B.; Tsien, R. Y. Improved Green Fluorescence. *Nature* **1995**, *373*, 663–664.
 434. Hill, A.; Bloom, K. Acquisition and Processing of a Conditional Dicentric Chromosome in *Saccharomyces Cerevisiae*. *Mol. Cell. Biol.* **1989**, *9*, 1368–1370.
 435. Butt, T. R.; Sternberg, E. J.; Gorman, J. A.; et al. Copper Metallothionein of Yeast, Structure of the Gene, and Regulation of Expression. *Proc. Natl. Acad. Sci.* **1984**, *81*, 3332–3336.
 436. Dittmar, J. C.; Reid, R. J. D.; Rothstein, R. ScreenMill: A Freely Available Software Suite for Growth Measurement, Analysis and Visualization of High-Throughput Screen Data. *BMC Bioinformatics* **2010**, *11*, 353.
 437. openFLIM-HCA
<http://www.imperial.ac.uk/photonics/research/biophotonics/instruments--software/openflim-hca/> (accessed May 27, 2019).
 438. openFLIM-HCA wiki <https://github.com/imperial-photonics/openFLIM-HCA/wiki/> (accessed May 27, 2019).
 439. Guo, W.; Kumar, S.; Görlitz, F.; et al. Automated Fluorescence Lifetime Imaging High-Content Analysis of Förster Resonance Energy Transfer between Endogenously Labeled Kinetochores in Live Budding Yeast Cells. *SLAS Technol.* **2019**, *28*, 2472630318819240.
 440. Waters, J. C. Accuracy and Precision in Quantitative Fluorescence Microscopy. *J. Cell Biol.* **2009**, *185*, 1135–1148.
 441. Grant, D. M.; McGinty, J.; McGhee, E. J.; et al. High Speed Optically Sectioned Fluorescence Lifetime Imaging Permits Study of Live Cell Signaling Events. *Opt. Express* **2007**, *15*, 15656–15673.
 442. FLIMfit <https://www.flimfit.org> (accessed May 27, 2019).
 443. OMERO platform <https://www.openmicroscopy.org/omero/> (accessed May 27, 2019).
 444. Kristoffersen, A. S.; Erga, S. R.; Hamre, B.; et al. Testing Fluorescence Lifetime Standards Using Two-Photon Excitation and Time-Domain Instrumentation: Rhodamine B, Coumarin 6 and Lucifer Yellow. *J. Fluoresc.* **2014**, *24*, 1015–1024.
 445. Payne, A. D.; Dorrington, A. A.; Cree, M. J.; et al. Image Intensifier Characterization. *Proc. Image Vis. Comput. New Zeal.* **2006**, *Proceeding*, 487–492.

446. Pelet, S.; Previte, M. J. R.; Laiho, L. H.; et al. A Fast Global Fitting Algorithm for Fluorescence Lifetime Imaging Microscopy Based on Image Segmentation. *Biophys. J.* **2004**, *87*, 2807–2817.
447. Beechem, J. M. Global Analysis of Biochemical and Biophysical Data. *Methods Enzymol.* **1992**, *210*, 37–54.
448. Wagnières, G. A.; Star, W. M.; Wilson, B. C. In Vivo Fluorescence Spectroscopy and Imaging for Oncological Applications. *Photochem. Photobiol.* **1998**, *68*, 603–632.
449. Stotz, A.; Linder, P. The ADE2 Gene from *Saccharomyces Cerevisiae*: Sequence and New Vectors. *Gene* **1990**, *95*, 91–98.
450. Sheff, M. A.; Thorn, K. S. Optimized Cassettes for Fluorescent Protein Tagging in *Saccharomyces Cerevisiae*. *Yeast* **2004**, *21*, 661–670.
451. Shaner, N. C.; Lin, M. Z.; McKeown, M. R.; et al. Improving the Photostability of Bright Monomeric Orange and Red Fluorescent Proteins. *Nat. Methods* **2008**, *5*, 545–551.
452. Dean, K. M.; Lubbeck, J. L.; Binder, J. K.; et al. Analysis of Red-Fluorescent Proteins Provides Insight into Dark-State Conversion and Photodegradation. *Biophys. J.* **2011**, *101*, 961–969.
453. Goedhart, J.; von Stetten, D.; Noirclerc-Savoye, M.; et al. Structure-Guided Evolution of Cyan Fluorescent Proteins towards a Quantum Yield of 93%. *Nat. Commun.* **2012**, *3*, 751.
454. Rines, D. R.; Thomann, D.; Dorn, J. F.; et al. Live Cell Imaging of Yeast. *Cold Spring Harb. Protoc.* **2011**, *2011*, pdb.top065482-pdb.top065482.
455. Bastiaens, P. I.; Van Hoek, A.; Wolkers, W. F.; et al. Comparison of the Dynamical Structures of Lipoamide Dehydrogenase and Glutathione Reductase by Time-Resolved Polarized Flavin Fluorescence. *Biochemistry* **1992**, *31*, 7050–7060.
456. Hanley, Q. S.; Subramaniam, V.; Jovin, D. J. A.; et al. Fluorescence Lifetime Imaging: Multi-point Calibration, Minimum Resolvable Differences, and Artifact Suppression. *Cytometry* **2001**, *43*, 248–260.
457. Szabelski, M.; Ilijev, D.; Sarkar, P.; et al. Collisional Quenching of Erythrosine B as a Potential Reference Dye for Impulse Response Function Evaluation: *Appl. Spectrosc.* **2009**, *63*, 363–368.
458. Szabelski, M.; Luchowski, R.; Gryczynski, Z.; et al. Evaluation of Instrument Response Functions for Lifetime Imaging Detectors Using Quenched Rose Bengal Solutions. *Chem. Phys. Lett.* **2009**, *471*, 153–159.
459. Luchowski, R.; Gryczynski, Z.; Sarkar, P.; et al. Instrument Response Standard in Time-Resolved Fluorescence. *Rev. Sci. Instrum.* **2009**, *80*, 33109.
460. Chib, R.; Shah, S.; Gryczynski, Z.; et al. Standard Reference for Instrument Response Function in Fluorescence Lifetime Measurements in Visible and near

- Infrared. *Meas. Sci. Technol.* **2016**, *27*, 27001.
461. Kolber, Z. S.; Barkley, M. D. Comparison of Approaches to the Instrumental Response Function in Fluorescence Decay Measurements. *Anal. Biochem.* **1986**, *152*, 6–21.
 462. Boens, N.; Ameloot, M.; Yamazaki, I.; et al. On the Use and the Performance of the Delta Function Convolution Method for the Estimation of Fluorescence Decay Parameters. *Chem. Phys.* **1988**, *121*, 73–86.
 463. Zuker, M.; Szabo, A. G.; Bramall, L.; et al. Delta Function Convolution Method (DFCM) for Fluorescence Decay Experiments. *Rev. Sci. Instrum.* **1998**, *56*, 14–22.
 464. Gauduchon, P.; Wahl, P. Pulsefluorimetry of Tyrosyl Peptides. *Biophys. Chem.* **1978**, *8*, 87–104.
 465. Talbot, C. B.; Lagarto, J.; Warren, S.; et al. Correction Approach for Delta Function Convolution Model Fitting of Fluorescence Decay Data in the Case of a Monoexponential Reference Fluorophore. *J. Fluoresc.* **2015**, *25*, 1169–1182.
 466. Boens, N.; Qin, W.; Basarić, N.; et al. Fluorescence Lifetime Standards for Time and Frequency Domain Fluorescence Spectroscopy. *Anal. Chem.* **2007**, *79*, 2137–2149.
 467. Martin, M. M.; Lindqvist, L. The PH Dependence of Fluorescein Fluorescence. *J. Lumin.* **1975**, *10*, 381–390.
 468. Doughty, M. J. PH Dependent Spectral Properties of Sodium Fluorescein Ophthalmic Solutions Revisited. *Ophthalmic Physiol. Opt.* **2010**, *30*, 167–174.
 469. Liu, M.; Jia, M.; Pan, H.; et al. Instrument Response Standard in Time-Resolved Fluorescence Spectroscopy at Visible Wavelength: Quenched Fluorescein Sodium: *Appl. Spectrosc.* **2014**, *68*, 577–583.
 470. Santos, A. F.; Zaltsman, A. B.; Martin, R. C.; et al. Angiogenesis: An Improved in Vitro Biological System and Automated Image-Based Workflow to Aid Identification and Characterization of Angiogenesis and Angiogenic Modulators. *Assay Drug Dev. Technol.* **2008**, *6*, 693–710.
 471. Maslanka, R.; Kwolek-Mirek, M.; Zadrag-Tecza, R. Autofluorescence of Yeast *Saccharomyces Cerevisiae* Cells Caused by Glucose Metabolism Products and Its Methodological Implications. *J. Microbiol. Methods* **2018**, *146*, 55–60.
 472. Sinnecker, D.; Voigt, P.; Nicole Hellwig, and; et al. Reversible Photobleaching of Enhanced Green Fluorescent Proteins. *Biochemistry* **2005**, *44*, 7085–7094.
 473. Wilcoxon, F. Individual Comparisons by Ranking Methods. *Biometrics Bull.* **1945**, *1*, 80.
 474. Dunn, O. J. Multiple Comparisons among Means. *J. Am. Stat. Assoc.* **1961**, *56*, 52–64.
 475. Joglekar, A. P.; Kukreja, A. A. How Kinetochore Architecture Shapes

- the Mechanisms of Its Function. *Curr. Biol.* **2017**, *27*, R816–R824.
476. Jenni, S.; Dimitrova, Y. N.; Valverde, R.; et al. Molecular Structures of Yeast Kinetochore Subcomplexes and Their Roles in Chromosome Segregation. *Cold Spring Harb. Symp. Quant. Biol.* **2017**, *82*, 83–89.
 477. Hinshaw, S. M.; Harrison, S. C. Kinetochore Function from the Bottom Up. *Trends Cell Biol.* **2018**, *28*, 22–33.
 478. Yan, K.; Zhang, Z.; Yang, J.; et al. Architecture of the CBF3-Centromere Complex of the Budding Yeast Kinetochore. *Nat. Struct. Mol. Biol.* **2018**, *25*, 1103–1110.
 479. Wei, R. R.; Schnell, J. R.; Larsen, N. A.; et al. Structure of a Central Component of the Yeast Kinetochore: The Spc24p/Spc25p Globular Domain. *Structure* **2006**, *14*, 1003–1009.
 480. Russell, I. D.; Grancell, A. S.; Sorger, P. K. The Unstable F-Box Protein P58-Ctf13 Forms the Structural Core of the CBF3 Kinetochore Complex. *J. Cell Biol.* **1999**.
 481. Herrero, E.; Thorpe, P. H. Synergistic Control of Kinetochore Protein Levels by Psh1 and Ubr2. *PLoS Genet.* **2016**.
 482. Perriches, T.; Singleton, M. R. Structure of Yeast Kinetochore Ndc10 DNA-Binding Domain Reveals Unexpected Evolutionary Relationship to Tyrosine Recombinases. *J. Biol. Chem.* **2012**, *287*, 5173–5179.
 483. McGill, R.; Tukey, J. W.; Larsen, W. A. Variations of Box Plots. *Am. Stat.* **1978**.
 484. Keppler, A.; Gendreizig, S.; Gronemeyer, T.; et al. A General Method for the Covalent Labeling of Fusion Proteins with Small Molecules in Vivo. *Nat. Biotechnol.* **2003**, *21*, 86–89.
 485. Stagge, F.; Mitronova, G. Y.; Belov, V. N.; et al. Snap-, CLIP- and Halo-Tag Labelling of Budding Yeast Cells. *PLoS One* **2013**, *8*, e78745.
 486. Eisen, J. A.; Sweder, K. S.; Hanawalt, P. C. Evolution of the SNF2 Family of Proteins: Subfamilies with Distinct Sequences and Functions. *Nucleic Acids Res.* **1995**, *23*, 2715–2723.
 487. Flaus, A.; Martin, D. M. A.; Barton, G. J.; et al. Identification of Multiple Distinct Snf2 Subfamilies with Conserved Structural Motifs. *Nucleic Acids Res.* **2006**, *34*, 2887–2905.
 488. Petukhova, G.; Sung, P.; Klein, H. Promotion of Rad51-Dependent D-Loop Formation by Yeast Recombination Factor Rdh54/Tid1. *Genes Dev.* **2000**, *14*, 2206–2215.
 489. Van der Aart, Q. J.; Barthe, C.; Doignon, F.; et al. Sequence Analysis of a 31 Kb DNA Fragment from the Right Arm of *Saccharomyces Cerevisiae* Chromosome II. *Yeast* **1994**, *10*, 959–964.
 490. Klein, H. L. RDH54, a RAD54 Homologue in *Saccharomyces Cerevisiae*, Is Required for Mitotic Diploid-Specific Recombination and Repair and for Meiosis.

Genetics **1997**, *147*, 1533–1543.

491. Shinohara, M.; Shita-Yamaguchi, E.; Buerstedde, J. M.; et al. Characterization of the Roles of the *Saccharomyces Cerevisiae* RAD54 Gene and a Homologue of RAD54, RDH54/TID1, in Mitosis and Meiosis. *Genetics* **1997**, *147*, 1545–1556.
492. Petukhova, G.; Van Komen, S.; Vergano, S.; et al. Yeast Rad54 Promotes Rad51-Dependent Homologous DNA Pairing via ATP Hydrolysis-Driven Change in DNA Double Helix Conformation. *J. Biol. Chem.* **1999**, *274*, 29453–29462.
493. Van Komen, S.; Petukhova, G.; Sigurdsson, S.; et al. Superhelicity-Driven Homologous DNA Pairing by Yeast Recombination Factors Rad51 and Rad54. *Mol. Cell* **2000**, *6*, 563–572.
494. Sugawara, N.; Wang, X.; Haber, J. E. In Vivo Roles of Rad52, Rad54, and Rad55 Proteins in Rad51-Mediated Recombination. *Mol. Cell* **2003**, *12*, 209–219.
495. Solinger, J. A.; Kiiianitsa, K.; Heyer, W.-D. Rad54, a Swi2/Snf2-like Recombinational Repair Protein, Disassembles Rad51:DNA Filaments. *Mol. Cell* **2002**, *10*, 1175–1188.
496. Dresser, M. E.; Ewing, D. J.; Conrad, M. N.; et al. DMC1 Functions in a *Saccharomyces Cerevisiae* Meiotic Pathway That Is Largely Independent of the RAD51 Pathway. *Genetics* **1997**, *147*, 533–544.
497. Shinohara, M.; Gasior, S. L.; Bishop, D. K.; et al. Tid1/Rdh54 Promotes Colocalization of Rad51 and Dmc1 during Meiotic Recombination. *Proc. Natl. Acad. Sci.* **2000**, *97*, 10814–10819.
498. Chi, P.; Kwon, Y.; Seong, C.; et al. Yeast Recombination Factor Rdh54 Functionally Interacts with the Rad51 Recombinase and Catalyzes Rad51 Removal from DNA. *J. Biol. Chem.* **2006**, *281*, 26268–26279.
499. Prasad, T. K.; Robertson, R. B.; Visnapuu, M.-L.; et al. A DNA-Translocating Snf2 Molecular Motor: *Saccharomyces Cerevisiae* Rdh54 Displays Processive Translocation and Extrudes DNA Loops. *J. Mol. Biol.* **2007**, *369*, 940–953.
500. Kwon, Y.; Seong, C.; Chi, P.; et al. ATP-Dependent Chromatin Remodeling by the *Saccharomyces Cerevisiae* Homologous Recombination Factor Rdh54. *J. Biol. Chem.* **2008**, *283*, 10445–10452.
501. Holzen, T. M.; Shah, P. P.; Olivares, H. A.; et al. Tid1/Rdh54 Promotes Dissociation of Dmc1 from Nonrecombinogenic Sites on Meiotic Chromatin. *Genes Dev.* **2006**, *20*, 2593–2604.
502. Maga, G.; Hubscher, U. Proliferating Cell Nuclear Antigen (PCNA): A Dancer with Many Partners. *J. Cell Sci.* **2003**, *116*, 3051–3060.
503. Burgess, R. C.; Sebesta, M.; Sisakova, A.; et al. The PCNA Interaction Protein Box Sequence in Rad54 Is an Integral Part of Its ATPase Domain and Is Required for Efficient DNA Repair and Recombination. *PLoS One* **2013**, *8*, e82630.
504. Zhang, X.-P.; Janke, R.; Kingsley, J.; et al. A Conserved Sequence Extending

- Motif III of the Motor Domain in the Snf2-Family DNA Translocase Rad54 Is Critical for ATPase Activity. *PLoS One* **2013**, 8.
505. Arbel, A.; Zenvirth, D.; Simchen, G. Sister Chromatid-Based DNA Repair Is Mediated by RAD54, Not by DMC1 or TID1. *EMBO J.* **1999**, 18, 2648–2658.
 506. Nimonkar, A. V.; Amitani, I.; Baskin, R. J.; et al. Single Molecule Imaging of Tid1/Rdh54, a Rad54 Homolog That Translocates on Duplex DNA and Can Disrupt Joint Molecules. *J. Biol. Chem.* **2007**, 282, 30776–30784.
 507. Shah, P. P.; Zheng, X.; Epshtein, A.; et al. Swi2/Snf2-Related Translocases Prevent Accumulation of Toxic Rad51 Complexes during Mitotic Growth. *Mol. Cell* **2010**, 39, 862–872.
 508. Chi, P.; Kwon, Y.; Visnapuu, M.-L.; et al. Analyses of the Yeast Rad51 Recombinase A265V Mutant Reveal Different in Vivo Roles of Swi2-like Factors. *Nucleic Acids Res.* **2011**, 39, 6511–6522.
 509. Kateneva, A. V.; Konovchenko, A. A.; Guacci, V.; et al. Recombination Protein Tid1p Controls Resolution of Cohesin-Dependent Linkages in Meiosis in *Saccharomyces Cerevisiae*. *J. Cell Biol.* **2005**, 171, 241–253.
 510. Kateneva, A. V.; Dresser, M. E. Sister Chromatid Cohesion Remodeling and Meiotic Recombination. *Cell Cycle* **2006**, 5, 467–471.
 511. Chan, Y.-L.; Zhang, A.; Weissman, B. P.; et al. RPA Resolves Conflicting Activities of Accessory Proteins during Reconstitution of Dmc1-Mediated Meiotic Recombination. *Nucleic Acids Res.* **2018**, 47, 747–761.
 512. Tsaponina, O.; Haber, J. E. Frequent Interchromosomal Template Switches during Gene Conversion in *S. Cerevisiae*. *Mol. Cell* **2014**, 55, 615–625.
 513. Haber, J. E.; Braberg, H.; Wu, Q.; et al. Systematic Triple-Mutant Analysis Uncovers Functional Connectivity between Pathways Involved in Chromosome Regulation. *Cell Rep.* **2013**, 3, 2168–2178.
 514. Tosato, V.; Sidari, S.; Bruschi, C. V. Bridge-Induced Chromosome Translocation in Yeast Relies upon a Rad54/Rdh54-Dependent, Pol32-Independent Pathway. *PLoS One* **2013**, 8, e60926.
 515. Sims, J.; Bruschi, C. V.; Bertin, C.; et al. High Reactive Oxygen Species Levels Are Detected at the End of the Chronological Life Span of Translocant Yeast Cells. *Mol. Genet. Genomics* **2016**, 291, 423–435.
 516. Santa Maria, S. R.; Kwon, Y.; Sung, P.; et al. Characterization of the Interaction between the *Saccharomyces Cerevisiae* Rad51 Recombinase and the DNA Translocase Rdh54. *J. Biol. Chem.* **2013**, 288, 21999–22005.
 517. Mersaoui, S.; Gravel, S.; Karpov, V.; et al. DNA Damage Checkpoint Adaptation Genes Are Required for Division of Cells Harboring Eroded Telomeres. *Microb. Cell* **2015**, 2, 394–405.
 518. Ferrari, M.; Nachimuthu, B. T.; Donnianni, R. A.; et al. Tid1/Rdh54 Translocase

- Is Phosphorylated through a Mec1- and Rad53-Dependent Manner in the Presence of DSB Lesions in Budding Yeast. *DNA Repair (Amst)*. **2013**, *12*, 347–355.
519. Tanaka, K.; Kagawa, W.; Kinebuchi, T.; et al. Human Rad54B Is a Double-Stranded DNA-Dependent ATPase and Has Biochemical Properties Different from Its Structural Homolog in Yeast, Tid1/Rdh54. *Nucleic Acids Res.* **2002**, *30*, 1346–1353.
 520. Miyagawa, K.; Tsuruga, T.; Kinomura, A.; et al. A Role for RAD54B in Homologous Recombination in Human Cells. *EMBO J.* **2002**, *21*, 175–180.
 521. Yasuhara, T.; Suzuki, T.; Katsura, M.; et al. Rad54B Serves as a Scaffold in the DNA Damage Response That Limits Checkpoint Strength. *Nat. Commun.* **2014**, *5*, 5426.
 522. Berry, L. K.; Ólafsson, G.; Ledesma-Fernández, E.; et al. Synthetic Protein Interactions Reveal a Functional Map of the Cell. *Elife* **2016**, *5*, e13053.
 523. Ólafsson, G.; Thorpe, P. H. Synthetic Physical Interactions Map Kinetochore-Checkpoint Activation Regions. *G3 (Bethesda)*. **2016**, *6*, 2531–2542.
 524. Eden, E.; Navon, R.; Steinfeld, I.; et al. GOrilla: A Tool for Discovery and Visualization of Enriched GO Terms in Ranked Gene Lists. *BMC Bioinformatics* **2009**, *10*, 48.
 525. Costanzo, M.; VanderSluis, B.; Koch, E. N.; et al. A Global Genetic Interaction Network Maps a Wiring Diagram of Cellular Function. *Science* **2016**, *353*, aaf1420–aaf1420.
 526. Usaj, M.; Tan, Y.; Wang, W.; et al. TheCellMap.Org: A Web-Accessible Database for Visualizing and Mining the Global Yeast Genetic Interaction Network. *G3 (Bethesda)*. **2017**, *7*, 1539–1549.
 527. Cejka, P.; Plank, J. L.; Dombrowski, C. C.; et al. Decatenation of DNA by the *S. Cerevisiae* Sgs1-Top3-Rmi1 and RPA Complex: A Mechanism for Disentangling Chromosomes. *Mol. Cell* **2012**, *47*, 886–896.
 528. Deshpande, I.; Seeber, A.; Shimada, K.; et al. Structural Basis of Mec1-Ddc2-RPA Assembly and Activation on Single-Stranded DNA at Sites of Damage. *Mol. Cell* **2017**, *68*, 431–445.e5.
 529. Wozniak, R.; Burke, B.; Doye, V. Nuclear Transport and the Mitotic Apparatus: An Evolving Relationship. *Cell. Mol. Life Sci.* **2010**, *67*, 2215–2230.
 530. Mossaid, I.; Fahrenkrog, B. Complex Commingling: Nucleoporins and the Spindle Assembly Checkpoint. *Cells* **2015**, *4*, 706–725.
 531. Scott, R. J.; Cairo, L. V.; Van de Vosse, D. W.; et al. The Nuclear Export Factor Xpo1p Targets Mad1p to Kinetochores in Yeast. *J. Cell Biol.* **2009**, *184*, 21–29.
 532. Cairo, L. V.; Ptak, C.; Wozniak, R. W. Dual Personality of Mad1: Regulation of Nuclear Import by a Spindle Assembly Checkpoint Protein. *Nucleus* **2013**, *4*, 367–373.

- 533. Heim, R.; Tsien, R. Y. Engineering Green Fluorescent Protein for Improved Brightness, Longer Wavelengths and Fluorescence Resonance Energy Transfer. *Curr. Biol.* **1996**, *6*, 178–182.
- 534. Shi, X.; Basran, J.; Seward, H. E.; et al. Anomalous Negative Fluorescence Anisotropy in Yellow Fluorescent Protein (YFP 10C): Quantitative Analysis of FRET in YFP Dimers. *Biochemistry* **2007**, *46*, 14403–14417.
- 535. Vogel, S. S.; Nguyen, T. A.; van der Meer, B. W.; et al. The Impact of Heterogeneity and Dark Acceptor States on FRET: Implications for Using Fluorescent Protein Donors and Acceptors. *PLoS One* **2012**, *7*, e49593.
- 536. Kim, J.-A.; Haber, J. E. Chromatin Assembly Factors Asf1 and CAF-1 Have Overlapping Roles in Deactivating the DNA Damage Checkpoint When DNA Repair Is Complete. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 1151–1156.