

Imperial College London
Department of Physics

**Development of oblique plane
microscopy and its application to
time-lapse high-content imaging of
live oncogene-expressing spheroids**

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Declaration of originality

I hereby declare that this thesis and the work reported herein was composed by and originated entirely from me. Information derived from the published and unpublished work of others has been acknowledged in the text and references are given in the list of sources.

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Abstract

Oblique plane microscopy (OPM) is a technique characterised by its ability to image 3D specimens with low photodamage while being compatible with standard sample-mounting techniques. Here, instrumentation and automation for OPM is presented to achieve automated four-colour imaging, compared to two-colour in the previous implementation. Additionally, an existing sample pre-find method was developed to locate samples in 3D using custom autofocus and spheroid detection scripts.

This OPM setup was used in a collaborative project to acquire four-channel, 3D time-lapse image data of 170–180 spheroids comprising up to three populations of breast cell lines, each expressing different oncogenes: *HER2WT*, *BRAFV600E* and *HER2D16*. An analysis pipeline was developed that segmented nuclei in 3D using Cellpose and performed cell-level, time-resolved analysis of the progression of each cell population. This marks the first instance of studying the competition between cells expressing different oncogenes in ~170 spheroids in parallel.

Finally, the light collection efficiency of OPM and dual-view OPM (dOPM) systems was investigated using a vectorial ray-tracing simulation. Unlike previously published methods, this simulation models the tilt of the mirror and a thin-film mirror coating for the first time. For the dOPM system considered (1st objective 60×/1.2 NA water, 2nd/3rd objective 50×/0.95 NA air) with an OPM angle of 35°, the calculated collection efficiencies relative to the primary microscope alone were (to 3 decimal places) 0.471, 0.331 and 0.329 for excitation polarised in the illumination sheet plane, perpendicular to the plane, and unpolarised respectively. Despite additional losses at the polarising beamsplitter in dOPM, for OPM these were lower at 0.298, 0.248 and 0.277.

Preliminary experimental measurements of relative collection efficiency showed trends similar to the simulations but were 22% lower than simulation after including manufactures' transmission data for the additional dOPM optics. Further experiment is needed to determine the source of the residual losses.

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Links

opmGeneralAnalysis

<https://github.com/ImperialCollegeLondon/opmGeneralAnalysis/tree/master>

Link #	Relative path of file
1.1	20200730_dotPhotonAndReslice/reslicing/OPMReconstruction/src/opmreconstruction/OPMReconstruction.java
1.2	20200730_dotPhotonAndReslice/dotphotonReslice.m
1.3	20200730_dotPhotonAndReslice/compressToDasTimestamp.m

mmgr-opm-scripts

<https://github.com/LeoRoweBrown/mmgr-opm-scripts>

Link #	Relative path of file
2.1	single_cam_4ch_fw_acq_xz_correction.bsh
2.2	prefind/find_coverslip_and_spheroid_z_nolabview_new.bsh
2.3	prefind/com_4x_pf_test.bsh
2.4	utils/excel positions 384 plate d4 underscore.bsh

mmgr-FW103H

<https://github.com/LeoRoweBrown/mmgr-FW103H>

OPMsim

<https://github.com/LeoRoweBrown/OPMsim/tree/refactor>

Link #	Relative path of file
3.1	notebooks/opm_dopm_simulation_thesis2023.ipynb
3.2	notebooks/anisotropy notebook.ipynb

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List of abbreviations

AOTF	Acousto-optic tuneable filter
APOM	Axial plane optical microscopy
BFP	Back focal plane
CE	Collection efficiency
CLSM	Confocal laser scanning microscope
diSPIM	Dual-view inverted SPIM
DOF	Depth of field
dOPM	Dual-view OPM
Dox	Doxycycline
DSLM	Digitally scanned light-sheet microscopy
EE	Emission efficiency
eSPIM	Epi-illumination SPIM
FACS	Fluorescence-activated cell sorting
FFP	Front focal plane
FOV	Field of view
FP	Fluorescent protein
FWHM	Full width at half maximum
GCE	Geometric collection efficiency
GRCE	Geometric relative collection efficiency
H2B	Histone H2B
HCA	High content analysis
HCS	High content screening
HILO	Highly inclined and laminated optical sheet (microscopy)
LSFM	Light-sheet fluorescence microscopy
NA	Numerical aperture
NI-DAQ	National Instruments Digital Acquisition box
NODO	Non-orthogonal dual-objective
obSTORM	Oblique stochastic optical reconstruction microscopy
ODO	Orthogonal dual-objective
OPM	Oblique plane microscopy
OS-SIM	Optical sectioning SIM
PALM	Photoactivatable localisation microscopy
PBS	Polarising beamsplitter
PSF	Point spread function
QWP	Quarter waveplate
RCE	Relative collection efficiency
SBR	Signal to background ratio
SCAPE	Swept confocally-aligned planar excitation (microscopy)
SDCM	Spinning disk confocal microscopy

SIM	Structured illumination microscopy
SLM	Spatial light modulator
SNR	Signal to noise ratio
SOPi	Scanned oblique plane illumination (microscopy)
SPIM	Selective plane illumination microscopy
STORM	Stochastic optical reconstruction microscopy
TIRFM	Total internal reflection microscopy

Supplementary media

Videos

Video 4.1 – Time-lapse ortho-MIP montage of live plate 1:

<https://imperialcollegelondon.box.com/s/jh7co9ux2dzqf6ml2pzb3i9qg6k6jdmu>

Video 4.2 – Time-lapse ortho-MIP montage of live plate 3:

<https://imperialcollegelondon.box.com/s/nc2rmoy1cetp9qc46pfm5kweyszl原因9p>

Video 4.3 – Time-lapse ortho-MIP montage of live plate 4:

<https://imperialcollegelondon.box.com/s/4snxsau8i73cb0l6ewl49mpgkd1te4au>

Video 4.4 – Time-lapse ortho-MIP montage of live plate 5:

<https://imperialcollegelondon.box.com/s/0vxiej1cn2lz3852pbp4kg04uryjsfdh>

Video 4.5 – Time-lapse ortho-MIP montage of live plate 6:

<https://imperialcollegelondon.box.com/s/7scx54jv73mc70e4el0hk8t7ehljl原因>

Chapter 4 full analysis data

Live plate 4: <https://imperialcollegelondon.box.com/s/dge7qxrha hdfu750nozala2ndj19j3w4>

Live plate 6: <https://imperialcollegelondon.box.com/s/75ibyfdm0o2jt94dhu9hk pddjynpbpwf>

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A thankyou is due to all the teachers who have helped me grow over the years and shared their wisdom. Someone who likely made a lot of difference is a certain primary school teacher, Mrs. Perry, who always believed in me.

Thanks to Mum, Dad, Kate and Max and friends for their general support, and especially my parents for housing me and cooking for me while I've been writing up; not to mention simply everything that they've done for me throughout my life. Maybe once I get this thesis done, I can do some elaborate cooking for everyone...

Finally, I wish to make a dedication to George. Thank you for enriching my life, your immense kindness, good company, and em dash suggestions. I can't wait to make more great memories together soon, and hopefully that will be much easier soon! We should think of a

good way to have an extravagant celebration; I'm sure food will be involved somehow.
Maybe warmed up pork pie? SüKü!

Overview

In this thesis, I will present my work on developing techniques and instrumentation for oblique plane microscopy (OPM).

OPM is a light sheet technique which achieves optical sectioning of fluorescent biological samples; in the first chapter of this thesis, I will introduce the literature surrounding light sheet fluorescence microscopy and OPM itself. The stage-scanning OPM system that I improved and used throughout my PhD was originally developed by members of our group, including Sunil Kumar, Nathan Curry and Vincent Maioli; Vincent Maioli describes much of the development in his thesis. the optical design was then updated by Nathan Curry, and I helped build this new optical setup at the beginning of my PhD. This current form of the OPM system is introduced in Chapter 2.

In Chapter 2, I also present my work developing the OPM instrumentation and acquisition control software to achieve automated (sequential) four-channel imaging, while the original setup could only image two channels in a single acquisition cycle. I describe the changes to the digital acquisition (DAQ) setup and LabVIEW code, the implementation of a motorised filter wheel in MicroManager and the writing of new control scripts in MicroManager for time-lapse acquisition.

Further improvements to OPM instrumentation are presented in Chapter 3, namely the methods, collectively referred to as pre-find, to automatically locate samples to streamline the process of conducting imaging experiments with multi-well plate formats. Although OPM is well-suited to high-throughput live cell imaging such as with 384-well plates, manually locating hundreds of samples is slow and laborious. In the case of Chapter 4, where the imaging plate had to be removed from the microscope frame, treated, and replaced, this meant locating the samples all over again. I will show that the pre-find methods I developed for OPM can successfully centre more than 90% of the 180 spheroids (imaged in Chapter 4) in x, y and z within the OPM field of view.

In Chapter 4, I present time-lapse OPM imaging of up to 180 breast cancer-mimicking spheroids in parallel, with each spheroid consisting of mixtures of between one and up to four (three in later experiments) cell lines with different mutations; this work was performed in collaboration with Nina Moderau. The instrumentation upgrades I implemented in Chapter 2 allowed automated four-channel, time-lapse imaging, and each cell line was identified via a fluorescent protein localised to the cell nucleus with a different emission wavelength. This allowed us to study different cancer populations expressing different oncogenes separately over the full duration of the experiment – up to 8 days. These results represent the first time that the competition between up to three different cancer cell lines has been studied in 170–180 spheroids in parallel at the individual cellular level over time. Nuclear segmentation and quantitative analysis of this rich dataset will also be presented here, including the cell counts of each population, expression levels, and nuclear volume.

Finally, in Chapter 5, I investigate the light collection efficiency of OPM and dual-view OPM (dOPM) systems through a vectorial ray tracing simulation that I developed. Previous work simulating dOPM does not fully model the effect of the tilt of the remote mirror and did not include the effects of protective oxide coating layers present on commercially available metallic mirrors; the method I developed is the first to achieve this. Due to the oblique plane of illumination, OPM and dOPM suffer from an inherent light loss due to pupil clipping when the tilted image of the sample is corrected further along the optical path. In OPM, this is caused by the physical angle between two objectives, while in dOPM it is from a tilted mirror placed in the focal plane of the secondary objective that reflects the light cone into a new direction. Here, I show that the excitation polarisation affects the collection efficiency of both OPM and dOPM, and that dOPM can increase the collection efficiency by increasing the collection of the tilted light cone with a higher NA secondary/tertiary objective. Namely, we will see that light sheets polarised in-plane have better performance than those polarised out-of-plane for both OPM and dOPM systems by up to 39%.

1 Introduction

The humble magnifying glass can be thought of as the most basic form of light microscope—a device that forms a magnified image of an object at a finite distance. In about 1600, Zacharias Janssen was said to have built the first compound microscope, which consists of an objective lens placed close to the object to form an intermediate image, which is then remagnified by the eyepiece to create a virtual image at infinity, resulting in far higher magnification than that with a single lens. Robert Hooke is then credited with being one of the first to observe microorganisms in *Micrographia* (1665), using his own compound microscope along with Antonie van Leeuwenhoek who observed single-cell organisms (1676) with single lens microscopes. The latter were made of hand-ground lenses with magnifications that surpassed any in the field at the time.

Optical microscopy is a powerful tool used today by research biologists and by clinicians in diagnosis, and many of these systems still resemble the compound microscope system with objective lenses and eyepieces. These modern microscopes include a range of modalities such as standard brightfield or transmission, fluorescence, and phase contrast, to name a few. By using fluorescence in particular, features and structures of a specimen can be identified both with higher specificity and higher contrast than other methods when fluorescent labelling techniques are used; for example, some stains such as DAPI label DNA, while the technique of immunofluorescence allows the use of antibodies to label specific proteins within a cell.

In this chapter, we introduce the concept of fluorescence and fluorescence microscopy, how ‘optical sectioning’ enables microscopy in three dimensions, and more specifically, light sheet fluorescence microscopy. Finally, oblique plane microscopy (OPM) itself is introduced along with the plethora of related techniques which have arisen from the original concept.

1.1 Fluorescence

The phenomenon of fluorescence is described by the excitation of the electronic ground state of an atom or molecule to a higher energy quantum state through the absorption of a photon, and subsequently, the emission of another (in general) lower energy photon as the system spontaneously transitions back down to the ground state.

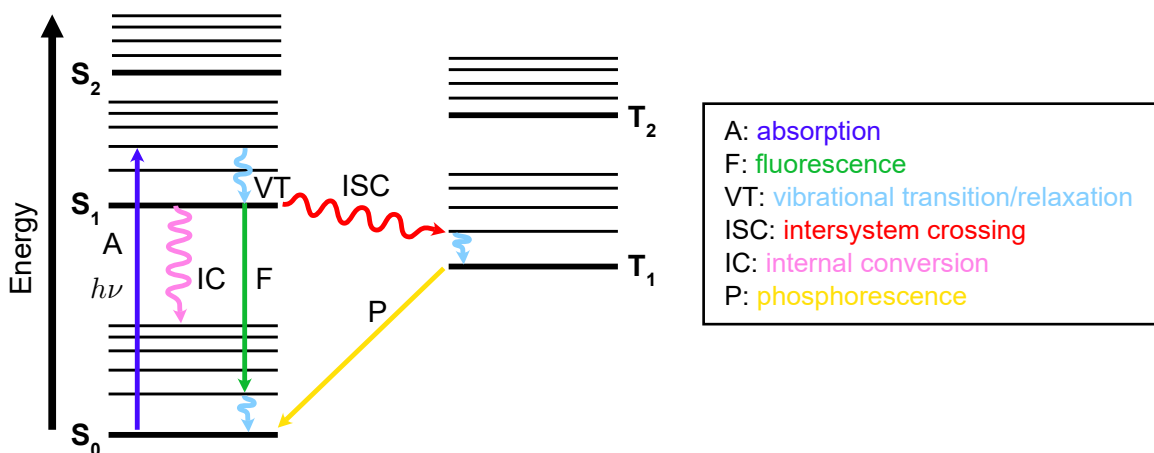


Figure 1.1 – Exemplar Jablonski diagram for a generic fluorophore. Horizontal thick black solid lines indicate the lowest vibrational level of electronic states and the thinner lines indicate vibrational states.

These transitions can be illustrated by a Jablonski diagram, see Figure 1.1, which shows the electronic energy levels and sublevels which represent molecular vibrational and rotational states. Under excitation with a photon with energy $E = h\nu$, where ν is the frequency of the photon and h Planck's constant, the energy must exceed the energy separation between the (electron) ground state S_0 and the excited state S_1 , where 0 and 1 are the principal quantum numbers, i.e. $h\nu > S_1 - S_0$. The absorbed energy may also excite the fluorophore into higher vibrational states. In this case, the fluorophore rapidly decays ($\sim$ ps) to the lowest vibrational state of S_1 , and then transition from S_1 to S_0 results in radiative fluorescence decay (10^{-8} s– 10^{-9} s) emitting a photon with an energy equal to $S_1 - S_0$.

It is usually true that the energy of the emitted photon is independent of the excitation photon energy since excitation to higher vibrational levels of S_1 or higher electronic states (S_2 and higher) become more closely spaced in energy. The close spacing of the energy levels corresponds to wavefunctions that have significant overlap such that the transitions down to the S_1 state happen in a much shorter time than transitioning directly to the ground state. This is formally known as Kasha's rule, which states that for a given multiplicity (the total number of possible spin orientations for a given total spin quantum number, S), the only appreciable contribution to the fluorescence is from the lowest vibrational state of the first excited state S_1 to S_0 (for single spin states).

In a filled orbital in the ground state, two paired electrons exist with opposed spins so that the total spin number $S = 0$. Due to the selection rule for electronic transitions $\Delta S = 0$, the excitation of an electron into the triplet state (where $S = 1$), corresponding to the unpairing of these opposed spins, is forbidden (associated with a very low probability). It is, however, in the presence of spin-orbit coupling, relatively more probable that a non-electronic transition can occur which changes the singlet state to a triplet state known as an intersystem crossing. For the same reason that direct excitation into the triplet state is very

unlikely, the emission of a photon and relaxation into the singlet ground state from a triplet state is also unlikely. This means that triplet states often correspond to a long-lived dark state before emission occurs, and the emission associated with the long-lived triplet state is called phosphorescence.

The time scales of each of these processes are summarised in Table 1.1, and these can vary greatly between molecules, depending on the internal structure affecting electronic configuration and physical dynamics. In particular, the fluorescence lifetime τ characterises the average time for a certain molecule to emit fluorescence and is defined as the average time taken for the fluorescence intensity signal from a fluorescence species to decay to $1/e$ of its initial value [1]. The concept of fluorescence lifetime has wide application to imaging with fluorescence lifetime imaging (FLIM): it can be used to distinguish fluorophores with spectral overlap that would otherwise be hard to resolve, as well as to probe the microenvironment of a biological sample since this affects the measured fluorescence lifetime [1].

In a real system, the absorption spectra are broadened by excitation into higher energy states, such as the additional vibrational states or excitation into S_2 (which may result in an absorption spectrum with multiple peaks). The emission spectra are also broadened due to relaxation into vibrational excited states of the ground state S_0 . Because of the broadening of the spectra, the emitted photon wavelength is often lower than the excitation photon and the wavelength difference between the peaks of the excitation and emission spectra is known as the Stokes shift, and an absorption and emission spectrum for an example fluorophore Rh6G is shown in Figure 1.2.

Table 1.1 – Typical time scales for transitions involved in fluorescence for a molecule in solution, adapted from Molecular Fluorescence: Principles and Applications, Valeur B. et al. [2].

Type of process	Transition	Radiative	Change in total spin quantum number, ΔS	Timescale
Absorption	$S_0 \rightarrow S_1$		0	10^{-15} s
Fluorescence	$S_1 \rightarrow S_0$	Yes	0	$10^{-10} - 10^{-7}$ s
Internal conversion	$S_1 \rightarrow S_0$	No	0	$10^{-11} - 10^{-9}$ s
Vibrational relaxation		No	0	$10^{-12} - 10^{-10}$ s
Intersystem crossing	$S_1 \rightarrow S_0$	No	1	$10^{-10} - 10^{-8}$ s
Phosphorescence	$T_1 \rightarrow S_0$	Yes	1	$10^{-6} - 1$ s

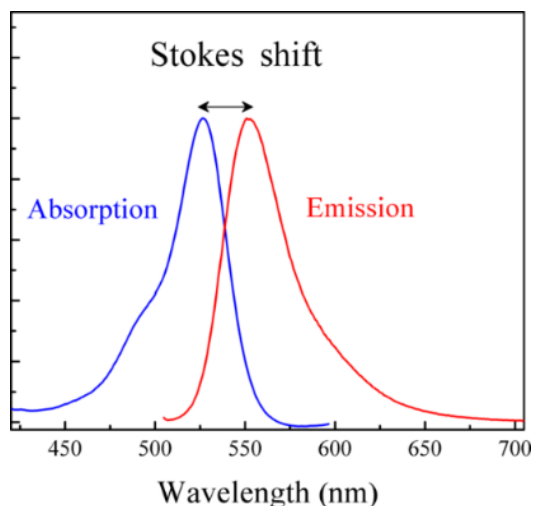


Figure 1.2 – Normalised absorption and emission spectrum for Rh6G (Sobarwiki, Public domain, via Wikimedia Commons) [3].

1.1.1 Phototoxicity and photobleaching

In fluorescence imaging of biological samples, phototoxicity and photobleaching, collectively known as photodamage, refer to sample damage due to the excitation light and are important considerations in microscopy. Photodamage is a complex phenomenon with a diverse range of causes and can vary widely based on the general photochemistry of the fluorophore, its environment and the excitation wavelength. Photodamage often occurs when fluorophores are excited into long-lived triplet states which, because they are long-lived, increases the probability of the excess energy being transferred to the environment and secondary excitations. This energy transfer can result in reactive oxygen species (ROS) being produced such as singlet oxygen [4], which can result in oxidative damage to a broad range of biomolecules and disruption of biological processes, as well as causing damage to the fluorophore itself.

In addition, UV wavelength light can result in phototoxicity from UV-induced apoptosis and can cause DNA damage from direct absorption [5], [6]—for this reason, fluorophores which are excited by longer wavelengths are preferable when setting up a fluorescence experiment. Longer wavelengths also can contribute to phototoxicity, however, since a wide range of wavelengths are absorbed by the many molecules in cells, this may result in heating of the sample and denaturing of proteins.

Photodamage appears to be strongly affected by peak excitation intensity. In general, lower intensity excitation—and correspondingly longer exposure times to maintain overall signal level—reduces photodamage [6], [7]. However, pulsed excitation where the pulses are separated by more than the triplet-state lifetime can also reduce photodamage [8]. In fixed samples, phototoxicity is not usually an issue, and so the microscope technique can be optimised to minimise photobleaching in this case.

1.1.2 Fluorescence microscopy

A common scheme for fluorescence microscopy is a widefield microscope with epi-illumination, with the schematic of a setup shown in Figure 1.3. These setups are often the same as standard brightfield (transmission) microscopes, albeit with the excitation light source introduced using a dichroic beamsplitter (mirror) into the same light path as the detection and an emission filter to separate the fluorescence from any reflected excitation light. In commercial epifluorescence microscopes, filter cubes incorporate the dichroic mirror, excitation, and emission filters all in one unit, with multiple filter cubes occupying a carousel to switch between different fluorescent channels.

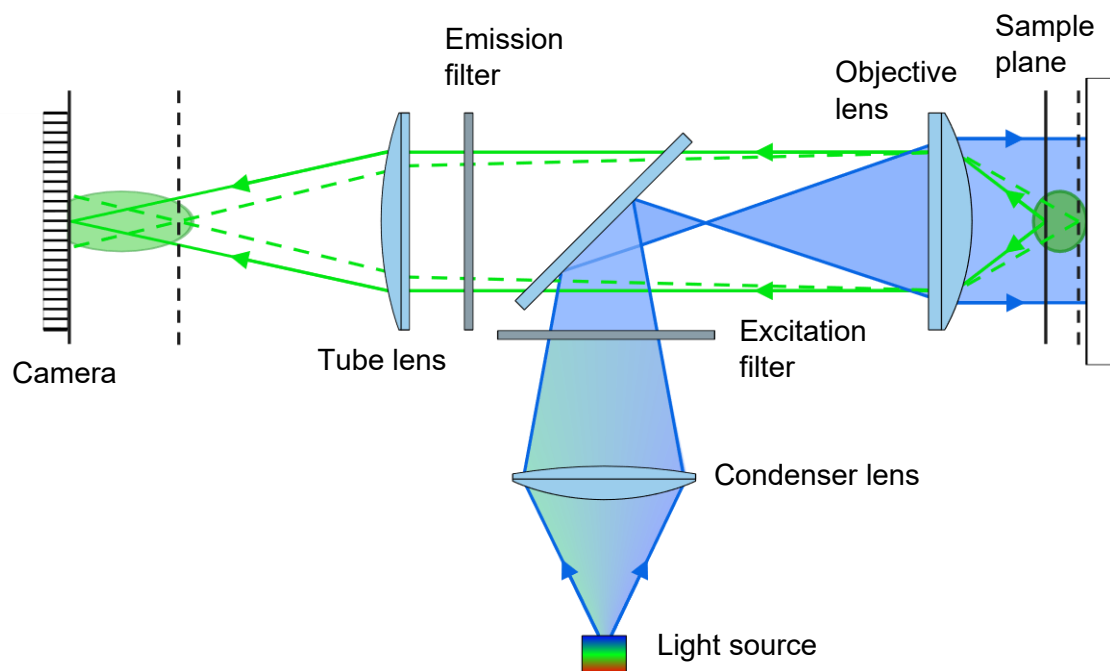


Figure 1.3 – Standard widefield microscope with epi-illumination. The camera plane is conjugate to the focal plane of the objective lens and an in-focus image of this plane is captured by the camera; however, for a sample with axial extent, out-of-plane fluorescence is out of focus at the camera plane (conjugate planes shown by dashed lines) and contributes to background. The system shown uses an infinity-corrected objective lens, which has an image distance set to infinity and is then paired with a tube lens to form an image.

Widefield fluorescence microscopy with incoherent sources such as LEDs is widely used in biomedical research but has diffraction-limited performance formally defined by Ernst Abbe and the Abbe limit. The Abbe limit describes the maximum grating spatial frequency that can be imaged, which requires a first-order maximum (and the central maximum) from the diffraction pattern produced by the grating to be collected and therefore depends on the maximum collection angle of the lens.

Rather than in terms of spatial frequency, the Abbe diffraction limit is often considered in terms of the minimum resolvable object separation d , and is commonly written as

$$d = \frac{\lambda}{2n \sin \theta} \quad 1.1$$

where the numerical aperture (NA) is defined as $NA = n \sin \theta$, where θ is the collection half-angle of the lens, λ is the (collected) wavelength of light.

The diffraction-limited resolution, which for an NA of 1 with green light (500 nm) is 250 nm, is sufficient for resolving compartments within individual mammalian cells which are of the order of a few microns in size; however, the diffraction limit remains an obstacle in studying finer sub-cellular structure or organelles in much detail.

Super-resolution microscopy techniques that can surpass the diffraction limit, however, were developed, and the 2014 Nobel Prize in Chemistry was awarded to Eric Betzig, Stefan Hell and William E. Moerner for their contributions to the first concepts and techniques. These super-resolution microscopy techniques included photoactivated localization microscopy (PALM) [9], [10], stochastic optical reconstruction microscopy (STORM) [11] and stimulated emission depletion microscopy (STED) [12], [13].

PALM and STORM are both single-molecule localisation microscopy (SMLM) techniques, in which many individual image frames are acquired with only a small subset of fluorophores emitting in each frame, allowing individual molecules to be detected and localised with a precision beyond the diffraction limit. Images are then computationally reconstructed with these localised points. PALM and STORM differ by the mechanism with which they activate the fluorescence subset of fluorophores: in the first implementation of STORM, activator and reporter dyes are used to induce stochastic blinking, while direct STORM (dSTORM) uses buffer solutions to induce stochastic photoswitching [14]. PALM, on the other hand, may use photoactivatable fluorescent proteins which are actively switched on and off by a specific activation wavelength.

STED is a modification to a confocal microscope (described in Section 1.2.1) which, along with the normal excitation beam, incorporates a donut-shaped depletion beam to quench fluorescence spatially around the excitation spot, resulting in an effective excitation spot which is smaller than would be possible with diffraction-limited optics.

The field of super-resolution microscopy is also usually taken to include super-resolution structured illumination microscopy (SR-SIM) [15], in which images of the sample are acquired under structured illumination (with multiple pattern phases or orientations) and computational processing is used to extract higher spatial frequency information. A related method is that of optically sectioned imaging by structured illumination, which was first implemented by Mark Neil et al. [16] to achieve optical sectioning and is introduced in the following section.

Another limitation of the widefield setup shown in Figure 1.3 is that the entire sample, including out-of-focus planes, is illuminated, and there is no scheme to reject light originating from planes that are out of focus. These out-of-focus planes result in contributions to the background of the image, reducing the signal-to-background ratio (SBR), i.e. the contrast of the image. Although to first order this background is constant, there is an associated photon shot noise that can overwhelm the signal from the in-focus plane, reducing the signal-to-noise ratio (SNR).

For these reasons, quasi-2D samples are often used in widefield microscopy and this may include cells cultured on flat substrates in a monolayer or thinly sectioned tissue, e.g. with a microtome, the latter being a destructive process. Methods which preserve some of the 3D structure of tissue or enable the imaging of 3D cell cultures are desirable since they are increasingly used in assays due to their better modelling of *in vivo* conditions. Optical sectioning provides a non-destructive way to image these 3D structures, and such methods are described in the following sections.

1.2 Optical sectioning

The term optical sectioning originates from the process of physically sectioning tissue for microscopy and describes any methods which can acquire clear images of separate planes of a thick sample through optical techniques alone. When using a high-NA lens in widefield microscopy, the depth of field (DOF) is reduced such that $\text{DOF} \propto 1/\text{NA}^2$. However, this is not the same as optical sectioning; whilst only a thin plane of interest may be in focus, out-of-focus haze still reduces the SBR (and SNR), and this is particularly an issue in samples much thicker than the DOF.

A common method of explicitly rejecting out-of-focus light in imaging is by using a confocal detection scheme which was first proposed by Marvin Minsky and patented in 1957 [17].

This was before the invention of the laser, making the technique difficult to realise with sufficient signal, and the first confocal laser scanning microscope (CLSM) was constructed by M. D. Egger and P. Davidovits at Yale University [18].

1.2.1 Confocal laser scanning microscopy

CLSM is a point-scanning technique as opposed to widefield microscopy, meaning that images of the sample are acquired point-by-point through scanning. In CLSM, illustrated in Figure 1.4, excitation light is focused onto a point on the sample, using a laser as the source of excitation, and the point of fluorescence is focussed back to the pinhole. Out-of-focus fluorescence which originates away from the sample plane is blocked at the pinhole which is in a conjugate plane, or confocal, to the sample plane; this greatly improves the axial sectioning ability which defines the axial resolution. Depending on its size, the pinhole also slightly enhances the lateral resolution [19]. Both the lateral resolution and the optical sectioning ability depend on the pinhole size. The smaller the pinhole size, the closer (better) the lateral and axial resolution come to the maximum (best) resolution achievable with a confocal, but the detected signal decreases and there is a trade-off between resolution and

signal. Photomultiplier tubes (PMT) or avalanche photodiodes (APDs) are often used for detection in confocal microscopy due to their fast response time and low noise.

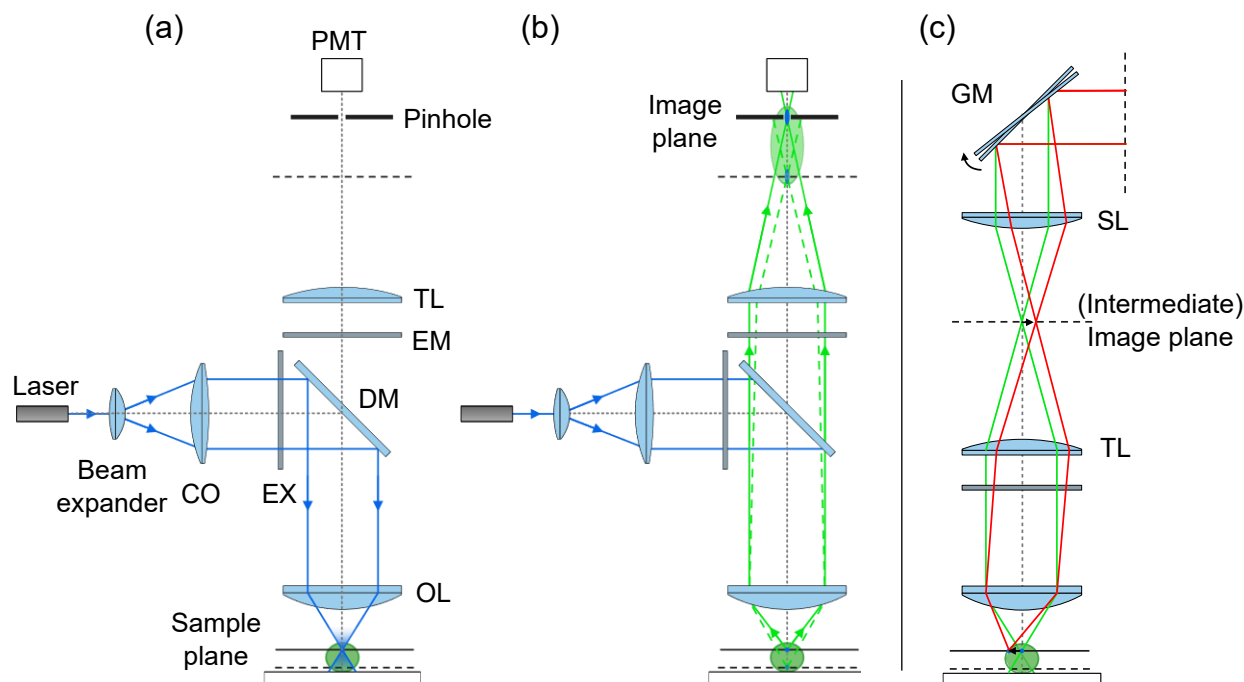


Figure 1.4 – (a,b) Confocal detection scheme used in confocal laser scanning microscopy (CLSM). DM – dichroic mirror, OL – objective lens, TL – tube lens, PMT – photomultiplier tube. (a) Illustrates the point illumination and (b) illustrates the confocal detection; blue lines indicate excitation and green detection. (c) One-axis galvo scanning in CLSM. Rotation of the galvo mirror (GM) which is in the back focal plane of the scan lens (SL) results in a lateral shift in the intermediate image plane which is conjugate to the sample plane.

In order to scan the excitation point over the sample, the sample itself can be translated laterally with a motorised stage and the objective can be translated in the axial direction, but this can be prohibitively slow. A faster scanning solution uses galvanometer (galvo) mirror scanning systems, which work by placing a galvo in the back focal plane (BFP) of a scan lens, which allows rotation of the galvo to correspond to the translation of the illumination spot in the front focal plane (FFP) of the scan lens. In CSLM, this scanning system would be positioned such that the FFP of the scan lens is at the (intermediate) image plane, see Figure 1.4I, and the dichroic and pinhole detection is upstream from the scanning system which results in simultaneously scanning of the excitation spot and de-scanning of the fluorescence spot. Galvo scanning is often used to perform lateral x and y scanning, and after a complete lateral scan, the objective is translated to acquire a different z plane of the sample to allow 3D imaging.

Confocal microscopy, however, can reach high peak excitation intensities as the scanning speed is increased—this is because with lower pixel exposure times (pixel dwell times), to acquire the same signal, the laser power must be increased. This has been shown to be associated with higher photodamage (see Section 1.1.1), and there is therefore a motivation to increase the acquisition rate of confocal techniques so that lower peak intensities can be used for the same acquisition time. This is achieved with Nipkow spinning disk microscopy,

which is described in the following section. An alternative method of increasing the scanning speed of CLSM is by using line-illumination and slit detection [20], [21] rather than point illumination and pinhole detection. In this case, the rejection of out-of-focus light is performed only along one axis which results in a trade-off between sectioning ability and scanning speed.

Another consequence of confocal microscopy that is inherent to its illumination geometry is the exposure of the full axial extent of the sample for a given point of illumination to the excitation laser. Although the illumination is focussed onto a single point, there is non-zero intensity out-of-plane and the resulting fluorescence is thrown away when it is blocked by the pinhole. This results in appreciable out-of-plane excitation and (considering the linear regime of photodamage), the entire sample is effectively exposed to the same dose of excitation light per XY scan of the sample.

1.2.2 Nipkow spinning disk microscopy

As mentioned in the previous section, confocal microscopy in the form of CLSM requires higher peak excitation intensities to achieve reasonable imaging rates. In spinning disk confocal microscopy (SDCM), a Nipkow disk containing an array of pinholes is used to parallelise the process of CLSM by scanning multiple beams across the sample in parallel, and this reduces imaging time compared to CLSM with the same excitation power. The concept of using a Nipkow disk for confocal microscopy was patented in 1966 by Mojmir Petran and Milan Hadravsky [22], before even the first realisation of laser scanning confocal microscopy.

Today, SDCM is often implemented together with another matched disk containing microlenses to focus incident planar excitation light into each of the pinholes on the other disk, which improves the transmittance of the excitation light by an order of magnitude [23]. In the same way as in CLSM, the choice of pinhole diameter is a trade-off between signal and sectioning (and resolution), but the pinhole separation must also be considered. For a given point, it is possible for out-of-focus light to enter another pinhole, and this is known as crosstalk and increases as the pinhole separation decreases. However, as pinhole separation increases, the disk rotation speed must be increased to maintain the scan time and the peak intensity at the sample increases.

1.2.3 Multiphoton microscopy

Multiphoton microscopy approaches the issue of optical sectioning from the excitation side rather than the detection side. In confocal microscopy, although illumination is focused to a point, fluorescence is still generated out-of-plane; multiphoton microscopy relies on using excitation wavelengths that require multiple photons must simultaneously be absorbed, i.e. the excitation photon energies sum to the fluorophore excitation energy. The simplest case is having excitation wavelengths that are multiple times that with single photon excitation, and this excitation scheme is illustrated in Figure 1.5.

Two-photon microscopy was first published by Winfried Denk in 1990 [24], and it showed that photobleaching was confined to the focal plane of the illumination (unlike in confocal) due to the non-linear relationship of absorption to illumination intensity and the fact that intensity is increased at the focus. This can be explained by the probability of absorption of two simultaneously incident photons being proportional to the square of the probability of one if the two photons are independent. For n -photon excitation, the emission intensity is proportional to the n^{th} power of the excitation intensity, allowing very localized regions of excitation with higher-order absorption processes, i.e., optical sectioning by excitation. It is worth noting however that, even though overall photodamage is reduced by not exciting out-of-plane, photodamage in the focal plane may be increased [25].

An additional advantage of multiphoton microscopy is that the longer wavelengths of excitation light are scattered less within the sample than with single-photon excitation, allowing higher penetration depth [26]; scattering on the detection side, however, is unchanged, but as the excitation light can be assumed to have originated from the excitation focus, a detection pinhole is not required for multiphoton excitation.

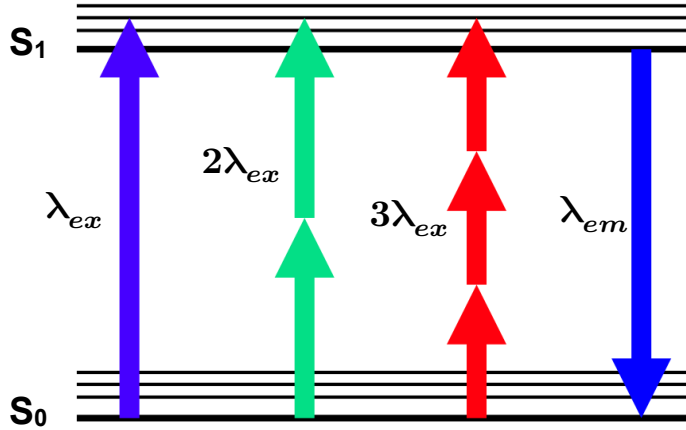


Figure 1.5 – Jablonski diagram for single-photon, two-photon and three-photon excitation, each resulting in fluorescence with the same wavelength but with excitation wavelengths of λ_{ex} , $2\lambda_{ex}$ and $3\lambda_{ex}$ respectively, with the energy of these photons given by $E_n = h/nc\lambda_{ex}$ where n is the order of the excitation scheme.

1.2.4 Structured illumination for optical sectioning

Mark Neil et al. [16] demonstrated optical sectioning with a widefield imaging geometry by using spatially varying illumination in a single direction and acquiring images for three different phases of the illumination pattern in a technique known as optically sectioning structured illumination microscopy (OS-SIM). The concept behind this is that the structured component of the patterned illumination is attenuated in the image for out-of-focus planes, and therefore, by only retaining the modulated term of the imaging the in-focus plane can be recovered to provide optical sectioning similar to that with confocal.

After acquiring three different images for each illumination pattern with different spatial phases, the non-modulated optically sectioned image is recovered computationally for each pixel individually by

$$I_{section} = \frac{\sqrt{2}}{3} \sqrt{(I_1 - I_2)^2 + (I_2 - I_3)^2 + (I_3 - I_1)^2} \quad 1.2$$

where I_1 , I_2 and I_3 are the three different acquired images.

Although the original paper is implemented for reflected-light imaging, OS-SIM was also demonstrated with fluorescence using a conventional microscope (brightfield, fluorescence), and in this case, the illumination pattern was produced via interference [27].

As previously mentioned in Section 1.1.2, structured illumination also provides a method of super-resolution imaging, known as SR-SIM which is distinct from OS-SIM but also uses patterned illumination [15]. In this case, the patterned illumination modulates the spatial frequencies present in the object such that the passband in Fourier space is shifted laterally, allowing higher frequencies to be captured or sampled. Again, multiple images are taken with different (periodic) illumination patterns such as rotations or phase shifts, and this is done to sample the full 2D Fourier space symmetrically.

1.2.5 Total internal reflection fluorescence microscopy

In total internal reflection, although there is no transmitted energy at the interface between two media, an evanescent field is produced which decays exponentially in the direction of the interface normal, and the field becomes negligible at depths larger than ~ 100 nm. Total internal reflection fluorescence microscopy (TIRFM) exploits the evanescent field formed by inclined laser illumination at the coverslip-specimen interface to localise the illumination within a ~ 100 nm thick region [28]. This provides an optical sectioning capability many times better than with confocal microscopy, which provides an axial resolution of 500 nm in optimal conditions [29], but only at the coverslip interface. TIRFM is therefore a highly specific tool for imaging single thin planes of a specimen with significantly improved SNR provided the sample is close enough to the coverslip.

TIRFM can be implemented with a standard widefield microscope by introducing the laser off-axis in the back focal plane of an objective with a sufficiently high NA, resulting in a highly inclined laser beam that is totally internally reflected at the coverslip-medium interface, with the requirement that the refractive index of the medium n_s is lower than that of the glass n_g . The fluorescence resulting from excitation by the evanescent field is then collected as it would be in standard widefield microscopy with epifluorescence excitation.

1.3 Light sheet fluorescence microscopy

The concept of illuminating samples with a sheet of light was first introduced by Zsigmondy in 1902 [30], for which the Nobel Prize was awarded in 1926. The system was referred to as an ultramicroscope setup which focused light from a slit to form a light sheet in a sample of colloids and observed the scattered light perpendicular to this light sheet.

This illumination scheme was later adapted to light sheet fluorescence microscopy (LSFM) by Voie et al. with orthogonal-plane fluorescence optical sectioning (OPFOS) [31], which uses a cylindrical lens to illuminate a fluorescent sample with a light sheet and an objective lens perpendicular to the light sheet propagation direction to collect the fluorescence. This then led to selective plane illumination microscopy (SPIM) [32], illustrated in Figure 1.6 below, which increases the numerical aperture of the illumination by using an additional objective after the cylindrical lens, yielding sub-cellular resolution seen in modern LSFM.

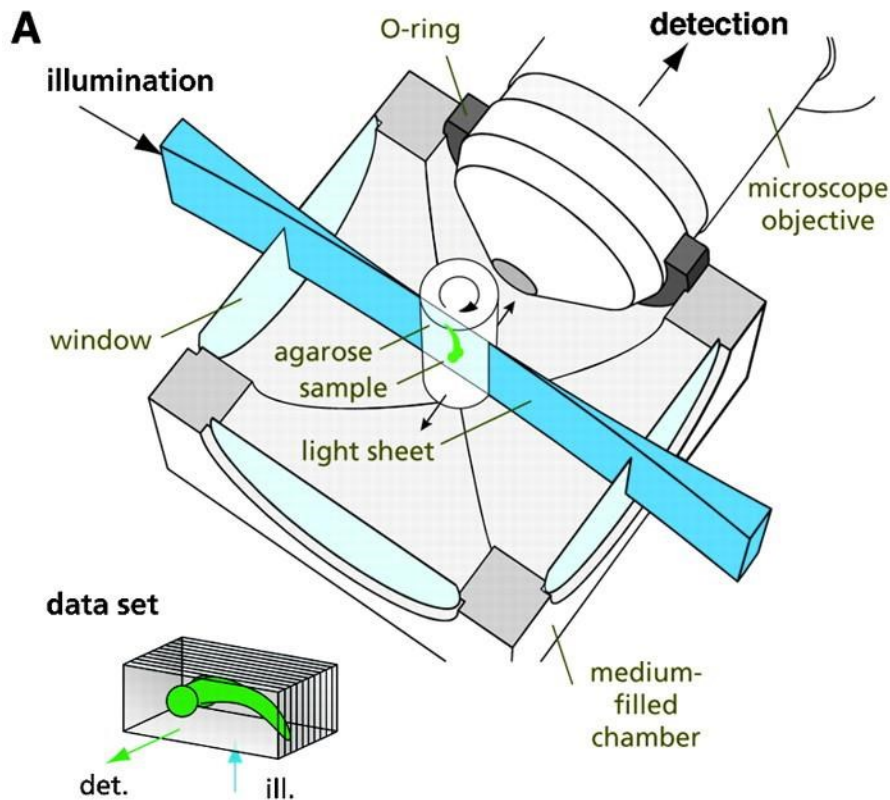


Figure 1.6 - SPIM configuration for LSFM, which mounts the detection objective onto a medium-filled chamber, within which a sample embedded in agarose is held. The agarose-embedded sample is extruded from a syringe which is held on a mechanical translation and rotation stage. Figure reproduced with permission from [32] (<https://www.science.org/content/page/reprints-and-permissions>).

The light-sheet directly optically sections the sample on the illumination side, since only a single plane is illuminated by the light sheet. In the standard configuration, such as in SPIM, images are acquired as they are with widefield fluorescence microscopy using the detection objective lens placed perpendicular to the illumination light-sheet. Being a camera-based technique, LSFM therefore has the benefit of illuminating and imaging an entire plane at once, making it possible to acquire at far higher rates than with standard confocal. In addition, the selective illumination only illuminates one plane at a time instead of exposing the entire axial extent of the sample per scan.

It is important to state that the overall detection PSF and therefore axial resolution of LSFM depends on the light-sheet thickness (sectioning ability) and the detection PSF

(determined by NA)—the overall PSF is given by the product of the excitation distribution and the detection PSF. If the light-sheet is thinner than the axial extent of the detection PSF, then the light-sheet thickness dominates the axial resolution. If the light-sheet is thicker than the axial extent of the detection PSF, the NA becomes important; however, the effect of reduced out-of-plane fluorescence background on image contrast is still significant and one of the main benefits of LSFM [33]. The light-sheet thickness is determined by excitation objective NA and can be freely chosen but the width profile of higher NA (thinner) light-sheets rapidly increases away from the waist, reducing usable FOV; this is also discussed in Section 1.3.2.

LSFM is therefore often referred to as a rapid and ‘gentle’ microscopy technique with relatively low photodamage compared to some other optical sectioning techniques, allowing it to be used in prolonged or high-time-resolution timelapse studies with samples that are sensitive to phototoxicity such as mouse embryos [33]. The relative speed combined with the gentle nature of LSFM also enables live volumetric imaging, for example, functional imaging of the whole zebrafish brain at 0.8 volumes per second has been demonstrated [34]. Additionally, time-lapse LSFM imaging has been documented to result in lower phototoxicity compared to time-lapse (spinning disk) confocal imaging in the case of the development of the zebrafish craniofacial bone [35]. Furthermore, LSFM does not suffer as significant scattering with imaging depth as in confocal, since the pinhole detection in confocal microscopy renders the point illumination and detection sensitive to small perturbations in the light path [36].

There are, however, limitations to SPIM. One is the restrictions on the sample mounting method imposed by the orthogonal illumination and detection objectives; conventional SPIM often require the detection (or both) objectives to be fixed to sample chamber, and the samples to be mounted in a liquid-filled cuvette or within solid agarose. The sample mount (e.g. syringe, cuvette) is mounted on a translation-rotation mount and translated to achieve 3D imaging and rotated about its vertical axis for multi-view reconstruction (to improve penetration depth), but high-throughput imaging of multiple samples is not straightforward in a standard SPIM setup. A common approach is to array multiple specimens in a line, and this approach was demonstrated with 20 mouse embryos [37]. There are many other approaches to improve the performance, throughput and sample mounting flexibility in LSFM, and these are presented separately in the following sections.

1.3.1 Digital scanning of light sheet

Instead of forming light sheets directly, a circular beam can be scanned laterally to produce effective light sheet illumination of the sample, which is referred to as digitally scanned light sheet microscopy (DSLM) [38], see Figure 1.7. This approach has the advantage of having more uniform illumination intensity across this digitally scanned region and higher illumination efficiency compared to a standard light sheet formed by a cylindrical lens. This is because when using a Gaussian laser beam and a cylindrical lens, the extent of the light sheet is often chosen to be much greater than the size of the field of view (FOV) to reduce

the variation of illumination intensity across the field of view, and this wastes light in the process.

When using a sCMOS camera, pixels can be read out independently from each other and it is possible to acquire with a rolling shutter mode so that a few adjacent lines of pixels can be read out at a time. The lines of pixels can be read out in such a way that it is synchronized with the DSLM beam scanning, resulting effectively in confocal slit detection [39]. This rejects out-of-focus, scattered light to improve optical sectioning and signal-to-noise ratio (SNR). An additional advantage of DSLM is that the signal from each illumination beam scan position is integrated incoherently on the camera, which reduces the impact of shadows in the illumination beam due to scatterers and absorbers compared to fully coherent light sheet illumination.

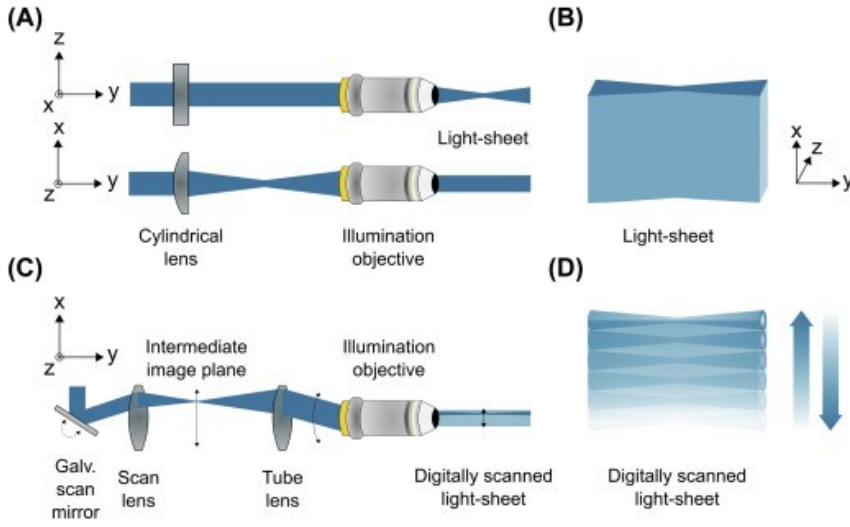


Figure 1.7 – (A) Light sheet illumination and (C) digitally scanned light sheet illumination setups. Light sheet illumination is conventionally generated by a cylindrical lens (and objective), while (D) digitally scanned light sheets involve scanning an illumination beam laterally – achieved in (C) using a galvo in a plane conjugate to the back focal plane of the illumination objective – to provide effective sheet illumination. Reproduced with permission from [40].

1.3.2 Non-Gaussian light sheet illumination

Both conventional light sheets formed using a cylindrical lens and digitally scanned light sheets are produced using a Gaussian beam from a laser. When Gaussian beams or light sheets are focussed onto the sample, the higher the NA of the focussing lens the smaller the spot size but the smaller the depth of field (DOF)—this limits the ability to generate light sheets that are thin over a large FOV. The Gaussian beam (or sheet) waist is focussed onto the sample, and the confocal parameter, equal to twice the Rayleigh length, defines the approximate range over which the width remains close to the width at the waist.

Bessel beams, which are non-diffracting beams with a Bessel intensity profile, can maintain a small beam profile width as the beam propagates, effectively having a significantly larger DOF in comparison to Gaussian beams—see Figure 1.8(b). Approximate Bessel beams can be generated by illuminating the back focal plane of an objective with a thin annulus pattern

or by using an axicon, and the use of Bessel beams has been demonstrated in generating digitally scanned light sheets with better optical sectioning over a wide FOV by Fahrbach et al. [41]. Bessel beams are also ‘self-healing’ and obstructions to the beam propagation do not significantly affect their profile, making them ideal for scattering samples and they help solve the problem of shadowing.

Bessel beams, however, have significant energy in their sidelobes which causes significant excitation out-of-plane. A number of approaches exist to address this such as confocal slit detection from the rolling shutter method employed in DSLM or by using multiphoton excitation [42] [43]. As is the case with any form of multiphoton microscopy, however, the fluorophore choice is limited in this illumination scheme by the availability of lasers with the appropriate wavelengths.

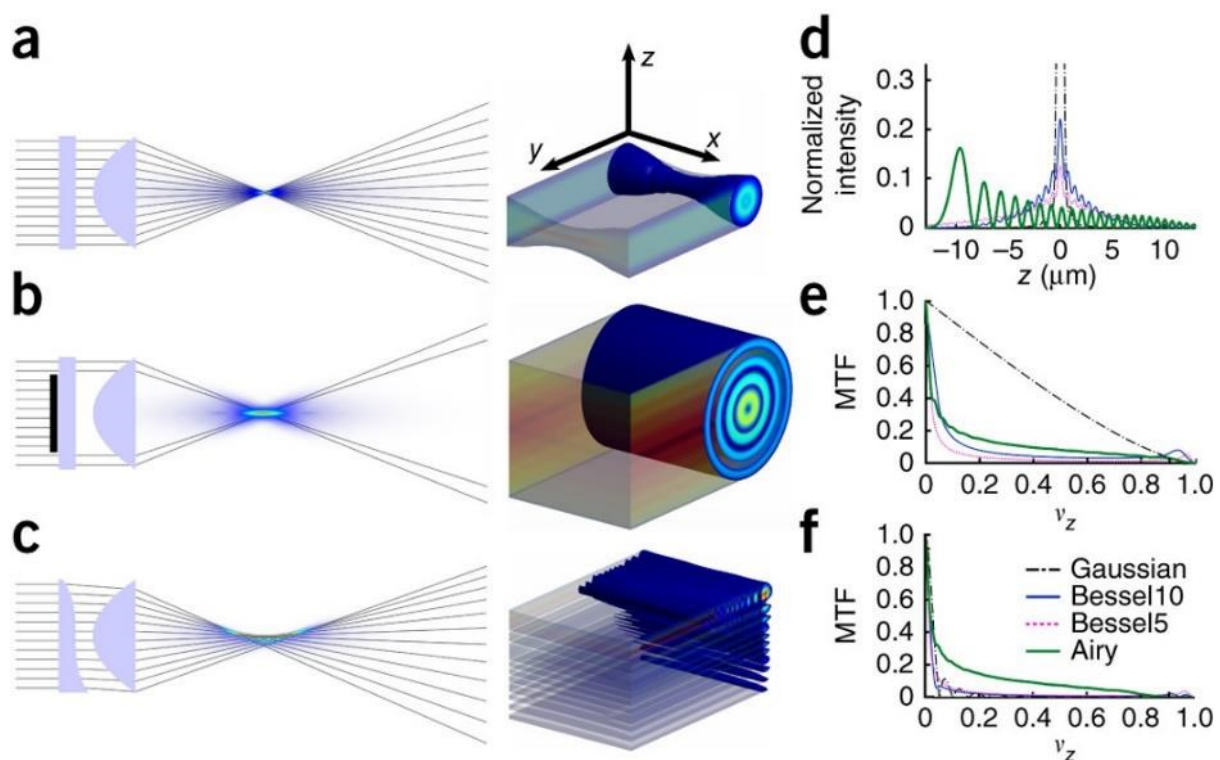


Figure 1.8 – Comparison of illumination beam profiles used in LSFM. (a) Gaussian beam, (b) Bessel beam, (c) Airy beam. (d) normalised intensity of the beams at the waist against I(e) z-axis modulus transfer functions (MTF) at the waist and (f) 25 μm from the waist. The beams Bessel5 and Bessel10 are formed with a ratio of annulus width to objective back aperture width of 0.05 and 0.10 respectively. Obtained with permission from [44].

Airy beams, see Figure 1.8(c), have been proposed as an alternative to the propagation-invariant Bessel beams for digitally scanned light sheets and have been demonstrated experimentally to have significantly lower beam waist, but most importantly the profile remains thin further from the waist more so than with both Gaussian and Bessel beams [44]. The Airy beams were generated by modulating the phase of a Gaussian profile in the back aperture by a cubic polynomial function of the Cartesian pupil coordinates ($x^3 + y^3$). Deconvolution of the final volume can correct for the self-bending properties of the Airy

beam in addition to the significant side lobes in the Airy beam; since the sidelobes are asymmetric about the main lobe, this contributes to the information and thus SNR of the image (after deconvolution). Along with the profiles of Gaussian, Bessel and Airy beam, the beam intensity at the waist against axial position (z) and axial modulus transfer functions for the different beams are compared—this characterises the axial resolution imaging under these different illumination schemes.

1.3.3 Axially swept light sheet

As seen in the previous section, the thickness of standard, diffracting light sheets (e.g. Gaussian) increases significantly away from the beam waist. This means that the optical sectioning strength of LSFM is lower further from the waist, and this is especially true for tightly focussed, thin light sheets. Axially swept light sheets aim to utilise the strong sectioning at the beam waist by scanning a light axially at the sample and synchronising acquisition within a window that corresponds to the beam waist region using a rolling shutter acquisition on the camera [45], see Figure 1.9. This allows high NA light sheets with thin waists to be used without the optical sectioning strength degrading along the propagation direction.

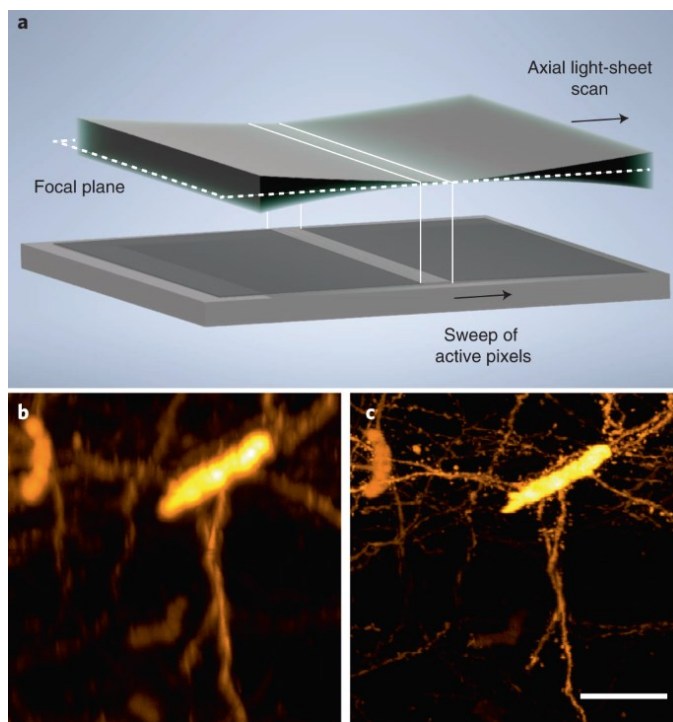


Figure 1.9 - Axially swept light sheet (ASLM). (a) Light sheet is scanned in the axial direction synchronously with the rolling shutter of a sCMOS camera. (b) LSFM and (c) ASLM cross-sectional XZ projections of cleared cortical Thy1-eYFP neurons. Scale bar: 50 μm . Reproduced with permission from [46].

The setup uses the standard orthogonal excitation and detection objective geometry, but the excitation objective forms part of a refocusing system, with a mirror translated by a piezo in the remote space. The linearly polarised light sheet itself is introduced into the remote space and coupled out using a system of a quarter waveplate (double pass) and polarising beamsplitter.

1.3.4 Lattice light sheet

An alternative approach to Bessel beam-generated light sheets is the lattice light sheet, which was pioneered by Eric Betzig’s lab. Lattice light sheet aims to suppress the sidelobes seen in single Bessel beams through destructive interference by generating a lattice of Bessel beams with a spatial light modulator (SLM) [47].

Bessel beams can be generated with thin annular illumination in the back focal plane of an objective and in the same way and more generally, optical lattices can be generated which are propagation invariant by confining a general illumination to this annular mask. Two such examples are the square lattice and hexagonal lattice—see Figure 1.10, which shows the resulting intensity distribution in sample space. These optical lattices are scanned in the same way as beams in standard digitally scanned light sheets, producing scanned light sheets that are thin over a longer range in the propagation direction than those produced with Gaussian beams, and reduced energy in the side lobes compared to Bessel beams, all without the need to deconvolve. The structure of the lattice illumination has also been exploited to achieve SIM by acquiring a set of images where the illumination pattern is stepped by a fraction of the lattice period [47].

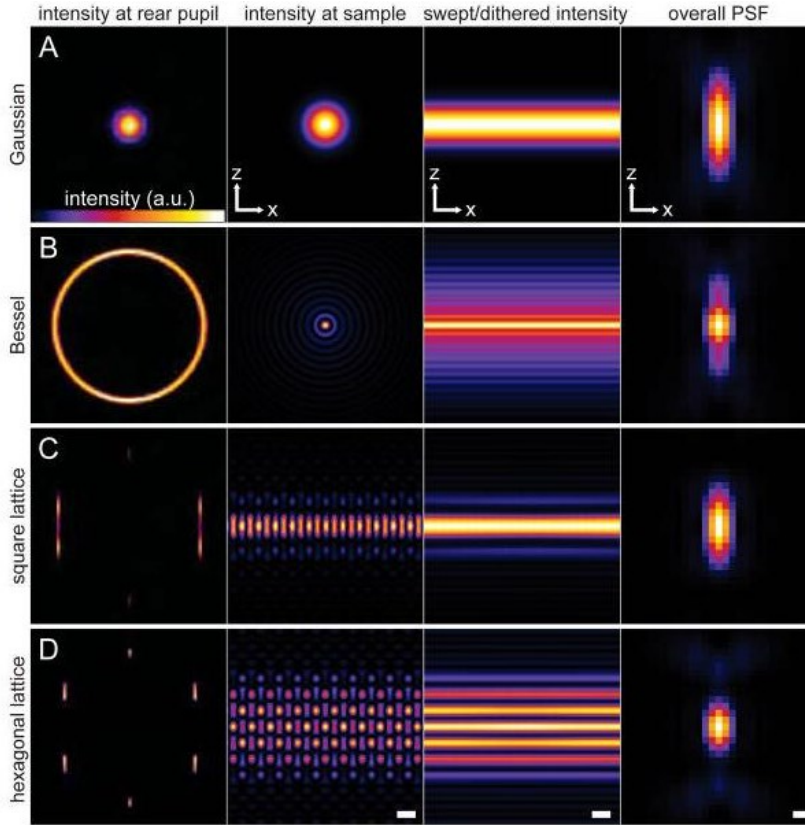


Figure 1.10 – Gaussian and optical lattice patterns used in lattice light sheet microscopy, from left to right: intensity in Fourier space, intensity in direct space, intensity when dithering, overall/detection PSF (theoretically: imaging PSF $\otimes$ illumination PSF). (A) Gaussian beam, which is the standard profile used in light sheet (B) Bessel beam, which is associated with significant energy in the side lobes about the central narrow core, (C and D) bound optical lattices which reduce these sidebands by destructive interference. The square lattice (C) optimises the reduction in sidebands, while the hexagonal lattice (D) optimises the axial thinness. Reproduced with permission (<https://www.science.org/content/page/reprints-and-permissions>), from [47].

However, the benefits of lattice illumination have been questioned, with B. Chang et al. suggesting that there is no discernible difference in the light sheet performance in terms of thickness and confocal parameter between Gaussian and lattice light sheets [48]. On the other hand, other publications suggest that the effective resolution should be considered in frequency space, since in direct space, low-frequency components dominate calculations [49].

1.4 High-throughput light sheet fluorescence microscopy

The term high-content screening (HCS) refers to methods used to extract multiple readouts from a biological assay simultaneously and this includes, and usually refers to, image-based assays. HCS often incorporates automation and tools to increase throughput so that data from multiple parallel samples can be read out in a single acquisition cycle.

In standard implementations of LSFM, such as seen with SPIM, there are limitations in the ability to perform high-throughput imaging due to sample mounting constraints. In this section, various approaches to LSFM which enable high-throughput imaging are presented, with many of these methods allowing the use of conventional HCS tools such as multi-well plates, while others use microfluidics.

1.4.1 Highly inclined and laminated optical sheet

Although not explicitly a light sheet technique, in highly inclined and laminated optical sheet (HILO) [50] microscopy, a laser beam is introduced near the edge of the pupil of the objective which generates an angled laser beam that is refracted at the coverslip-specimen interface to form thin and highly inclined planar illumination of the sample in a setup similar to TIRFM, see Figure 1.11. Unlike in TIRFM, the illumination laser is not totally internally reflected but is transmitted and refracted by the coverslip, and this refracted, flattened beam therefore illuminates within the sample at a high angle rather than just the surface like in TIRFM. Since it is a single-objective technique, conventional microscope frames can be used and three-dimensional images of the sample are collected through a z-scan of the objective or sample holder, reconstructing the volume from the z-planes of the sample. Since the angle of the illumination is non-zero, however, much of the illuminated plane is defocused, restricting the effective FOV to a small central region of 15-40 μm , with an illumination thickness of 5-10 μm .

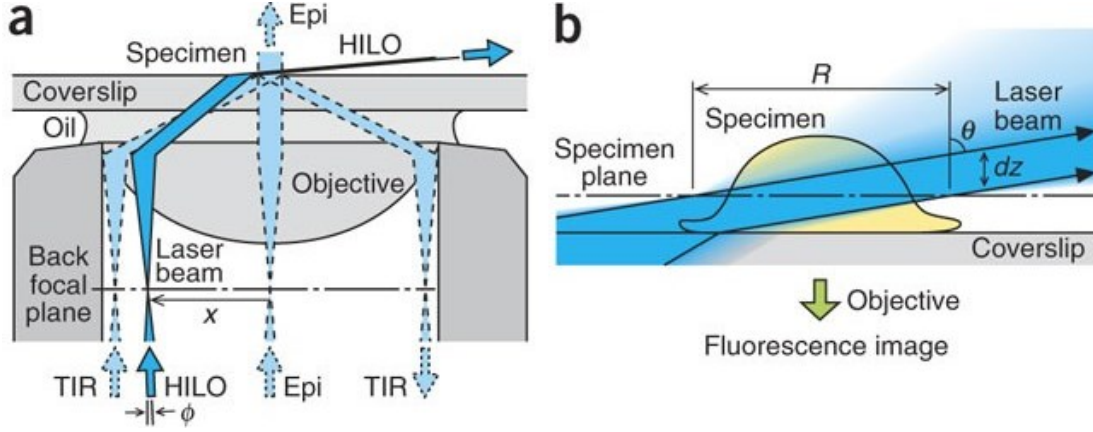


Figure 1.11 – (a) HILO illumination scheme compared to standard epi-illumination and TIR illumination. (b) Highly inclined planar illumination at the sample, inclined at an angle described by θ , which is the angle between the plane and the optical axis. The plane has an illumination axial thickness dz which is determined by the width of the illumination R and the angle of the plane. Reproduced with permission from [50].

1.4.2 Inverted SPIM

A light sheet illumination module that can be affixed to a standard microscope frame with a custom pillar is demonstrated by inverted SPIM (iSPIM) [51], and this uses a configuration which illuminates and detects at 45° to the sample plane. The light sheet-generating objective and detection objective are mounted onto the custom microscope pillar at 90° to one another, and this mounting assembly is motorised along with galvo scanning of the light sheet accompanied with axial piezo scanning objective in sync with the galvo for sample scanning. The iSPIM system was then expanded to allow either objective (both $40\times$ 0.8 NA water) to be used for illumination in dual-illumination SPIM (diSPIM) to sequentially image two orthogonal views for spatially isotropic resolution after deconvolution, as opposed to standard light sheet which is usually limited by the light sheet axial thickness [52]. This setup demonstrated imaging of 200 planes per second with dimensions 480×432 px², corresponding to $480 \times 432 \times 100$ (width $\times$ length $\times$ depth) px³ volumes in 1 s with a FOV of $78 \times 70 \times 50$ μm^3 , with a resolution of ~ 330 nm both laterally and axially after deconvolution.

Due to geometrical constraints when using the two objectives close to the sample, however, this either requires long working distance objectives or immersing the objectives in a large reservoir of immersion media. The former restricts the NA of the objectives since the NA reduces when the working distance increases, while the latter can cause inter-sample contamination and drift, as well as limiting the compounds used in tissue clearing so as to not damage the objectives [53].

1.4.3 Open-top light sheet microscopy

A similar approach to diSPIM is open top light sheet (OTLS), which rather than introducing the illumination and detection objectives from above the sample, positions them below and images through coverslip/coverglass of the sample holder. This implementation of LSFM also uses long work distance objectives, and in initial implementations by McGorty et al. [54],

required a ‘water prism’ to couple the light to and from the air objectives to the sample media without incurring significant aberrations from refractions at the air-coverslip interface. Astigmatism induced by the water-coverglass interface because of the 45° angle between the coverglass of the sample holder and the optical axis of the objectives (because of the non-equal refractive indices of water and glass) is partially corrected with a weak cylindrical lens in the detection path. Although this system is limited to imaging aqueous solutions, the open-top geometry enables the use of multi-well plates or other methods used with conventional commercial inverted microscopes to enable high-throughput and high-content imaging.

Another OTLS system, illustrated in Figure 1.12, was then developed by Glaser et al. [53] which included a multi-immersion detection objective and was capable of imaging with various sample media to facilitate imaging with different clearing methods. This is accomplished by sealing the detection objective within a reservoir containing changeable immersion fluid matched to the refractive index of the sample media, and in turn, matching the sample holder material refractive index; however, this does add complexity requiring custom or a limited range of specific sample holders to minimise aberrations. The illumination is coupled to the reservoir by an air objective and a plano-convex lens, or solid immersion lens (which is referred to as a SIMlens), which matches the lens and wavefront curvatures to reduce off-axis and spherical aberrations at the air-reservoir interface [55].

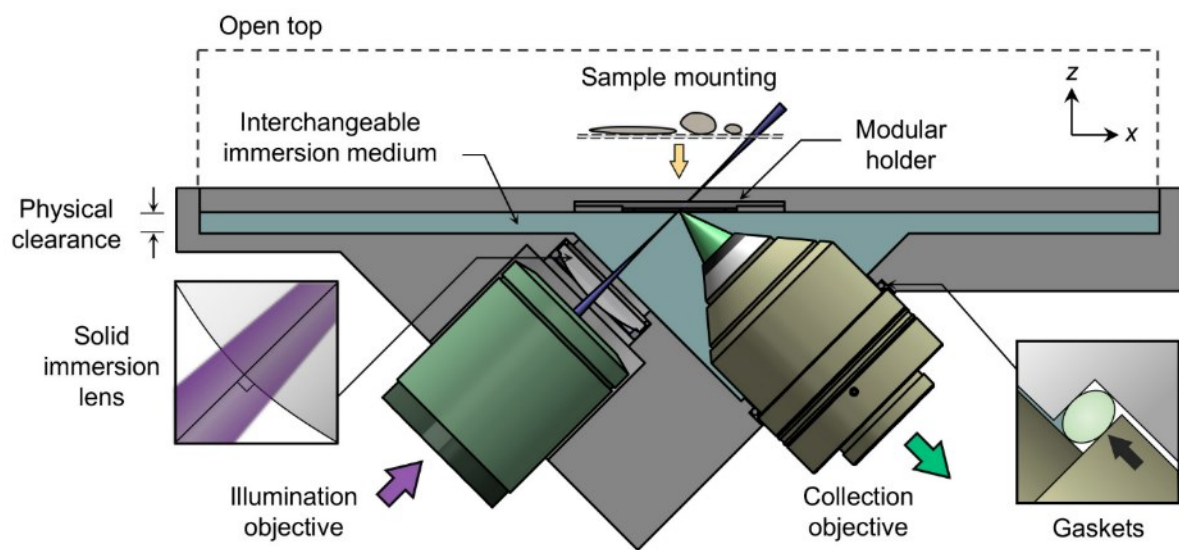


Figure 1.12 – Multi-immersion open-top light sheet (OTLS) microscope. Illumination and collection objectives are located beneath the sample holder and are optically coupled to a liquid reservoir filled with an interchangeable immersion medium. This medium and the sample holder material are chosen to be index-matched with the imaging medium (e.g. clearing solution) to minimise aberrations due to refractions at each interface. Adapted from Glaser et al. [53] ([CC-BY 4.0](#) licence).

In each case here, long working distance objectives are used and the angle of the objectives relative to the coverglass reduces the effective working distance further, limiting the NA and imaging resolution. The limited NA makes this system more suited for larger FOV imaging rather than sub-micron imaging.

A more recent system, however, implements a third objective with conventional orientation to the sample plane for high-NA detection which is described as a non-orthogonal dual-objective (NODO) configuration [56], see Figure 1.13. In this hybrid setup, the NODO configuration is combined with the original OTLS detection scheme—referred to as orthogonal dual-objective (ODO)—to achieve multi-scale imaging. The orthogonal objectives for ODO are both low NA ($NA < 0.4$) air objectives and are each paired with the SIMlenses, and the high-NA objective for NODO provides a NA of 0.483 in air which scales up to 0.75 with $n = 1.56$ immersion media. The ODO modality can image 12×7.5 cm planes at ~ 5 μm resolution, and the high-NA at approximately 0.5 μm lateral and 3 μm axial resolution with ~ 0.4 mm planes.

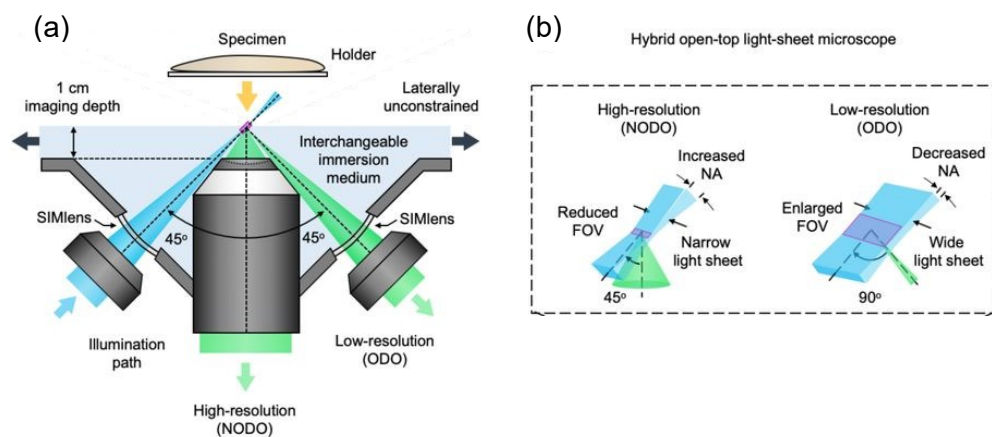


Figure 1.13 – (a) Open-top light sheet illumination and collection optics at the sample which comprise the lower NA orthogonal illumination and detection (ODO) imaging and higher NA non-orthogonal (NODO) modalities. The custom SIMlens couples the illumination and detection in the ODO configuration and minimises refractive aberrations by matching the curvature of the wavefront. (b) comparison of light sheet and collection NAs for ODO and NODO modalities. Reproduced from A. Glaser et al. [57] ([CC-BY 4.0](#) licence).

This NODO detection however requires a relay system consisting of a pair of microscopes to create a remote image of the plane, mitigating issues with anisotropic magnification of the imaged plane and severe aberrations from imaging out of plane. This method of non-orthogonal or oblique detection was first devised by Chris Dunsby with oblique plane microscopy (OPM) [58], which will be described in more detail below, and this relay system—originally intended for remote refocusing in high NA systems—was demonstrated by Tony Wilson’s group in Botcherby et al. [59], [60].

1.4.4 Oblique plane microscopy

Oblique plane microscopy (OPM) [58] removes the constraint on sample mounting methods by employing the use of a single high NA objective at the sample to provide angled, planar illumination at the sample in a similar manner to HILO. OPM, however, does not rely on refraction of an illumination beam at the coverslip-sample interface to achieve a beam that is as highly inclined as possible, but rather generates an obliquely angled (30° - 45°) light sheet generated by a cylindrical lens that generates a short line of illumination at the edge of the objective pupil.

High NA (> 0.6) microscope objectives generally have short (few mm) focal lengths—and therefore provide high magnification—and are designed to minimise aberrations when imaging an object placed in a specific plane in front of the objective lens. They are therefore generally designed to satisfy the Sine condition [61]. Imaging away from the designed object plane will generally introduce severe spherical aberration, and the axial position of the image plane will move significantly since the axial magnification of a system scales as the square of the lateral magnification. This is a problem in OPM since the illuminated plane of the sample is angled with respect to and does not lie wholly within the object plane.

The remote refocusing system first demonstrated by Tony Wilson’s group [59] overcomes the problem of how to remotely refocus a microscope image by creating an intermediate image of the object where the magnification is isotropic, i.e. the lateral and axial magnifications are equal. They demonstrated that this approach can be considered to be aberration-free according to the Strehl condition over a $70\text{ }\mu\text{m}$ axial refocusing range for a $1.4\text{ NA } 60\times$ oil-immersion primary objective [60]. Their refocusing system consists of two back-to-back microscopes and includes the primary microscope that illuminates and collects fluorescence from the sample along with a demagnifying microscope to produce total magnification equal to the ratio of the immersion media refractive indices of the primary and secondary objectives, $O1$ and $O2$ respectively, see Figure 1.14 below. A tertiary detection microscope re-images this intermediate image onto a camera to provide the desired lateral magnification of the sample.

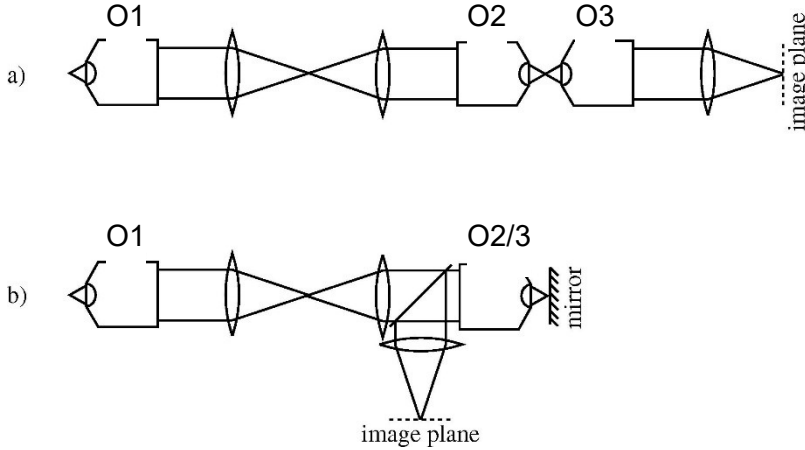


Figure 1.14 – Diagrams of Botcherby et al. systems used for ‘aberration-free’ remote refocusing. A volume region in object space (before $O1$) is stigmatically imaged in remote refocusing space between $O2$ and $O3$ and is re-imaged by $O3$ which may be translated to refocus on the remote space image. (a) Three objective setup and (b) folded setup which re-uses the second objective to function as both $O2$ and $O3$. Reproduced with permission from [60].

A remote refocusing system can also be used to solve the issue with the tilted OPM titled image plane having anisotropic resolution and strong aberrations. In OPM, a refocusing system forms a remote image with isotropic resolution and corrected aberrations, and a third microscope for detection is tilted relative to the optical axis of the refocusing system. The

tilt angle is set to be equal to the angle of the oblique illumination light sheet, defined as the OPM angle.

In the first iteration of OPM, the light sheet is introduced by a dichroic beamsplitter placed between objective O1 and the tube lens of O1 (TL1), but the second iteration of OPM [62] more conveniently introduces the light sheet in the remote space at a 90° angle to the detection microscope. This method of introducing the light sheet allows the illumination and detection ‘arms’ to be mounted on the same breadboard and rotated by the OPM angle and mostly be aligned in tandem; the fine alignment of the axial position of the lens that images the light sheet in the remote space and the detection objective, O3, are performed separately. In this iteration of OPM, volumetric imaging of a sample was demonstrated at 21 volumes per second (VPS) by axially scanning O2 with a piezo-electric objective actuator, which translates the intermediate image relative to the detection optics along the optical axis of O2. Volume acquisition via stage-scanning was introduced in OPM later by Vincent Maioli, as depicted in Figure 1.15, to allow the automated imaging of well plates populated with spheroids for high-content imaging, in addition to allowing volumes to be acquired with unconstrained size in the scan direction [63].

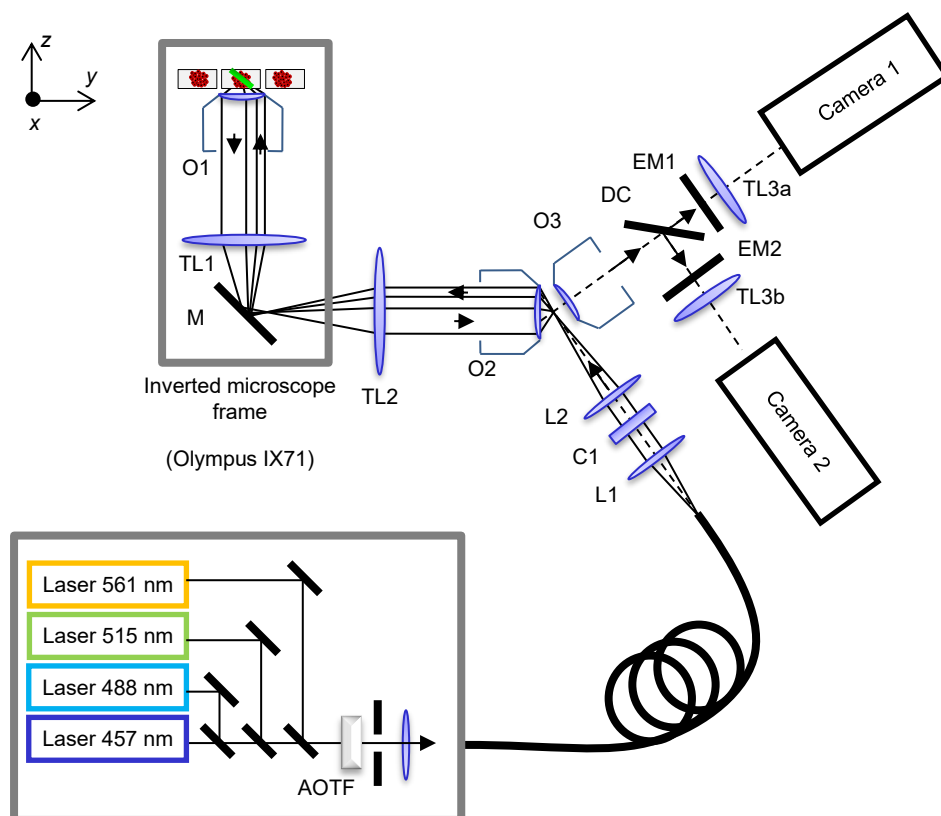


Figure 1.15 – Stage scanning OPM setup according to Maioli et al. [63]. O – objective, TL – tube lens, M – mirror, DC – dichroic mirror/beamsplitter, EM – emission filter, L – spherical lens, C – cylindrical lens, AOTF – acousto-optic tuneable filter. This microscope implements four laser/spectral channels and simultaneous OPM imaging on two cameras.

OPM, however, suffers from light loss at the interface between O2 and O3 due to the relative angles of the two objectives. The tilt between O3 and O2 often results in

asymmetrical clipping of the light cone emitted by O2 which, as well as reducing system collection efficiency, also results in an asymmetrical effective (collection) NA and resolution. This can theoretically be mitigated with a higher NA O3, but O3 must have a sufficiently long working distance to ensure that the two objectives do not physically collide. The collection efficiency of OPM calculated in Chapter 5 using a ray-tracing simulation, and the factors which contribute to collection efficiency are explained in detail there.

In the second iteration of the OPM system shown here with an OPM angle of 30° and 1.2NA/water objective for O1, the effective horizontal/longitudinal NA is 0.76 and the effective vertical/latitudinal NA is 0.80. Notwithstanding the light collection (and NA) loss, the inherent low photodamage benefits of light sheet microscopy still apply to OPM, which are particularly significant when acquiring volumes with a large number of z-planes and where a confocal imaging system would expose the whole sample for every image plane acquired. In the following sections, systems described as OPM or single objective light sheet (SOLS) systems are presented which were developed to directly address the light collection issues seen in standard OPM.

1.4.5 Axial plane optical microscopy

Axial plane optical microscopy (APOM) similarly uses a single objective to both illuminate a plane within the sample and collect the fluorescence, but instead of illuminating with an oblique plane, APOM illuminates a plane along the optical axis of the primary objective, which is equivalent to an OPM angle of 90° . Then, a remote refocusing system forms an intermediate image of the axial plane onto a mirror oriented at 45° which rotates the axial plane into a horizontal plane which is then imaged onto a camera. Since the light sheet runs along the axial direction, the axial resolution is improved, but at the expense of one of the lateral directions in addition to significant light loss due to O3 effectively being at 90° to O2. Volumes can be acquired with APOM by scanning the sample laterally through the axial plane of illumination to create y-stacks.

1.4.6 SPIM with microfluidics

Although the ordinary SPIM configuration does not facilitate high-throughput imaging, it can be adapted to implement fluidics to image 3D samples such as spheroids [64], [65], [66], [67], [68], replacing the agarose block in SPIM with a capillary tube. Automating sample loading from a multi-well plate has been demonstrated with SPIM-fluid, which could be applied to high-throughput, drug screening studies [64].

1.5 Variations on OPM

Since the advent of OPM, many variations of the technique have been developed: some with a focus on improving light collection and NA, and some to implement rapid scanning—similar to what is seen in the galvo scanning used in CLSM. Other techniques incorporate super-resolution modalities such as STORM and SIM, while others instead opt for OPM with larger FOVs and lower NA. Here, some of these variations are presented and explained in more detail.

In some of these techniques, different coordinate definitions may be used in the literature to describe the coordinates in the sample plane. To avoid confusion, the terms ‘vertical’ and ‘lateral’ are used here, where the ‘lateral’ direction lies in the plane of rotation of the detection arm, and the ‘vertical’ direction is the axis about which the rotation occurs. In the case of the original OPM paper, the vertical direction is defined as x and the lateral as y .

1.5.1 OPM rapid scanning techniques

To acquire three-dimensional volumes in OPM, multiple planes of the sample must be acquired, and this can be accomplished by, for example, laterally scanning the sample itself (stagescanning OPM) or by remotely scanning the intermediate image in the axial direction. The former requires the movement of a motorised stage, while the latter only requires the actuation of a single objective and is therefore capable of scanning much faster. Even so, an objective cannot physically be scanned as fast as rotating a mirror galvanometer (galvo); galvos are often used in confocal microscopes to scan the excitation beam and de-scan the imaged point rapidly.

Remote scanning OPM

As previously described in Section 1.4.4, objective O2 can be scanned rapidly using a piezoelectric motor to image a $36.3 \times 72.6 \times 36.1 \mu\text{m}^3$ volume in lab coordinates (lateral, vertical, axial) at 21 volumes per second (VPS) using EMCCD camera technology (20 frames per volume). This however is limited by the camera framerate, and faster cameras would enable these volumes to be imaged at up to 110 VPS.

SCAPE

Swept confocally-aligned planar excitation (SCAPE) microscopy [69], developed in E. Hillman’s group, uses an OPM-like setup with the addition of a polygon mirror mounted on a galvanometer motor for scanning the illumination light sheet and detection plane, see Figure 1.16(a). The mirror is placed close to conjugate to the back focal plane of the primary imaging objective to both rapidly scan the light-sheet illumination with one face of the polygonal mirror and de-scan the emission with another face, such that the illumination and detection planes remain stationary with respect to one another. The use of a polygon mirror rather than galvos, which have a rotation axis that lies in the plane of the mirror, results in imperfect rotational scanning which leads to light sheet scanning that is partially lateral, but with increasing inclination with increasing scan angle at the sample. This tilt-variant lateral scanning then requires a complex reconstruction procedure and means there is anisotropic sampling of the specimen. This first iteration of SCAPE achieves imaging at 20 volumes per second (VPS) for a $430 \times 1,330 \times 134 \mu\text{m}^3$ volume in lab coordinates (80 frames per volume) using an sCMOS camera.

Scanning window

Light-sheet scanning can also be achieved by using a galvo to tilt a refractive optical window [70] which is positioned in the back focal plane of the primary tube lens, e.g., TL1 in OPM, see Figure 1.16(b). This achieves lateral and linear (tilt-invariant) scanning of the light-sheet

over a range of $82\text{ }\mu\text{m}$, however, it was only demonstrated at a rate of 0.4 VPS for a $105 \times 91 \times 80\text{ }\mu\text{m}^3$ volume in lab coordinates (160 frames), limited by the camera framerate. Theoretically, galvo-limited scanning speed in this setup is far higher than 0.4 VPS. The refractive scanning window, however, introduces tilt-variant aberrations into the imaging path, namely spherical and chromatic aberrations due to refraction and dispersion respectively.

SOPi and SCAPE 2.0

To address the tilt-variation scanning issue seen in SCAPE that involves lateral and rotational movement of the light-sheet at the sample, a single mirror scanning galvanometer can be used instead of a polygon mirror for both scanning the light sheet and de-scanning the image, see Figure 1.16(c). The centre of rotation of the galvo mirror can then be positioned conjugate to the back focal plane of the primary objective. This was demonstrated experimentally by Yang et al. in Bo Huang's lab [71] (first as a preprint [72]), M. Kumar et al. in Y. Kozorovitskiy's group [73] and Hillman et al. [74] at about the same time.

Following the developments of SOPi, the Hillman lab developed SCAPE 2.0, which implements the galvo scanning method together with other improvements such as using a Powell lens for more uniform light sheet formation and a Plössl scan lens to reduce field curvature of the detection [75]. SCAPE 2.0 achieves imaging at greater than 100 VPS in imaging of a zebrafish heart, and 321 VPS for volumes of approximately $200 \times 200 \times 50\text{ }\mu\text{m}^3$ (127 and 57 frames per volume respectively).

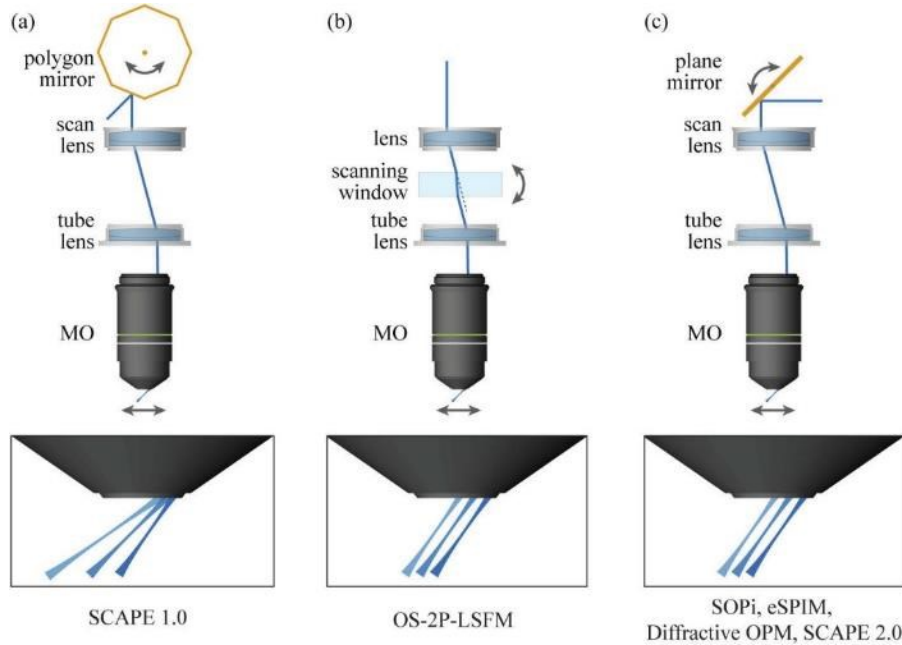


Figure 1.16 – Rapid light sheet scanning techniques. (a) tilt-variant scanning of SCAPE, (b) scanning with a refractive optical window [70], which results in imperfect de-scanning of the image (b) tilt-invariant scanning and de-scanning with SOPi. MO: microscope objective. Figure obtained with permission from M. Kumar et al. (2020) [76] (OSA Open Access Licence).

1.5.2 Improving NA and light collection of OPM

In standard OPM, O3 is a long working distance 0.6NA objective, which limits the collection efficiency and effective NA of the system. The theoretical best possible collection occurs when the NA of O3 approaches 1 and this is the approach used by epi-illumination SPIM (eSPIM) [71] and by the use of the AMS-AGY ‘snouty’ lens [77], [78], and these are described in the following sections.

eSPIM

Epi-illumination SPIM was developed by Bo Huang’s group at UCSF by Yang et al. and describes an OPM-like setup with a galvo mirror for sample scanning but with the detection objective, O3, a 1.0 NA water objective—see Figure 1.17. A custom-built water-filled prism with a microscope coverslip as the cap is mounted on O3 to provide the immersion fluid and O2 forms the image on the surface of the coverslip. Effectively, this approach achieves a lens with an NA close to 1 and with a working distance of zero. When a water immersion objective with an NA of 1.27 is used for O1, the effective detection NA of the system was estimated to be ~ 1.20 vertically (y , or out of the page in Figure 1.17) and ~ 1.06 laterally (x'), compared to 0.8 and 0.76 in the original OPM system that uses an objective with an NA of 1.2 as O1 (calculated using code described in Section 5.1.1). The FOV for a diffraction-limited volume imaged by eSPIM, however, is limited to approximately $\sim 100 \times 70 \times 20 \mu\text{m}^3$ (lateral, vertical, axial), which may be a result of asymmetric aberrations (astigmatism, coma) in particular.

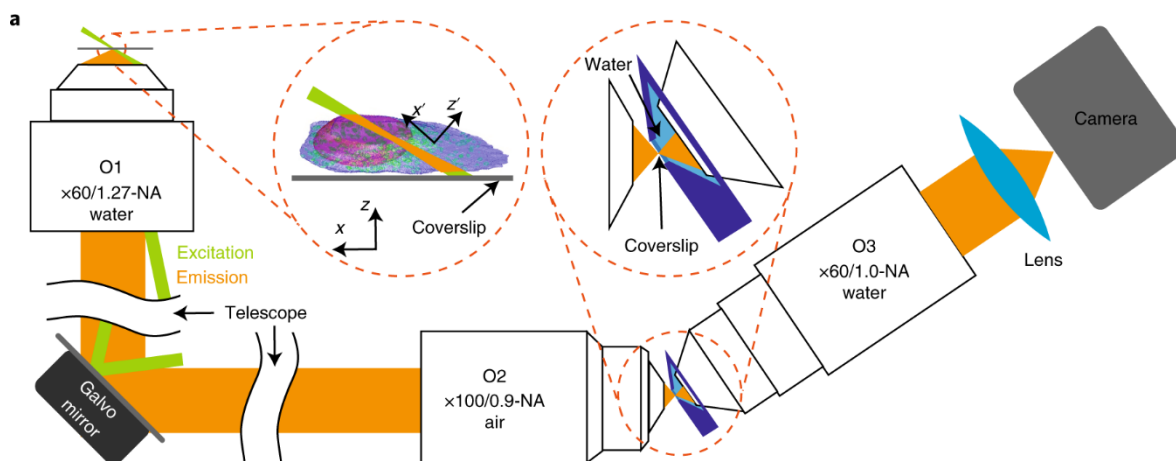


Figure 1.17 – Schematic of eSPIM system, which uses a 3D-printed water prism for the 1.0NA water immersion detection objective O3. Figure obtained with permission from [71]. A galvo mirror is used for lateral scanning of the light sheet and descanning of the image.

Glass-tipped ‘Snouty’ objective

Andrew G. York and Alfred Millett-Sikking at Calico Life Sciences followed a similar approach to eSPIM to increase light collection and effective NA of OPM systems but instead, a custom objective lens (AMS-AGY v1.0, Applied Scientific Instrumentation) with an NA of 1.0 and a working distance of slightly less than zero was used as O3 [77]. The custom objective, referred to as the snouty lens, has a carefully shaped glass tip that allows

the lens to be placed close to and at an angle to O2, see Figure 1.18, and the intermediate image is formed just inside the surface of the glass frustum. In work with Reto Fiolka and Kevin M. Dean, an OPM system using the AMS-AGY v1.0 lens was built and quoting a FWHM resolution of 299 ± 21 nm, 336 ± 16 nm, and 731 ± 21 nm in the vertical, lateral and axial directions respectively, within an FOV of $180 \times 180 \mu\text{m}^2$ in the light sheet plane. These FWHM measurements became 203 ± 24 nm, 209 ± 33 nm, and 523 ± 60 nm when the images were deconvolved with Richardson-Lucy deconvolution.

This FOV was limited by the snouty lens itself, and this was later improved upon with the second generation snouty, or ‘King Snout’ objective (AMS-AGY v2.0, Applied Scientific Instrumentation), shown in Figure 1.18. claims a $450 \mu\text{m}$ diffraction-limited FOV ($750 \mu\text{m}$ ‘usable’) in intermediate image space, corresponding to a sample-space FOV of approximately $340 \mu\text{m}$ ($560 \mu\text{m}$) with a water immersion O1 ($n=1.33$). The use of the ‘King Snout’ for larger FOV imaging is demonstrated in OPM with tiled FOVs of $800 \times 500 \times 200 \mu\text{m}^3$ through dual-axis galvo scanning [79] or with galvo-stabilised stage-scanning in DaXi, which is presented in Section 1.5.5.

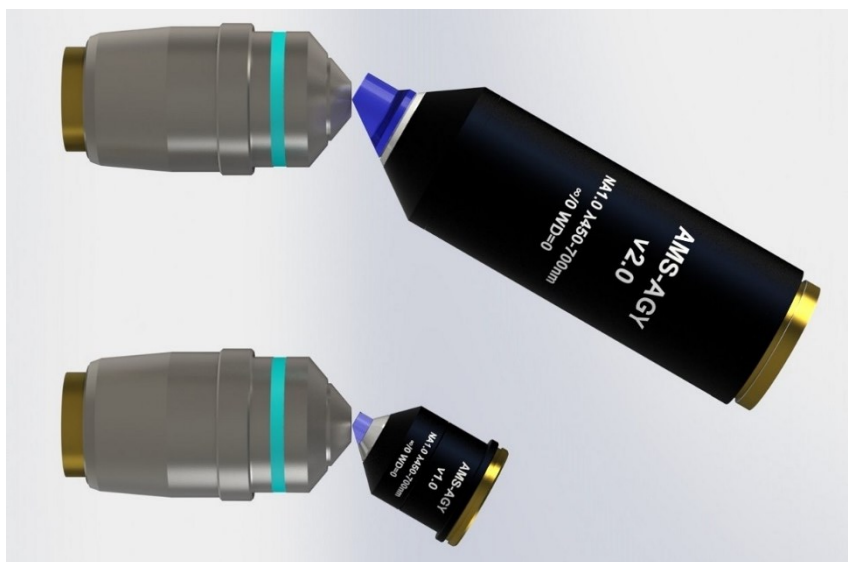


Figure 1.18 – 3D CAD render of snouty lenses. Top, AMS-AGY v1.0 (Snouty); bottom, AMS-AGY v2.0 (King Snout). Reproduced from [77] ([CC-BY 4.0](#) licence).

1.5.3 obSTORM

Single-molecule localisation microscopy (SMLM) techniques achieve sub-diffraction limit resolution (super-resolution) by acquiring images in multiple cycles with only a subset of fluorophores emitting in a single frame, such that each fluorophore can be localised individually. Photoactivation Localization Microscopy (PALM) and Stochastic Optical Reconstruction Microscopy (STORM) are two main SMLM techniques [9], [10], [11], which are described previously in Section 1.1.2.

Oblique-plane single-molecule localization microscopy, or obSTORM [80], achieves optical sectioned STORM on an OPM setup and is illustrated in Figure 1.19. Previous

demonstrations of SMLM in LSFM have generally been made by employing some form of small mirror near the sample to reflect the light sheet so it is in the front focal plane of the objective [81], [82]. The benefit of obSTORM is that it allows the flexibility to image samples beyond whole-cell thickness with the use of any standard stage and sample mount like in OPM. In obSTORM, a folded remote refocus geometry is used similar to one of the configurations shown in Botcherby et al. and demonstrated in APOM. In obSTORM, the angle of the remote mirror was set to 22.5° for an OPM angle of 45° , see Figure 1.19.

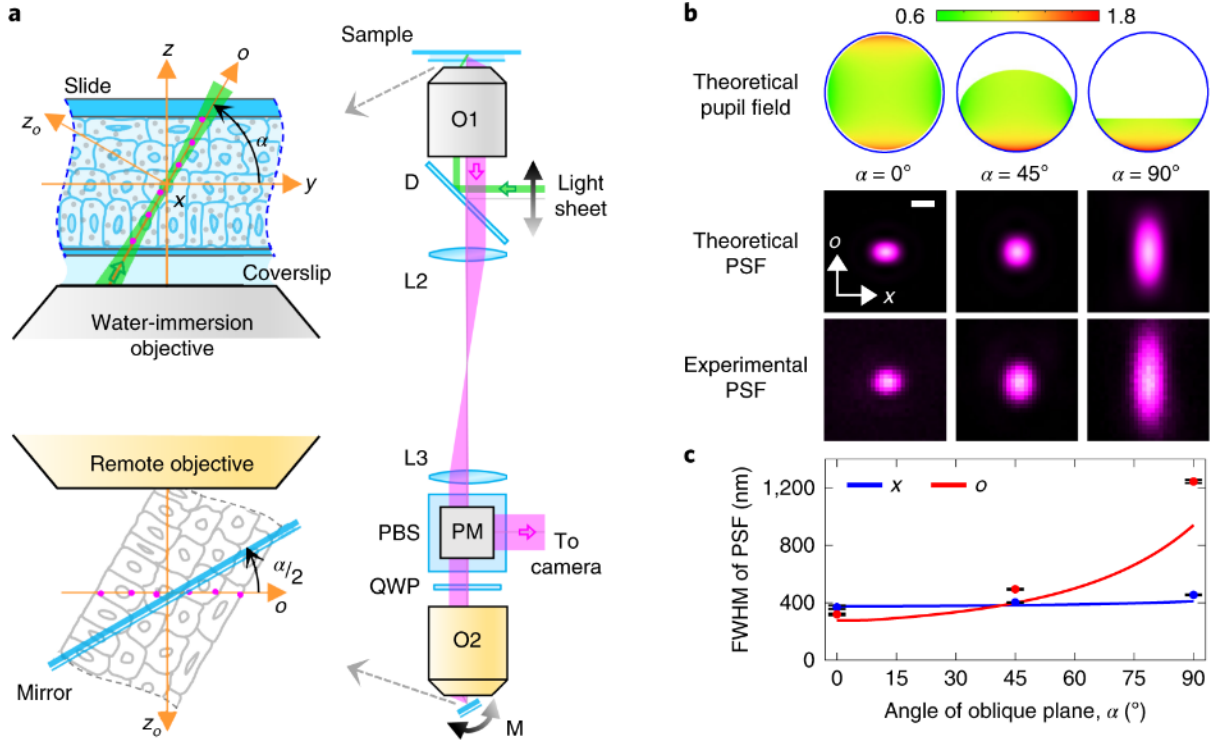


Figure 1.19 – (a) Layout of obSTORM. D – dichroic mirror, L – (tube) lens, PBS – polarising beamsplitter, PM – periscope mirror, QWP – quarter waveplate, M – mirror angled at $\alpha/2$. (b) Theoretical simulated intensity distribution in exit pupil for different OPM angles, and the corresponding simulated and measured PSFs. The exit pupil is ‘clipped’ as the angle increases, reducing the effective light collection and NA as seen in OPM in general. (c) Comparison simulated and measured PSF FWHM in the lateral and vertical directions as a function of light sheet angle – here, o is the clipped direction. Reproduced with permission from [80].

The advantage of this arrangement is that a long working distance detection objective, O3, does not need to be used, and the high-NA O2 is used both in the refocusing system and as the detection objective. To outcouple the fluorescence, however, a polarising beamsplitter (PBS) is used; this transmits linearly polarised light which is then rotated 90° by a double pass of the quarter waveplate (QWP) before being reflected by the same beamsplitter now that the linear polarisation is crossed with the transmission. When ignoring the potential for partial polarisation of the fluorescence, this results in a 50% collection loss at the beamsplitter. The increased NA, or geometrical collection, can compensate for the loss at the PBS in the overall collection efficiency—this is quantified in the following section of dOPM and is explored in depth with calculations and simulations in Chapter 5.

1.5.4 dOPM

In SPIM, the sample can often be physically rotated with ease and multiple views of the sample can be acquired and the views merged—this has the benefit of suppressing shadowing artefacts where the light sheet is locally scattered or absorbed in the sample. Dual-view OPM (dOPM) [83] is a technique that can acquire two OPM views of the sample, and in the same way, these can be merged to reduce shadowing but also increase the isotropy of the resolution, especially after deconvolution. This setup is shown in Figure 1.20, along with the resulting parallelepiped volumes acquired for each view. In dOPM, the FWHM of deconvolved fluorescent bead images were $0.35 \pm 0.04 \text{ }\mu\text{m}$, $0.39 \pm 0.02 \text{ }\mu\text{m}$ and $0.81 \pm 0.07 \text{ }\mu\text{m}$ in the vertical, lateral, and axial directions respectively, while the FOV is larger than that of standard OPM in this case since here a $40\times$ objective is used. Deconvolution was shown to greatly improve the optical sectioning strength, measured in the axial direction, with the individual views showing this to be $\sim 5 \text{ }\mu\text{m}$, and after deconvolution, the optical sectioning became $1.33 \pm 0.45 \text{ }\mu\text{m}$.

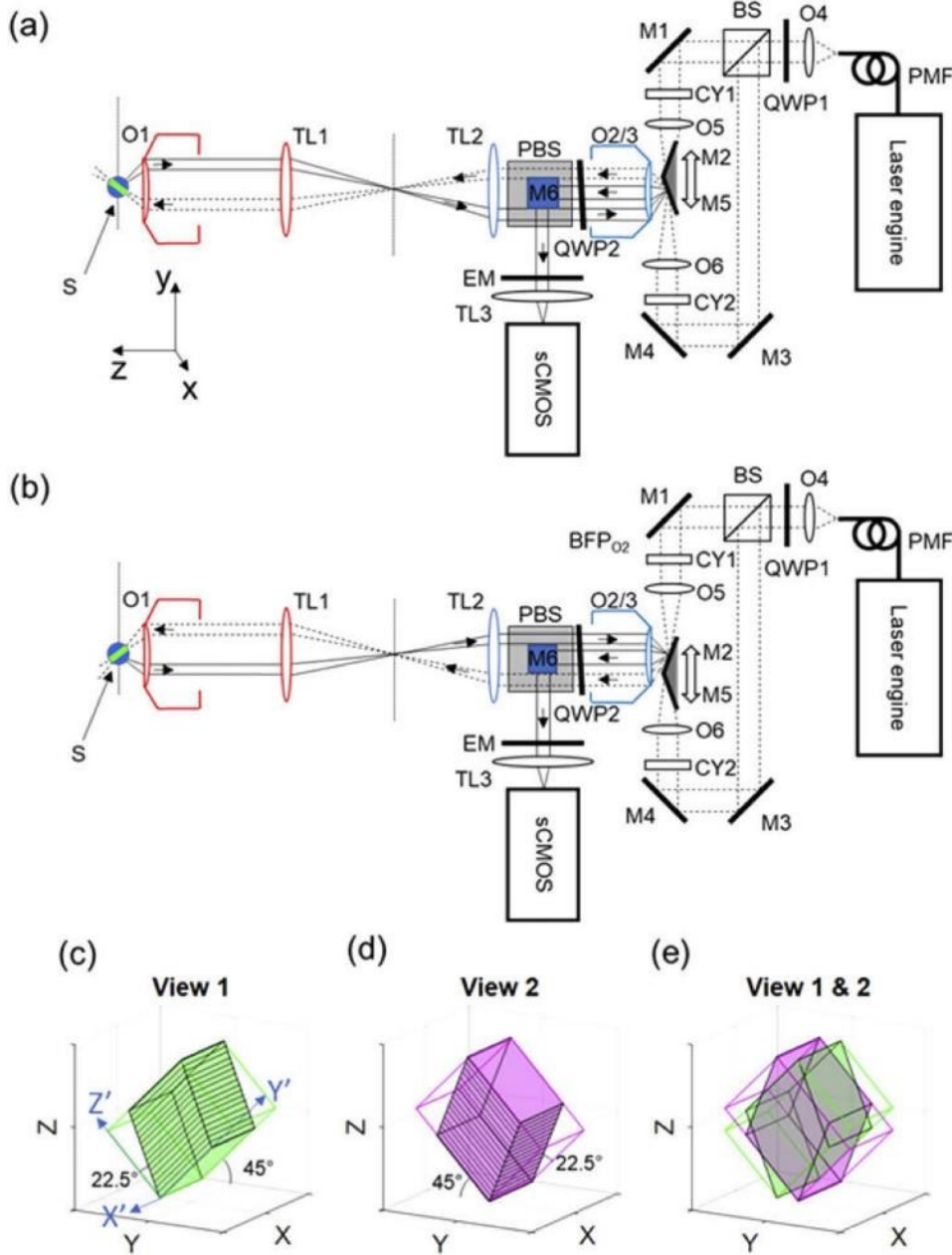


Figure 1.20 - (a) dOPM setup for view 1 and (b) for view 2, noting the translation of the mirror pair (M2 and M5). O – objective, TL – tube lens, PBS – polarising beamsplitter, EM – emission filter, QWP – quarter waveplate, M – mirror, CY – cylindrical lens, BS – beamsplitter, PMF – polarisation maintaining fibre. (c) The stack of planes obtained through lateral scanning of the mirror pair for view 1 and (d) view 2, and the two orthogonal views fused (e). Reproduced with permission from [83] ([CC-BY 4.0](#) licence).

dOPM uses a folded configuration similar to that used by obSTORM, with the angled mirror implemented as a pair of prism mirrors attached together on a prism holder mounted on a single-axis actuator. The illumination is also introduced at the prism mirror, unlike in other similar folded geometries such as obSTORM and APOM. The prism mirrors are mounted in the holder at, e.g., at $\pm 22.5^\circ$ for an OPM angle of 45° , and the actuator translates the mount laterally to switch between the mirror in the light path, corresponding to switching the views. The actuator additionally scans the prism mirrors laterally to acquire volumes for each of the views through remote scanning. The remote scanning method is similar to that

seen with remote scanning OPM, but the angled mirror is scanned laterally which results in the plane being refocused normal to the mirror surface instead of along the optical axis.

Recently, preliminary measurements (unpublished) of the light collection efficiency relative to epi-illumination measurements have been made by Hugh Sparks for a dOPM setup with a 1.2NA 60 $\times$ water objective—the same as used in collection efficiency measurements in the OPM setup which was presented in Vincent Maioli’s thesis. Here, for an OPM angle of 35°, the relative collection efficiency was 0.18 and 0.23 for unpolarised and partially polarised excitation respectively, which is similar to the 0.21 measured for the similar OPM setup. This shows that the light loss at the PBS is partially compensated by the increased effective NA, and the efficiency is higher still when the excitation is polarised. In dOPM, light sheets are polarised in the light sheet plane, and this results in partially polarised emission [84]. Therefore, the nominal 50% light loss at the PBS can be theoretically reduced if the PBS is oriented favourably; this is the focus of Chapter 5, where a full polarisation ray-tracing simulation that I developed is presented to calculate the theoretical light collection efficiency.

1.5.5 DaXi

A dual view OPM was also demonstrated by the authors of the snouty lens-based OPM system which uses an optical image flipper system consisting of 2 galvos and 3 plane mirrors to switch between orthogonal views at an OPM angle of 45°. The use of galvos here increases the speed with which the views can be switched compared to dOPM. The volumes are acquired using a novel combination of the tilt-invariant lateral scanning method paired with stage scanning to achieve rapid scanning with an unconstrained FOV in the scan direction—this is faster than tiling volumes with galvo scanning or stage scanning alone. This scanning method is called light sheet stabilized stage scanning, and it utilises the speed of galvos to counter the motion of stage scanning to keep the sample stationary in the FOV while a frame is acquired, before rapidly stepping the FOV by resetting the galvo position to acquire the next frame, thus avoiding motion blur—this scheme is illustrated in Figure 1.21. DaXi demonstrates FWHM values from a measured PSF from bead volumes of 479.9 ± 28.0 , 379.2 ± 20.9 and $1,864.9 \pm 174.3$ nm in the vertical, lateral, and axial directions respectively.

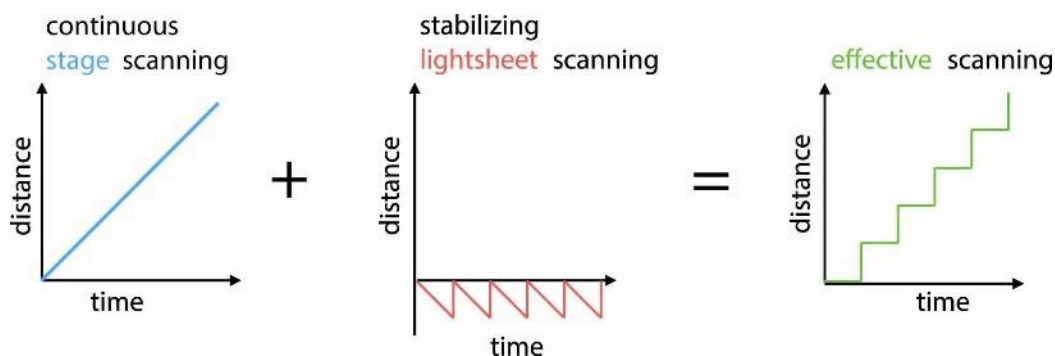


Figure 1.21 – Light sheet stabilized stage scanning method used in DaXi which results in effectively stepwise scanning which mitigates motion blur from continuous stage scanning. Reproduced from [85] ([CC-BY 4.0](#) licence).

1.6 Summary

In this chapter, the concept of optical sectioning to acquire images of thin sections of 3D samples was introduced, summarised at the end of this chapter in Table 1.2. Several techniques exist to achieve this, and the method most commonly applied to biological imaging is the confocal microscope. Confocal microscopy is a point illumination technique that is usually implemented with fluorescence and laser illumination and uses a pinhole in front of the detector placed conjugate to the sample plane to reject out-of-focus light. This is an effective method of optical sectioning; however, it exposes regions of the sample out of the imaging plane to unnecessary light, which is then discarded at the pinhole. The confocal laser scanning implementation also requires high peak laser powers for reasonable signal and imaging rate compared to widefield techniques but spinning disk confocal aims to address this by parallelising the process of point scanning. Other methods of optical sectioning with lower associated photodamage are presented, including multiphoton microscopy, optical sectioning structured illumination microscopy (OS-SIM), total internal reflection fluorescence microscopy (TIRFM) and light sheet fluorescence microscopy (LSFM)

LSFM is an approach to optical sectioning that achieves low photodamage optical sectioning and is potentially faster than OS-SIM, has far greater possible imaging depth than TIRFM and is less limited by laser choice than multiphoton microscopy. The basic principle of LSFM involves illuminating the specimen with a thin sheet of laser light in the sample plane, while capturing fluorescence emitted from the illuminated plane using a separate detection objective, usually positioned at 90° to the illumination axis, and the plane is imaged onto a camera. This illumination scheme does not result in photodamage caused by out-of-focus excitation in confocal methods (including spinning disk) and earns it the description of being a gentle optical sectioning technique.

In this chapter, LSFM techniques were explored and presented in detail including various approaches to light-sheet generation which improve optical sectioning such as digital scanning of the light-sheet (with confocal slit detection); Bessel, Airy, and Lattice light sheet illumination; and axially swept light sheet microscopy. These different light-sheet generation methods are summarised in Table 1.3. Originally, the standard implementation of LSFM was SPIM, which has limited imaging throughput and is incompatible with multi-well plates used in high content screening (HCS) which is essential for drug screening assays and biological research that desires multiple parallel conditions and repeats. Techniques which aim to enable HCS with LSFM by employing alternative geometries are described and summarised in Table 1.4, some of which illuminate and detect fluorescence at 45° to the sample plane such as with inverted SPIM (iSPIM), dual illumination SPIM (diSPIM) and open top light sheet (OTLS). These techniques are generally associated with lower NA due to the use of long working distance objectives, particularly iSPIM and diSPIM, while OTLS has recently implemented a higher NA (up to 0.7) imaging modality. Since OTLS illuminates and collects fluorescence below the sample, it is also compatible with multi-well plates, which is not possible with iSPIM or diSPIM.

Other high-throughput LSFM techniques employ the use of a single objective at the sample, allowing conventional microscope frames to be used along with conventional sample holders, including multi-well plates. One such technique is oblique plane microscopy (OPM), in which a short line of illumination is introduced at the pupil edge of the objective to generate obliquely angled (30° – 45°) light sheet illumination at the sample. OPM, the focus of this thesis, is notable for having the benefits LSFM in a single objective configuration which allows maximum possible sample holder compatibility, making it a powerful tool for HCS and the method of choice for the high-throughput, multi-gene cancer spheroid experiments in Chapter 4.

Since OPM was introduced, variations have spawned. SCAPE used a galvo-mounted polygon mirror to increase scanning speed, but this resulted in a scan-position-dependent light sheet angle. This was corrected by using single plane mirror galvos positioned so the rotation axis was conjugate to the back focal plane of the primary objective (objective at the sample), as demonstrated in eSPIM, SOPi, and SCAPE 2.0. Other variations aim to improve the light efficiency and effective NA by maximising the light collection at the O2-O3 interface. This includes eSPIM, which uses a water prism mounted on a 1.0NA water objective, and the ‘snouty’ glass frustum-tipped 1.0NA objective with a working distance slightly less than 0. OPM systems which provide multiple views of the sample for more isotropic resolution and reduced shadowing artefacts are also demonstrated, including dOPM and DaXi.

An alternative configuration for OPM is the folded configuration, which is seen in obSTORM and dOPM. In these systems, the fluorescence signal is coupled out using quarter waveplates and a polarising beamsplitter which nominally reduces the light collection efficiency by 50% but is theoretically less in the case of partially polarised emission. In Chapter 5, I present my polarisation ray-tracing model to calculate the collection efficiency of OPM-like systems and dOPM-like folded systems.

Table 1.2 - Summary of optical sectioning microscopy techniques. Techniques are determined to be high-throughput if they allow a large number of samples to be imaged, and if the technique is camera based or common implementations of rapid point-scanning methods exist, e.g. Nipkow spinning disk confocal (SDCM). Advantages and disadvantages represented by + and – respectively.

	High-throughput?	Advantages and disadvantages
CLSM	(Generally) no due to point-scanning time	+ Relatively easy to implement on conventional microscope system + Optical sectioning (and improved lateral resolution) by rejecting out-of-focus light with pinhole - Photodamage from out-of-plane fluorescence - Point-scanning makes CLSM slow, requiring higher peak excitation power for appreciable imaging rates, increasing photodamage
SDCM	Yes	+ Optical sectioning (and improved lateral resolution) by rejecting out-of-focus light with pinholes + Disk with array of pinholes increases imaging speed/throughput, allowing lower peak excitation powers to be used - Photodamage from out-of-plane fluorescence - Crosstalk between pinholes reduces signal to background/contrast; increases with pinhole density
OS-SIM	Yes	+ Lower photodamage than confocal-based methods (CLSM, SDCM) + Widefield/camera-based method, so is faster than most confocal-based methods (but requires at least 3 images per plane)
Multiphoton	(Generally) no due to point-scanning time	+ Lower photodamage than confocal-based methods away from imaging plane -/+ Originally a point-scanning technique [24], but can be fast if implemented in LSFM, for example - Potentially non-linear/higher photodamage at imaging plane - Multiphoton absorption requirement restricts fluorophore choice
TIRFM	Yes	+ Sub-micron sectioning strength + Widefield/camera-based method, so is faster than most confocal-based methods - Imaging depth limited to coverslip surface
LSFM (SPIM)	Not usually, possible with microfluidics [64], [66] or different geometries (see Table 1.4)	+ Only excites imaging plane, minimising photodamage + Widefield/image-based method, so is faster than most confocal-based methods - Conventional LSFM has limited sample mounting options, restricting sample size and potential throughput

Table 1.3 - Summary of alternative light-sheet generation techniques (techniques other than Gaussian light-sheets generated with cylindrical lens). Note that resolution and FOV can vary for a single technique from the choice of lenses. Most crucially, there is usually a trade-off between beam waist and usable FOV since Gaussian beams have a rayleigh length (distance where cross-sectional area of beam is twice that at the waist) that is inversely proportional to the square of the beam waist. Technically, the light-sheet generation should have no impact on ability for a LSM technique to be high-throughput. Advantages and disadvantages represented by + and - respectively.

Variations on light-sheet generation	
Method	Advantages and disadvantages
Digitally scanned light-sheet	+ More uniform illumination than light-sheets generated with cylindrical lens + Effective confocal slit detection from rolling shutter can improve contrast and optical sectioning + Timed with camera rolling shutter, does not reduce imaging speed - Requires careful synchronisation
Axially swept light-sheet	+ Only illuminates with thinnest part of light sheet (waist) for best possible resolution (e.g. isotropic, 380 nm) + Timed with camera rolling shutter, does not reduce imaging speed - Reduced collection duty cycle, increasing potential photodamage (fluorescence away from waist rejected) - Requires extra optics for axial scanning, including refocussing system which limits (diffraction-limited) scan range, and careful synchronisation
Bessel beam	+ Bessel beams remain thin for better sectioning in a larger FOV - Side lobes cause unwanted fluorescence
Airy beam	+ Airy beams remain thin for better sectioning in a larger FOV, even more so than Bessel beams - has side lobes like Bessel beams +/- Self-bending beam means deconvolution required, but side lobes may contribute to resolution due to curved profile
Lattice light-sheet	+ Thin profile over long distance like Bessel + Square lattice suppresses unwanted side lobes, while hexagonal lattice increases axial resolution (but with stronger side lobes than square lattice) + Structure allows SIM-mode lattice light-sheet imaging for super-resolved imaging - Performance claims are disputed, Fiolka et al. stipulated that Gaussian beams have equivalent performance [48]

Table 1.4 – Summary of light-sheet microscopy techniques with setups that employ alternative geometries, e.g. non-orthogonal excitation and collection. * Indicates where quantities for OPM-like systems have been calculated/estimated using our MATLAB code (described in Section 5.1.1), namely NA and collection efficiency. As before, many techniques will have different objective/lens configurations meaning that in general the NAs, resolutions and FOVs will not be the same.

Alternative light-sheet system geometries		
Method	High throughput?	Advantages and disadvantages
diSPIM	With custom sample mounts	+ Geometry allows large sample imaging and custom multi-well mounts to be used + Acquiring two views allows suppression of scatter effects and fusion and deconvolution for enhanced, isotropic resolution (improved axial) compared to standard SPIM - Water dipping objectives for high NA imaging make direct contact with sample media and may result in contamination, sample drift or objective corrosion.
Open top light sheet (with NODO)	Yes, but with coverglass material requirements	+ Capable of multi-scale imaging with ODO and NODO modalities: larger FOV vs higher resolution + Compatible with multi-well plates and large samples - To avoid aberrations, sample holder coverglass material must be index matched to sample media
HILO	Yes, conventional sample mounting	+ Built on conventional microscope frame, maximum possible compatibility with sample mounting techniques - Defocus from angled illumination limits FOV to 15-40 μm region
OPM	with commercial frames	+ Built on conventional microscope frame, maximum possible compatibility with sample mounting techniques - Reduced light collection efficiency ($\sim 30\%$ relative collection efficiency) and NA due to relative tilt of objectives: 0.76* lateral NA and 0.8* vertical effective NA with 1.2NA primary objective, $\theta_{\text{OPM}} = 32^\circ$
SCAPE		See OPM + Faster scanning than OPM - Tilt-variant scanning means non-uniform sampling of volume with different local coordinates
SCAPE 2.0/SOPi		See OPM + Faster scanning than OPM + Tilt invariant scanning
APOM		+ Built on conventional microscope frame, maximum possible compatibility with sample mounting techniques + Light-sheet propagates axially relative to detection for sub-micron axial resolution

		- 45° mirror and beamsplitter reduces light collection efficiency (<25%* relative collection, including 25% loss from beamsplitter) and effective NA (with 1.4NA objectives: 0.6* lateral, 1.3* vertical effective NA)
eSPIM		+ Built on conventional microscope frame, maximum possible compatibility with sample mounting techniques + Water prism increases NA (to 1.0) of O3 to improve light-collection and effective NA compared to OPM: ~1.06 lateral and 1.20 vertical effective NA with 1.27NA primary objective, $\theta_{\text{OPM}} = 30^\circ$ - Water prism hard to align practically - Prism interface may introduce asymmetric aberrations
Snouty		+ Built on conventional microscope frame, maximum possible compatibility with sample mounting techniques + Custom “snouty” objective increases NA (to 1.0) of O3 to improve light-collection and effective NA compared to OPM: see eSPIM + Custom objective allows aberrations (i.e. those in eSPIM) to be balanced in the lens design itself - Custom objective increases cost
DaXi		DaXi uses snouty objective; see Snouty + Acquiring two views allows suppression of scatter effects and fusion and deconvolution for enhanced, isotropic resolution - Two galvos in image flipper system increases cost
dOPM		+ Built on conventional microscope frame, maximum possible compatibility with sample mounting techniques + Acquiring two views allows suppression of scatter effects and fusion and deconvolution for enhanced, isotropic resolution + Folded setup allows remote space objective (O2/3) to be higher NA than in OPM, increasing effective NA: 1.02 lateral and 1.2 vertical effective NA with 1.2 NA - folded system means additional 30-50% light-collection loss when out-coupling fluorescence

2 OPM instrumentation

2.1 Introduction

As shown in the previous chapter, as an extension to the original OPM designed by Chris Dunsby, the stage scanning OPM system was developed through additional contributions of Vincent Maioli, Sunil Kumar and Nathan Curry. I inherited this system at the beginning of this PhD.

Many of the details of the initial system are described in Chapter 2 of Vincent Maioli’s thesis [86] and will be briefly presented here, and following this, the improvements to this system made during this PhD. The first improvements enabled three-camera imaging to increase the imaging throughput when channels are imaged simultaneously; these improvements were implemented through the optical and mechanical designs of Nathan Curry, and I was involved in building this design and aligning the system. The three-camera upgrade, however, was not fully utilised during this PhD and rather the system and software development was focussed on facilitating automated 4-colour imaging on a single camera for the time-lapse experiments described in Chapter 4.

Methods to automatically locate samples in 384 multi-well plates were also developed, and these are presented separately in Chapter 3, while this chapter focuses on introducing the existing hardware and methods for the OPM system, hardware upgrades and the associated low-level, i.e., acquisition software upgrades.

2.2 Initial setup of OPM

The OPM system at the start of this PhD was published as stage-scanning OPM [63] which utilises stage-scanning and hardware-triggered camera acquisition to obtain 3D OPM volumes—the setup of this system is shown in Figure 2.1.

The full hardware details, instrumentation and data acquisition processes of this system, shown in Figure 2.1 and described by the flowchart in (b), are explained in the following sections. Many of these features are unchanged in the current form of the OPM system, except for certain optical design and optomechanical changes described later.

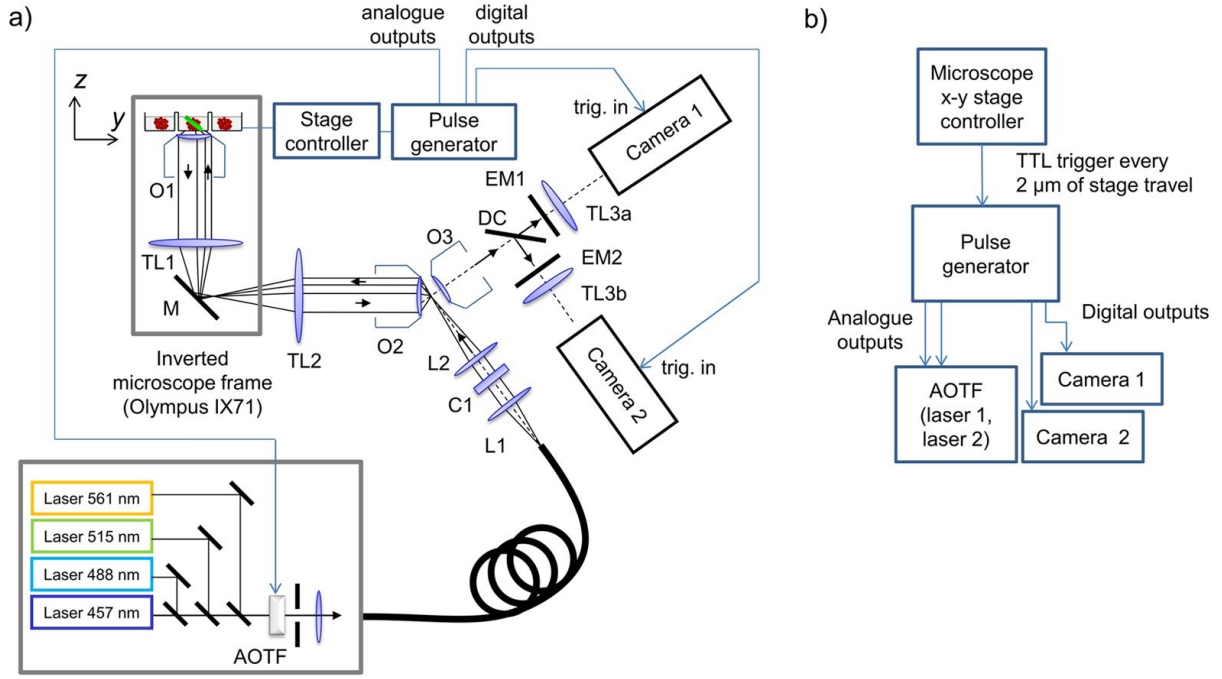


Figure 2.1 – Stage scanning OPM diagram. O – objective, TL – tube lens, L – lens, DC – dichroic mirror, EM – emission filter, C – cylindrical lens, M – mirror.

2.2.1 Optical and mechanical design

OPM imaging

The OPM system consists of three arms: the illumination arm, the refocusing system, and the detection arm. Both the illumination and detection arms are mounted on a breadboard fixed to a rotation-translation stage and supported by ball bearings on the optical table, which allows the OPM angle to be adjusted as shown in Figure 2.2.

Inside a laser combiner box, an AOTF (AA Opto Electronic) is used to select between solid-state lasers at 457 nm, 515 nm, 561 nm (Cobolt), 488 nm and 642 nm (Spectra-Physics), and a voltage input modulates the output power of the laser from the AOTF. The laser output from the AOTF is coupled to a single-mode polarisation maintaining fibre, leading to the illumination arm where the output of the fibre is collimated by a 10X 0.3NA (UPlanFLN, Olympus) objective (L1). The light sheet is then generated with a 20 mm cylindrical lens (C1) in the back focal plane of a 25 mm spherical lens (L2).

The light sheet is formed in the intermediate image space between O2 and O3 with the illumination arm oriented such that an angle of $90^\circ - \theta_{OPM}$ is subtended by the optical axes of O2 and L2. The light sheet is then coupled to O2 where it is re-imaged into object space by the remote refocusing system which comprises microscope 1 (O1, TL1) in the IX71 microscope frame and microscope 2 (TL2, O2) which is mounted on the left port of the IX71 (see Appendix A.2.1). In object space, the light sheet is then formed at an angle of θ_{OPM} measured from the focal plane of O1 (or the horizontal in lab coordinates).

The subsequent tilted plane of fluorescence from the sample is magnified by microscope 1 and demagnified by microscope 2 with a total magnification of 1.33 according to the

requirements for aberration-free remote refocusing [59], [60] when using a water immersion objective for O1 ($n = 1.33$). In remote refocusing space, the resulting image is re-imaged by microscope 3 (O3 and TL3), which is mounted on the rotatable breadboard along with the dichroic mirror, emission filters and cameras, forming what is called here the detection arm. The breadboard mounted on a rotation-translation mount is rotated such that the optical axis of the detection arm is at the OPM angle, θ_{OPM} , relative to that of O2. The breadboard can easily be rotated to facilitate OPM imaging at a range of different OPM angles (depending on the desired axial FOV, resolution, etc.), alignment requiring only adjustments to the translation of the rotation-translation stage, followed by axial adjustment of O3 and the lens that forms the light sheet.

In this initial setup, in the detection arm, the signal is split by a dichroic mirror to enable simultaneous or interleaved multi-channel imaging with two cameras. For each camera, there is a tube lens (TL3a and TL3b) and manual filter wheel on each output of the dichroic. Further details of the optical elements in the system are summarised in Table 2.1 and the total magnification of this system with these elements is 42.67.

The alignment of the refocusing system is crucial to its performance and is necessary for OPM to negate the issue of anisotropic magnification and severe spherical aberrations of the tilted plane in detection space. Therefore, O2 and TL2 are each on XY translation mounts which allows them to be aligned to be coaxial. To align O2 and TL2, the breadboard is rotated to (an OPM angle of) 0° , and the full alignment process is described in Section 2.4.1. With the breadboard rotated to 0° , the remote focusing system performance can also be characterised using transmitted light and a resolution target, see Section 3.6 of Vincent Maioli’s thesis.

The frame was also equipped with a motorised focus drive (Thorlabs)—which was later changed to a Märzhäuser focus drive—mounted to the fine focus knob which allows the objective (O1) to be translated in z within the MicroManager software. A motorised mechanical XY-stage (Märzhäuser) allows automatic movement between wells, also within MicroManager, and hardware-triggered stage-scanning acquisition which is described in Section 2.2.2.

Widefield imaging

The trinocular port on the IX71 frame is used for brightfield imaging with a white lamp (Olympus) and epi-illumination imaging with a set of LEDs (Thorlabs). The LED (Thorlabs) is fixed to the rear upper port of the IX71, and the dichroic filter cube carousel allows epifluorescence imaging with the 430 nm, 505 nm, 565 nm and 625 nm LEDs.

The IX71 allows the light path in the trinocular port to be diverted from the eyepieces to a camera port, onto which a demagnifying relay built from two achromatic doublets of $f = 80$ mm and $f = 30$ mm (Thorlabs) respectively is mounted. This relay images the sample onto an Andor Luca EMCCD camera which allows brightfield and epifluorescence imaging, and live visualisation of the eyepiece view with a camera.

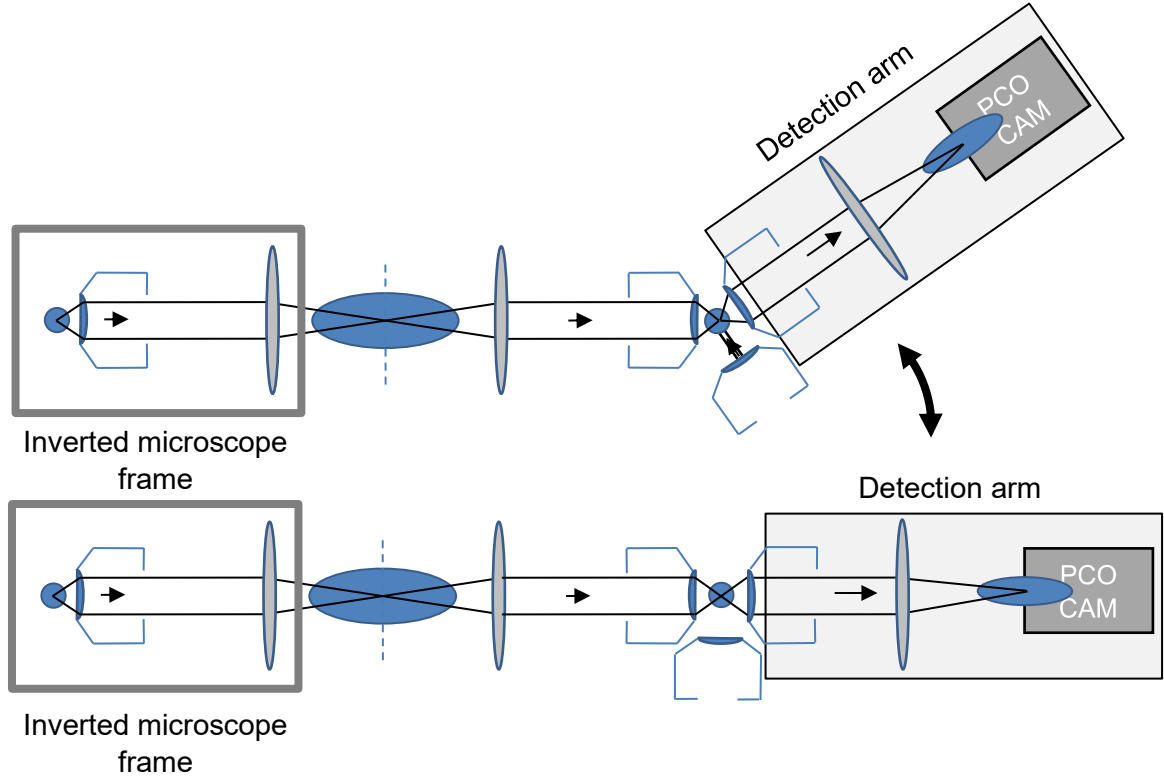


Figure 2.2 – OPM with breadboard un-rotated at 0° (bottom), and OPM after breadboard is rotated to 35° (top).

Table 2.1 – Lenses used in original OPM setup.

Label in Figure 2.1	NA/magnification/focal length/immersion medium	Description
O1	$60\times$ / 1.2NA Water	Olympus objective
TL1	$f = 180$ mm	Olympus tube lens
TL2	$f = 162$ mm	Multi-element tube lens (Thorlabs): -500 mm aplanatic meniscus and 150 mm achromatic doublet separated by 97 mm
O2	$50\times$ / 0.95 Air	Olympus objective
O3	$40\times$ / 0.6 Air	Nikon objective
TL3	$f = 160$ mm	Tube lens (Comar achromatic doublet)

2.2.2 Data acquisition

To automatically acquire OPM volumes, a stage-triggering-based method is used as described in Vincent Maioli's thesis [86], which uses a National Instruments data acquisition system (DAQ) (USB-6229) for signal generation (on the DAQ internal clock), triggered by the XY stage. This was realised by LabVIEW code originally written by Sunil Kumar.

Volumes are acquired in what we call a trigger loop, and this is described in Figure 2.1(b). In the trigger loop, the XY stage is translated in y by y_{distance} which determines the y -FOV

of the volume, and a TTL pulse is generated by the Tango stage controller at distance intervals, y_{trigger} , and the TTL pulse is used as an input to the DAQ to trigger the generation of pulse signals that control the laser and the cameras. The pulse generation occurs in the LabVIEW code, which allows interleaved or continuous imaging and generates separate pulses for each of the two lasers and cameras, with each pulse signal output from the DAQ and wired to the AOTF controller amplitude pin and camera trigger input respectively. The durations (exposure time) and heights (laser amplitude) of these pulses are chosen depending on the required signal or fluorophore brightness and are determined by the user settings in a MicroManager acquisition script. These are saved into shared **.txt** files that are polled by the LabVIEW code.

During the acquisition trigger loop, the camera buffer is simultaneously read by MicroManager in a while loop, and the code waits for n_{img} frames to be acquired (or the timeout is reached), with

$$n_{\text{img}} = \lfloor y_{\text{distance}}/y_{\text{trigger}} \rfloor. \quad 2.1$$

If the actual number of acquired frames is less than n_{img} by more than the user-set maximum number of dropped frames, an error is raised. This occurs when the duration of the pulses approaches the time between the pulses meaning that the camera is still exposing when it receives the TTL trigger from the stage and so the trigger is ‘missed’. To avoid this, the scan speed is reduced accordingly to increase the time between pulses. This means that if fast imaging is required, the exposure time must be reduced (which may in turn require higher laser amplitudes) and vice versa.

In this setup, there are two cameras which are triggered by the DAQ system, called the ‘reflected’ and ‘straight’ cameras, named as such from the light paths from the dichroic mirror. To image additional fluorescent channels, the dichroic filter may need to be swapped, and the BNC cables connecting the DAQ laser-controlling output to the AOTF may need to be swapped so different lasers can be controlled. There are 4 10V analogue BNC outputs on the DAQ, with one used as the AOTF digital blanking channel which switches all lasers on (voltage high input) or off (voltage low input), so only 3 lasers can be connected to the DAQ with the BNC cables at once in this configuration.

2.2.3 Reslicing raw data

The acquired frames are in a skewed coordinate space and consequently, so is the OPM volume; this raw data must be converted to lab coordinates for intuitive interpretation and visualisation of the data—known as reslicing. The reslicing process involves the interpolation of voxels in lab coordinates, and the interpolation is calculated from raw OPM frames in the direction orthogonal to the frames as detailed in the thesis of Vincent Maioli [86]. The initial implementation was written in MATLAB by Vincent Maioli but was then written in Java to increase processing speed by Ian Munroe [87]—the source code can be found in the **opmGeneralAnalysis** GitHub repository (see link 1.1). The Java code further bins the data (usually by 4, into $1 \mu\text{m}^3$ voxels) and is called by a MATLAB script (see link 1.2), which

automates the process of reslicing, for example, data from multi-well plates and time-lapse data.

A stage-top incubator was built by the group workshop team (Simon Johnson, Martin Kehoe) which is mounted on and encloses the stage. Heated air is supplied to provide a constant in-well temperature of 37°C for 384-well plates, measured with a digital thermopile. In live biological samples, the bicarbonate system requires a constant supply of 5% CO₂ in air, and this is provided through an enclosed stage-top plate insert (ASI) with pre-mixed CO₂ supplied via a PVC tube. The pre-mixed CO₂ is first hydrated by being bubbled through a water-filled beaker (with a porous ceramic fitting to the pipe) to prevent drying out the sample, and then a second empty beaker to collect excess water. The pre-mixed 5% CO₂ is switched on via a canister and regulator, with the pin valve adjusted so that an appropriate flow rate of CO₂, i.e. when bubbles originate from 2-3 sites from the porous ceramic submerged in the water. A custom-made objective water cap (manufactured by our workshop) is used to prevent the water immersion objective from drying out during imaging, and this is fed with ultrapure water (Milli-Q) using a peristaltic pump. Due to the size of the water cap and plate enclosure, the outer three wells of a 384-well plate could not be imaged since the cap would collide with the plate enclosure. The live-cell imaging apparatus described is depicted in Figure 2.3.

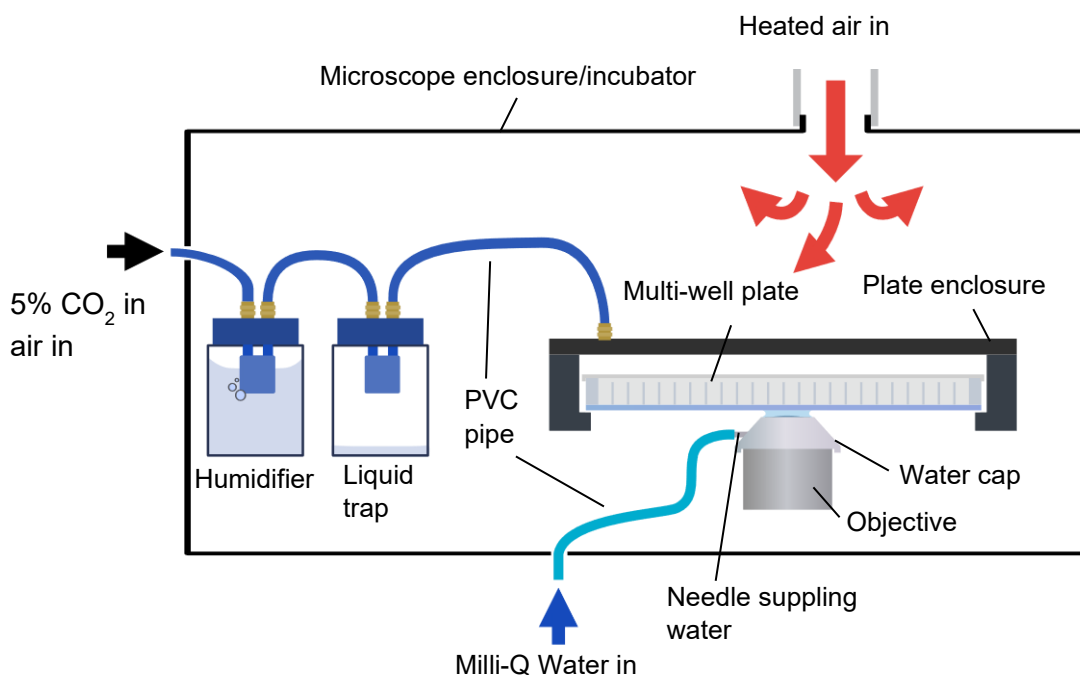


Figure 2.3 – Live imaging apparatus used in live-cell imaging experiments. The incubator built by the group workshop team is mounted on and surrounds the IX71 microscope frame, and hot air is supplied through inlet holes. 5% CO₂ in air is bubbled through water and supplied directly to a plate enclosure mounted on the XY stage, in which the well plate is held.

2.3 Current OPM setup

2.3.1 Optical and mechanical design

Through the work of Nathan Curry, a new optical design and configuration was developed that would enable three cameras on the detection arm. The system was then rebuilt using this design at the beginning of the PhD, and this is now the current OPM setup. Each of the changes is described in this section, with the CAD rendering of the current optical setup and the system diagram shown in Figure 2.4 and Figure 2.5 respectively.

In terms of mechanical design, the previous OPM system was built with a rod/cage system (Thorlabs) supported by adjustable height optical posts, and the current system was changed to have fixed height optical posts to improve alignment stability; these were machined by our workshop to the specifications on the CAD design.

Firstly, the illumination replaces L2, originally an achromatic doublet, with a $4\times$ objective (UPlanFLN, Olympus) lens for better chromatic performance since axial chromatic aberration of the light sheet was an issue in the previous system. For the desired light sheet NA, the objective must be underfilled and a demagnifying $4\times$ system consisting of L1a and L2b is used to achieve this; this does, however, still introduce some chromatic aberration. In the illumination arm, there is also a periscope system with two adjustable right-angle kinematic mirror mounts (Thorlabs) followed by another right-angle (fixed) mirror in the back focal plane of the cylindrical lens. The final mirror can then be replaced with a scanning galvanometer mirror to enable angle dithering of the light sheet which reduces shadowing artifacts in light sheet images. Light sheet dithering results in a time-averaged illumination over a small range of light sheet angles which suppresses any effect of the light sheet being impeded by absorbing and scattering regions of the sample, since a small angle change may avoid this obstacle.

The aberration performance of TL2 was improved through a manual search for an achromatic doublet pair to replace the -500 mm aplanatic meniscus and 150 mm achromatic doublet lens pair. Pairs of stock achromatic doublets which provided an overall focal length of 162 mm were tested in Zemax, and the best performance was found for a Linos $f = 300$ mm (model no. 322305000) and a Thorlabs $f = 300$ mm lens pair. In recent work by Wenzhi Hong [88], the procedure of finding the best lens combinations from stock optics for custom tube lenses has been automated by searching the Zemax library for existing optics and testing their performance. In addition to TL2, the $f = 100$ mm doublet used for TL3 was replaced with two Thorlabs $f = 200$ mm doublets with a 5 mm spacing, and this also improves aberration performance in addition to decreasing the overall magnification by approximately 1.6 . The total magnification is therefore 27.01 , which results in an object-space pixel size with a PCO edge 4.2 camera of $0.24\text{ }\mu\text{m}$, while the approximate lateral resolution of the OPM system is $0.5\text{ }\mu\text{m}$ [63].

On the detection arm, there are two dichroic mirrors which allow three cameras to be mounted: one each for the reflection from DC1, the reflection from DC2 and the

transmission from DC2. Each light path has a filter wheel and tube lens TL3, followed by a port to mount a camera. To date, only Camera 1 is in use and the filter wheel for Camera 1 is motorised (FW103H, Thorlabs) which is used to allow fully automated four-channel imaging when DC1 is replaced with a normal mirror, and this is described in further detail in the next section. The updated optics list for the current setup is shown in Table 2.2.

Table 2.2 – Lenses used in the current OPM setup.

Label in Figure 2.5	NA/magnification/focal length/immersion medium	Description
O1	$60\times$ / 1.2NA Water	Olympus objective
TL1	$f = 180$ mm	Olympus tube lens
TL2	$f = 162$ mm	Linco $f = 300$ mm and Thorlabs $f = 300$ mm separated by 38 mm
O2	$50\times$ / 0.95 Air	Olympus objective
O3	$40\times$ / 0.6 Air	Nikon objective
TL3	$f = 101.3$ mm	Two Thorlabs $f = 200$ mm lenses spaced by 5 mm

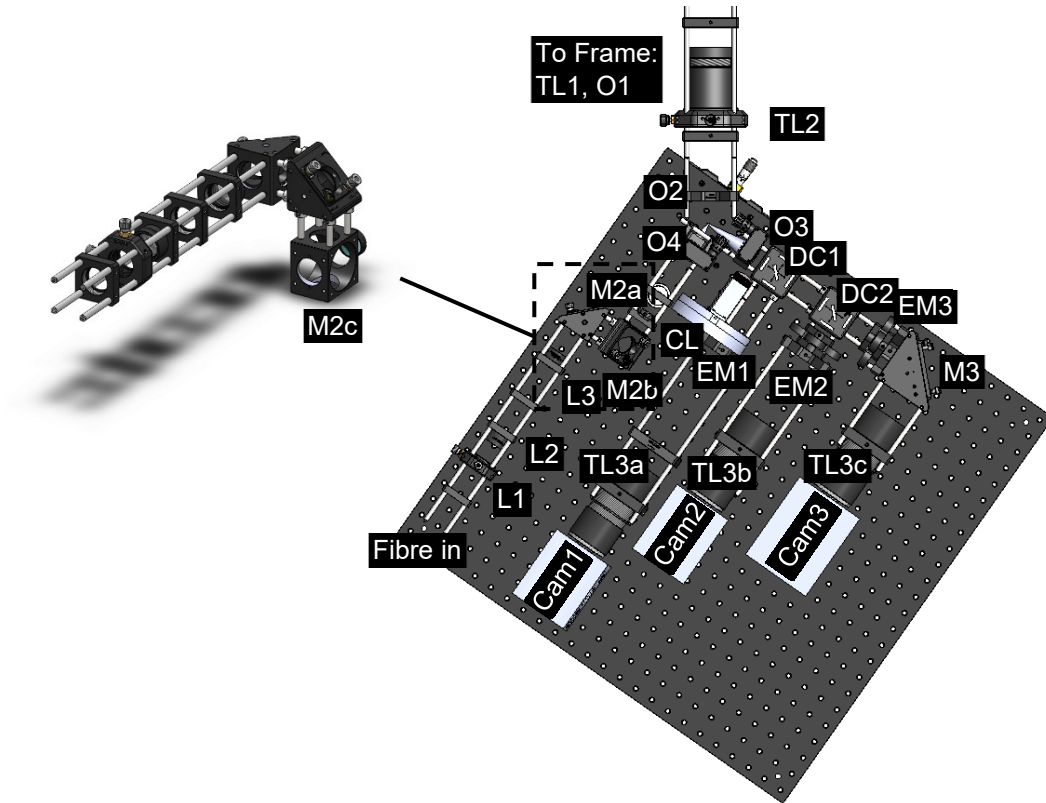


Figure 2.4 – CAD rendering of the current OPM setup breadboard. This breadboard is mounted on a rotation-translation stage allowing the OPM angle to be changed and the breadboard realigned with respect to the refocusing system as required; here it is shown rotated to $\theta_{OPM} = 35^\circ$.

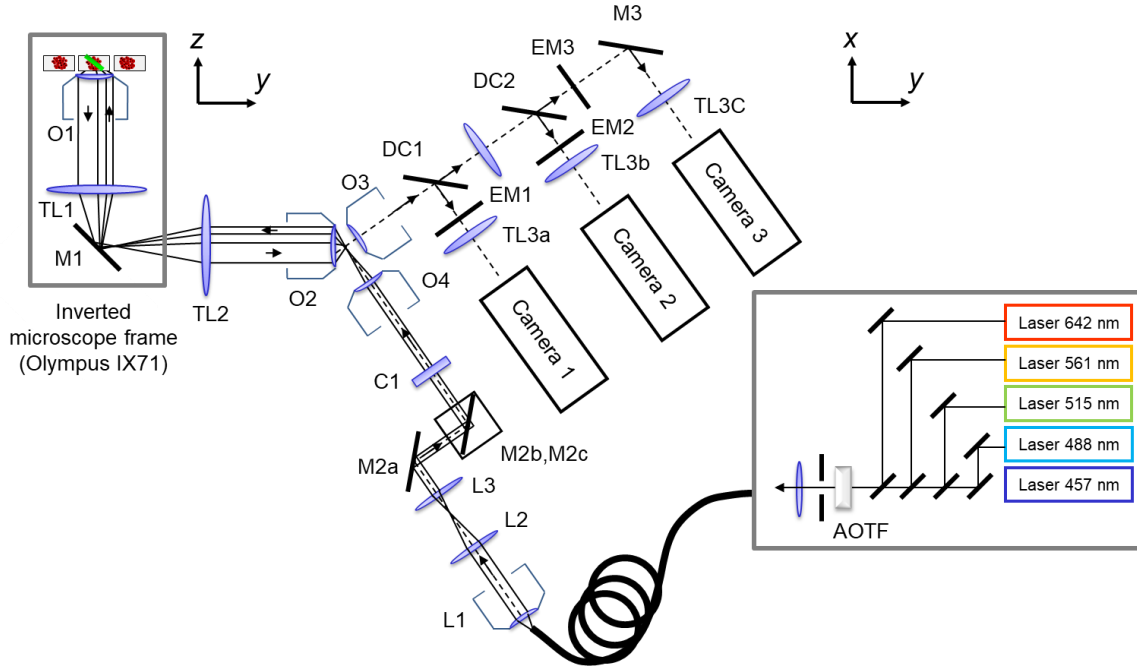


Figure 2.5 – Optical diagram of current OPM setup with three cameras. Currently, only camera 1 is used (PCO edge 4.2) with EM1 being a motorised filter wheel for automated multi-channel imaging. O – objective, TL – tube lens, L – lens, DC – dichroic mirror, EM – emission filter, C – cylindrical lens, M – mirror. M2b and M2c form a periscope (see Figure 2.4), with the intention to use a scanning galvanometer mirror in place of M2c for angle dithering.

2.3.2 Updated processing pipeline with compression

A standard raw OPM volume of dimensions $1280 \times 1000 \times 358$ px ($320 \times 705 \times 144 \mu\text{m}^3$) is 878 MB, and with multi-channel time-lapse imaging with multi-well plates, these datasets rapidly scale in size up to ~10 TB in the experiments presented in Chapter 4. It is therefore pertinent to find methods of image compression to save storage space on our server, ideally lossless compression. While developing the optical design of the three-camera OPM setup, Nathan Curry implemented Jetraw compression (Dotphoton) in the data processing pipeline: acquire data, compress with Jetraw, transfer to the server, decompress and perform reslicing and binning/downsampling. The compression is only used to store raw data, with the resliced data usually significantly smaller (62 MB compared to 878 MB for 4-factor downsampling) than the unresliced raw data. The compression stage of this updated pipeline is handled by a MATLAB script (**compressToDasTimestamp.m**) found in the same GitHub repository as the Java reslicing code, see link 1.3 on page 9. The script reads timestamp data (encoded with pixel values in the top-left corner of the frames), saves it in a **.mat** file and then calls the command-line Jetraw compression tool to compress the raw frames before copying it to our server. When reslicing the Jetraw-compressed data with **dotphotonReslice.m**, the **loadVolumStyle** variable is set to **'dotPhoton'** rather than **'matlab2020'** for reslicing raw data directly.

2.3.3 Enabling automated four-channel imaging

A Thorlabs Fast-Change FW103-H motorised filter wheel was added before the first camera, while manual filter wheels were used on the other camera arms. There was no MicroManager device adapter when the motorised filter wheel was added to the setup, so to control the motorised filter wheel in MicroManager and automatically during acquisitions, I wrote a device adapter in C++ which is available on GitHub, compiled for Windows x64 and MMDevice API version 33 (v0.2). The filters in the motorised filter wheel are listed below in Table 2.3.

Table 2.3 – Filters in FW103-H motorised filter wheel and the associated excitation laser wavelength.

Filter position	Centre wavelength/bandwidth	Associated laser wavelength	Model
Position 0	Empty (scatter)	N/A	N/A
Position 1	731/137 nm	642 nm	FF01-731/137-25 (Semrock)
Position 2	605/70 nm	561 nm	ET605/70m (Chroma)
Position 3	550/49 nm	515 nm	FF01-550/49-25 (Semrock)
Position 4	525/50 nm	488 nm	FF03-525/50 (Semrock)
Position 5	482/25 nm	457 nm	FF01-482/25-25 (Semrock)

The OPM has a laser box that combines 5 lasers, with the interlock system limited to using 4 lasers at a time, but initially, only 3 could be controlled by the NIDAQ (USB-6229) box at one time. This is because the acousto-optic tuneable filter (AOTF) (AA Opto Electronic), by default, controls laser intensity by an analogue voltage input that ranges from 0-10 V. The DAQ box has only 4 analogue voltage outputs with 10 V with BNC connectivity with one already used for the AOTF digital blanking channel which switches all laser lines on or off. This blanking channel pin on the AOTF has the same input voltage range as the laser intensity pins, and the digital pins on the DAQ only supply up to 5 V, so cannot be used when 10 V is the logical high. Each laser has a BNC connector that corresponds to a pin on the AOTF electronics board, so to image a sample which requires more than 3 different laser lines, imaging is carried out first with 3 laser cables connected, and then unplugged and replaced with the other laser(s) before running the imaging again. The motorised filter wheel therefore allows automatic three-channel imaging on a single camera but is otherwise limited by the DAQ outputs.

It is possible, however, to switch to 0-5 V input mode on the AOTF. We can therefore instead of using the analogue BNC connectors, use 1 of the 8 digital I/O BNC connectors for the blanking channel, with one being used to trigger the camera acquisition. The stage trigger output is wired to PFI2 (input), and the camera is wired to P0.0 via the USER1 BNC terminal and configured as an output. See details of the triggering system in Section 2.2.2.

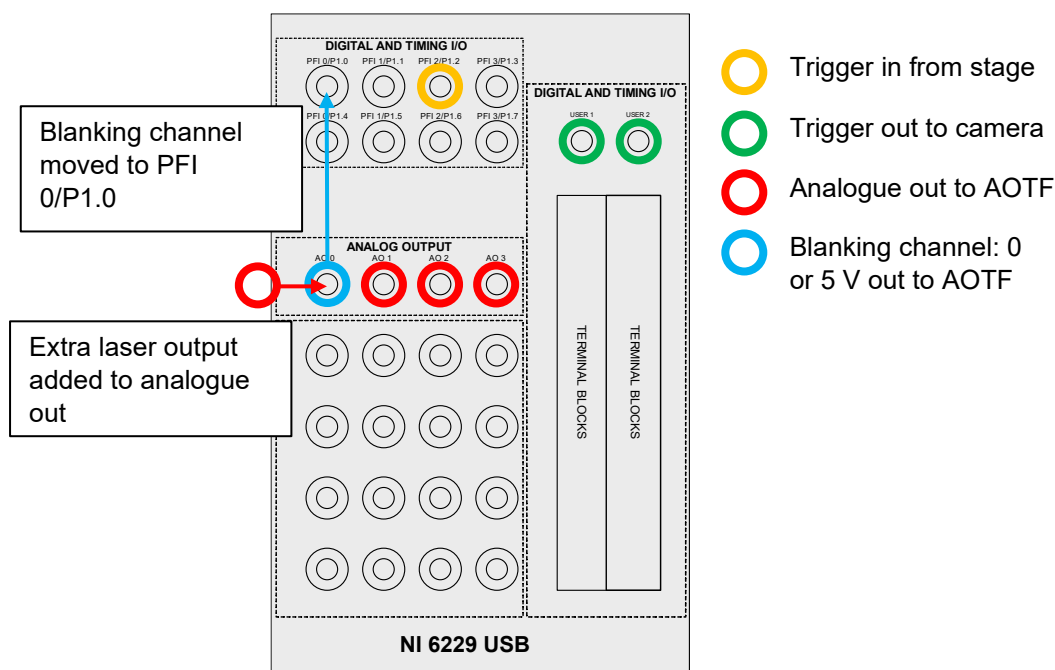


Figure 2.6 – Changes to DAQ wiring of inputs and outputs to allow all four lasers to be connected for automated four-channel imaging. AOTF blanking channel was originally wired to an analogue output, but after changing the AOTF input voltage range to 0-5 V it was wired to a digital output, freeing an analogue output for the fourth laser.

It was also necessary to make updates to the LabVIEW code such as limiting to output to 5 V to avoid damage the AOTF box, adding the ability to manually control all 4 lasers outside the acquisition loop, and allowing 1 of the 4 lasers to be selected from an array in the acquisition loop. The system of selecting the desired laser in the acquisition loop was achieved by writing to a text file **las_channels.txt** in MicroManager an integer from 0-3 indicating the laser to use, then reading this within LabVIEW to select from the array of laser analogue outputs to which the laser-controlling pulses were sent.

2.3.4 Time-lapse acquisition code upgrades

Within MicroManager, a time-lapse acquisition script was written in BeanShell by Sunil Kumar along with functions to control the XY stage and Z-drive within MicroManager and initiate the trigger loop described in Section 2.2.2 to acquire volumes. This script acquires volumes for each position in the MicroManager positions list (using a loop) and can also be repeated at time intervals defined by the user to perform automated time-lapse imaging.

The MicroManager acquisition script was then adapted to allow four-channel imaging, now using arrays for the laser channels, laser power, filter position, exposure time, and stage scan speed (for stage-scanned acquisition), with their length depending on the number of channels/filter combinations imaged. With the original two-camera setup, the two channels were obtained simultaneously or with interleaving within the LabVIEW code, and separate variables were set for each of the channels. With the single-camera setup with the motorised filter wheel, the multi-channel imaging was performed sequentially with an additional loop

for the laser and filter wheel position combination nested in the position list loop and time-lapse loop.

Following the first live imaging experiment shown in Chapter 4, several issues were encountered with the updated script, such as skewed volumes, missing data, incorrect FOVs and frame-dropping errors which caused the time-lapse imaging script to abort. Changes to the acquisition script and the hardware-control functions were therefore made to make the automated time-lapse imaging more robust.

The skewed volumes appeared to be affected in the y-direction, the scan direction, as seen in Figure 2.7 and hence it appears that the trigger distance y_{trigger} was set incorrectly (too low) for the XY stage such that there is oversampling in y (and the scan ends early). This was resolved by writing code to query the XY stage and ensure that the trigger distance is correct, and setting the trigger distance again until the correct trigger distance is reported back by the stage.

For the incorrect FOVs and missing data, it was believed that this was caused by the XY stage not reaching the position in time and incorrectly reporting that it had finished moving. this would happen when the XY stage speed was not updated from the scanning speed which was of the order 0.1 mm/ms, compared to 10 mm/ms used to move between wells. The stage position was therefore probed directly and the acquisition would wait until the correct position was reached, using a timeout so that the code would not hang.

Other miscellaneous issues that occurred randomly throughout the time-lapse acquisition, included frame drops, failures to set device properties with serial commands (e.g., stage triggering, stage scan speed) and erroneous serial commands received from the devices. These issues were mitigated by extensive error handling and retrying the current volume acquisition up to a maximum number of times before skipping onto the next volume. The script will abort if the maximum number of skips is exceeded.

Many of these issues point towards noisy serial connections, and it may be pertinent to investigate this by monitoring and logging these serial connections. However, the error handling method was sufficient for our needs, and the time-lapse code can now run for multiple days without error since implementing these issues. Before this, experiments would sometimes halt overnight, and this would result in missing timepoints and require the user to restart the acquisition. The current version of these MicroManager functions and acquisition scripts is hosted on GitHub (see mmgr-opm-scripts repository links on page 9).



Figure 2.7 - MIPs of y-skewed volumes in live plate 1 data with erroneous trigger distance setting for the XY stage.

2.4 Alignment procedures

The OPM performance is characterised extensively in Chapter 3 of Vincent Maioli’s thesis [86], including characterisation of the refocusing system performance, light sheet thickness, and the total detection PSF. Here, the alignment procedures developed in the PhD that are not already described in the previous literature and would aid a reader in the construction and maintenance of an OPM system are documented. These include the alignment of O2 and TL2 with O1 and TL1 to optimise refocusing system performance, and the coupling of the light sheet to the refocusing system and optimising its position within the OPM FOV and its focus.

2.4.1 Aligning O2 and TL2 in refocusing system

To ensure that the refocusing system forms an isotropic replica image of the sample space object, it is essential to ensure that O2 and TL2 are aligned laterally, and their optical axes coaxial. A lateral shift between O2 and TL2 results in a rotation of the rays which causes the image to be displaced laterally with a dependence on the axial position, hence forming a skewed replica image. This is therefore an important step in aligning an OPM system and a formalised process was developed with the assistance of Nathan Curry.

The alignment is done with the OPM set to zero degrees by rotating the breadboard and using brightfield illumination of a resolution/USAF target in the sample plane, carrying out the following procedure:

1. Using the MicroManager live view of the OPM (PCO) camera, bring the USAF target into focus (Figure 2.8(a)) and draw a selection box around a well-defined region on the USAF target, e.g., a set of lines. Choose two Z-stage positions over a suitable range e.g., 100 μm so that $\Delta Z = \pm 50 \mu\text{m}$, over which to change the z position of the objective.
2. After changing the Z-stage position in one direction by ΔZ , refocus with the detection options (adjust the axial actuator on O3) to bring the target back into focus (see Figure 2.8(b)).
3. Adjust X and Y actuators on O2 (or TL2) to bring the object into the selection box.
4. Move to the other extreme of focus (Figure 2.8(c)), refocus with O2 again in the opposite direction and use instead the TL2 (or O2) X and Y actuators to bring the object back into the box.

5. Iterate with steps 3 and 4 until the object remains centred in the selection box for both foci. Check this again for the nominal zero defocus position in Figure 2.8(a).

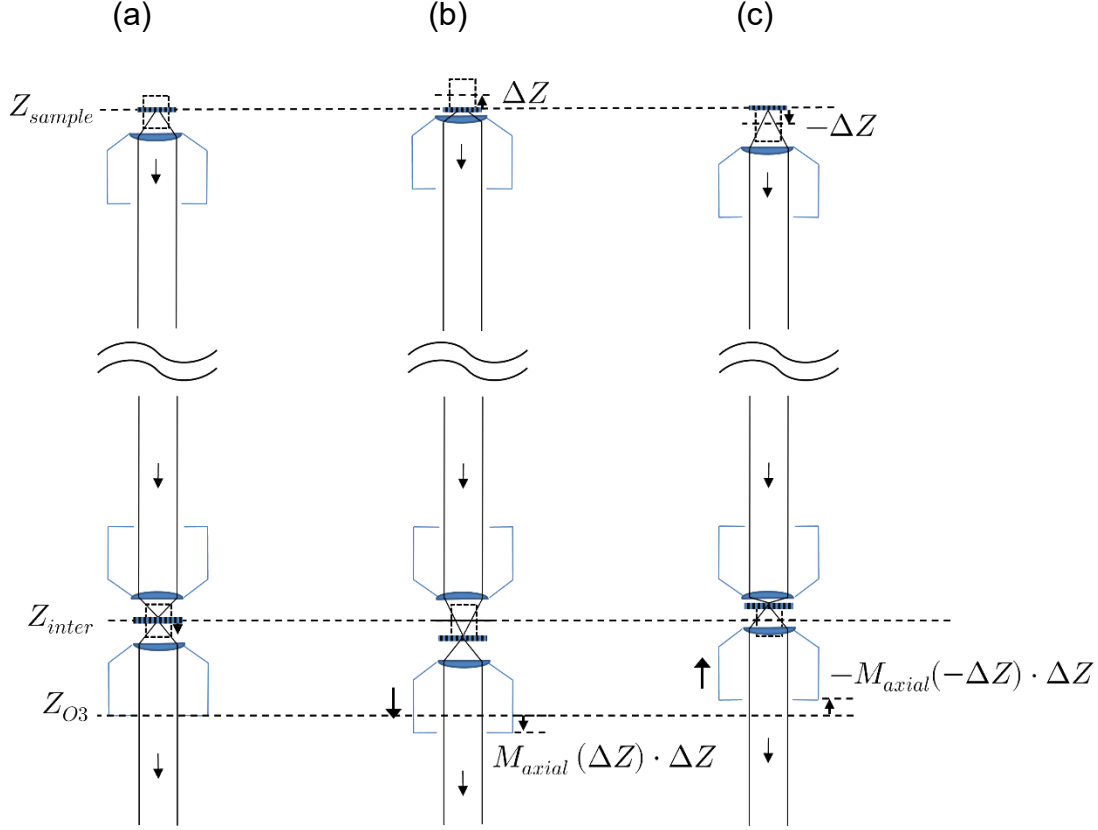


Figure 2.8 – Procedure for refocusing sample onto OPM camera with O3 when O1 is defocused, using a resolution target as the sample. Procedure is used both for pupil alignment and magnification characterisation of the refocusing system. (a) Sample is placed at Z_{sample} and is in focus for microscope eyepieces as well as on the OPM camera. Intermediate image plane is Z_{inter} and nominal O3 axial position Z_{O3} . (b) when O1 is defocused by ΔZ , the axial position of O3 is changed to refocus the remote image onto the OPM camera. The O3 refocus distance is $M_{axial}(\Delta Z) \cdot \Delta Z$ where M_{axial} is the axial magnification of the refocusing system as a function of defocus (which is roughly constant within the refocusing range). (c) Same as (b), but with defocus in the opposite direction. The dashed square indicates the approximate region where there is ‘aberration-free’ refocusing.

The procedural method of optimising TL2 and O2 each for different object z positions is iterative and should reliably converge on the best possible alignment; it is similar in nature to the technique of walking a beam onto a new path with a pair of mirrors through a pair of irises on the new beam path.

By optimising both the positions of TL2 and O2, with each optimised for different object z positions, we ensure that we have aligned for both jointly. If the optical axis of either O2 or TL2 is not aligned with the optical axis of O1, there will be an inherent rotation of the rays. Because this results in a lateral displacement of the remote image that changes with O1’s defocus, and there would be no possibility of maintaining the object in the same position in the FOV (in the selection box).

2.4.2 Coupling light sheet to O2 and light sheet optimisation

From the OPM set at 0° , before the breadboard is rotated to the desired OPM angle (35° for all imaging in this thesis) it is first necessary to translate the breadboard (in the axial direction) to allow physical clearance of the objectives before rotation—for this reason it is necessary to realign the system each time the breadboard is rotated to couple the light sheet into O2.

Ordinarily, a thin fluorescent sheet sample, e.g. a thin sheet of rhodamine, is used to view the fluorescence from the light sheet and it appears as a strip/band of fluorescence in the OPM camera. The following steps are required when the breadboard has been rotated, or the system has become misaligned such that fluorescence from the light sheet cannot be observed in the OPM camera.

1. A surface of the fluorescent sheet is first brought into focus for the eyepieces using LED.
2. In the OPM view now, using a laser below the ELV, translate the breadboard so that the light sheet formed by CL and O4 is visible on the front surface on O2, and translate to optimise the light sheet thinness observed in the intermediate image space between TL1 and TL2.
3. At this point, a (perhaps wide) band fluorescence should be observed on the OPM camera – if not, a Chroma fluorescent slide may also be used initially, and the breadboard position optimised to maximise fluorescence signal on the camera before switching back to the thin sheet.
4. The band of fluorescence can then be brought into the centre of the field of view with the actuator which translates the breadboard towards O2 (axially).
5. Using the axial translation actuators on O3 and O4 in tandem focus the light sheet such that the thickness of the line on the camera is minimised, as the sheet is focused, ensure laser power is low e.g., 0.25-0.5 V to avoid photobleaching.

Now that the light sheet has been coupled, the focus and position of the light sheet can be optimised. The following steps should also be performed before any imaging experiment and involve ensuring the waist of the light sheet is in the centre of the FOV of the OPM camera, and that the centre of the OPM camera FOV is also conjugate with the centre of the FOV of the eyepieces.

6. Find and bring into focus a small feature on the fluorescent sheet with the eyepieces using epifluorescence, e.g., a dark spot as per the configuration in Figure 2.9(a).
7. If not done already, translate the breadboard in the axial direction to ensure the light sheet is in the centre of the OPM camera FOV—shown in Figure 2.9(e)—then translate the breadboard laterally until the feature coincides with the light sheet—shown in Figure 2.9(d).

8. Optimise for minimum thickness of the light sheet with the axial actuators on O3 (Figure 2.9(c)) and O4 (Figure 2.9(b)) using the plot profile tool in MicroManager for the light sheet, maximising for peak height. Repeat step 7 if necessary.

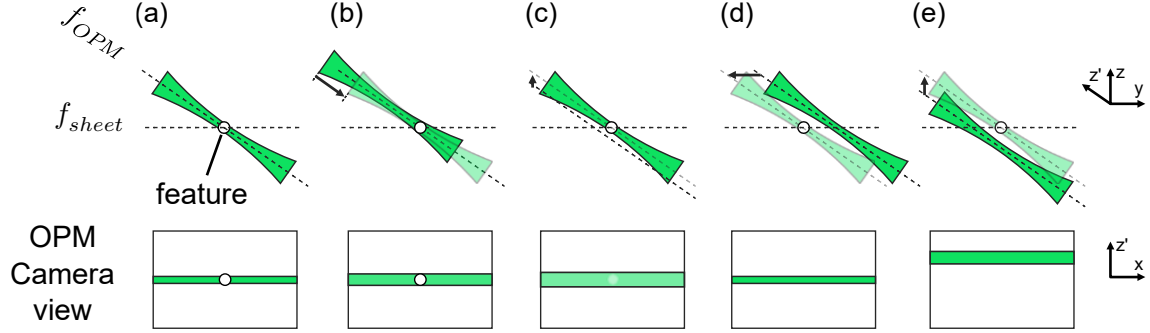


Figure 2.9 – Cases of light-sheet and/or camera defocus and how they are observed on a camera. A fluorescent sheet sample is in the plane f_{sheet} , such that the fluorescent sheet sample and a distinguishable feature, e.g., a localised area without fluorescence, are in focus for the eyepieces under epifluorescence. The image plane f_{OPM} for the OPM view is indicated by the dashed line. (a) Correctly aligned OPM setup—feature in focus for eyepieces and for the OPM camera view, and light sheet waist is positioned on the feature. (b) Effect of axial misalignment of O4: the light sheet waist is not positioned in the centre of the OPM FOV and not on the feature. (c) Axial misalignment of O3 means the OPM image plane is not in the light sheet plane. The effect of (d) horizontal and (e) axial translation of the breadboard. When the breadboard is misaligned (d) horizontally (with respect to the view of the eyepieces) the OPM FOV is not centred on the feature. (e) Axial translation of the breadboard (i.e. towards O2) is equivalent to axial scanning O2 as utilised in remote scanning OPM. The axial position of the breadboard should have the waist of the light sheet positioned in the plane of the fluorescence sheet sample, f_{sheet} . In each case the arrow indicates the necessary translation to correct for the misalignment/defocus.

2.5 Conclusion

In this chapter, the initial stage scanning OPM setup as it was at the beginning of the PhD was presented in detail, along with the existing data acquisition techniques. The optical and mechanical changes to the OPM according to the designs by Nathan Curry were then described. These changes included extending the detection optics to allow a third (OPM) camera, adding a periscope in the illumination arm to allow a galvo scanner to be added for light sheet dithering, and implementing a motorised filter wheel for one of the cameras. The extra cameras and galvo scanner are not yet implemented; at this point, only the single camera with the motorised filter wheel is used for imaging. Nathan Curry and I built this new design together to replace the initial setup, and I made further instrumentation and software upgrades to enable automated four-channel time-lapse imaging for the experiments described in Chapter 4. This included writing a device adapter for the filter wheel, changing the wiring of the DAQ system, and updating the LabVIEW and MicroManager code accordingly. In addition to this, error handling was added to the acquisition script in MicroManager to prevent the script from crashing mid-experiment and issues with missing data and skewed volumes were fixed.

Finally, alignment protocols were described that are not yet documented in literature, and these methods were formalised with the help of Nathan Curry. The first of these was the

procedure for aligning the objective O2 and tube lens TL2 with the primary microscope so the optics in the refocussing system were all coaxial, and this ensures isotropic magnification performance of the refocusing system. The other procedure that was presented was the step-by-step method for coupling the light sheet illumination to O2 and optimising the focus and position of the light sheet.

3 Spheroid pre-find

In the experiments presented in chapter 4 of this thesis, 384-well microplates are utilised to perform live 3D imaging of 180 wells over 8 days on our OPM setup. The wells in these microplates have base dimensions of $3.3 \times 3.3 \text{ mm}^2$, while the spheroids themselves start at $100 \text{ }\mu\text{m}$ in diameter and the field of view (FOV) of the $60\times$ objective lens used for OPM imaging is $440 \text{ }\mu\text{m}$. The FOV is calculated using the Olympus microscope specifications [89] to be

$$\text{Field of View} = \frac{\text{field number}}{\text{magnification}} \quad 3.1$$

where the field number is the maximum diameter of the intermediate image (that can be observed through an eyepiece).

This FOV is significantly smaller than the dimensions of the well, and therefore the spheroid is likely to be outside the FOV for a given position within the well. It is therefore necessary as a first step to locate the spheroids within the well in a process we call pre-find. Although it is possible to do this manually, the process is time-consuming for a large number of wells, motivating its automation to maximise the potential of the high-throughput platform. The techniques presented here are likely applicable to samples other than spheroids, but since the methods have been tested on and applied to spheroid samples, we present this as the pre-find process for spheroids in particular.

At the beginning of my PhD, pre-find methods were already implemented with higher FOV, lower magnification epifluorescence imaging with a $4\times$ objective. These low magnification pre-find methods found the approximate lateral (x and y) position of spheroids so that they are at least within the FOV of the $60\times$ objective, but the process of finding the correct focus and centring the spheroids in the $60\times$ FOV had to be done manually.

In this chapter, the pre-existing low-magnification (2D) pre-find method in its original implementation is introduced as the initial pre-find, followed by my extensions to this method. Initially, the method was adapted for samples expressing four different fluorophores by analysing epifluorescence images of each well taken in multiple channels, but this was later unused when the experiments in Chapter 4 began using a common channel (for segmentation).

After the common channel was introduced, a new low-magnification pre-find method was devised with increased automation and improved workflow. In addition to the low magnification pre-find, autofocus by detecting the coverslip height and a 3D spheroid pre-find process was developed, both using the $60\times$ objective. These two new processes removed the need to manually find correct focus and centre the spheroid in 3D within the OPM FOV.

The current, full pre-find process using the two different magnifications is summarised by the flowchart in Figure 3.1, and in the following sections, each step will be described in detail. In addition to being used in the pre-find, the coverslip detection and 3D spheroid pre-

find processes are also repurposed to automatically correct for small changes in plate height and sample drift respectively throughout a time-lapse experiment.

The scripts used throughout the current pre-find process are written in BeanShell for MicroManager 2.0 and are available in the GitHub repository under the pre-find folder (see **mmgr-opm-scripts** links on page 9).

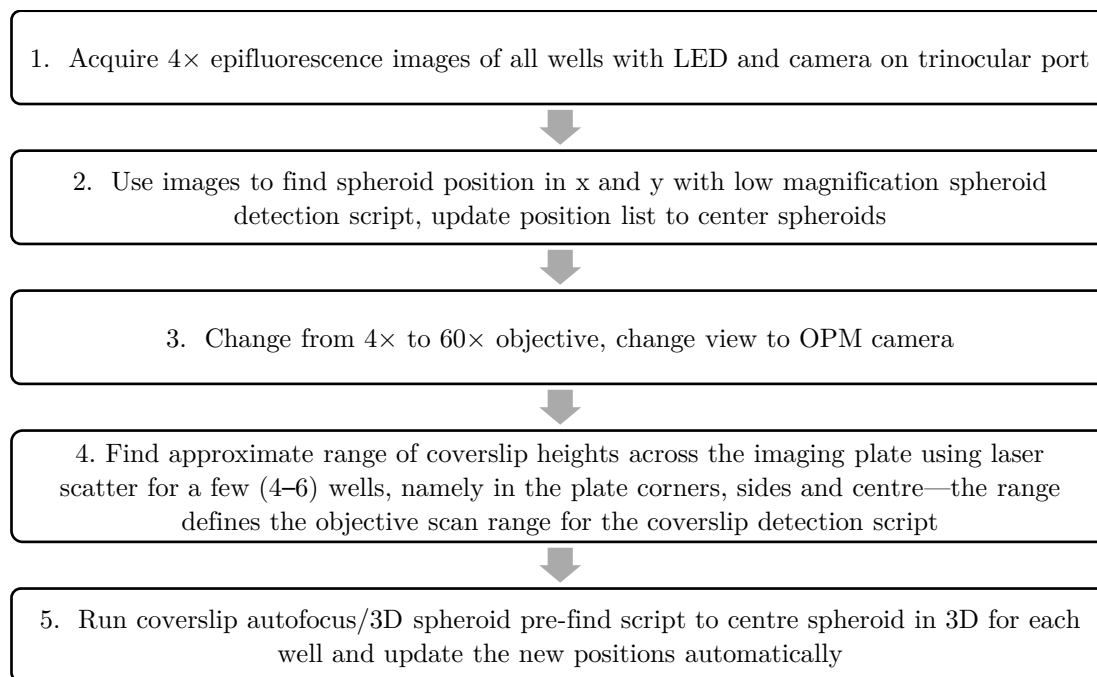


Figure 3.1 – Flowchart for stages of the full, current pre-find of spheroids in multi-well imaging plates. Note that the initial pre-find method only includes Stages 1 and 2.

3.1 Initial pre-find of spheroids in widefield

3.1.1 Low-magnification pre-find

Before starting work on the pre-find process, low-magnification pre-find had already been developed by Ranjeet Kumar, Nathan Curry and Chris Dunsby. This original procedure only included Stages 1 and 2 of the current full pre-find process shown in Figure 3.1, while the rest was performed manually. The epifluorescence imaging modality described in Section 2.2.1 was used with LED (Thorlabs) illumination, the 4× objective (Olympus, UPlanFl 0.13NA) and the Andor camera, which was mounted on the trinocular port of the microscope frame.

The initial pre-find procedure is described in this section for a 384-well plate. Starting with Stage 1, the procedure consists of the following steps:

1. Bring a known well into the centre of the epifluorescence camera (Andor) FOV as a reference position—D4 is used here since this avoids issues of the sample holder and water-cap clearance as discussed in Section 2.2.4.
2. With the stage at the reference position, use the BeanShell script **excel positions 384 plate d4 underscore.bsh** (see link 2.4), generate a

positions list according to the plate layout defined in an Excel file **position.xls**, which marks occupied wells with ones and empty wells with zeroes in a 24×16 grid.

3. Using an LED and dichroic filter cube appropriate to the fluorophore(s) in the sample, find an occupied well and bring the image into focus on the epifluorescence camera.
4. At current focus, i.e., objective z position, use the multi-dimensional acquisition (MDA) tool in MicroManager to acquire images of all wells selected in step 1. Repeat the MDA with defocus (typically $\pm 100 \mu\text{m}$) in both directions from the initial focus to account for the variation in spheroid and coverslip heights across the plate.

In Stage 2 of the original procedure, this epifluorescence data is processed by the spheroid detection code (written in MATLAB by Ranjeet Kumar and Chris Dunsby) which finds the spheroid positions in each well. The output is used to update the MicroManager position list. The specific processes are as follows:

1. For each well, the 3 images at different foci are combined using a median z-projection.
2. The median fluorescence image is convolved with a spheroid-finding kernel with a positive circular central region of radius approximately equal to the expected spheroid size, surrounded by a ring of negative values (similar to a Laplacian kernel). The magnitude of the negative values is chosen so that the local image background surrounding the spheroid is averaged and effectively subtracted from the central region.
3. The brightest pixel (global maximum) of the convolved image, which is ideally the location of the spheroid, is found relative to the centre of the field of view (FOV) in pixels and then converted into x and y coordinates in microns.
4. For each position in the position list, the x and y positions of the spheroid from the centre of the FOV are added so that the new position has the spheroid in the centre of the FOV.

There were a few issues associated with the initial method. Firstly, the MATLAB code made false detections at the edge of the well—as shown in Figure 3.2—since the cell medium is slightly autofluorescent and this extends up to the edge of the well. When the spheroid detection kernel is applied, the convolved image therefore has a bright ring at this edge caused by the edge-detecting properties of the kernel. In addition, the method is sensitive to the manual choice of input parameters—when spheroid diameters are mismatched to the kernel diameter, or the fluorescence from the spheroid is dim, the spheroids are less likely to be detected (false negatives). If the detection threshold is reduced to increase the true positive detection rate, then false positive detections are more likely.

To acquire the images at three different levels of focus, three separate plate scans were acquired, and the focus was changed manually for each acquisition. The option to set up the MicroManager MDA plugin to acquire image sets at different z positions automatically

exists, however, using this option caused an error in MicroManager communication with the z-focus drive. This appears to be a software bug within MicroManager itself or in the MicroManager device adapters.

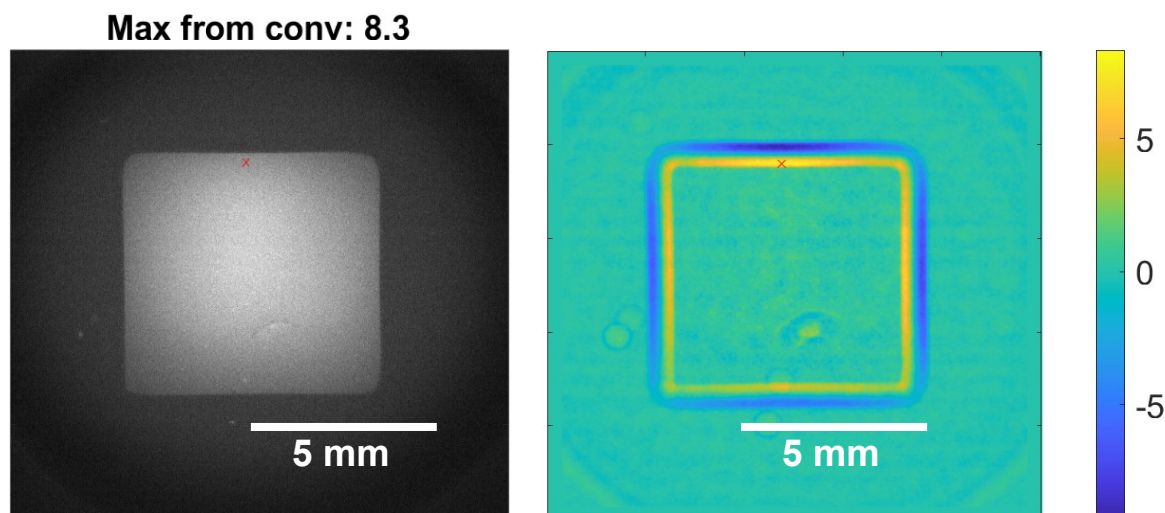


Figure 3.2 – Case of a false positive spheroid detection at the edge of the well in a fixed spheroid experiment due to the edge detection property of the spheroid-detecting kernel. Image of the well is acquired with the 4× objective and epifluorescence with the 625 nm LED.

Finally, there are cases where it is desirable to perform a pre-find in multiple fluorescent channels; for example, on a plate containing different samples that do not share a common fluorophore.

The next section describes changes made to the pre-find procedure, both in the instrumentation and software, to overcome issues in performing pre-find for multiple fluorescent channels, without a common fluorescent channel.

3.1.2 Multi-channel low-magnification pre-find upgrade

The initial setup had a single epifluorescence LED source mounted on the rear port of the frame and the light was collimated by a fixed-position lens before entering the microscope. Therefore, every time a different fluorescent channel was needed, the current LED had to be manually unscrewed and replaced on the back of the frame. This was time-consuming, risked damage to the LED cables over time if care was not taken, and different wavelength LED sources were collimated differently by the lens due to longitudinal chromatic aberration in this lens.

The experiments in Chapter 4 involved imaging spheroids composed of a combination of different cell lines. Each cell line expressed one of four different fluorescent proteins tagged to the nuclear H2B protein indicating the expression of a specific oncogene. In the first iteration of experiments, there was no single wavelength that could be used in the pre-find

step to excite fluorescence in all cells, and therefore required four LEDs to be used in the pre-find—one for each fluorophore.

For this reason, an assembly was developed to replace the single LED on the rear of the microscope with one that combines light from four LEDs using dichroic mirrors, see Figure 3.3(b). Each LED has its own collimating lens, and this allowed each to be collimated independently to avoid the axial (longitudinal) chromatic aberration that was in the original setup. In the original setup, the 430 nm LED source in particular resulted in the structure of the source being visible in the sample plane, see Figure 3.3(a).

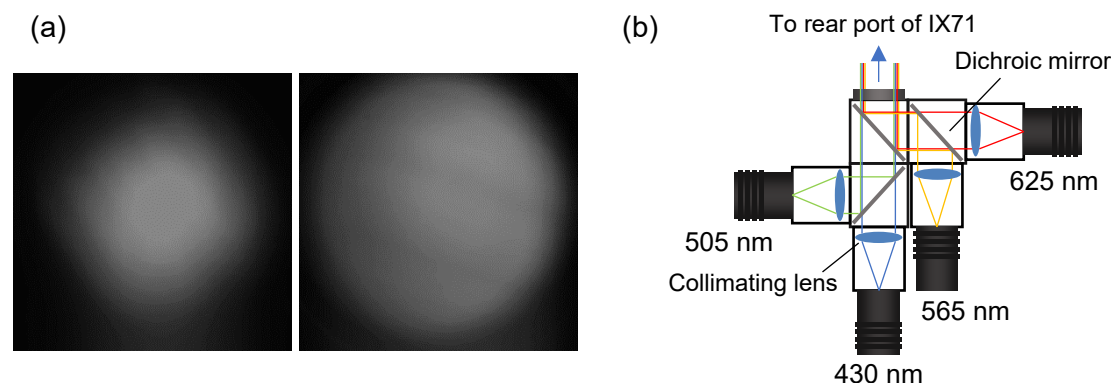


Figure 3.3 – (a) Images from illumination of a fluorescent sheet with the 430 nm LED, before (left) and after (right) the new assembly. (b) Diagram of four LED combiner assembly which combines the four LEDs using dichroic mirrors.

The initial pre-find code assumed a single fluorophore was common in all samples, and so the MATLAB code was also necessarily updated to include the images taken with the different LEDs. This was done using the same process as detailed in the previous section but repeating it for each channel, comparing the brightest pixel in the convolved image across all channels and selecting the channel in which it is brightest. The location of the brightest pixel in the convolved image was used to compute the necessary x and y location of the spheroid in the FOV as usual, and the spheroid location was used to update the position list.

These changes did not address the well-edge issue mentioned above, but the independent collimation of the different LED sources improved the illumination to contribute to better pre-find image data. Furthermore, changes described in the following section further increased the signal detected from the spheroid.

In another attempt to improve spheroid detection, an alternative approach to spheroid detection using a Circular Hough Transform was investigated. This resulted in fewer false detections, but also fewer positive detections and had to be manually and finely tuned to get an acceptable detection rate. Ultimately, ImageJ's Analyze Particles command was investigated for spheroid detection, described in more detail below.

3.2 New pre-find process

3.2.1 Low-magnification pre-find

While the new LED illumination assembly described above in Section 3.1.2 addressed the issues associated with a non-common fluorescent channel, it was found that the variation in the intensity of the four different H2B-labelling fluorophores was too large to allow reliable quantitative data analysis. In particular, there were concerns that differences in nucleus counting and segmentation between the different fluorophore channels might bias the resulting data. Therefore, it was decided that a common fluorescently tagged H2B protein would be used for segmentation, meaning that the pre-find could be performed using a single spectral channel—the multi-channel pre-find was no longer needed. The development of the system then focused on improving the workflow and continuing to improve the detection method to avoid false detections at the edge of the well.

The workflow was improved in two ways. Firstly, a BeanShell acquisition script that can acquire images at a number of different z positions was written to replace the Micromanager MDA. Ideally, we would have solved the bug that prevented the MicroManager MDA plugin from acquiring images at multiple z positions, but this was not possible at the time.

Secondly, the code for detecting the spheroids in the pre-find images was rewritten in BeanShell so that the pre-find image acquisition and spheroid detection scripts can be run sequentially within MicroManager, with the positions list updated automatically. In the initial implementation of the pre-find, the MATLAB code edited the MicroManager positions list file and the positions list file standards appeared to change between MicroManager versions; this was not robust. Later, MicroManager scripts would load an external file generated by MATLAB, but this was also less efficient than the current process where everything is executed within MicroManager.

The spheroid detection method used in the new low-magnification pre-find procedure employed the Analyze Particles plugin in ImageJ [90] along with image pre-processing to reduce false detections. While the uniformity of the fluorescence illumination was improved through the ability to adjust the LED collimation, it was found that further flat-fielding was required. This was achieved by subtracting the average (mean) image of all FOVs from each image. This relies on there being a sufficiently large number of FOVs in the data set so that bright spots in any individual FOV do not cause a problem. It also relies on the position of the sample being random within the FOV to avoid the estimated background being too high in regions where samples are more likely to be. Generally, around 200 FOVs were imaged and the sample location within a well (FOV) appeared sufficiently random for these conditions to be met. Eventually, however, the median was implemented instead of the mean average which should suppress bright spots, but it was not used during any experiments presented in this thesis.

Noting that steps 1 and 2 are unchanged from the initial pre-find procedure, the new low-magnification pre-find procedure is as follows:

1. Bring a known well into the centre of the FOV of the epifluorescence camera as a reference position—D4 is used here.
2. With the stage at the reference position, generate a position list according to the plate layout defined in an Excel file **position.xls**, which marks occupied wells with ones and empty wells with zeroes in a 24×16 grid.
3. Using an appropriate LED and dichroic filter slot on the filter wheel carousel, find a well containing a spheroid and bring the image into focus on the epifluorescence camera.
4. At the current z position, run the pre-find acquisition script, defining the number of z positions per well and z-scan (i.e. focus) range.

The pre-find acquisition code acquires z-stacks of images for each well—having images taken at a range of z positions accounts for the variation in coverslip height and spheroid z positions across the plate. The new spheroid detection script, see link 2.3, is then used to update the xy positions for each well to centre the FOV on a spheroid. The new spheroid detection process for the pre-find is illustrated in Figure 3.4, and the step-by-step process is as follows:

1. For each slice calculate the histogram of the image (Figure 3.4(a)).
2. Threshold the image using the histogram, retaining the k highest pixel values in ImageJ (Figure 3.4(b)). The value of k is tuned to the size of spheroids in the images to ensure all potential spheroid candidates are detected and is supplied to the script as **spheroid_px_diameter** (equal to the square root of k , i.e. k is proportional to the spheroid area in the image).
3. Run ‘Set Measurements’ in ImageJ (under the Analyze menu in the GUI) to choose the stats Max, Mean, Area, Center of Mass and enable thresholding by Area.
4. Use ImageJ’s Analyze Particles (Figure 3.4(c)) to get the stats (defined in step 3) on connected regions of pixels.
5. Select the most likely spheroid candidate using the region with the highest mean value (biggest and brightest spheroid), maximum value (brightest spheroid) or maximum area (biggest spheroid), depending on user choice for metric used.
6. Repeat the process for each slice, using the slice which contains the region with overall best metric and use the centre of mass (XM, YM in Analyze Particles) as the spheroid position to update the xy position in positions list. The centre of mass is calculated using

$$XM = \frac{\sum_i^A I(x_i, y_i) \cdot x_i}{\sum_i^A I(x_i, y_i)}, \quad YM = \frac{\sum_i^A I(x_i, y_i) \cdot y_i}{\sum_i^A I(x_i, y_i)}, \quad 3.2$$

where $I(x_i, y_i)$ is the pixel value for the pixel at (x_i, y_i) , i denotes a pixel within the connected region detected by Analyze Particles which contains A pixels.

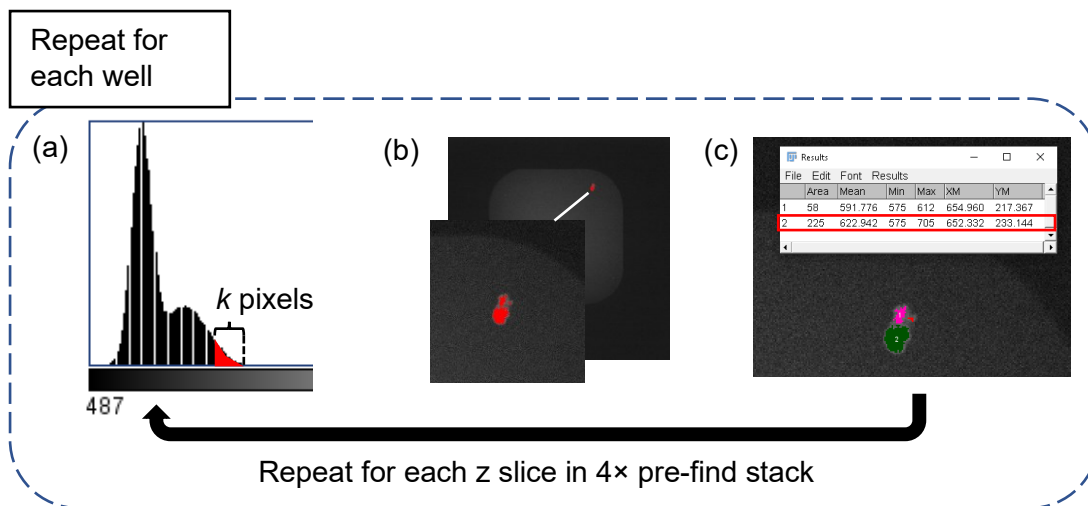


Figure 3.4 – New spheroid detection process using epifluorescence imaging under low magnification, applied in a loop over the positions list. (a) Find the k pixels with the highest values and (b) select these with the threshold tool. (c) Use Analyze Particles plugin in ImageJ to detect regions and, depending on the method used, select the “brightest”, “biggest” or “brightest and biggest” region (red box).

This new pre-find procedure would locate nearly all spheroids if visible by eye, although fluorescent fibres sometimes caused false positives.

3.2.2 Coverslip detection (autofocus)

The coverslip base of imaging multi-well plates is typically not flat, resulting in variation in coverslip height as a function of well position and FOV position within each well.

Furthermore, plates may not be mounted entirely flat, and some amount of tip or tilt may add to the intrinsic variation of the surface to cause coverslip height variations across the plate as much as 200 μm such as seen in Figure 3.5, which is real coverslip height data taken during a live imaging experiment. There is also variation in the height of the spheroid in the Matrigel matrix relative to the coverslip, and occasionally, spheroids are unimageable from being located beyond the working distance of the microscope.

The working distance of our primary objective O1 (Olympus 60× 1.2NA water UPLSAPO60XW) is 280 μm , which is similar to the potential coverslip height range. The variation in coverslip height and spheroid height relative to coverslip makes it likely that the range of absolute spheroid heights is beyond the working distance of the objective. Therefore, care must be taken to prevent the front surface of O1 colliding with the plate coverslip when moving between wells and while performing the 3D pre-find to locate spheroids to image.

The 3D spheroid pre-find involves searching for spheroids at increasing heights, moving the objective towards the coverslip sequentially within the working distance range. Due to the coverslip height variation, the range of acceptable absolute z positions of O1 depends on the coverslip height at the current position on the plate.

The z position O1 is therefore constrained by the condition

$$z_{\text{coverslip}} < z < z_{\text{coverslip}} + WD, \quad 3.3$$

where WD is the working distance and $z_{\text{coverslip}}$ is the absolute z position (height) of the coverslip-sample medium interface; in our case this is the top surface in our inverted configuration. Therefore, to search for spheroids in the correct, safe range, $z_{\text{coverslip}}$ must be found using the autofocus procedure described in this section.

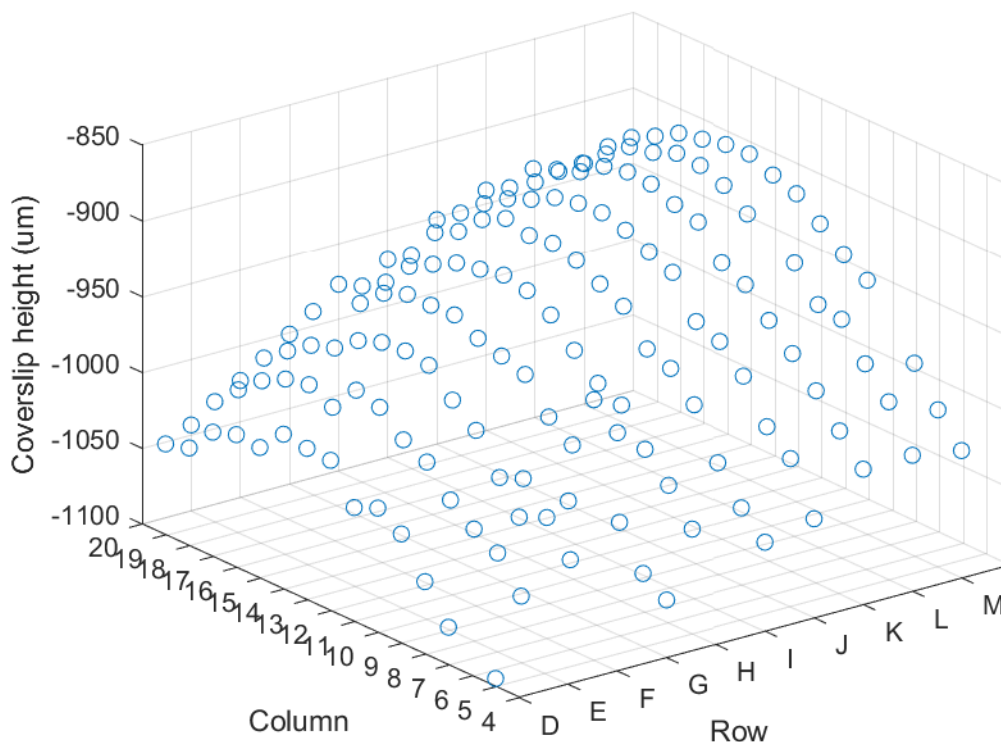


Figure 3.5 – Height variation of coverslip across 384-well microplate (water-glass surface), measured using automated coverslip detection.

Modern commercially available microscope frames, e.g. Olympus IX83, Nikon Eclipse Ti, implement hardware-based coverslip autofocus, namely TruFocus and Perfect Focus respectively. Both of these commercial autofocus methods can be used to find initial coverslip height, $z_{\text{coverslip}}$, for each field of view, but also have a continuous mode which detects fluctuations or changes in coverslip height. In time-lapse imaging applications, drift in coverslip height such as due to thermal expansion or the slippage of the focus drive can be significant, and if we want to maximise our effective z-FOV, we want to minimise the effect of this [91].

The IX71 is not compatible with the Olympus TruFocus module, so in our setup, a custom autofocus method was devised by imaging the laser reflection from the coverslip-water interface. In OPM, the reflection is observed as a line (see Figure 3.6), the height of which indicates the height of the coverslip and together with the current objective z-position $z_{\text{coverslip}}$ can be found. This method is used both in the pre-find and to compensate for focus drift during time-lapse imaging after every time-point—this is not as frequently as the

commercial systems which can detect drift immediately before imaging a well but met our needs sufficiently. The implementation of this method in MicroManager is described in the following section.

3.2.3 Custom coverslip detection method

Automatic coverslip detection for the OPM setup was achieved using a BeanShell script within MicroManager which communicated with LabVIEW to control the laser power and acquire images of reflection from the bottom surface (surface closest to the objective) of the coverslip while changing the focus until the coverslip is detected (or a maximum height is reached).

The script requires input parameters for the lowest and highest z position of the objective (furthest and closest to coverslip respectively) which define the range over which to search for the coverslip—this is to ensure that the objective is not driven to collide with the plate if no coverslip is detected. The lowest z and highest z positions, $z_{\min}$ and $z_{\max}$, are found by manually measuring the coverslip height for a range of positions over the plate, e.g., the middle, corners, and mid-points of the plate edges. The values of $z_{\min}$ and $z_{\max}$ aim to approximate the full range of coverslip heights across the imaging plate.

Coverslip height is measured while monitoring scattered light images obtained using the 642 nm laser at low power and with the emission filter wheel in the empty position. The user starts with a low objective z position, e.g., 500 μm from where the well is in focus when viewed in brightfield image through the eyepieces. Using the laser and OPM camera, the z position is increased until the line of reflection from the bottom surface of the plate is seen in the centre of the FOV of the camera. The scan range used in the automatic coverslip height detection script is increased by ~ 50 μm either side compared to the range found with $z_{\min}$ and $z_{\max}$ to account for extra possible variation, see **starting_z** and **max_z** in Table 3.1, which lists the parameters used in the automatic coverslip detection script.

Within MicroManager (and in BeanShell scripts), it is important to note that the direction that moves the objective towards the sample is defined as negative, and that the units inside the Märzhäuser software for the z -drive (which reports to MicroManager) are such that 100 units equals 10 μm . For clarity and to follow conventional notation, in this thesis all further calculations will be in microns and the direction towards the sample (upwards) will be defined as positive. Furthermore, unless otherwise stated, there is an implicit conversion from pixels to microns in the following sections, and this conversion is given by the factor $\text{pixel_size}_{\mu\text{m}} \cdot M$, where $\text{pixel_size}_{\mu\text{m}}$ is the pixel size of the detector in microns, and M the magnification of the microscope system.

The automatic coverslip detection procedure is illustrated in Figure 3.6 and involves the following steps:

0. At the first well at a position 150 μm below the minimum z position, set laser to low power and filter wheel to empty position, snap image with OPM camera to define the background image, this step is done once.

The code then begins a loop over well positions in the position list, within which there is a loop over objective z position (to scan the objective).

1. Starting at the minimum objective z position, **starting_z**, set laser to low power and filter wheel to empty position, snap image with OPM camera and subtract background image acquired in step 0.
2. Threshold the image (manually defined) to create a binary image and erode with 2×2 kernel. The erosion step is included to make the process more robust by removing any individual pixels that exceeded the threshold but were not part of the reflected line from the coverslip.
3. Extract a vertical line profile of the binary image in ImageJ which sums across the columns of pixels and find the distance $\Delta z'$ of the first non-zero value from the bottom of the FOV. If there are no non-zero pixels and the maximum z position is not reached, add **z_increment** to the current z position, (and move to this position) and return to step 1. If maximum z position is reached, coverslip detection has failed and move to next well, move objective z position to the minimum and return to step 1.

4. After successfully detecting coverslip position within the FOV, $\Delta z'$, convert to the corresponding z value (lab coordinates) using

$$z_{\text{relative}} = \Delta z' \cdot \sin(\theta_{\text{OPM}}) \quad 3.4$$

where $\Delta z'$ is the vertical position of the detected coverslip in microns from the bottom of the field of view, θ_{OPM} is the OPM angle.

5. Record the value of $z_{\text{coverslip}}$ for this well, which is defined as

$$z_{\text{coverslip}} = z_{\text{current_focus}} + z_{\text{relative}} \quad 3.5$$

where $z_{\text{current_focus}}$ is the current z position of the objective.

6. Move onto the next well and repeat steps 1–5 using $z_{\text{current_focus}}$ for the objective z position rather than starting from **starting_z**, since nearby wells will have similar coverslip heights. If the coverslip is not found, retry from steps 1–5 starting with the **starting_z**.

In this procedure, as stated in step 6, after a coverslip is successfully detected and the code moves on to the next well, step 1 is performed from the previous objective z position, since the coverslip height is likely to be similar between adjacent wells (well positions are contiguous in the positions list except for when some are omitted in previous steps of the pre-find). If the coverslip is not found, the inner loop over objective z position is restarted

from the starting z position (**starting_z**). In the future, instead of restarting from **starting_z** when no coverslip is found, it may be faster to first retry the detection (steps 1–5) for positions one step either side of the previous objective z position. The process currently takes ~10 mins for 180 (adjacent) wells.

In time-lapse imaging experiments, acquisition could be configured to run the coverslip detection code and update the coverslip conditions accordingly to correct for drift. This was used in experiments presented in Chapter 4, along with spheroid centring code which is presented in the next section used to correct for sample drift.

Table 3.1 – Parameters used in coverslip finding code. $z_{\min}$ and $z_{\max}$ are the approximate minimum and maximum coverslip heights found by manually measuring coverslip heights across the plate.

Variable	Example value in code	Description
starting_z	$z_{\min} - 50$ (μm)	Lowest objective height to search for coverslip
max_z	$z_{\max} + 50$ (μm)	Highest objective height to search for coverslip. Typically, the scan range is ~ 250 μm
step_z	-30 (μm)	Amount to move in z between coverslip detection attempts
cslip_exp	2 (ms)	Exposure time for OPM camera detecting coverslip
minfind_otsuthresh	5900 (camera digital numbers)	Manual threshold value—name based on old technique

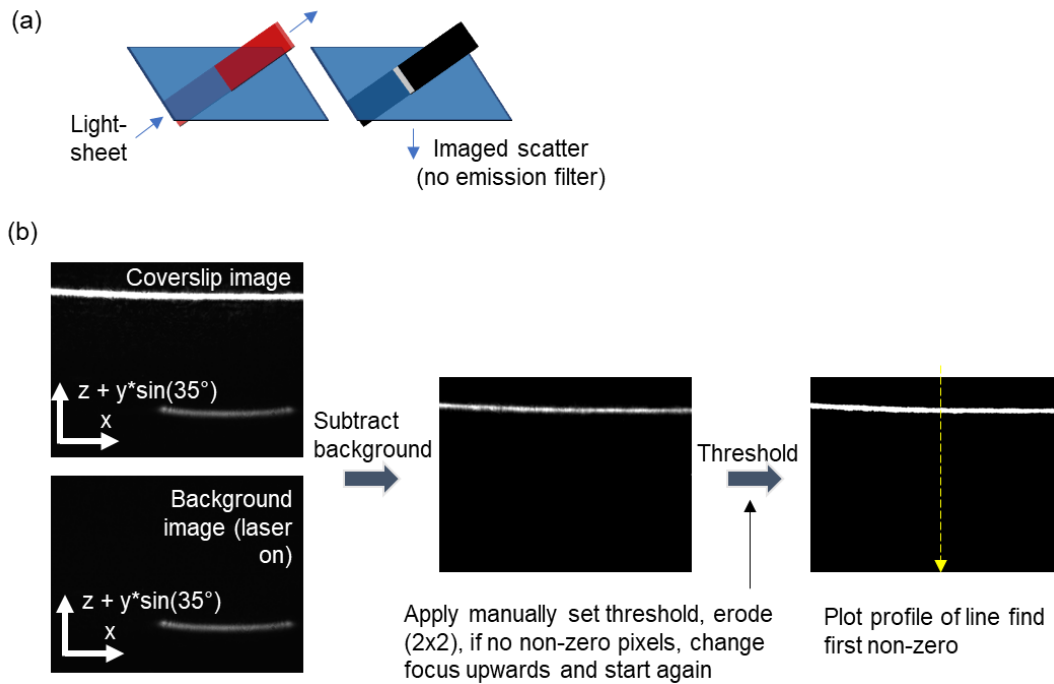


Figure 3.6 – Automatic coverslip detection using reflection of the light sheet. (a) Light-sheet propagates through coverslip and partially reflects off the bottom (water-glass) coverslip surface, which appears as line on the camera. (b) Flowchart process describing the automatic coverslip detection code.

3.2.4 3D pre-find of spheroids in OPM view

Once the coverslip detection process has been completed and the coverslip heights are known across the plate, it is now possible to safely perform 3D sample pre-find with the 60× objective (O1). The 3D pre-find is performed using the OPM imaging modality, acquiring volumes at increasing objective z positions to search for spheroids over a user-defined z range less than the full working distance of the O1.

A 3D pre-find script, also written in BeanShell for MicroManager, was implemented together with the coverslip detection code into a single combined script (see link 2.2); the combined procedure is illustrated in Figure 3.7. In the combined procedure, for each well, the bottom surface of the coverslip is found, the focus is moved to the top surface of the coverslip (i.e. by ~170 µm, the coverslip thickness), then the 3D pre-find is performed.

In addition to the parameters used in the coverslip-detection script (see Table 3.1), the combined script includes parameters for the minimum spheroid size and a manual threshold value—the full list of user parameters is shown in Table 3.2. Since the coverslip-detection procedure was already presented, the 3D pre-find procedure itself is outlined by the following steps:

1. For a given well and objective z position (starting with the top surface of the coverslip at the bottom of the FOV), acquire a stage-scanned OPM volume (y-stack) with coarsely spaced slices (10 µm instead of the standard 1.4 µm). The volume is passed to a custom analysis function written in MicroManager and pixels are calibrated into µm.
2. On the stack of N slices, apply a manual threshold (**mip_spheroid_otsuthresh**) to the stack and calculate the centre of mass in y (y_{com}) to estimate the spheroid position in y, which is given by

$$y_{\text{com}} = \frac{\sum_i^N I_i^{xy} \cdot y}{\sum_i^N I_i^{xy}} \quad 3.6$$

where I_i^{xy} is the sum of pixels for an image slice i , and y is the slice position.

3. On the same thresholded y-stack, calculate the maximum intensity projection (MIP) of the stack.
4. Run Analyze Particles to find objects above the minimum size in µm² (**min_size**) in the MIP. If no particles are found above the minimum size, then translate objective by **z_step_spheroid** and repeat from step 1 (if current focus is not beyond the maximum allowed, in which case, skip the well).
5. On the output of Analyze Particles, select the largest region and use the centre of mass in x of this particle to define the x position of the spheroid, this is given by

$$x_{\text{com}} = \frac{\sum_i^A I(x_i, z'_i) \cdot x_i}{\sum_i^A I(x_i, z'_i)} \quad 3.7$$

where $I(x_i, z'_i)$ is the value of a pixel at (x_i, z'_i) in the MIP, x_i is the x position of pixel i , and A is the number of pixels in the region detected by Analyze Particles.

The x_{com} defines the x-shift necessary to centre the spheroid in the FOV.

6. Calculate the distance from the bottom of the FOV to the bottom of the bounding box calculated by Analyze Particles, $\Delta z'$. The required objective z-shift in lab coordinates is given by

$$z_{\text{shift}} = (\Delta z' - 0.05 \cdot \text{FOV}_{z'}) \cdot \sin(\theta_{\text{OPM}}) \quad 3.8$$

where θ_{OPM} is the OPM angle and $\text{FOV}_{z'}$ is the z' FOV of the image. The shift includes an offset of 5% of the maximum z' FOV to move the spheroid slightly closer to the centre of the FOV and ensure the bottom of the spheroid is imaged.

7. Since the non-orthogonal coordinates are skewed compared to lab coordinates (the imaged volume is a parallelepiped), the actual XY stage y-shift required to centre the spheroid depends on z_{shift} as well as y_{com} and this y_{shift} is given by

$$y_{\text{shift}} = y_{\text{com}} + z_{\text{shift}} \cdot \tan(\theta_{\text{OPM}}). \quad 3.9$$

This OPM imaging geometry that causes the y-shift to be dependent on the z-shift is illustrated and explained in Figure 3.7(d).

8. Combine x, y and z-shift and apply to the positions list, then move onto the next well to restart the coverslip detection and spheroid detection process from step 1.

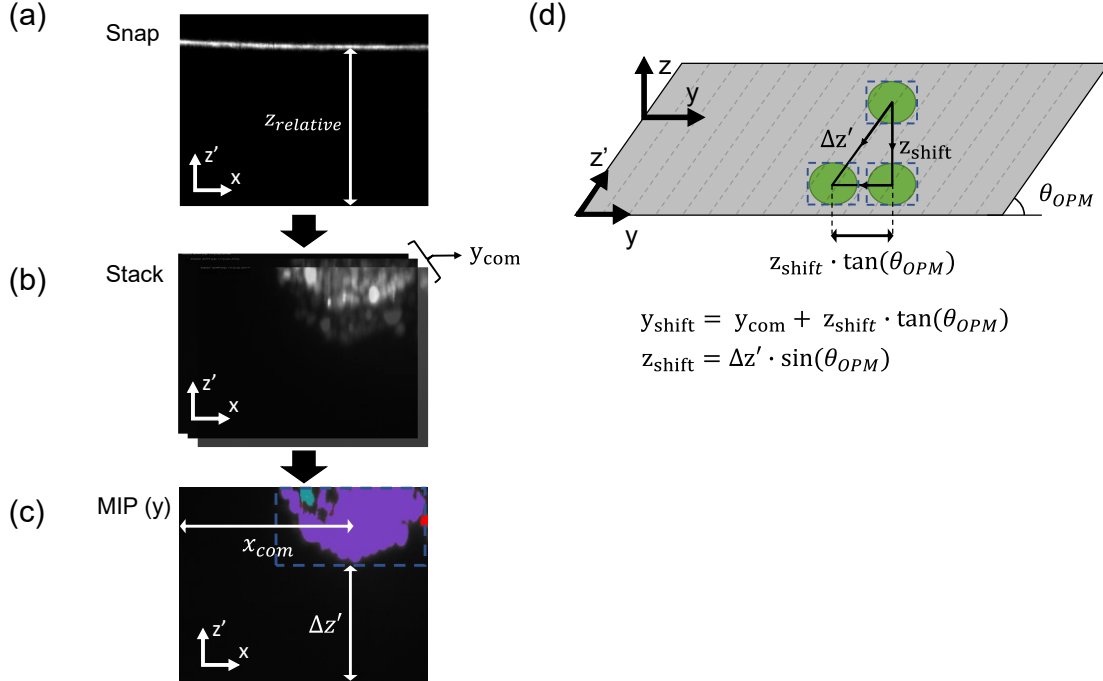


Figure 3.7 – Automated spheroid pre-find process in 3D using OPM view, including coverslip detection. (a) Firstly, the coverslip position, $z_{\text{coverslip}}$, is found. (b) The focus is moved upwards by approximately the coverslip thickness, then a coarsely sampled OPM volume is acquired from which the y centre of mass, y_{com} , is extracted. (c) The MIP is calculated along the y-axis and from this, the x centre of mass x_{com} is calculated along with the bounding box, indicated by box with dashed lines. The distance of the spheroid bounding box bottom edge from the bottom of the FOV, $\Delta z'$, is measured. The y_{shift} and z_{shift} (lab coordinates) are calculated according to the geometry shown in (d) using the measurements in the images (which are in the skewed OPM coordinate space).

Table 3.2 – Parameters used in 3D spheroid detection and coverslip detection code.

Variable	Example value in code	Description
min_size	5500 (μm^2)	Minimum size of spheroid in MIP in μm^2
z_step_spheroid	-400 (μm)	Amount to move in z between spheroid detection attempts
coverslip_thickness	1400 (< 1700 to avoid missing spheroid) (μm)	Coverslip thickness: distance to move objective from bottom surface of coverslip to top (in order to image inside well)
duty_cycle	0.5	Fraction of time between exposure and time spacing between images
nimgs	10	Number of OPM images to take in stack
y_scanwidth_um	200 (μm)	y-range over which to acquire OPM volume for pre-find
spheroid_las_amplitude	1 (V)	Laser amplitude (voltage into AOTF, 0-5V)
mip_spheroid_otsuthresh	300 (camera digital numbers)	Manual threshold applied to OPM images

We found that when this 3D pre-find process was used in experiments with 180 spheroids, at least 170 of them would be successfully detected and centred with no need for manual correction. Typically, there were less than 5 false negatives per ~ 180 wells, corresponding to a $\sim 97\%$ rate of success.

One cause of inaccuracy in the 3D pre-find method is if the xy position of the spheroid is not fully within the 3D FOV of the pre-find imaging volume. In this case, there is the potential for incorrect x and y-shifts to be calculated. The z-positioning is comparatively robust since it uses the bounding box of the image, but is still susceptible to this effect, shown in Figure 3.8.

Ideally, the low magnification pre-find process finds sufficiently accurate x and y positions for the spheroid and then the effect illustrated in Figure 3.8 is not observed. This emphasizes the importance of an optimized low magnification pre-find process. In the future, it may be possible to improve the algorithm to detect whether the spheroid is partially outside the 3D pre-find imaging volume in x or y.

The spheroid detection/centring algorithm used to process these OPM pre-find volumes is also applied to data acquired during the time-lapse experiment. Along with the coverslip autofocus, this algorithm is currently run immediately after the acquisition of a time-point using the image data that was just acquired. Sample drift that occurs between this timepoint and the next is not fully corrected for, and so this approach is only able to correct for small or gradual changes in sample position. The more frequent the time points, the better this

method is at accounting for drift, but increasing imaging frequency also increases the light dose on the sample.

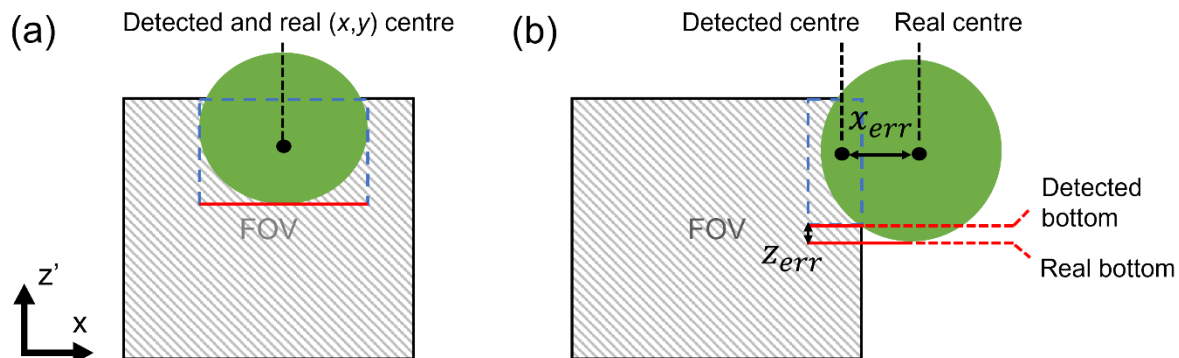


Figure 3.8 – Potential spheroid pre-find inaccuracy when spheroid is partially out of the FOV. (a) Case where the calculated x centre of mass accurately describes spheroid location. (b) The centre detection method cannot include the regions of the spheroid that are outside the FOV, resulting in the detected centre being different to the real centre and the required x -shift is underestimated. For illustrative purposes, this effect is only shown here for x , but it can also occur for y .

In the future, it may be possible to acquire the coarse 3D pre-find volumes and correct for sample drift before the acquisition of each time point, although this would increase the light dose applied to the sample. An alternative, lower light dose method of (high magnification) pre-find would be to use LED excitation and widefield imaging which would have worse performance in z but would be sufficient in tracking x and y during the timelapse experiment, and this could be run before timepoints without causing significant photodamage. This is not possible with the current hardware, however, and requires a motorised filter carousel in the IX71 to switch from the empty filter to perform OPM imaging to the dichroic mirrors for epifluorescence.

3.2.5 Spheroid re-find

In cases where the imaging plate is removed and replaced, the orientation of the plate may be changed slightly or the samples themselves may have drifted or been disturbed, meaning the pre-find process must be repeated. In these cases, the current procedure is:

1. Acquire low magnification epifluorescence images with the $4\times$ objective in place (as per Section 3.2.1)
2. Run adapted pre-find code in MicroManager (also described in Section 3.2.1), but instead, optimising for the detected object that is closest to the centre of the FOV—ideally, the shift in positions should be small on the scale of the low-magnification FOV.
3. Run coverslip detection and 3D spheroid pre-find in OPM view (Section 3.2.2 and 3.2.4).

There are some situations, however, in which the re-find process can fail. For step 1, if there are multiple objects near the centre of the field of view, the wrong object can be detected. In step 3, the 3D spheroid detection code scans upwards, so in cases where there is a fluorescent

object that is large enough to be detected and accepted by the algorithm (e.g. bundle of cells that have broken away during the experiment), but is closer to the coverslip than the spheroid of interest, this can be detected before finding the spheroid and results in finding the wrong focus. To mitigate this, before removing the plate from the microscope, brightfield images are taken for all wells using the multi-dimensional acquisition tool (MDA) in MicroManager with brightfield imaging. These are then compared by eye with another set of brightfield images taken after the re-find to find cases where the re-find has failed. This can be done for both $4\times$ magnification and $60\times$ magnification.

3.3 Summary and Future work

3.3.1 Summary

In this chapter, the improvements to low-magnification pre-find that was inherited at the beginning of the PhD were presented. At first, the pre-find was adapted for the use of samples fluorescent in multiple spectral channels and without a common channel. It was no longer used after a common spectral channel was added to the experiments presented in Chapter 4, and the focus was shifted to improving single-channel pre-find performance and workflow.

In addition to this, coverslip-detecting autofocus was developed so that 3D pre-find could be performed, which automated the process of finding the spheroid z position and centred the spheroid in the FOV with higher precision than the low-magnification methods. The coverslip detection (autofocus) method scans the objective upwards until the reflection of the light sheet from the bottom coverslip surface is observed on the camera. After the coverslip height detection, the 3D spheroid pre-find is then performed by imaging within the well at increasing objective z positions until a spheroid is detected, and the stage position is updated to centre the spheroid within the FOV. In addition to being used in the pre-find, the coverslip detection was used to compensate for plate and focus drift during time-lapse experiments, while the spheroid-centring methods used in the 3D spheroid pre-find also allowed corrections for small shifts/drift in spheroid position. We noted that the sample drift was most significant in the first two days.

Although the current procedure allows the successful full pre-find of $>90\%$ of spheroids (a minimum of 170/180 wells in recent experiments), there are further improvements that could be made; these will be discussed in the following section about future work. The low magnification pre-find took approximately 10 minutes for 180 wells, while the combined coverslip detection and 3D spheroid pre-find process took approximately 30 minutes. This does not include the time taken to set up the acquisition and scripts, with the estimation of the coverslip heights across the plate necessary for the latter process. However, we estimate that the entire process took approximately 1 hour, which was faster (and requires less effort) than manually locating and centring spheroids in the OPM FOV which would take approximately 3 hours or more.

3.3.2 Future work

In the current implementation, one issue is that highly fluorescent fibres present within the sample can lead to incorrect determination of spheroid location. One factor in this is that the low magnification pre-find does not consider the circularity of the ROI detected. In the future, the circularity of the ROI could be used to reject ROIs that are affected by fibres. The adjustments to the code would be straightforward to implement since the Analyze Particles plugin in ImageJ provides this as an output, but this would then need to be tested with a range of spheroid plates to see if this helps solve the issue.

For the coverslip detection, code could be simplified by once again utilising the Analyze Particles tool in ImageJ/MicroManager to process the image of the coverslip scatter, instead of using both an erosion and line profile. Analyze Particles reports the pixel positions of a region in a binary image and can easily reject a smaller, bright region that is not the coverslip, or choose the largest region—this is what the erode step attempts to solve, but the Analyze Particles plugin is more tuneable than the current method and this may offer improved performance.

The coverslip searching method also puts strain on the Z-drive/focus wheel axle since it repeatedly moves the objective large distances over 180 wells. Small changes could be made to reduce the movement in z: at the moment, after finding a coverslip and moving onto the next well, the z position is unchanged and an image is taken, but if there is no coverslip detected, it drives the z back to the minimum position and scans all the way back up. A simple solution to this is to move the objective one z step from the original z downwards (away from the coverslip), then, if the coverslip is not found, one step higher than the original z, and this will most likely find the coverslip in most cases.

4 OPM timelapse imaging of heterogeneous breast cancer-mimicking spheroids

4.1 Biology introduction

4.1.1. Intratumoural heterogeneity in cancer

Cancer is a complex and dynamic disease, often involving various populations of cancer cells which evolve over time, generally increasing in heterogeneity [92]. Indeed, a tumour will often not just comprise a single population of cells with a specific oncogene (cancer causing mutation), but instead, multiple populations of cells with a diverse set of phenotypes and genotypes. Current cancer treatments often target specific molecular signatures unique to an oncogene and are necessarily ineffective against other populations of cancer cells which may be present; this can result in the proliferation of the surviving, treatment-resistant cell lines and thus poor patient survival. It is therefore important to accurately assess heterogeneity in tumours for successful treatment through techniques such as multi-regional sequencing and single-cell sequencing [93], [94].

These sequencing methods, however, are limited to the single time-point of a biopsy (which cannot be practically performed regularly) or autopsy and these methods therefore cannot know the tumour lineage and evolutionary trajectory, accurately forecast the progression of tumours or the therapy efficacy.

4.1.2. Cancer models for heterogeneity

Cancer models can be used to study this genomic evolution in the presence of heterogeneity, including patient-derived models and those from established cell lines (ECLs). Patient-derived models are particularly attractive to personalised medicine, and such models include patient-derived xenografts (PDXs), patient-derived organoids (PDOs) and patient-derived cell lines (PDCLs) [95].

ECLs are widely used for *in vitro* cancer research and drug discovery due to their ability to proliferate indefinitely in cell culture, making them comparatively scalable, low cost, and robust. For explorative studies of the phenotypes associated with different mutations, for example, cell culture models from ECLs provide a convenient option, since genetic manipulation can be easily performed to study a wide mutational landscape. There are disadvantages to using ECLs, however, since they have evolved often over decades, acquiring genetic and histological differences and losing clonal heterogeneity compared to the native tumour from which it was obtained due to selective pressures from culture [96]. The evolution of cell culture cancer models is easily studied through timelapse imaging, which can be automated and parallelised for simultaneous studies of multiple conditions—this is often referred to as high content analysis (HCA) or high content screening (HCS). Here, the term HCS will be used since it emphasises the high throughput.

PDXs involve implanting tissue harvested from a tumour into immunocompromised mice *in vivo*, which therefore closely resemble the biological characteristics of the patient tumour,

but the metabolism, physiology and therefore microenvironment in the mouse differs significantly from the native environment of the patient. Furthermore, PDX studies are expensive and difficult to scale and automate compared to cell culture models. PDOs are grown in 3D culture to mimic *in vivo* conditions with pluripotent stem cells or isolated organ progenitors obtained from the patient and provide a less expensive and time-consuming alternative to PDX without using animals (but more expensive than using ECLs) [97]. Since these are cell cultures, HCS can also be applied to studies of these organoid models, instead of manually sampling xenografts at different timepoints.

4.1.3. Imaging studies of cell culture models

As mentioned in the previous section, HCS of cell culture cancer models through timelapse imaging can be used to perform time-resolved, non-destructive studies of the evolution of cell culture models of tumours. HCS is characterised by the simultaneous readout of multiple parameters, and this is fulfilled by imaging techniques which obtain spatial information, and in the case of fluorescence microscopy, signal level and distribution e.g., to measure the expression level of fluorescently labelled proteins. HCS is often used in a high throughput setting, and in a standard microscope, this can be achieved using multi-well imaging plates to allow different conditions or drugs to be tested in the same experiment.

Although cell culture models are often 2D and have limited replication of *in vivo* conditions, just like with PDOs, there has been a recent focus on developing 3D models such as spheroids, organoids, or cultures with 3D scaffolds to remedy this [98], [99], [100].

Advantages of 3D models include better modelling of factors such as nutrient and gas exchange, extracellular matrix, cell-cell contact and polarity, with organoids also mimicking the structure and, to some extent, the function of the organ it is derived from. 3D cell culture models, however, demand optical sectioning microscopy techniques to both reject out-of-focus light and image 3D structure. Common imaging platforms capable of optical sectioning such as Nipkow spinning disk microscopy can perform time-lapse studies of 3D cell culture models, but its comparatively high light dose can result in phototoxicity and photobleaching compared to other optical sectioning techniques such as light-sheet fluorescence microscopy (LSFM).

As shown in Chapter 1, there are multiple LSFM modalities which are compatible with the HCS workflow including oblique plane microscopy (OPM). In this chapter, OPM is used to perform long-term (> 8 days) four-channel 3D timelapse imaging of a novel, multigene spheroid model of heterogeneous breast cancer tumours. The details of this spheroid model are presented, along with the results of the timelapse imaging and the quantitative analysis to measure clonal development and competition in 3D.

4.2 Four-oncogene spheroid model for heterogeneous breast duct tumour

4.2.1 Introduction

The work in this chapter was partly funded by the Integrated Biological Imaging Network (IBIN) and was carried out as a collaboration between the Department of Surgery and Cancer and the Photonics Group at Imperial College London. In this work, a novel spheroid model of heterogeneous breast duct tumours was developed by Nina Moderau and Sandesh Meyler to be imaged with the OPM system (presented in Chapter 2). Initial work on this project was carried out by Hugh Sparks, who optimised the choice of fluorescent proteins and overall assay design, and Nathan Curry, who developed the image compression pipeline and led the initial imaging experiments of the four-oncogene spheroids. These spheroids model heterogeneity by consisting of multiple different cell lines, each established from the HB2 cell line and expressing a unique oncogene along with a complementary fluorescent protein (FP) tagged to Histone 2B (H2B) that causes fluorescence to localise in the nucleus. Different combinations of these cell lines were cultured and combined to grow 3D spheroids in ultra-low attachment (ULA) microplates, before being imaged under time-lapse with the OPM as shown in Figure 4.1, which describes the current workflow.

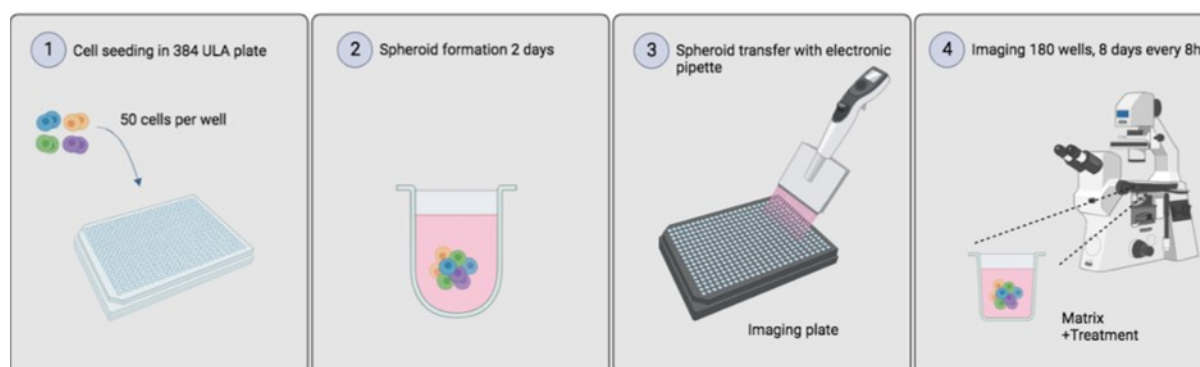


Figure 4.1 – Experimental process for formation and time-lapse imaging of the breast duct tumour spheroids. Obtained with permission from Nina Moderau.

4.2.2 Oncogene choice

The choice of the oncogenes was determined by the Moderau lab after they screened a subset of known or putative breast cancer oncogenes described in literature [101]. Here, a panel of mutations, amplifications or deletions was mimicked using the overexpression system based on the pInducer21 gateway system [102] which integrates the oncogene into a cell line with a stable transduction and with activatable expression. The panel of mutations was initially reduced to four: overexpression of wild type (WT) *ERBB2*, *ERBB2D16*, *BRAF V600E* and *KRAS G12V*. As described in more detail in the following section, four cell lines were generated from the HB2 cell line, each expressing one of the four oncogenes and stably coexpressing a different fluorescent protein tagged to H2B. Since the erbB-2/HER2, BRAF and KRAS proteins all interact with the MAPK signalling pathway, we hoped to see clear

incidences of competition and collaboration between mutations, which is another motivation for this panel. The text below provides a brief background to each oncogene.

The receptor tyrosine-protein kinase *erbB-2* is a protein found in the cell membrane encoded by the *ERBB2* gene and is involved in promoting cell proliferation through activating pathways including MAPK/ERK and PI3K/Akt, and in humans this protein is commonly referred to as human epidermal growth factor receptor 2 (HER2).

Amplification/overexpression of the *ERBB2* gene is linked to an aggressive subtype of breast cancer, associated with poor prognosis, increased metastasis, and disease recurrence.

However, only about 50% of HER2-positive patients respond to targeted therapies. *ERBB2* overexpression is also relatively common, with an estimated 11-30% prevalence in primary tumours [103]. Understanding the biology and drug susceptibility mechanisms of HER2 is crucial for improving cure rates, and it is therefore pertinent to include it in this spheroid model.

The *ERBB2D16* splice variant is a mutant of WT *ERBB2* (written here as *ERBB2WT*) characterised by the skipping of exon 16. It has been reported in breast cancer patients and HER2-positive human cancer cell lines [104]. This variant leads to the formation of stable and active HER2 homodimers on tumour cells and consequently influences tumour initiation, aggressiveness, cancer stem cell properties and epithelial-mesenchymal transition (EMT) i.e., invasiveness. It serves as a novel and potentially useful biomarker for risk assessment and treatment decisions in HER2-positive disease [105].

The *BRAF V600E* mutation is a genetic alteration in the *BRAF* gene that leads to continuous activation of the BRAF protein kinase. The BRAF protein is a key component of the MAPK/ERK signalling pathway that plays a crucial role in regulating cell growth, differentiation, and survival. Albeit rare, with *BRAF* altered in 1.38% of breast carcinoma patients, and *BRAF V600E* present in 0.11% of breast carcinoma patients, recent genomic studies using whole-genome sequencing of breast cancer metastasis identify mutations that have significant prevalence in metastases compared to primary tumour tissue [106]. This included mutations in the *BRAF* gene which had a prevalence of 19% in brain metastases [107]. The *BRAF V600E* mutation was also detected at a higher frequency in circulating tumour cells than in the primary tumour [108] and higher in secondary tumours in the brain [109], suggesting that *BRAF V600E* expressing cells are a likely candidate for brain cancer breast metastasis (BCBM).

It is currently unclear whether the discrepancy between primary tumour and secondary tumour prevalence is due to selective pressure in the secondary tumour, or evolution in the primary tumour. In preliminary breast carcinoma spheroid models developed by Dr Nina Moderau, when *BRAF V600E* is combined with other mutations, these cells are observed to disrupt and break through the basal membrane and spread into the surrounding matrix—suggesting the latter.

Furthermore, *BRAF V600E* may be of potential therapeutic interest, since good results have been obtained in other tumour types [110], [111]. Vemurafenib is one of these therapeutics which interrupt the MAPK/ERK pathway in the presence of the *BRAF V600E* mutation, and we use this as a proof of concept for a drug screening platform, with drug response studies planned.

Another member of the MAPK/ERK signalling pathway is the K-Ras protein, encoded by the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene which belongs to the rat sarcoma (RAS) family of oncogenes. *KRAS* is one of the most frequently mutated genes in cancer with a 2.1% occurrence in breast cancer [112]. Within mutated *KRAS* variants, 83% present a G12 mutation with G12V being found in 26% of all cases. Little is known about the function of *KRAS* and its mutations in breast cancer, therefore, to analyse its behaviour in multiclonal environments is crucial to understand its role.

4.2.3 Methods

Cell line design

Each of the four FP-oncogene pairs were integrated into different populations of HB2 to generate four different cell lines by stable transduction using a lentivirus system, making them identifiable and distinguishable from each other by the spectral channel that the FP emits into. The cell lines are combined in different combinations to form spheroids, and a spheroid containing all four cell lines is depicted in Figure 4.2 to explain how the different FPs allow the individual cell lines to be identified.

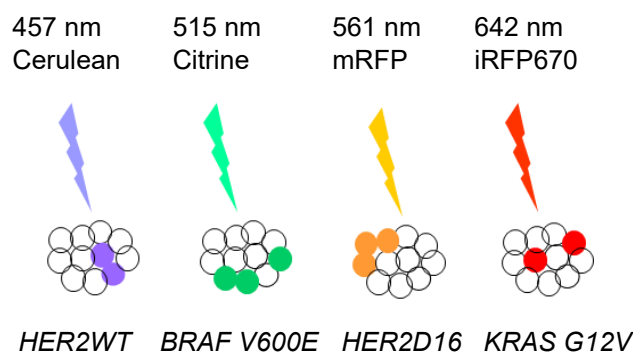


Figure 4.2 – Illustration of four-cell line combination spheroid setup, where spheroids can contain up to four different cell lines, each expressing a unique oncogene coexpressed with an identifying FP.

In this lentivirus system, the transfer plasmid—which contains the DNA to be integrated into the cell line—along with packaging and envelope plasmids are transfected into a separate HEK-293T cell line culture to generate the lentivirus. The lentivirus is then extracted from the HEK-293T cell culture and used in the transduction of the HB2-derived cell lines.

The transfer plasmid, shown in Figure 4.3, includes the oncogene, FP-tagged histone (H2B) and the reverse transactivator protein rtTA for the Tet-on system. Since the FP is

integrated as a complex with H2B, the fluorescence is localised to within the nucleus. The Tet-on system based on the pInducer21 design [113] allows the oncogene expression to be modulated separately from the FP, induced by the addition of the antibiotic doxycycline (Dox). This means that the oncogene is only expressed in the presence of Dox, allowing the effect of the oncogene on the spheroids' phenotypical behaviour to be directly compared to a non-induced control case where the oncogene is not expressed. Note, from this point the transfer plasmid will be referred to as just the plasmid for brevity.

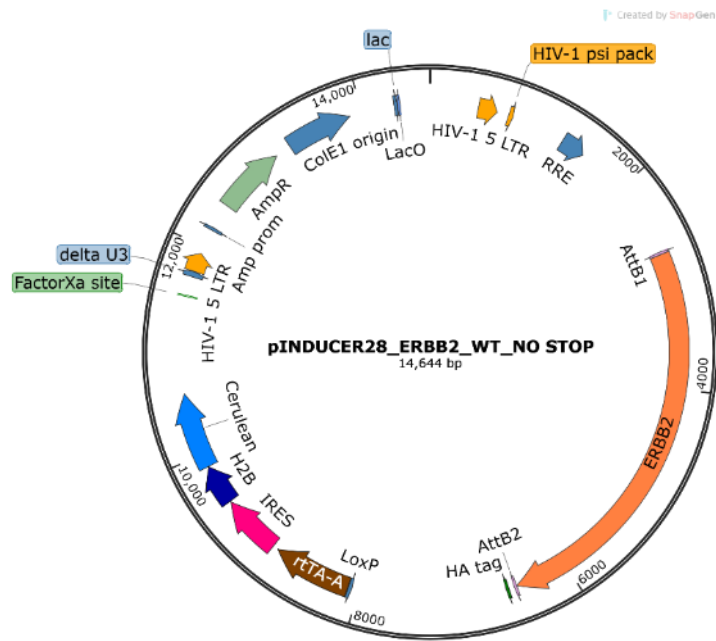


Figure 4.3 – Representation map of a (transfer) plasmid based on the pInducer21 system used in transduction to incorporate Dox-inducible oncogene and H2B-tagged FP into cell line; in this example, *ERRB2* WT and H2B-Cerulean. The transgene that is integrated into the cell lines is between the two LTR sequences. In the presence of Dox or tetracycline, the reverse transactivator protein rtTA (encoded by the plasmid here) binds to TetO sequences within the tetracycline response element (TRE) which consists of several TetO sequences with a minimal promoter. The binding of rtTA to the TRE upstream from the *ERRB2* initiates its transcription.

The transduction process is non-deterministic, resulting in variability in the number of plasmid integrations into the genome across cells, and hence there is additional heterogeneity in oncogene expression and FP expression within each cell line. Since the oncogene and FP are coexpressed, the number of integrated oncogene copies directly correlates to the number of FP copies and the number of oncogene copies therefore correlates with the fluorescence intensity, see Figure 4.4. This also allows the heterogeneity to be maintained deliberately in each cell line to mimic natural selection more accurately using fluorescence-activated cell sorting (FACS), with selection and outgrowth of single clones being avoided within each cell line.

This additional heterogeneity is a source of biological interest, and by measuring the fluorescence intensity in the nuclei, it allows comparison of the phenotypical behaviour of populations of cells (clones) within the same cell lines with different numbers of oncogene copies, in addition to comparisons between the different cell lines.

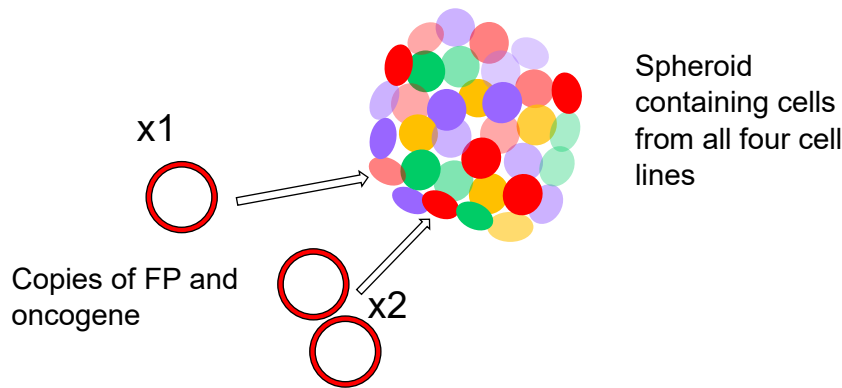


Figure 4.4 – Diagram of spheroid containing the four cell lines, each containing additional clones with different levels of oncogene/FP expression, illustrated by the brightness of the colours in the diagram. Here, two ERBB2d16-iRFP670-expressing nuclei are indicated, the nucleus with two copies of the FP gene and oncogene is brighter than the nucleus with one copy of the FP gene and oncogene. The red circles indicate the number of copies of the plasmid incorporated.

Cell culture

The HB2 cell line is derived from subclones of the MTSV1-7 mammary luminal epithelial cells isolated from human milk, and these were immortalized via the introduction of the gene encoding simian virus 40 (SV40) large T antigen [114]. All cells were regularly checked for mycoplasma contamination and all experiments were performed with mycoplasma-free cells.

HEK-293T was cultured in DMEM medium, 10% FBS, and the HB2 was cultured in DMEM medium, 10% FBS, 5 µg/mL Hydrocortisone, 25 µg/mL Insulin. All cell lines were kept at 37°C, 95% humidity and 5% CO₂.

Spheroid formation/plate preparation

In preparation for the live imaging experiments, 384-well, repellent, U-bottom culture plates (Greiner #787979) were seeded with approximately 50 cells, spheroids were grown to 100-200 cells in size over two days before being transferred in a volume of 30 µL to the 384-well imaging microplates (Thermofisher #142761, untreated) and the spheroids were embedded in 2.5% Matrigel to a maximum volume of 80 µm³ along with an initial dose of doxycycline (Dox) at 2 µg/mL if applicable. From the start of imaging, spheroids consisted of approximately 125-175 cells and the imaging duration aimed to grow spheroids to roughly fill the 320×605×144 µm (*x, y z*) FOV.

Each column of the imaging plate contained spheroids with different combinations of the different cell lines as described in

Table 4.1, with rows E to H induced with Dox (+Dox) and rows I to K non-induced (-Dox). This corresponds to a total of 128 occupied wells, each containing one imaged spheroid. The surrounding empty wells were filled with PBS or medium to reduce evaporation from experimental wells.

Table 4.1 – Description of the experimental plate layout for live plate 1, i.e., the arrangement of the different spheroid treatments and cell combinations. Treatment with +Dox is indicated by shaded cells. H – *ERBB2WT*; B – *BRAFV 600E*; d16 – *ERBB2d16*; K – *KRAS G12V*. Spheroids aim to contain equal proportions of each cell line, but there is variation in the pipetting in the seeding process.

	Column(s)															
Rows	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
E	H	B	d16	K	H, B	H, d16	H, K	B, d16	B, K	d16, K	H, B, d16	H, B, K	H, d16, K	B d16, K	H, B, d16, K	H, B, d16, K
F																
G																
H																
I	H	B	d16	K	H, B	H, d16	H, K	B, d16	B, K	d16, K	H, B, d16	H, B, K	H, d16, K	B d16, K	H, B, d16, K	H, B, d16, K
J																
K																
L																

-Dox	+Dox
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Time-lapse imaging procedure

As described in section 2.2.4, the sample stage of the IX71 frame is enclosed in a custom incubator which is supplied with hot air and is configured to achieve a sample well temperature of 37°C [86]. The stage top sample chamber is supplied with 5% CO₂ in air from a pre-mixed source that is humidified by bubbling it through water. A peristaltic pump and custom water-immersion cap continuously supplies water to the 60× 1.2NA/W objective O1 so that the water immersion does not evaporate due to the heating from the incubator. The optical design of the OPM system is as described in section 2.3.1, and all OPM images are acquired on a single PCO.edge (4.2) camera with an automated filter wheel, labelled as Camera 1 in Figure 2.1.

In this experiment, the 4× objective was used to perform the multi-channel pre-find procedure described in Section 3.1 to locate a spheroid in each well and update the MicroManager positions list to centre them in the FOV. The 60× objective is then used with brightfield illumination with the updated position list and the spheroids are manually centred more accurately in x and y. The optimum focus position is found using the live OPM view using a channel that the spheroid is fluorescent in, with a preference for longer wavelengths to reduce phototoxicity. The excitation laser is manually enabled while manually scanning the spheroid in the scan direction (y) and adjusting the focus position until the bottom of the spheroid is slightly above the bottom of the OPM field of view (FOV). This step was not ideal, as the laser illumination was on continuously, introducing a risk of photodamage. Therefore, the minimum possible laser power was used for the shortest possible time. In the later experiments (live plates 2–6) the light dose is reduced by implementing an improved pre-find imaging procedure described in Section 3.2.

A BeanShell script (see link 2.1 on page 9) as described in Section 2.3.3 is used to automate the acquisition of the OPM stacks for each of the well positions and the imaging sequence is

automatically repeated every 8 hours. In this script, the laser and filter combinations, laser power, laser illumination duration, camera exposure time, and time between acquisitions are defined. After 5 days, the plate holder—with the plate retained within it—is removed from the stage to allow the cell culture medium (DMEM), Dox and Vemurafenib to be replenished. When the plate holder is placed back on the stage, the plate holder will be slightly translated and rotated compared to before it was removed and therefore the spheroid positions are also shifted, sometimes out of the FOV of the 60× objective; the positions must therefore be found again using the same pre-find procedure described above, but taking care to ensure that the spheroid found is the same as the one imaged before.

In parallel, widefield fluorescence time-lapse imaging of an identical plate was conducted by Nina Moderau on an *Incucyte*® Live-Cell Analysis System in the ICTEM lab to be compared to the OPM plate in order to identify issues in the OPM instrument incubator or photodamage.

4.2.4 Data processing and analysis

Reslicing raw data

As described in section 2.3.2, the timestamps are read from the raw images in the acquired OPM stacks, and the stacks are then compressed with Dotphoton’s Jetraw and transferred from the acquisition computer to a server. Stacks are resliced from the skewed OPM coordinates into lab coordinates and voxels are rebinned to a final size of $1 \times 1 \times 1 \mu\text{m}^3$ using the reslicing script.

Orthogonal cuts, MIPs and montages

From resliced volumes, central slices in x-y, x-z, and y-z planes are arranged into a thumbnail image which we refer to as an ortho-cut. Similarly, maximum intensity projections (MIPs) are calculated in orthogonal directions to produce views in the same x-y, x-z, and y-z planes. These are also arranged into a thumbnail image and are referred to as ortho-MIPs.

For each volume (which corresponds to a well in the multi-well plate), each of these thumbnails is arranged according to the well position on the plate to visualise the spheroids in 2D against the plate layout. In the time-lapse data, this is repeated for each timepoint to form montage movies, and ortho-MIP montage movies are included in the Supplementary Videos (see page 18).

Nucleus counting

One analysis of interest is the nucleus count for each of the cell lines. The nucleus counts can be used to measure the growth and competition between cell lines to see if certain oncogenes have a survival advantage.

A blob detection algorithm was developed in MATLAB and was inspired by trackMate [115], which uses the Laplacian of Gaussian (LoG) filter which approximates the 3D LoG function which is defined as

$$LoG(x, y, z) = \frac{1}{\sigma^2} \left(3 - \frac{x^2 + y^2 + z^2}{\sigma^2} \right) e^{-\frac{x^2 + y^2 + z^2}{2\sigma^2}}. \quad 4.1$$

The LoG is equivalent to applying both the Laplacian and Gaussian operators [116]. The Laplacian filter approximates the sum of 2nd spatial derivatives and has a strong response to blobs or peaks (strongly negative for bright blobs on dark background), while the Gaussian smooths the image to avoid detecting peaks in noise. The value of σ defines the radius of the filter's profile and the target size of the blobs to be detected.

OPM volumes were masked with manual thresholds so that blobs were not detected within the background noise and the LoG filter returned by **fspecial3** in MATLAB was applied to OPM volumes. Peaks beyond a user-defined minimum of prominence in the resulting LoG-filtered volume are detected using the MATLAB function **imextendedmin** [117], which uses the h-maxima transform. The number of individual connected components in the binary image that represents peak locations is counted to derive nucleus counts.

4.2.5 Results and discussion for four-oncogene spheroids

Out of the 128 wells that were prepared, only 70 were imaged due to wells where no spheroids were present in the well (true negatives) or where spheroids were missed in the pre-find stage (either false negatives or false positives that were subsequently manually rejected). A subset of time-points of the time-lapse ortho-MIP montage is shown in Figure 4.5, with the full data in supplementary video 4.1, and an example of an individual well (J18) is shown in Figure 4.6.

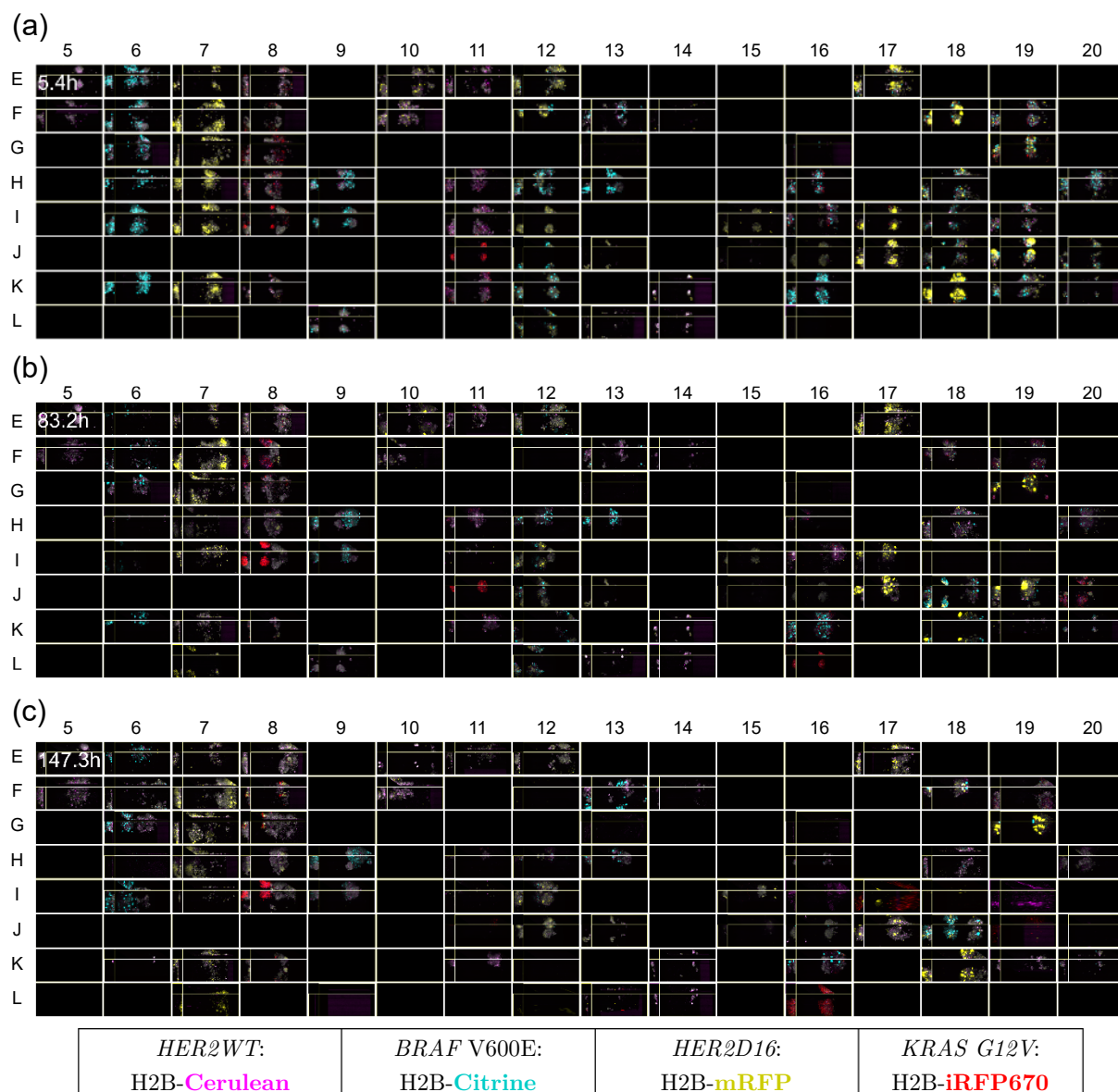


Figure 4.5 – Time-lapse ortho-MIP montage for live plate 1 at timepoints (a) 5.4 h, (b) 83.2 h and (c) 147.3 h. Each thumbnail contains MIPs for the x-y (bottom right), x-z (left) and y-z (top) views; each thumbnail represents a FOV of $302 \times 804 \times 144 \mu\text{m}^3$ (x, y, z). In cases where grey is seen, this is from the low-level autofluorescence observed in each of the channels, most visibly in the non-Citrine channels.

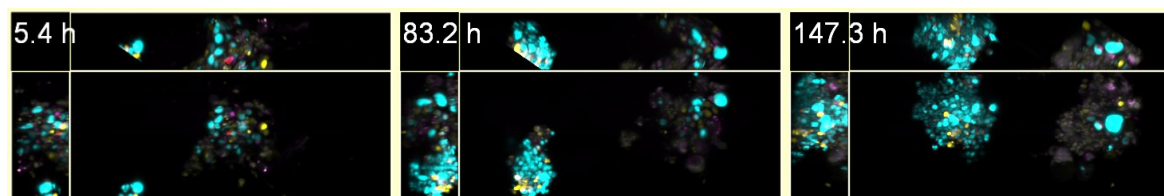


Figure 4.6 – Time-lapse ortho-MIP for well J18 in live plate 1 at times (left to right) 5.4 h, 83.2 h and 147.3 h

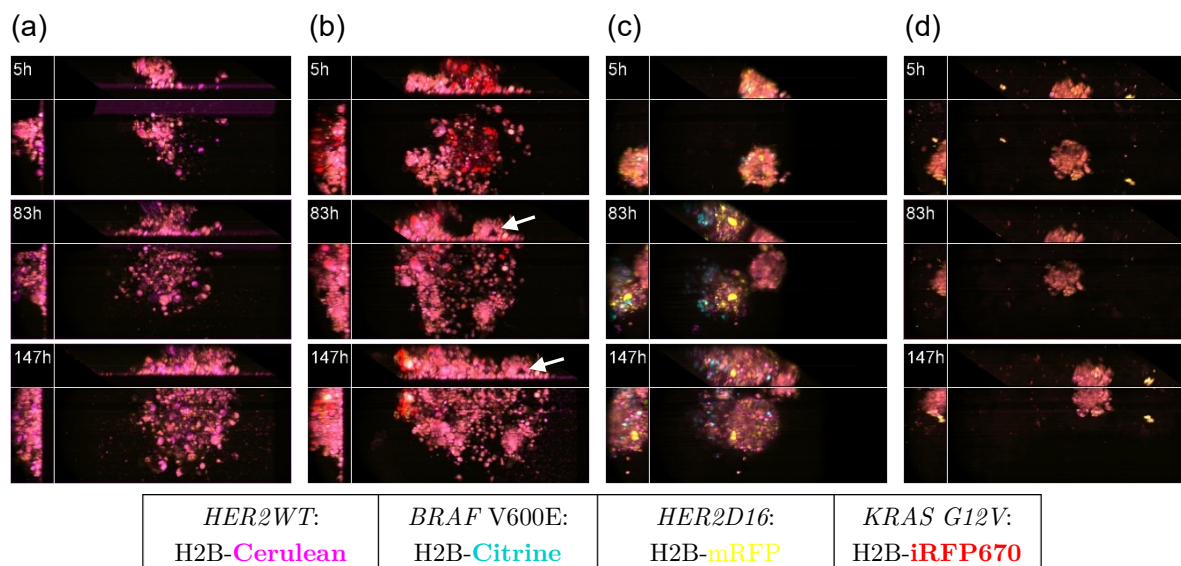


Figure 4.7 – Examples of issues encountered in the four-oncogene experiments. (a) Well F5 (*ERBB2WT*-Cerulean only)—cell attachment to coverslip, resulting in loss of the 3D spheroid structure. (b) Well G8 (*KRAS G12V*-iRFP670 only)—cell attachment, partial disintegration/splitting of spheroid, and partial death observed in a structure marked by the white arrow. (c) Well I12 (*BRAF V600E*-Citrine, *ERBB2D16*-mRFP combination)—spheroid splitting into two sub-spheroids. (d) Well I15 (*ERBB2WT*-Cerulean, *BRAF V600E*-Citrine, *KRAS G12V*-iRFP670 combination)—dead spheroid which has no visible morphological changes throughout the experiment. Note that significant autofluorescence is observed in the Cerulean and iRFP670 channels, with (a) and (b) examples of autofluorescence in the iRFP670 and Cerulean channels respectively.

In this time-lapse montage, many spheroids, including non-induced spheroids, display undesirable behaviour including significant cell fragmentation and death, spheroid flattening and loss of structure, and decreased spheroid viability (or death of the spheroid), with examples of each shown in Figure 4.7. Throughout these experiments, we chose to define spheroid death as when no significant visible morphological changes occur in 16 hrs. Sometimes, the death is observed in clusters within the spheroid while the rest of the spheroid remains viable for longer, and this is seen in G8 (Figure 4.7(b)). Cell shedding and flattening may indicate cell death, but it is also likely that cells are adhering to the coverslip despite the (uncoated) hydrophobic polystyrene coverslip surface which prevents cell attachment.

Spheroid death and cell death in general could be caused by incorrect CO₂ supply, incorrect temperature, or phototoxicity. Indeed, in this experiment, the laser power was set at the maximum available and the laser duration and camera exposure time was set to 50 ms for all 4 channels. This exposure time is significantly higher than typical exposure times used on this OPM system, which typically range from 2–20 ms, because the fluorescent proteins (FPs) in this sample except for Citrine had low levels of expression; the Citrine signal is an order of magnitude greater than that of the Cerulean signal, and 3–4 times that of the mRFP and iRFP670 signal, see Figure 4.8.

In plate 1, the low level of expression was compensated for by the longer exposure time and higher laser power, but this does not change the ratio of FP fluorescence to background

cellular autofluorescence. This was particularly visible in the Cerulean channel, where the exposure time was longest. Autofluorescence in dead cells was also visible in the iRFP670 channel; this can be seen in well F5 (*ERBB2*WT-Cerulean only) shown in

Figure 4.7(a), as well as in the later experiments, see Section 4.3.3 for a more complete discussion.

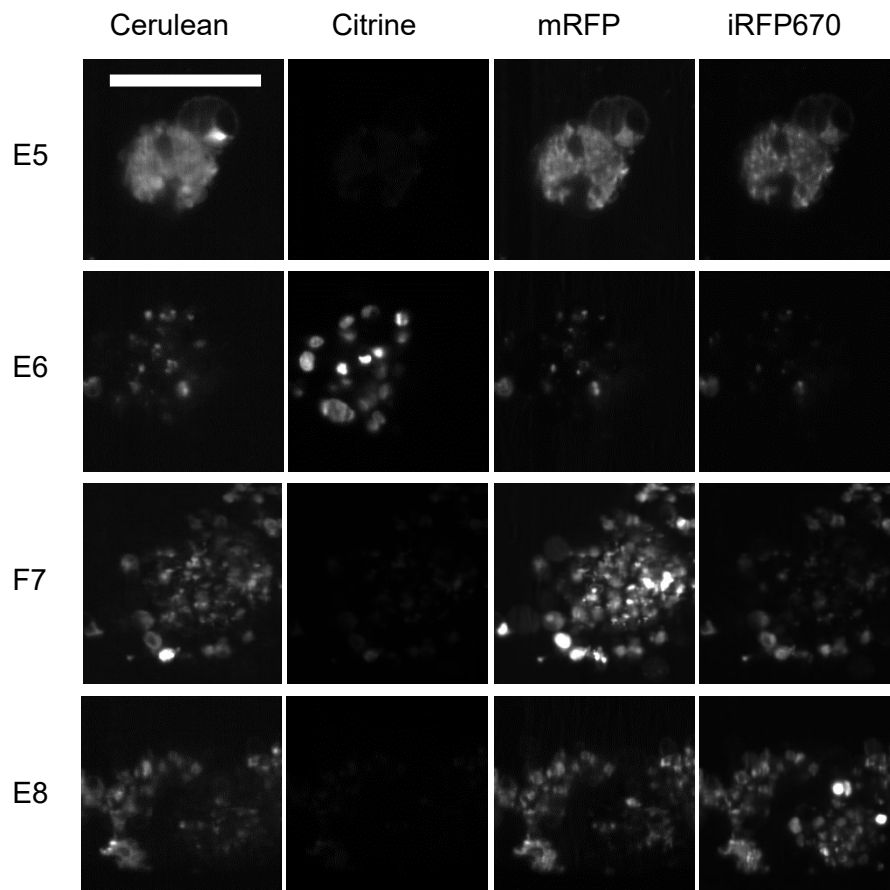


Figure 4.8 – Each row shows an example spheroid from live plate 1 which each contains a single cell line: *ERBB2*WT-Cerulean, *BRAF V600E*-Citrine, *ERBB2*D16-mRFP and *KRAS G12V*-iRFP670 only, from wells E5, E6, F7 and E8 respectively. The columns show the four spectral channels. Ideally, only the diagonal images would be bright. Each channel has the brightness scaled differently, and relative to the Citrine scaling, this is: Cerulean – 44 $\times$, Citrine – 1 $\times$, mRFP – 12 $\times$, iRFP670 – 18 $\times$. Signal in the non-diagonal channels is attributed to autofluorescence. Autofluorescence in the Citrine channel is lowest relative to the signal from the FP, followed by mRFP, while Cerulean and iRFP670 suffer from significant autofluorescence relative to their FP signals. Scale bar: 100 μ m.

The variation in fluorescence signal between FPs means that some cell lines will be quantified more accurately than others in the nucleus counting method. In Figure 4.8, the nuclei are resolved easily by eye in the Citrine channel, and less so in the other channels where the signal-to-background ratio (SBR) and signal-to-noise ratio (SNR) is lower and there are additional non-FP sources of fluorescence. We attributed the background fluorescence to autofluorescence rather than crosstalk: for the combination of lasers, filters and FPs, the crosstalk was <1% in all cases, calculated by the product of fluorophore excitation efficiency and filter collection efficiency. A further issue was noted with the

ERBB2WT-Cerulean construct, which appeared to leak into the cytoplasm rather than remain confined to the nucleus—this causes difficulty in detecting nuclei in the image data and therefore difficulty in counting nuclei/cells. The leaky expression was fixed by generating a new *ERBB2WT*/Cerulean cell line.

In this experiment, we considered that the signal level is simply insufficient to confidently count nuclei except for in the Citrine channel. For future experiments, we decided to try and boost the fluorescence signal by sorting the cell lines with FACS shortly before the experiment to select cells with bright nuclei.

In theory, if the SNR is sufficiently high, a balance of false positive and false negative nucleus detections can be achieved by tuning the threshold level to reject background for each channel. This, however, requires careful supervision to determine true positives, and care must be taken to choose the thresholds so that there is no bias between the channels (cell lines). For this reason, we decided to sacrifice one of the oncogenes and to use the mRFP channel as a common label present in all cell lines to assist with segmentation and to reduce any potential bias in nucleus detection between the different cell lines.

4.3 Three-oncogene spheroid model with segmentation channel

As determined in the four-oncogene experiments, the SNR for each fluorescent channel varied significantly, with some channels' signal rendering nucleus counting unviable. We therefore aimed to boost FP brightness by sorting with FACS before future experiments and introducing a common channel with the mRFP to avoid bias in nucleus counting between cell lines. The three different oncogene-FP combinations expressed by the three different cell lines used in subsequent experiments are therefore now *ERBB2WT*-Cerulean, *BRAF V600E*-Citrine, *ERBB2D16*-iRFP670. An illustration of a spheroid containing all three cell lines is shown in Figure 4.9.

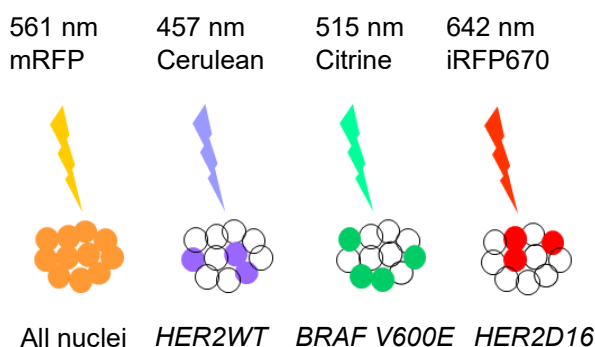


Figure 4.9 – Illustration of three-cell line combination spheroid setup which uses mRFP for a common channel expressed in all cell lines. All cell lines are now fluorescent in the mRFP channel in addition to the cell line-specific FPs.

4.3.1 Methods

In this experiment, many of the methods are shared with the four-oncogene experiment and are already presented. When this is the case, the methods will be referred back to the previous section, while changes to the methods are described here.

Cell line design

The cell line design and cell culture methods are mostly identical to that in Section 4.2.3, with the FP and oncogene integrated into the cell line with the same lentiviral transduction.

However, an additional plasmid which contains the complex H2B-mRFP (specifically the vector *LV-RFP* [118]), shown in Figure 4.10, is introduced into each cell line with the same transduction process. The transduction of the mRFP and the FP-oncogene pair is done separately such that the fluorescence of each is expressed independently.

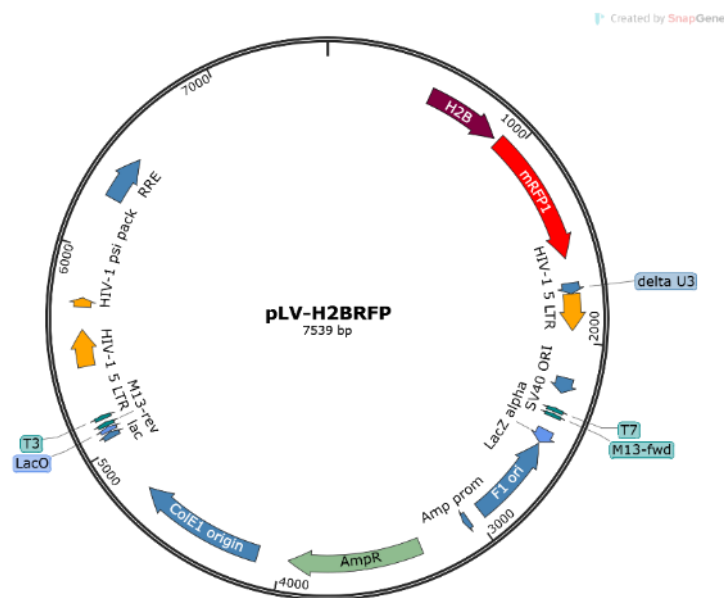
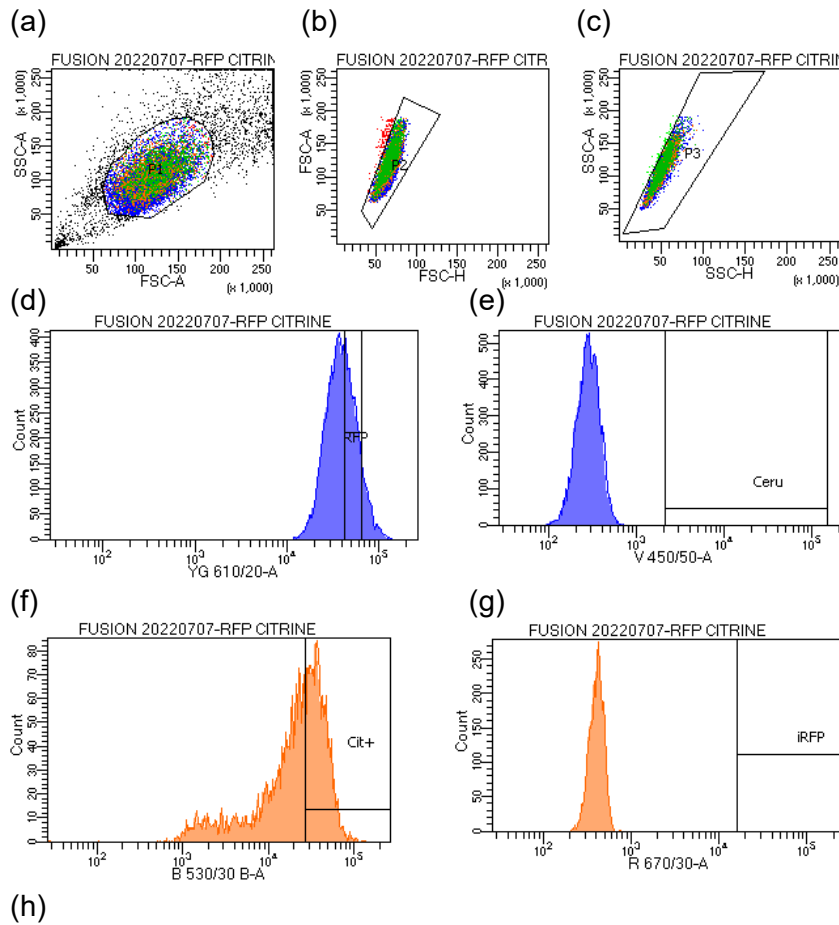


Figure 4.10 – Map of (transfer) plasmid used in transfection to integrate the gene that codes the common channel fluorescent protein (FP) mRFP into each of the cell lines. Unlike the plasmids containing Dox-inducible oncogenes (Figure 4.3), the Tet-on system is not used and the mRFP gene is always expressed. The plasmid shown in Figure 4.3 is used to transfect the oncogene-FP pairs as usual except for the KRAS G12V gene.

FACS was used to optimise for the FP signal specific to each cell line while keeping the mRFP signal within a narrow band of fluorescence. The latter aims to reduce variation in signal between cells, improve the consistency of segmentation and minimise any bias in segmentation between nuclei in different cell lines. Each cell line was sorted separately, and the first step of the FACS was to sort for a narrow band of mRFP signal in addition to removing debris and doublet cells through the forward scatter and side scatter signals. Following this, the resulting subpopulation was sorted again to maximise oncogene-labelling FP signal as shown by the example results of FACS in Figure 4.11.



Tube: RFP CITRINE			
Population	#Events	%Parent	%Total
All Events	13,355	####	100.0
P1	11,018	82.5	82.5
P2	10,867	98.6	81.4
P3	10,744	98.9	80.4
RFP	3,222	30.0	24.1
iRFP	0	0.0	0.0
Cit+	1,372	42.6	10.3
Ceru	0	0.0	0.0

Figure 4.11 – FACS data and plots for *BRAF V600E*-Citrine expressing cell line. In the first row of graphs, the forward and side scatter signal (FSC and SSC) under laser illumination is collected against time for each cell, with individual scattering events parameterised by the height (peak) -H of the distribution and area -A of the distribution. (a) The FSC signal from debris tends to be lower than for cells, and plotting FSC against SSC allows debris to be rejected by a manually drawn region. Doublet cells (two cells stuck together) result in FSC and SSC peaks which have higher height than area, and by plotting these metrics against one another for (b) FSC and (c) SSC, doublet cells can also be rejected. (d) The mRFP signal is selected within a narrow band of expression level (between the black vertical lines). Shown here is FACS for the *BRAF V600E*-Citrine expressing cell line, and therefore any false-positive or contaminant cells with fluorescence in the (e) Cerulean and (g) iRFP670 channels are rejected by discarding cells with high levels of cerulean signal, while (f) Citrine positive cells are kept and selected to be high in fluorescence. (h) the bottom table shows how cells are rejected and the percentage of the population kept in each stage of the FACS sorting (%Parent) and the percentage of the total population (%Total). FACS was performed by, and figures were generated by Nina Moderau.

Plate treatment and layout

The imaging plates were prepared using the same procedure outlined in Section 4.2.3. Compared to the four-oncogene experiment, in the three-oncogene experiment the combinations of cell lines decreased from 15 to 7, which allowed 10 repeats per condition instead of 5, and used all 18 columns available for different spheroid combinations and treatments.

In addition, the therapeutic Vemurafenib was introduced into experiments (live plates 4, 5 and 6) as an extra investigation into treatment response. Vemurafenib is usually used for the treatment of melanoma and targets *BRAF V600E* which frequently occurs in melanoma. We therefore included a Vemurafenib-treated column for cell line combinations including the cell line expressing *BRAF V600E*. The details of this experiment plate layout are shown in Table 4.2. As will be discussed later, the first two experiments with the three-oncogene model (live plate 2, and 3) were unsuccessful and the plate layout is omitted.

Table 4.2 - Experimental plate layout details for experimental plates 5, 6 and 7. Each column on the plate contained 10 replicate conditions (rows D-M), meaning the same combination of cell lines and same treatment (Dox/Vemurafenib). The + and - respectively indicate the presence and absence of Dox or Vemurafenib. E - *ERBB2WT*; B - *BRAF V600E*; d16 - *ERBB2d16*.

Oncogenes	B			H		d16		H; B			B; d16			H; d16		H; B; d16		
Column(s)	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Dox	-	+	+	-	+	-	+	-	+	+	-	+	+	-	+	-	+	+
Vemurafenib	+	-	+	-	+	-	-	+	-	+	+	-	+	-	-	+	-	+

Time-lapse imaging procedure

Since the three-oncogene model has a common fluorescent channel, the four-channel pre-find developed for the previous experiment was no longer utilised. Instead, a single-channel spheroid-finding method was developed as detailed in Section 3.2 which includes an updated low magnification pre-find, coverslip autofocus and a 3D spheroid-centring script to replace the manual processes. The same time-lapse acquisition scripts used in the three-oncogene experiment were used in this four-oncogene experiment and the DMEM, Dox and Vemurafenib was replenished after 4-5 days in the same procedure as before.

In the three-oncogene experiments, the re-find procedure presented in Section 3.2.5 is used to correct for the shifted positions when the plate holder is placed back on the stage. This uses an adapted version of the low magnification pre-find which finds spheroids that are close to the centre of the FOV rather than the biggest or brightest object—this attempts to make sure the same spheroid that was being imaged before is found, since some wells may contain multiple spheroids. This is then followed by the same coverslip autofocus and 3D spheroid-centring as used before.

4.3.2 Quantitative image analysis methods

In the three-oncogene experiments with the common mRFP segmentation channel, we used the mRFP signal to segment nuclei and then assigned each nucleus to a particular cell line using the levels of the other FP signals. This approach allows not only nucleus counting, but accurate calculation of the fluorescent signal within the nuclei and morphological analysis of the nuclei. In this subsection, the segmentation method and its development, the cell line assignment method, and the quantitative analysis of the nuclei are described.

Segmentation with classical watershed method

There are many techniques for image segmentation and a commonly used approach is the classical watershed transformation. The watershed transformation effectively uses an image input (often the gradient of the image) to represent a topographical map with pixel value defining height. Flooding is simulated originating from minima in the image which define catchment basins and the segmentation boundaries are defined along lines where the flooding sources meet.

This watershed algorithm is effective on high SNR data, but the classical form that uses minima as the initial markers is unusable on noisy data and will result in severe over-segmentation, since local minima are often detected in the noise. For this reason, image data or gradient data is often pre-processed to remove noise such as by applying median filters, but even so, this can be insufficient in solving the over-segmentation problem [119]. A common approach to overcoming this limitation is the seeded—also called marker-controlled—watershed segmentation approach, which uses information from another source to define the initial regions from which flooding is initiated.

A marker-controlled watershed script was therefore written in MATLAB (inspired by MathWorks' method [120]) to test the viability of the method. Markers are determined automatically using the LoG detection method described in Section 4.2.4, and the magnitude of the gradient of the volume is calculated as the input for the watershed method. In addition to the markers for the nuclei, the skeleton by influence zones (SKIZ) is calculated to provide a single connected region that marks the background and aims to demarcate the nuclei. The SKIZ is calculated by taking the distance transform of the binary mask (by Otsu threshold), calculating the watershed transform of the distance transform and the SKIZ is defined as a binary image where zeroes are set to 1 and other values to 0. Minima from the LoG-detected points, which estimate the locations of the nuclei, are imposed onto the gradient image with (**imimposemin**) to set the initial labels of the watershed together with the SKIZ image to label the background. The watershed transform of the gradient volume is then calculated.

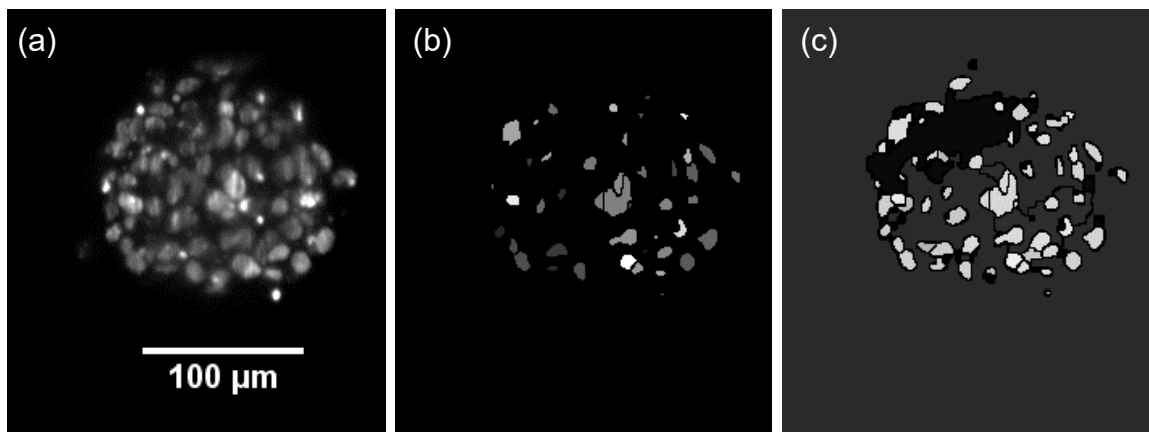


Figure 4.12 – Performance of marker-controlled/seeded watershed segmentation on induced three-oncogene live spheroid (a) mRFP channel. (b) Marker-controlled watershed segmentation with SKIZ to mark background region (c) marker-controlled watershed segmentation without marking background.

An example of the performance of the watershed segmentation script on a spheroid approximately 150 μm in diameter is shown in Figure 4.12. We see that many of the nuclei that are resolvable by eye failed to be segmented. The SKIZ used to mark the background was removed to see if the performance improved, and although more nuclei were segmented, the background was (predictably) not properly marked by a single label. Furthermore, over-segmentation was still present due to subcellular structure that is still not suppressed, possibly in the LoG detection step.

Ultimately, machine-learning-based methods were pursued to resolve these issues in segmentation. In addition to the issues mentioned, the classical methods are sensitive to hyperparameters, namely the threshold levels used in preprocessing that need to be determined manually. In addition, the spheroids vary in size, with deeper depths in the larger spheroids suffering from scattering within the sample that reduces the image contrast, and this means that the optimum hyperparameters may vary with imaging depth. This was another reason for pursuing a machine-learning method that can encode a multitude of variables to handle variations in SNR both between spheroids and between nuclei in the same spheroid.

Segmentation with deep learning

Deep learning-based segmentation algorithms StarDist [121] and Cellpose [122], [123], which are both based on a modified U-Net architecture, were investigated. StarDist uses a star-convex representation for segmentation shapes to derive the segmentation boundaries, with shapes in the star-convex domain defined such that for any point on the boundary of the shape, a line may be drawn to a common point within the shape without crossing the boundary. Cellpose uses a more general vectorial gradient-based representation and does not have such a constraint on segment shape, and it is shown in this section that Cellpose displayed the best performance and therefore was chosen as the final method.

More specifically, the Cellpose model generates a topological/spatial gradient representation of the image as is commonly done in the watershed segmentation, but unlike with the

watershed method, (smooth) gradient representations are generated by the Cellpose model that is trained on human-defined segmentations. The deep-learning approach to evaluating gradient should be better able to handle variability in nucleus sharpness and contrast. Moreover, this approach allows the effects of noise and fluorescence variations such as from chromatin condensation or other localisations of fluorescent proteins to be reduced if appropriate datasets containing examples of these fluctuations are included in the training data.

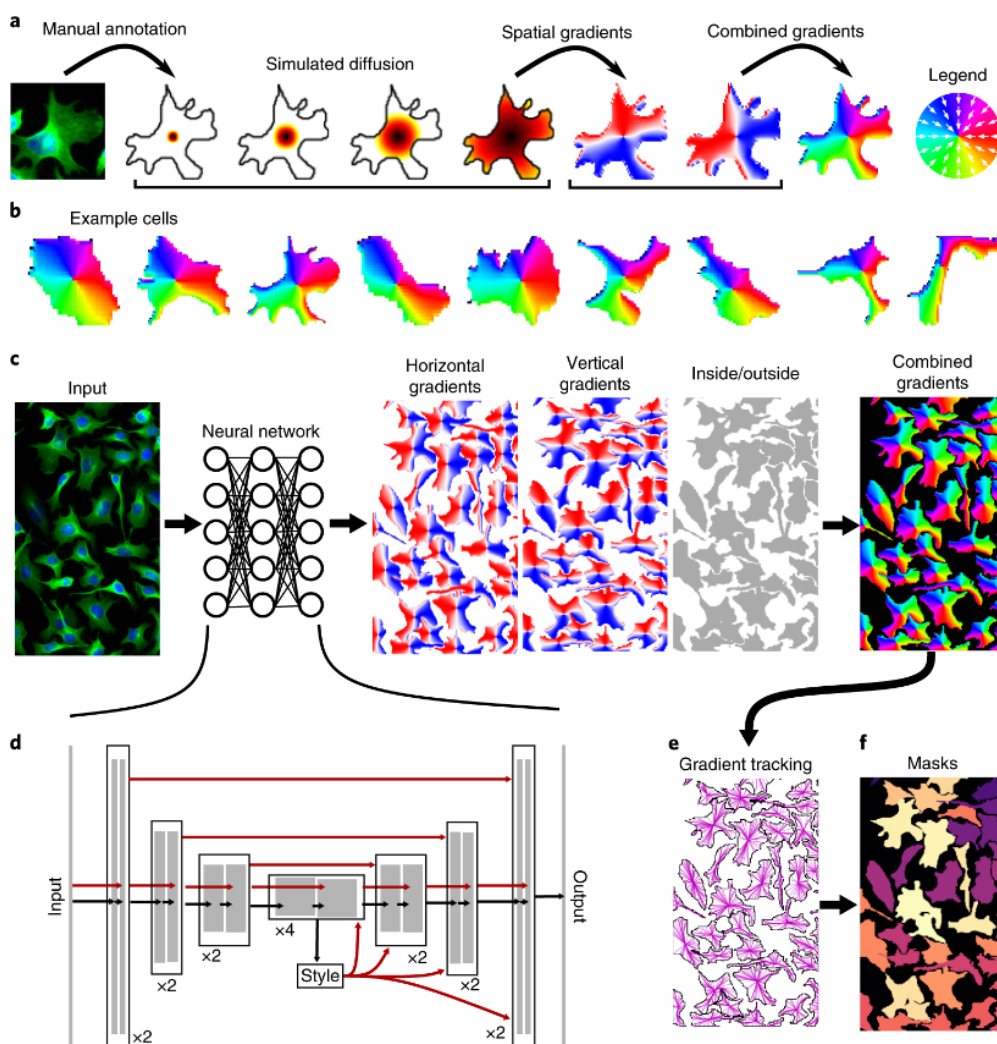


Figure 4.13 – Cellpose algorithm processes. (a) Generation vertical and horizontal spatial gradients from manual annotations which are then used as the ground truth to train the (d) U-Net-based neural network. The spatial gradient maps are combined into vectorial gradient fields. (b) Example combined vectorial gradients for various cell mask shapes. (c) Process of the estimating the binary mask and horizontal/vertical gradient representation of the image using the neural network. These outputs are combined to produce vectorial gradient fields representations. (e) Masks for each of the segments are derived by tracking the combined, vectorial gradients – (f) pixels are grouped into the same segment if the gradient tracking converges to the same point. Reproduced with permission from [122].

Although these spatial gradients are only computed in 2D for 2D images, Cellpose can predict segmentations in 3D for 3D volumes by predicting the horizontal and vertical gradient maps for 3 different views of the stack. The six predicted gradient maps (two each

for x , y and z) are averaged in each direction and combined into a single 3D gradient vector field. As is done in the 2D segmentation method, pixels are assigned to a segment by tracking the now 3D gradient vector field; the vector field is tracked from a pixel, and all pixels whose field lines converge to the same point are assigned to the same segment. The Cellpose method is summarised in Figure 4.13.

Cellpose segmentation performance

Cellpose (2.0 [123]) was initially used to segment spheroids volumes using Cellpose’s pre-trained ‘nuclei’ model. This performed unsatisfactorily, with false negatives clearly visible as well as under and over-segmentation as shown in Figure 4.14. Furthermore, in larger spheroids for which image SNR is lower due to more severe scattering of excitation and emission signal, false negatives were higher.

Retraining was therefore necessary. To achieve this, one spheroid from a fixed imaging experiment and one from a live experiment were manually annotated slice-by-slice using VAST lite [124]. The training was run for 150 epochs and the training data was augmented by a flip in the y -axis and a translation of $x = 20$, $y = 100$, resulting in 3 different orientations of the training data in total, which we found helped to suppress over-segmentation.

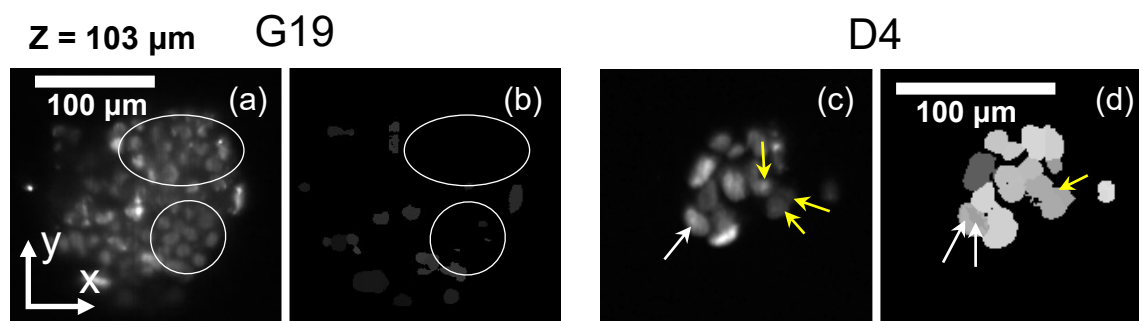


Figure 4.14 – Cellpose segmentation performance with pre-trained “nuclei model” for two example spheroids (mRFP channel). (a) three-oncogene spheroid, not induced, (b) segmentation output. White ellipses show regions of missed segmentations or false negatives. (c) Example spheroid with only *ERBB2*WT cell line, not induced, (d) segmentation output. Yellow arrows in (c) and (d) show under-segmentation of three nuclei as one segment, while white arrows show over-segmentation of one nucleus with two segments.

Initially, only the x - y slice direction was used to train Cellpose, however, since the training data volumes were annotated fully in 3D, the y - z and x - z slice directions could be used in the training too. It was found that this was important in improving segmentation since the Cellpose 3D segmentation process combines the output from each of these slice directions to predict the segments. The improvement was postulated to be due to scattering of the fluorescence signal being worse for larger spheroids and worse deeper into these spheroids, i.e., it has a z -dependence. This means that x - z and y - z stacks are likely to have a larger, more representative range of nucleus SNR within any given image compared to x - y stacks, and this may encourage more scattered nuclei to be segmented. Also, if the x - z and y - z stacks have different properties than the x - y slices and the model is only trained with x - y

stacks, it will not form accurate predictions for the resliced directions—i.e. for 2 out of 3 of the directions considered by Cellpose’s 3D segmentation method.

An example of the Cellpose segmentation achieved with the different models is shown in Figure 4.15 for an x-y slice taken at a depth of 103 μm from the coverslip where scattering is noticeable. This shows the clear increase in successful segmentations when the model is retrained on manually annotated data and when all three slice directions are included.

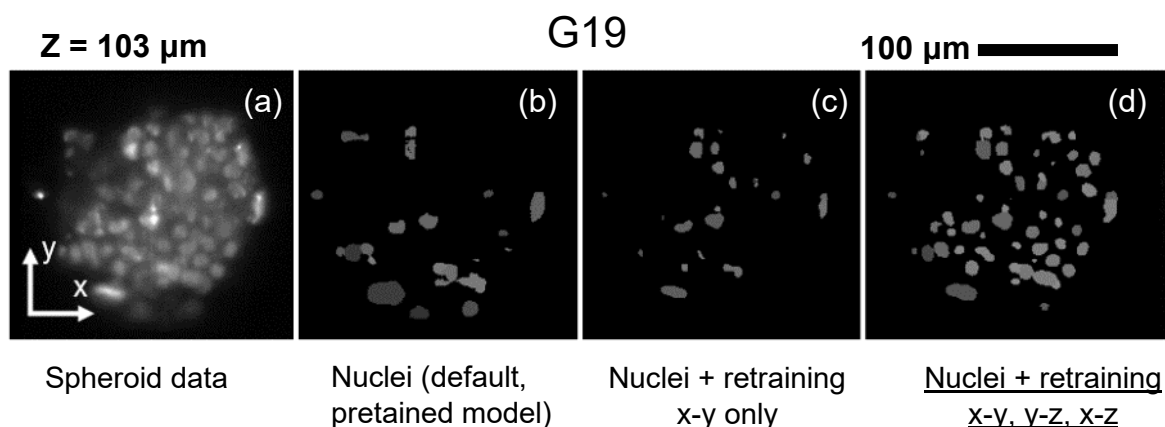


Figure 4.15 – Comparison of segmentation output for different Cellpose models for a three-oncogene non-induced spheroid. (a) x-y slice of mRFP segmentation channel at a depth from coverslip of 103 μm . Segmentation results are shown in (b) for the default pre-trained model, (c) for the re-trained model from the two manually annotated spheroids (x-y planes only), and (d) the re-trained model using planes from all three slice directions.

StarDist was also trained with the same manually annotated data as used for training the Cellpose model, and the results from each of the machine-learning-based segmentation methods (as well as the classical watershed method) were directly compared for a manually annotated non-induced spheroid (L19) from live plate 4, shown in Table 4.3, using the **seg_compare** Python package [125]. Segments are identified as positive detections, under-segmentations, over-segmentations, many-to-many or in-background segmentations depending on the overlap with the ground truth (manual annotation). The in-background segmentations indicate where the algorithm has segmented an area that is not annotated in the ground truth, but this is not necessarily a false positive since the manual annotations in this dataset were only ones that were confidently segmentable by eye. It may be pertinent in future performance characterisations to attempt manual annotations on these spurious nuclei that are difficult to determine by eye; furthermore, an induced spheroid should be used which has a wider variety of nuclear morphology.

In addition to assessing segmentation performance semi-qualitatively with these categories, the Jaccard index (JI) is calculated which quantifies the overlap of the ground truth and predicted segmentation. It is calculated as the intersection over the union of the ground truth and predicted segmentations. For any pair of ground truth and predicted segments this is given by

$$JI_{ij} = \frac{|S_{g,i} \cap S_{p,j}|}{|S_{g,i} \cup S_{p,j}|}, \quad 4.2$$

where $S_{g,i}$ is the ground truth segmentation for segment i , and $S_{p,j}$ the predicted segmentation for segment j . For this to be meaningful, the maximum JI for a given segment i is considered in order to pair the ground-truth segments with the appropriate predicted segment. The total JI over all i segments can then be calculated by

$$JI = \sum_i \max_j \frac{|S_{g,i} \cap S_{p,j}|}{|S_{g,i} \cup S_{p,j}|}. \quad 4.3$$

The volume-weighted JI (JIV) can also be calculated by

$$JIV = \frac{1}{V_i} \sum_i V_i \max_j \frac{|S_{g,i} \cap S_{p,j}|}{|S_{g,i} \cup S_{p,j}|} \quad 4.4$$

where V_i is the volume of the i^{th} ground-truth segment. The results, see Table 4.3, show that the pre-trained nuclei Cellpose model has significantly worse performance in all metrics compared to the trained machine-learning-based methods, and notably, worse performance than the classical marker-controlled watershed method. The StarDist segmentation appears to successfully detect more nuclei in the ground truth compared to the Cellpose models but had a lower value of JIV and a lower percentage of successful (not over or under or many-to-many segmentations) than the current trained Cellpose model at 78.5% compared to 82.9%. Indeed, under visual inspection, the StarDist algorithm appears to over-segment ellipsoidal nuclei with multiple more spherical shapes and in general the algorithm tended to segment with overly spherical shapes, as seen in Figure 4.16. This is perhaps a result of the star-convex constraint which would fail to correctly segment some “bean-shaped” nuclei, for example.

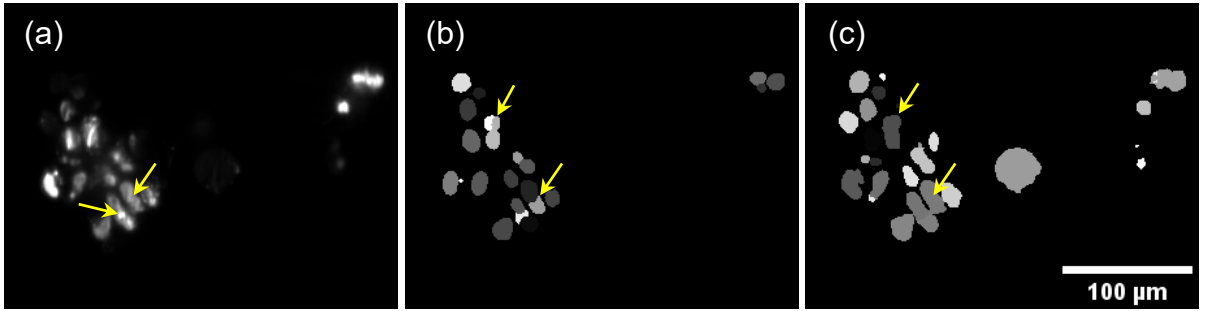


Figure 4.16 – (a) Slice of induced three-oncogene spheroid (mRFP channel). (b) StarDist segmentation showing failure of StarDist to correctly segment ellipsoidal objects compared to (c) Cellpose segmentation indicated by yellow arrows.

Table 4.3 – Table of segmentation method performances based on a non-induced three oncogene spheroid (L19) manually annotated ground truth from live plate 4. Numbers in bold indicate best performance across the techniques to be considered (StarDist and Cellpose).

	Marker-controlled watershed	StarDist	Cellpose		
Details	Post-processed to remove large regions	Trained, 2 volumes	Nuclei model	Retrained, 2 volumes, XY stacks	Retrained, 2 volumes, XY, YZ, XZ stacks
Manually segmented nuclei	159				
Percentage of manual nuclei detected	89.3%	93.7%	76.1%	89.9%	91.8%
Total nuclei detections	346	272	338	228	282
Percentage Segmentation in background	71.4%	46.7%	37.9%	46.2%	51.7%
Percentage of successful nuclei segmentations	76.1%	78.5%	61.2%	77.6%	82.9%
Percentage Under-segmented	0.7%	5.4%	2.5%	9.8%	12.14%
Percentage Over-segmented	17.6%	13.4%	36.36%	12.6%	3.57%
Percentage misc. incorrect segmentations	2.8%	1.3%	0.0%	0.0%	0.7%
Jaccard index (JI)	0.24716	0.4628	0.20785	0.50202	0.46112
Volume-weighted JI	0.28945	0.52796	0.17443	0.58406	0.54646

Nucleus assignment using FP signals

In order to enable the data to be analysed, each segmented nucleus must be assigned to a cell line population according to its fluorescent signal in the non-common spectral channels—these fluorescent signals are obtained by computing the average (mean) of the pixels in the non-common channels within the segmented volume obtained from the common mRFP channel.

A procedure was developed using MATLAB (ultimately as part of the full analysis script) by representing the fluorescent intensities of nuclei in a 3D point cloud with the axes being the intensity in each of the channels that correspond to an oncogene: Cerulean, Citrine and iRFP670. A scaling factor was applied to the values in each channel so that intensities were comparable, and three lines were initially defined along each of the channel axes in this 3D space, defined as

$$\mathbf{L}_1(x_1) = (x_1, b_2, b_3), \quad \mathbf{L}_2(x_2) = (b_1, x_2, b_3), \quad \mathbf{L}_3(x_3) = (b_1, b_2, x_3) \quad 4.5$$

where b_i are the respective constant background values in each channel (which may be set to 0 if appropriate) and x_i are variables which define the lines. In practice, these background values can be calculated iteratively by assigning nuclei (points) to each of the cell line populations and calculating the median values in the non-signal channels, i.e., the background values of group 2 and 3 are the median of the intensities in channels 2 and 3 for nuclei assigned to group 1.

By considering the minimum Euclidian distance of the points to each of the lines, the points are assigned to a particular FP/cell line. The minimum fractional distance metric $d_{j,i}$ for a given point j is used to quantify the distance to the lines (parameterised by i) and is given by

$$d_{j,i} = \frac{D_{j,i}}{D_{j,1} + D_{j,2} + D_{j,3}} \quad 4.6$$

where $D_{j,i}$ is the (minimum) Euclidian distance between a point j and line i . The value of i that corresponds to the minimum value of $d_{i,j}$ determines the cell line that point j is assigned to, but the segment is unassigned for values of $d_{j,i}$ over a threshold and if none of the intensity signal values are greater than a minimum threshold. A value of $d_{j,i}$ higher than the threshold represents a case where the assignment is ambiguous due to signal from multiple channels, and this can be due to under-segmentation (includes multiple nuclei expressing different FP), from autofluorescence, or even due to the loss of the FP gene. The point cloud of nuclei for a volume containing nuclei from all 3 of the cell line populations and the lines used to identify the nuclei are shown in Figure 4.17.

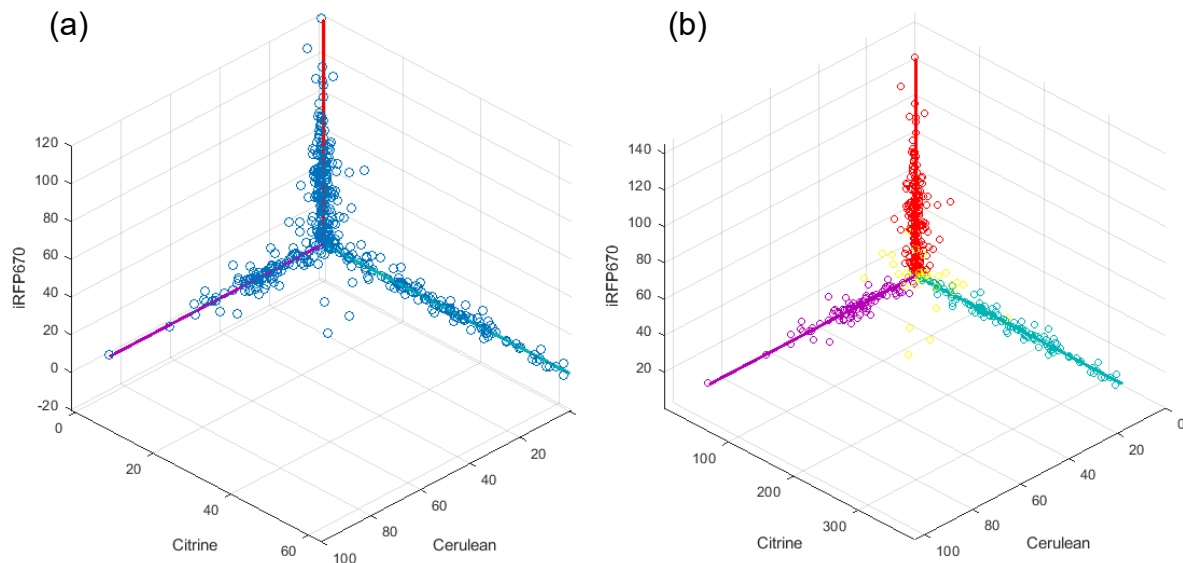


Figure 4.17 – Point cloud method for nucleus assignment. (a): Cloud of points where each point represents the fluorescence intensity of a single nucleus in each of the 3 channels; ideally nuclei only emit into one channel, and points should exist along each of the 3 lines away from the origin. (b): nuclei points after assignment, with the yellow group representing rejected segments due to ambiguous assignments or low fluorescent signal.

Measurements from segmented nuclei

By assigning nuclei to the corresponding cell line populations, it is possible to derive measurements of each spheroid on the nuclear level for each population independently. As mentioned previously, this allows the number of nuclei in each population to be calculated for each spheroid, but additionally, the mean nuclear signal within nuclei (as used in the previous section), nuclear volume/size, with the potential to extend this further to other measurements in the future.

Since this is done at the nuclear level, it also allows analyses of the distribution of nuclear properties and how they evolve over time, and this can be presented in the form of histograms at each time point. A summary of all the measurements and figures generated is presented in Table 4.4, and a subset of these analyses is presented in the next section.

Table 4.4 – Summary of nuclear measurements obtained from image data and the derived readouts from which analysis figures are generated. Here, nuclear signal is defined as mean nuclear intensity (in camera numbers) from within a segmented nucleus. Each metric is calculated for each timepoint, with the histograms visualised as 2D heatmaps to include the time dependence.

Measurement	Derived readout	Comments
Nucleus count	Nucleus count	Number of nuclei in each population within a single spheroid
	Fractional nucleus count	Number of nuclei in each population within a single spheroid, normalised by total number
	Nucleus count across column	Number of nuclei in each population averaged across spheroids under repeat conditions (i.e. mean is calculated over rows of plate for a given column)
Nucleus signal	Average signal across nuclei	For each cell line/channel, the mean nuclear signal calculated across nuclei from a single spheroid
	Average signal across column	For each cell line/channel, the mean nuclear signal calculated across all nuclei from spheroids under repeat conditions (i.e. mean is calculated over rows for a given column)
	Histogram of signal	For each cell line/channel, distribution of nuclear signals within a single spheroid
	Histogram of signal across column	For each cell line/channel, distribution of nuclear signals using all nuclei across repeat conditions (i.e. nuclei from the same column are combined into one histogram)
Nucleus size	Average size	For each cell line/channel, the mean nuclear volume calculated across nuclei from a single spheroid
	Average size across column	For each cell line/channel, the mean nuclear volume calculated over all nuclei from spheroids with repeat conditions (i.e. mean is calculated over rows for a given column)
	Histogram of sizes	For each cell line/channel, distribution of nuclear volume within a single spheroid
	Histogram of sizes across column	For each cell line/channel, distribution of nuclear volume using all nuclei across repeat conditions (i.e. nuclei from the same column are combined into one histogram)

Post-processing to remove spurious data

To reduce false positive detections and assignment of nuclei, and to reject data points in the subsequent analysis, thresholding and masking steps were applied as follows. For individual nuclei, we apply the following thresholds shown below. Table 4.5 shows the mRFP threshold and volume thresholds applied to all nuclei before assignment with the aim of rejecting poor segmentations.

Table 4.5 – Thresholds used to reject nuclei from analysis.

Threshold	Comment	Value used
mRFP channel pixel value	Reject objects with low mRFP signal to avoid poor segmentations. Applied after background subtraction.	70
Nucleus volume in px^3	Reject objects that are too small to be nuclei	150
FP [Cerulean, Citrine, iRFP670] pixel value	Applied after background subtraction (but before channel scaling in the assignment step)	[9 9 9]

4.3.3 Issues and iterations of experiment

A total of 5 live-cell experiments were conducted measuring the evolution of these three-oncogene spheroids, and the experiments were iterated upon after encountering issues in the experimental process. Out of these experiments, the analysis of two is presented, namely what are called live plate 4 and live plate 6, and the naming and descriptions of these plates are summarised in Table 4.6.

Table 4.6 – List of time-lapse experiments using the three-oncogene model. Initially, in live plates 2 and 3. Dox – Doxycycline, Vem – Vemurafenib.

Plate name	Comments	Treatments
Live plate 2	Spheroids died within 3 days due to lack of CO ₂	<u>Spheroids without</u> <u><i>BRAF V600E</i></u> :
Live plate 3 (supplementary video 4.2)	Widespread spheroid attachment to coverslip	-Dox, +Dox <u>Spheroids with</u> <u><i>BRAF V600E</i></u> : -Dox, +Dox, +Dox/Vem (20 µM)
Live plate 4 (supplementary video 4.3)	Results are presented here. Spheroid death beyond 180 h, High Vemurafenib concentration was generally toxic to cells	<u>Spheroids without</u> <u><i>BRAF V600E</i></u> : -Dox, +Dox <u>Spheroids with</u> <u><i>BRAF V600E</i></u> : -Dox/Vem (10 µM), +Dox, +Dox/Vem (10 µM)
Live plate 5 (supplementary video 4.4)	Spheroids did not grow; attributed to initial size being too small	<u>Spheroids without</u> <u><i>BRAF V600E</i></u> : -Dox, +Dox
Live plate 6 (supplementary video 4.5)	Results presented here	<u>Spheroids with</u> <u><i>BRAF V600E</i></u> : -Dox/Vem (5 µM), +Dox, +Dox/Vem (5 µM)

CO₂ supply

For the first of these experiments, named live plate 2 (live plate 1 is the initial four-oncogene experiment presented in Section 4.2), the spheroids died and lost viability within 3 days and the experiment was terminated. The phenol red solution in the culture medium indicated high pH at the end of the experiment and spheroid death was attributed to the CO₂ flow rate being set too low because 5% CO₂ is required to maintain the cell culture medium pH at 7.4. For subsequent plates, the 5% CO₂ in air flow rate was increased.

Cell attachment to coverslip

For the first two live experiments (live plates 2 and 3), Greiner 384-well Sensoplates (#781892) were used and treated (in-house by Nina Moderau) with BIOFLOAT™ (faCellitate) to prevent cell attachment and allow spheroids to grow in 3D rather than across the coverslip. However, in live plate 3, any spheroids situated close to the well edge appeared to still attach to the coverslip and well edges, causing them to grow rapidly and mostly in 2D, losing the spheroid shape as shown with the spheroid in K14 in Figure 4.18(a). It was thought that the coating did not sufficiently treat the well edges, as most spheroids nearer the centre of the well continued to grow as spheroids successfully. A few spheroids

that were not at the edges were observed to still attach to the coverslip such as with the spheroid in G9, and this is also shown in Figure 4.18(b).

In subsequent experiments, Greiner CELLSTAR® plates were used instead and we observed no cell attachment during 10 days of live imaging (live plates 4, 5 and 6).

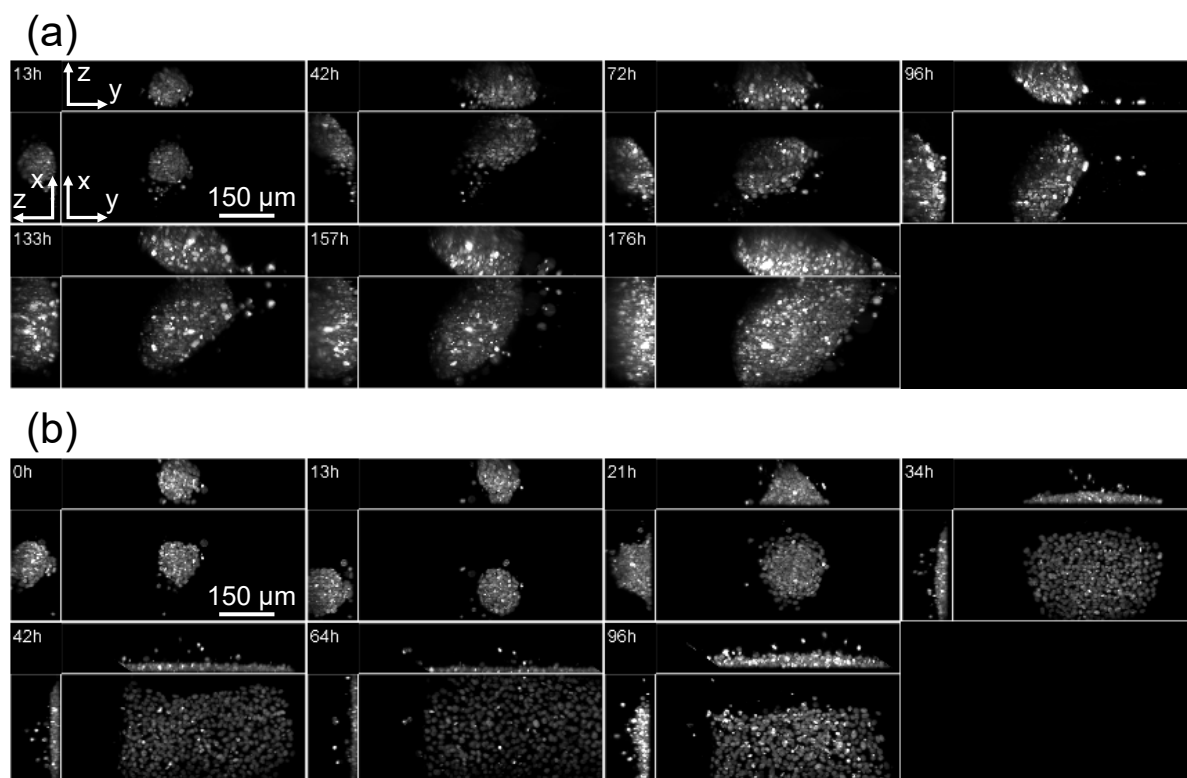


Figure 4.18 – Orthogonal maximum intensity projections (ortho-MIPs) at different time-points from OPM images taken in the live plate 3 experiment (mRFP channel). Top (a): well K14, an example of non-induced spheroid attaching at the corner of the well. Bottom (b): well G9, an example of non-induced spheroid attaching to coverslip while being situated away from the corner or edge of the well. Each example is non-induced, showing that the experimental setup is the cause rather than the phenotype due to the oncogene.

Vemurafenib cytotoxicity

The next issue encountered in these experiments was with Vemurafenib cytotoxicity in live plate 4. To provide an accurate control condition for wells treated with Dox (+Dox) and Vemurafenib, the associated non-induced wells without Dox (-Dox) were also treated with Vemurafenib, which should not have a targeted effect on the non-induced cells and inhibits B-Raf encoded by the *BRAF* V600E mutation only in induced cells.

In live plate 4, non-induced spheroids treated with Vemurafenib would die by approximately 9 days, while Vemurafenib should not have a measurable effect when spheroids are not induced, see Figure 4.19. We attributed this to a non-targeted effect of Vemurafenib and this introduces an uncontrolled variable for comparing +Dox and -Dox conditions. For further experiments, the concentration of the Vemurafenib solution was therefore reduced from 10 μ M in live plate 4 to 5 μ M in plates 5 and 6, intending to observe only targeted effects of Vemurafenib in the wells treated with both Dox and Vemurafenib i.e., within the

therapeutic window. There is, however, still valuable information contained in this dataset; it shows the cytotoxic effect of Vemurafenib at high doses, and, as exemplified in Figure 4.19, that there was more and earlier onset of cell death in the presence of both Vemurafenib and Dox than just Vemurafenib.

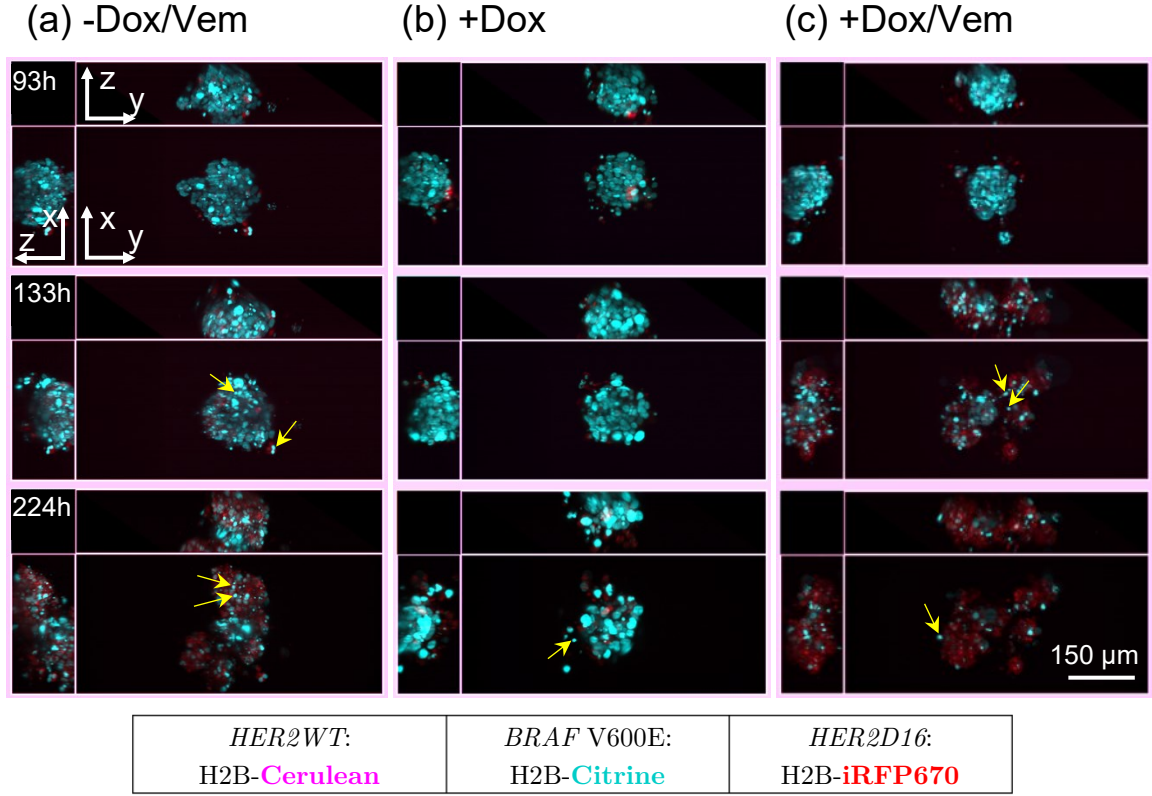


Figure 4.19 – Non-targeted cytotoxicity of Vemurafenib (Vem) in live plate 4. (a) Non-induced single oncogene (*BRAF V600E*) spheroid treated with Vemurafenib (well E6), (b) the induced case, not treated with Vemurafenib (E7) and (c) induced and treated with Vemurafenib (F8). Yellow arrows indicate dying cells which shrink, and the FP (Citrine) becomes more concentrated.

Cell death during imaging in small spheroids

For live plate 5 the experiment was initiated with smaller spheroids relative to previous experiments. The reduction in starting cell number was chosen to reduce the overall size of the spheroid by the end of the 8-day experiment to allow the spheroid to remain within the FOV. Therefore, after seeding wells with 50 cells, the spheroids were transferred into the imaging plate after one day instead of two days of growing. However, the reduction of starting cell number led to a premature spheroid death independent of treatment of genotype.

By the 7-day point (168 h), many of the spheroids (particularly the three-cell line combinations) disintegrated and flattened, indicating reduced viability of the spheroids. This is shown in Figure 4.20, which compares spheroids in column 20 (Dox-only, 3 cell-line combination) between live plate 4 and live plate 5. Furthermore (not shown here), by 180 h, the 3 cell-line spheroids in live plate 5 cease to have major morphological changes, again suggesting total loss of viability/death of the spheroid.

This was observed in wells not treated with Vemurafenib, so the cell death must be due to other factors such as incubator temperature, insufficient CO₂ supply, or phototoxicity. In this case, the starting spheroid sizes in live plate 5 were smaller than in live plate 4, but otherwise, the experimental conditions should be identical; for this reason, we hypothesise that larger spheroids are more resilient to the factors that contribute to unwanted cell death. The factor that is most readily controlled is the phototoxicity from imaging, and therefore to mitigate potential phototoxicity the imaging schedule for the first two days of the experiment was reduced for live plate 6, as well as allowing spheroids to grow larger before the imaging experiment is initiated.

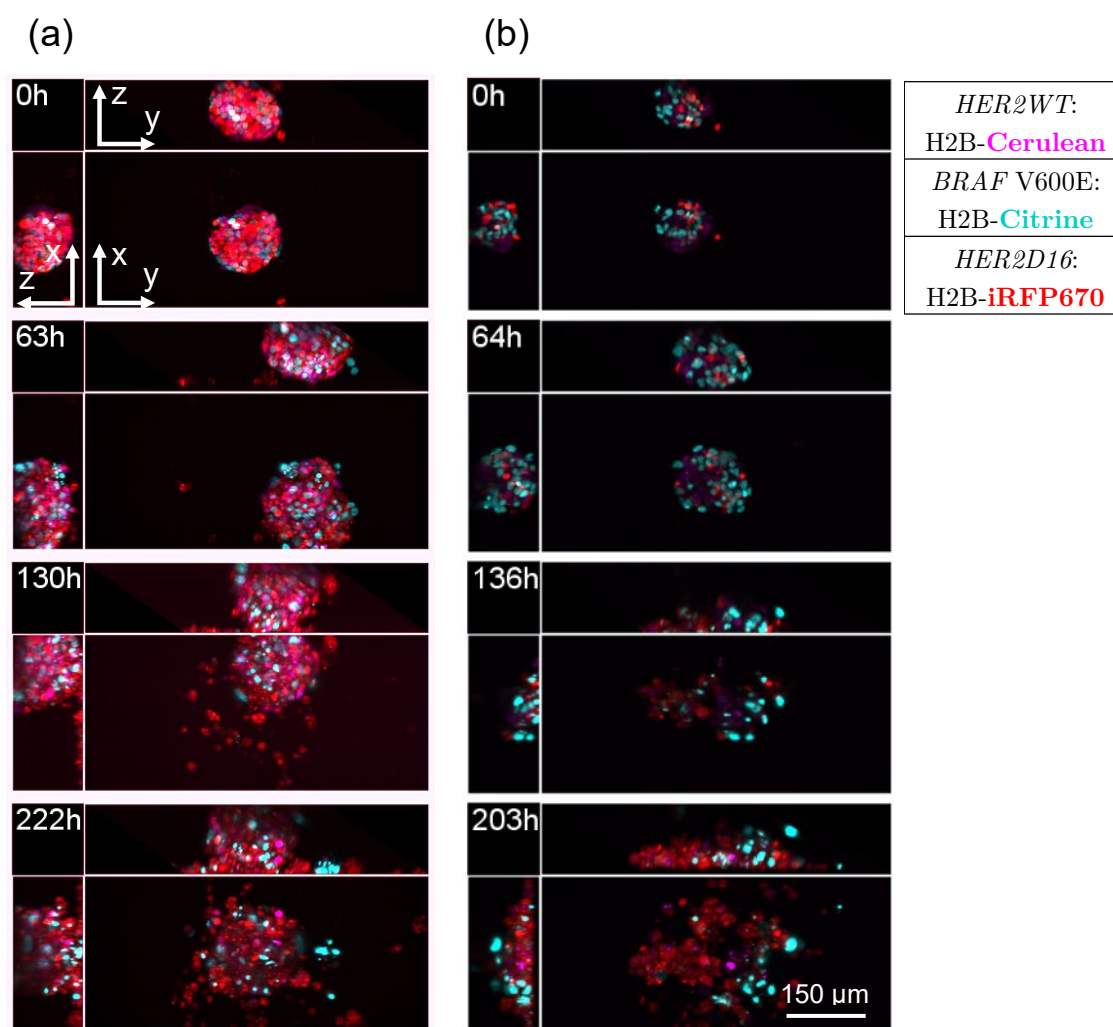


Figure 4.20 – Representative example of induced three-cell line spheroids at different timepoints from column 20 in live plate 4 (a) and live plate 5 (b); wells are K20 and I20 respectively. These spheroids are both a mixture of all three cell lines and expression of the oncogenes was induced by the addition of Dox in both cases. The spheroid from live plate 5 has a smaller starting size and starts disintegrating and flattening by 136 h, while the spheroid in live plate 4 retains a more spherical shape for longer.

False detections of dead cells in iRFP670 channel

In live plate 4, dead cells appear to display increased autofluorescence in the iRFP670 channel, as seen in Figure 4.19(c). This has the potential to cause issues with false-positive

assignments of dead cell nuclei to the *ERBB2D16* cell line population. However, as there is no significant autofluorescence in the mRFP segmentation channel, if the segmentation is accurate the dead cells should never be detected; yet in live plate 4, significant numbers of false detections of dead cells as *ERBB2D16* cells were observed by the 200 h time-point. On investigation, the segmentation model appeared to erroneously segment small dim regions in the mRFP channel which coincided with the autofluorescence in the iRFP670 channel; we see this effect when analysing the nuclear counts over time for live plate 4, see Figure 4.21. These graphs collate nucleus counts in each of the spheroids in column 8 (*BRAF V600E* cells expressing Citrine only, treated with Dox and Vemurafenib) and show an increase in detected iRFP670-expressing nuclei towards the end of the experiment when there should be no iRFP670 expressed. To resolve this, during analysis, segments with a volume below a threshold and with a mean mRFP signal below a threshold are rejected. The volume threshold chosen was 150 px^3 , and the mean mRFP signal threshold (above the background) was 70 camera digital numbers above background.

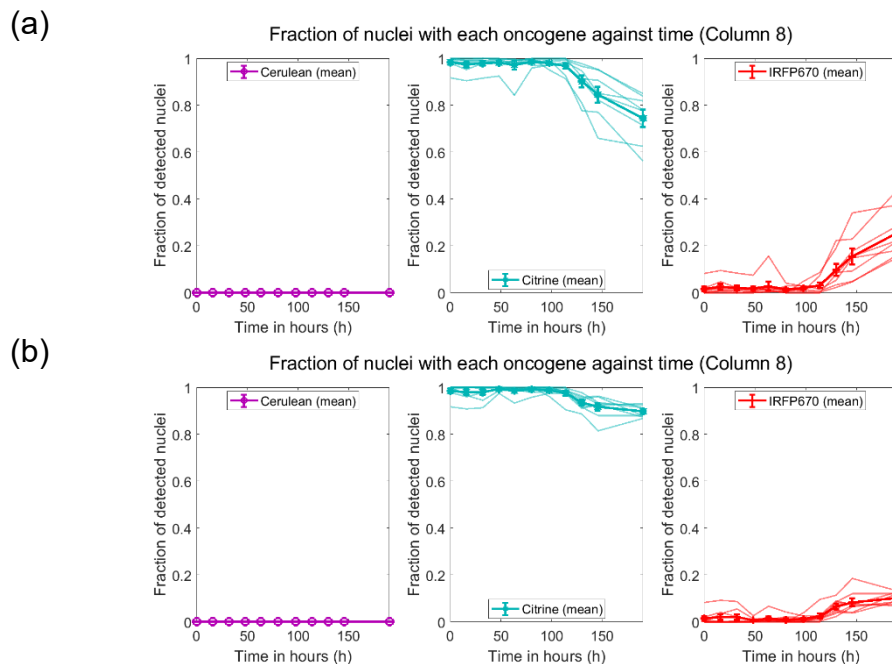


Figure 4.21 – Fractional nuclear counts for single cell line spheroids (*BRAF V600E* only) in column 8 (+Dox, 10 μM Vem) for live plate 4, (a) before applying thresholds to mRFP channel to suppress false positives and (b) after. Mean of nucleus count (thick line) is calculated across rows/repeats for each assigned cell population, and plotted with standard error error bars. Fractional cell count curves from each individual spheroid are also overlaid (thin lines).

4.3.4 Results and discussion for three-oncogene spheroids

We observed the growth and selective pressure of spheroids expressing up to 3 different oncogenes in 5 live imaging experiments with OPM, and of those, live plate 4 and live 6 were processed with the data analysis pipeline. This includes 3D segmentation of nuclei, assigning nuclei to one of the 3 cell line populations, and time-resolved analysis of nuclear count, signal intensity, and volume in px^3 . Three frames of the time-lapse ortho-MIP montage movies for live plate 4 and live plate 6 are shown in Figure 4.22 and Figure 4.24 respectively.

The analysis was first run on individual spheroids and then the results were grouped according to columns, since for a given column, the conditions are identical. This was done by computing the mean of each timepoint across the repeats, excluding values from spheroids where the number of nuclei is below a threshold of 10 to prevent the data being skewed by small clusters of cells (which don't represent spheroids) and missing timepoints. Additionally, these mean plots computed for each column are combined according to the combination of cell lines/oncogenes, to compare the effect of different conditions, i.e., Dox and Vemurafenib. All these figures are found in the Box cloud storage links (Chapter 4 full analysis data, page 18) with just the most relevant figures where the data is discussed in the text shown below in this chapter.

This is a large and rich dataset, and here the focus has been on analysing the data with a qualitative approach. In particular, I've looked for observations that are consistent between plates 4 and 6 in comparable cases i.e., without the effect of Vemurafenib since the concentration used was different between the two plates, as well as potential Vemurafenib dose-dependent differences. Of course, any consistency may still just be a statistical fluke, but it is important to identify trends that could be tested in any subsequent future-planned experiments which provide more biological repeats.

Care must be taken when interpreting these results, especially between experiments. The different cell lines have different proliferation rates, for example, particularly in live plate 4, the *ERBB2D16* cell line has a higher proliferation rate than the *ERBB2WT* cell line, and this is irrespective of the presence of the inducer. After this, the fast-proliferating *ERBB2D16* cell line was discarded and the original parental cell line was used which didn't show the increased growth. Indeed, spheroids in live plate 4 grow much larger and higher in cell count than in live plate 6 by 180 h and as such, future experiments may be run for longer until the spheroids fill the FOV. Therefore, for each experiment, the readouts (e.g. nucleus count) are considered relative to the non-induced, control spheroids within the same imaging plate as discussed in the following section. The two experiments—live plate 4 and live plate 6—were treated with different concentrations of Vemurafenib—10 μ M and 5 μ M respectively—which means that to assess repeatability between experiments, the *ERBB2WT* and *ERBB2D16* combination spheroids are analysed as these are not treated with Vemurafenib. The three-oncogene spheroids will also be presented and compared, and further analysis of the *BRAF V600E* cell line will be presented to investigate the effects of Vemurafenib.

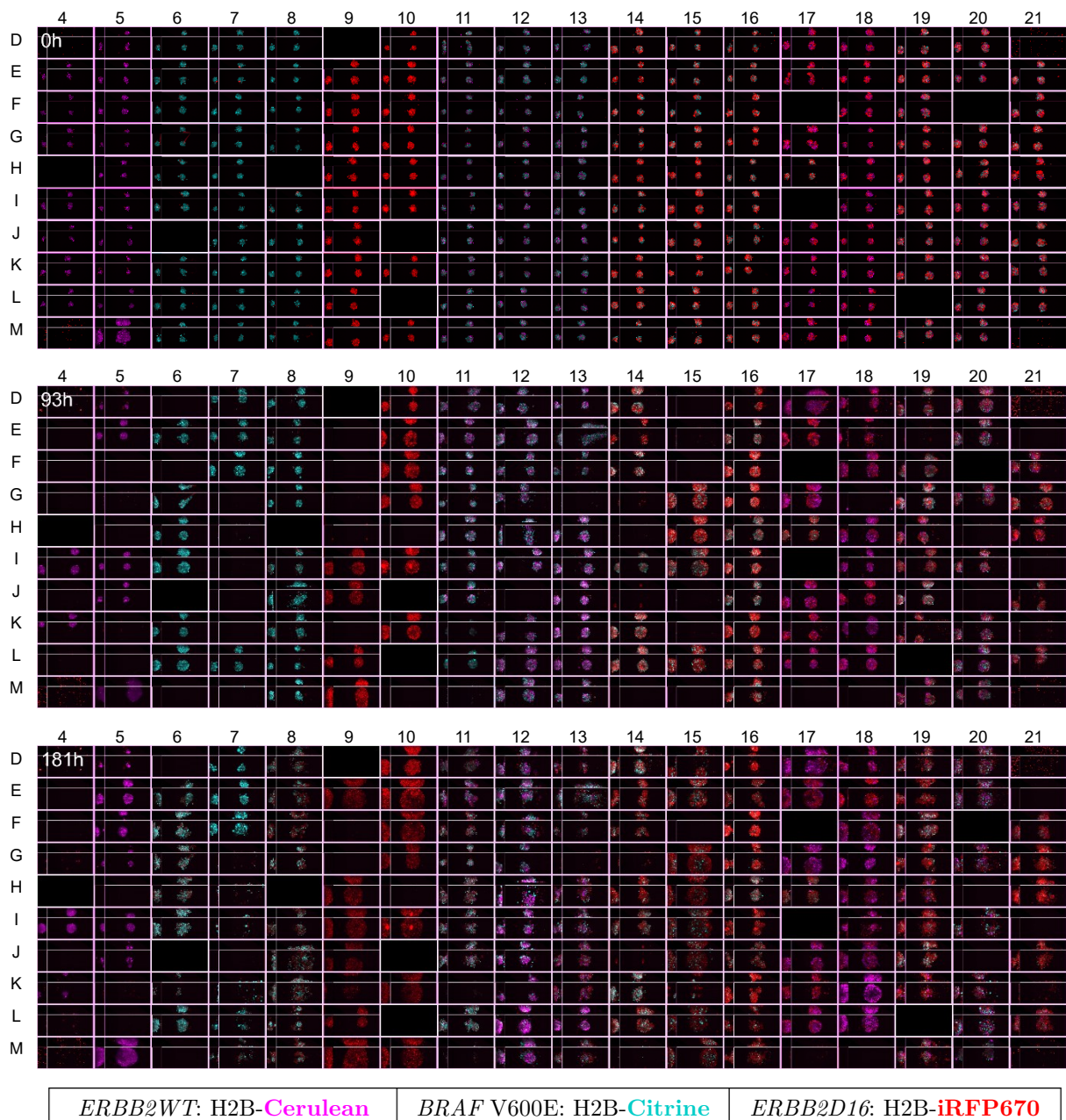


Figure 4.22 - Live plate 4 ortho-MIP montage at timepoints (a) 0 h, (b) 93 h and (c) 181 h. Magenta is *ERBB2*WT-Cerulean; cyan is *BRAF* V600E-Citrine; red is *ERBB2*D16-iRFP670. Each thumbnail represents an FOV of $302 \times 705 \times 144 \mu\text{m}^3$ (x, y, z). For full file see supplementary video 4.3.

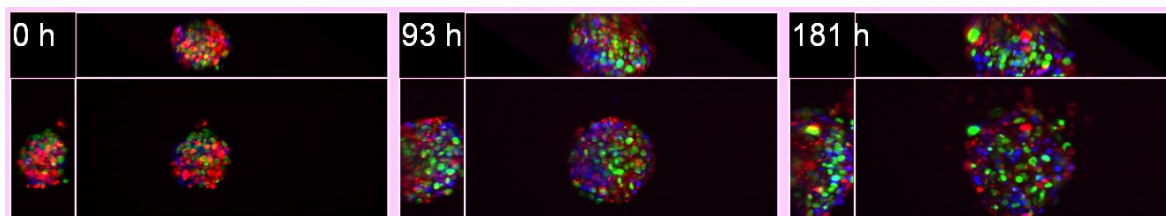


Figure 4.23 – Time-lapse ortho-MIP for well L20 in live plate 4 at times (left to right) 0 h, 93 h and 181 h

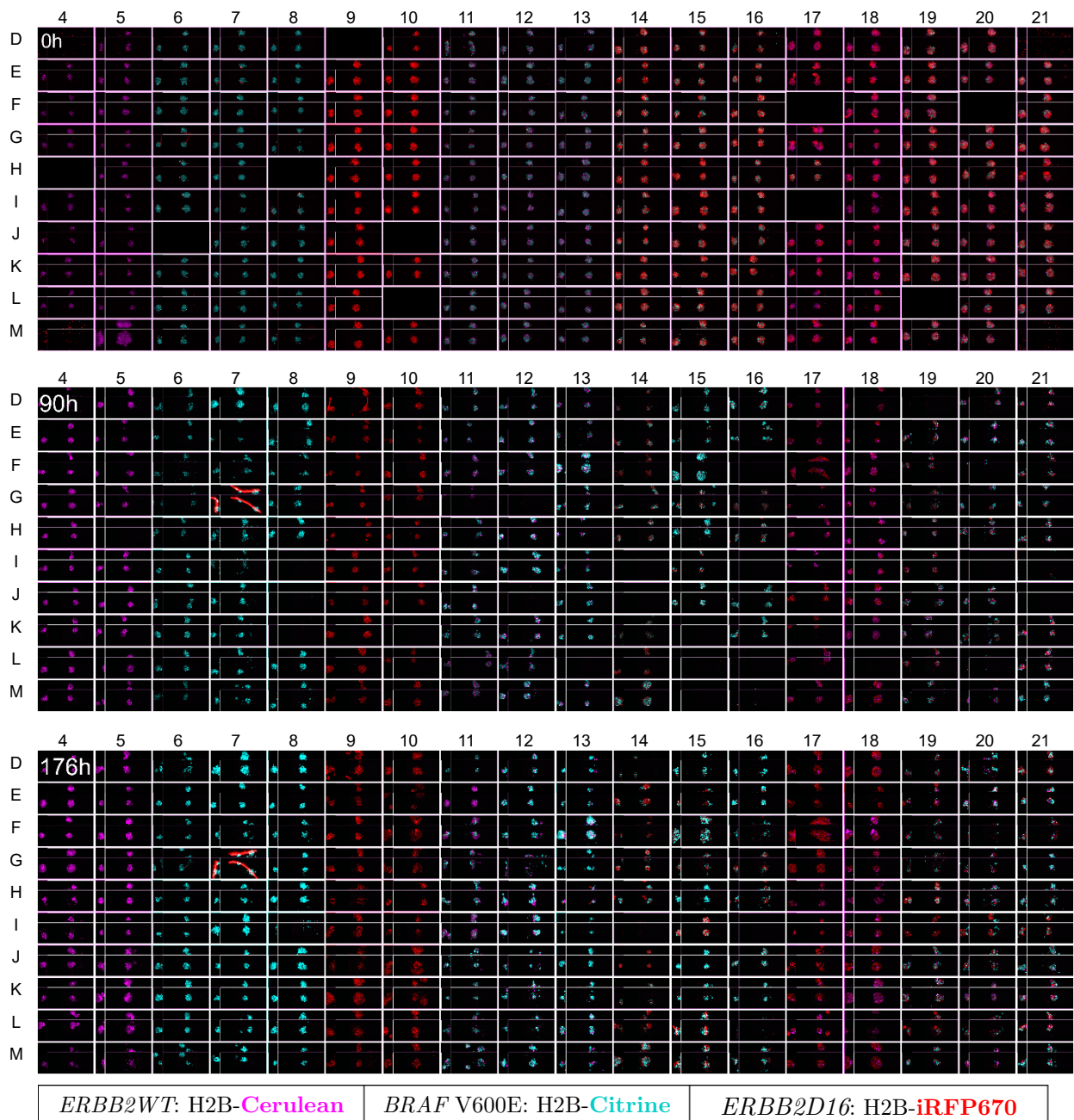


Figure 4.24 - Live plate 6 ortho-MIP montage at timepoints (a) 0 h, (b) 90 h and (c) 176 h. Magenta is *ERBB2*WT-Cerulean; cyan is *BRAF V600E*-Citrine; red is *ERBB2D16*-iRFP670. For full file see supplementary video 4.5.

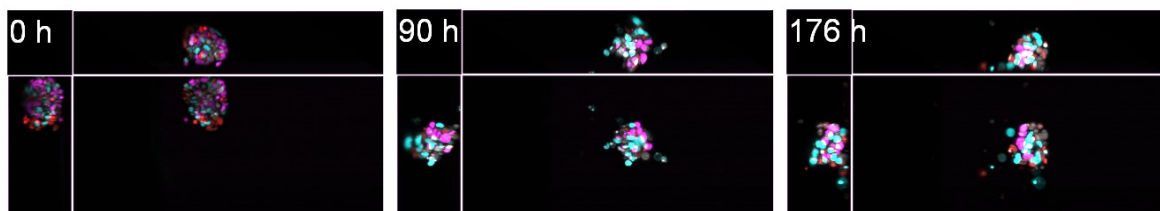


Figure 4.25 – Time-lapse ortho-MIP for well E20 in live plate 6 at times (left to right) 0 h, 90 h and 176 h

Fractional nuclear counts

For both live plate 4 and live plate 6, the number of nuclei (and therefore cells) in each of the cell line populations was analysed. From this, the fractional number of nuclei was also calculated, and this is useful for direct comparison of the nucleus counts from each of the cell lines as well as being robust to effects which reduce the total number of detected nuclei e.g., the sample drifting out of the FOV.

ERBB2WT and *ERBB2D16* combination spheroids

I looked first at spheroids composed of the *ERBB2WT* and *ERBB2D16* cell lines since these are not treated with Vemurafenib. This data is summarised in Figure 4.26 for live plate 4 and live plate 6, and this shows that in live plate 4 the fraction of *ERBB2D16* expressing cells is consistently lower in induced spheroids than non-induced, while the opposite trend is observed for the *ERBB2WT* cell population by necessity of the definition of the fractional nucleus count. In live plate 6, the difference between induced and non-induced is small but similar to the trend seen in live plate 4.

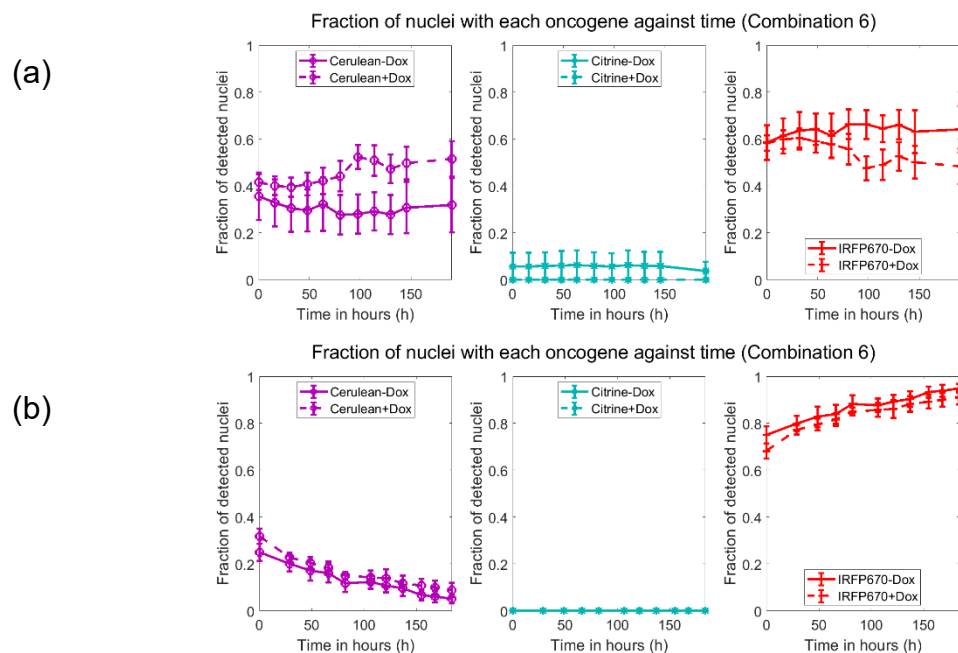


Figure 4.26 – Fractional counts, i.e., proportion of cells belonging to each cell line population for *ERBB2WT*-Cerulean and *ERBB2D16*-iRFP670 combination spheroids for (a) plate 4 and (b) plate 6, with non-induced (solid line) and induced (dashed line) treatments plotted on the same axis. The lines indicate the average (mean) over all spheroids in the dataset for a given spheroid cell line composition and treatment, while the error bars are the standard error across these spheroids, i.e. the same as shown in Figure 4.21.

Three-oncogene spheroids combination spheroids

The fractional nuclear counts for the three-oncogene combination spheroids are shown in Figure 4.27 for live plates 4 and 6. The trends seen for the *ERBB2WT* and *ERBB2D16* cell lines for the induced and non-induced three-oncogene spheroids are in agreement with the data discussed above for the two-oncogene spheroids. This suggests that the presence of the *BRAF V600E* expressing cells does not greatly alter the relative growth rate of the *ERBB2WT* or *ERBB2D16* cell lines either with or without inducer.

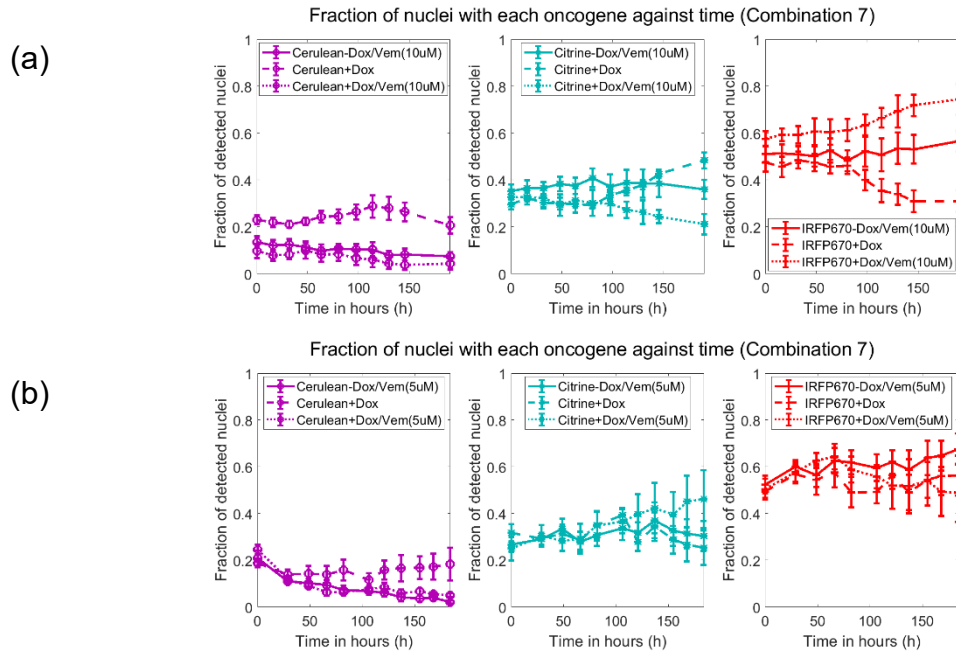


Figure 4.27 – Fractional counts for three-oncogene combination spheroids from live plate 4 (a) and live plate (6), comparing non-induced with Vemurafenib (solid line), induced (dashed line), induced with Vemurafenib (dotted line). The oncogene-FP pairs expressed in each cell line are *ERBB2WT*-Cerulean, *BRAF V600E*-Citrine, and *ERBB2D16*-iRFP670.

The trends seen in the fractional count of *BRAF V600E* cells differ between live plate 4 and 6; in live plate 4, the non-induced spheroids have a higher proportion of *BRAF V600E* cells than the induced spheroids until ~130 hours, after which (and by the final time-point) the opposite is seen. In live plate 6, the proportion non-induced of *BRAF V600E* cells was more similar to the induced spheroids throughout. Furthermore, the 10 μM Vemurafenib treatment of the induced spheroids in live plate 4 reduced the proportion of *BRAF V600E* cells compared to the non-induced and induced cases, while in live plate 6, the spheroids treated with 5 μM Vemurafenib has a higher fraction of *BRAF V600E* cells at the end of the experiment compared to the other two conditions. The higher Vemurafenib concentration in live plate 4 may explain the lower proportion of *BRAF V600E* cells by the end of the experiment in the non-induced case (with Vemurafenib) compared to the induced case (without Vemurafenib) since Vemurafenib appears to be generally cytotoxic at 10 μM . The increase of the fraction of *BRAF V600E* cells in the induced case with 5 μM Vemurafenib is, however, unexpected, and would need to be confirmed with additional experiments.

Mean nuclear signal

The fluorescent signal within each nuclear region was calculated (the mean of the pixel values within each region) for each of the three cell lines and from this, histograms can be generated to visualise the distribution of the mean nuclear signal for a given time-point. This data can be further distilled by calculating the mean nuclear signal of all nuclei in the spheroid at a given time-point to generate 1D graphs of mean nuclear (intensity) signal against time. The mean nuclear signal graphs can then illustrate trends towards brighter or

dimmer nuclei populations, and this indicates trends towards higher or lower expression of oncogenes present respectively.

***ERBB2WT* and *ERBB2D16* combination spheroids**

As in the previous section for the nucleus counts, the mean nuclear signal was calculated for the *ERBB2WT* and *ERBB2D16* combination and three-oncogene spheroids and the resulting plots are shown in Appendix A.4.2. The mean nuclear signal of the *BRAF V600E* population in the different cell line combinations is discussed in more detail in this section, since some trends were detected in live plate 6.

There appeared to be no trends in the induced vs non-induced average signal that were consistent between live plates 4 and 6. Although this may be expected for spheroids treated with Vemurafenib (since the concentration was different), the trends seen in the *ERBB2WT* and *ERBB2D16* combination spheroids also did not agree between plates, and these had identical treatments between the two experiments—see Figure A4.2 in appendix.

Further analysis of *BRAF V600E* nuclei

In live plate 6, there was a notable trend in the *BRAF V600E* average nuclear signal that was reproduced in spheroids with every different cell line combination containing *BRAF V600E*, see Figure 4.28. The trend is that the non-induced (-Dox treatment) *BRAF V600E* cells had a relatively consistent average signal, while for induced (+Dox treatment) spheroids (both with and without Vemurafenib) it increased with time. In live plate 4, however, this was not observed and instead, the trends in *BRAF V600E* average nuclear signal were inconsistent between the different cell line combinations.

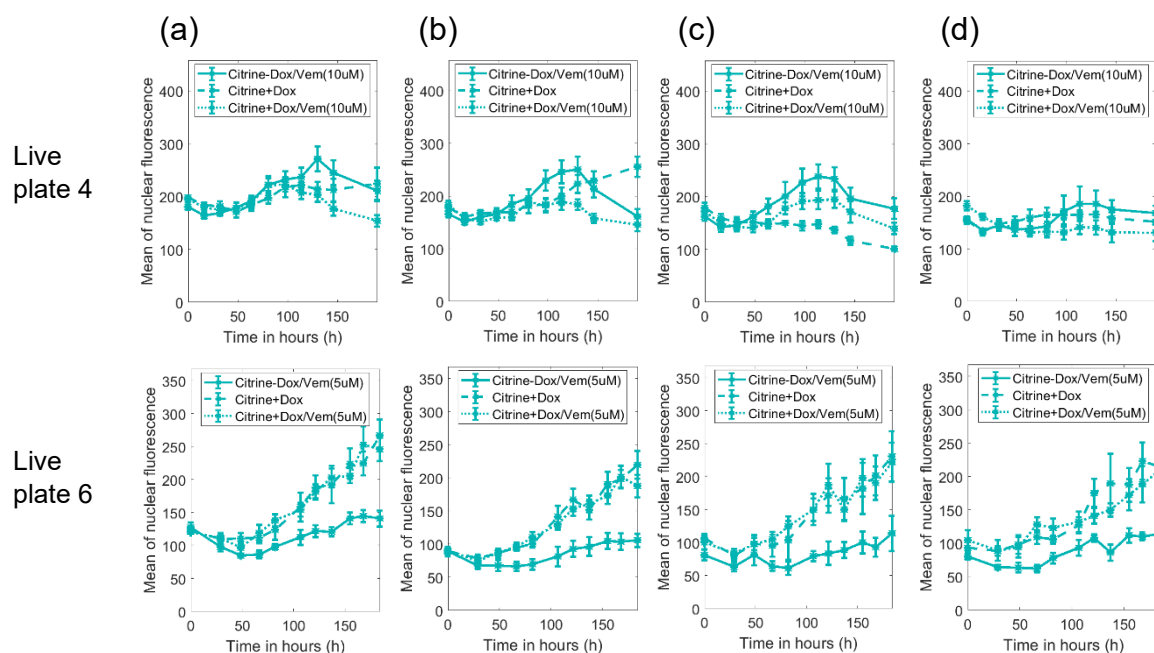


Figure 4.28 – *BRAF V600E*-Citrine cell line average nuclear signal for all cell line combinations containing the *BRAF V600E*-Citrine cell line in live plate 4 (above) and 6 (below): (a) single combination spheroids, (b) *BRAF V600E*-Citrine and *ERBB2WT*-Cerulean, (c) *BRAF V600E*-Citrine and *ERBB2D16*-iRFP670 and (d)

three-oncogene spheroids. The three different treatments are plotted on the same axis: non-induced with Vemurafenib (solid line), induced (dashed line), and induced with Vemurafenib (dotted line).

While the results in live plate 4 and 6 are not comparable for the Dox/Vemurafenib treatment since the concentration is different between plates, we should expect to see similar trends in the -Dox/Vemurafenib treatment results for the two different Vemurafenib concentrations—including the one seen here. This is because the effect of the Vemurafenib is non-specific since the *BRAF V600E* is not actively expressed, and therefore there is no selective pressure on the number of oncogene copies and therefore FP copies. In other words, the number of oncogene copies does not affect the cell phenotype and the effect of Vemurafenib.

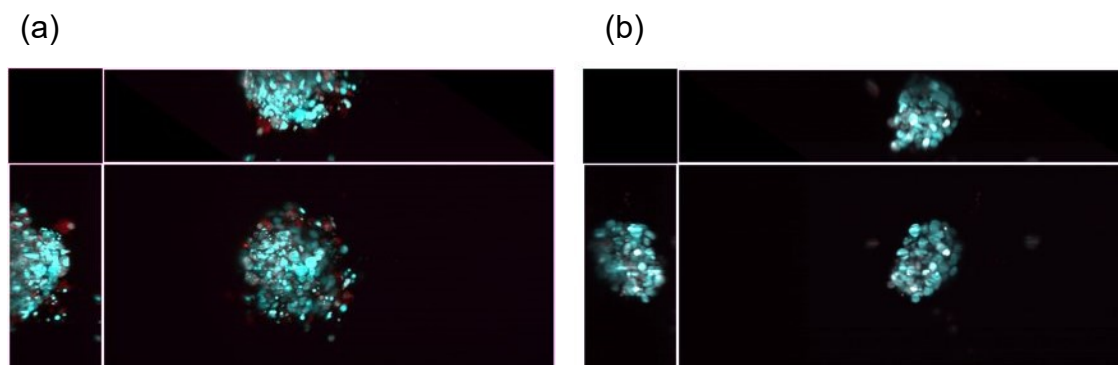


Figure 4.29 – Vemurafenib causing cells to die and shrink, resulting in small, bright nuclei which may skew the nuclear signal readout data, particularly in spheroids in live plate 4. Examples of *BRAF V600E*-Citrine only spheroids under the -Dox/Vemurafenib treatment in (a) live plate 4 with 10 μ M Vemurafenib, (well F6, 157 h) and (b) live plate 6 with 5 μ M Vemurafenib (well K6, 155 h).

The increased concentration of Vemurafenib in live plate 4 caused more cell death than live plate 6, and when cells die the nuclei shrink and condense the FP within the nucleus. This may explain the trend in live plate 6 not being seen in live plate 4; the shrinking nuclei and increased FP density may positively skew the average nuclear fluorescence (which is evaluated as a mean over the nuclear region) for the -Dox/Vemurafenib-treated spheroids compared to the Dox only spheroids, obfuscating the trend observed in live plate 6. This can be seen in the image data as shown in Figure 4.29 above with ortho-MIPs.

Ultimately, further experiments would be needed to establish whether the results from live plate 6 can be reproduced.

Distribution of nuclear signal

The *BRAF V600E* variation in the nuclear signal shown above in Figure 4.28 can be investigated in more detail via the distribution of nuclear signals in the whole population of nuclei for a given condition. These values are not calculated on the spheroid level, but from all nuclei from spheroids in a certain column, and each column corresponds to a specific spheroid oncogene combination and treatment. This may be considered analogous to using flow cytometry on all spheroids from a given column to measure the population of intensities.

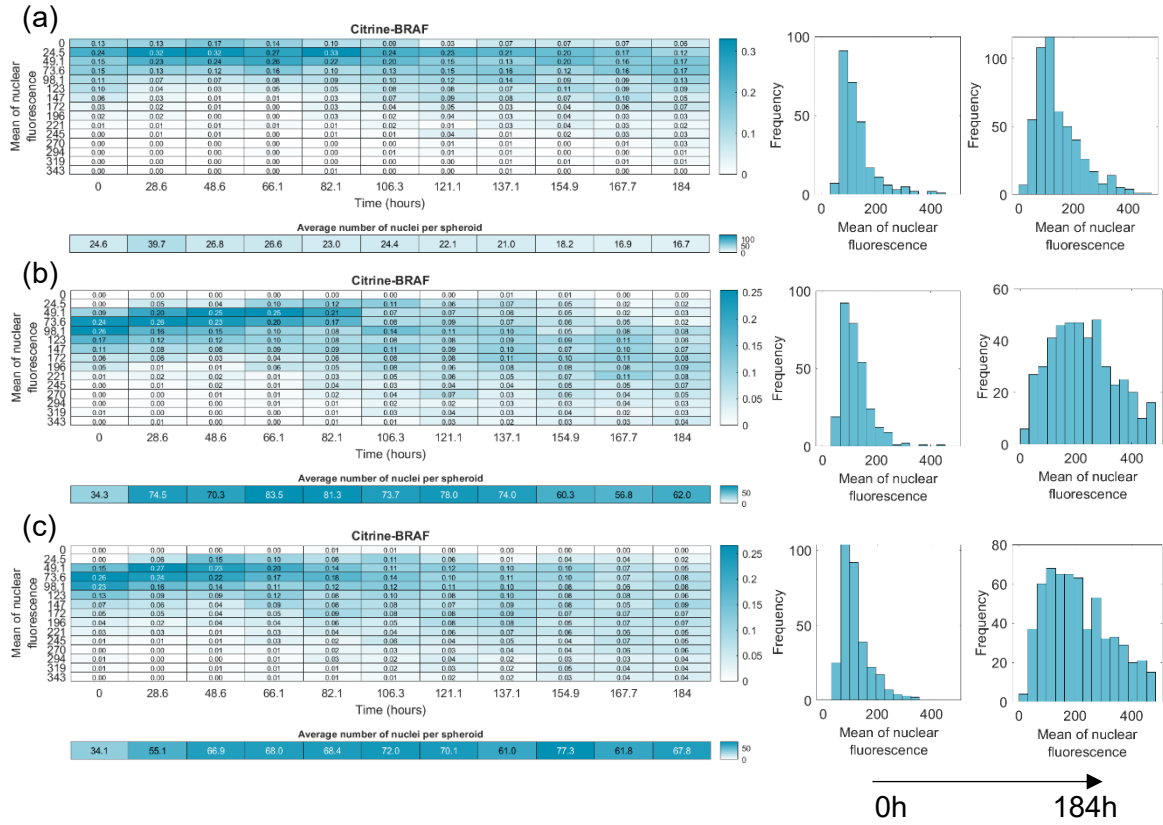


Figure 4.30 – 2D heatmaps to illustrate nuclear fluorescence intensity distribution against time for nuclei from spheroids of same cell line combination and treatment—data from live plate 6. The results are shown for (a) non-induced spheroids of the *BRAF V600E*-Citrine cell line only with Vemurafenib, (b) induced spheroids and (c) induced with Vemurafenib treatment. Here, the nuclei from each repeat are combined into one single dataset, shown by this 2D histogram/heatmap, and the average number of nuclei for each time-point that make up this dataset are plotted below each 2D histogram in a 1D heatmap. Histograms for the first and last timepoint are displayed on the right, and these represent line profiles of the 2D heatmap at $t=0$ h and $t=184$ h.

Using the *BRAF V600E* only spheroids in live plate 6 as an example, the mean plots seen in Figure 4.28(a) suggest that the mean nuclear fluorescence intensity is higher at the end of the experiment for induced spheroids compared to non-induced, but that there is no significant difference in mean nuclear fluorescent signal between the induced spheroids with and without the treatment Vemurafenib. The 2D histograms/heatmaps shown in Figure 4.30 show that indeed the induced population (Figure 4.30(b)) tends to a distribution with brighter nuclei and one with a larger spread compared to the non-induced (Figure 4.30(a)), and this is illustrated further by the 1D histograms at the final timepoint (184 h). Here, for each of the treatments, the distributions are asymmetric/skewed but have relatively distinct peaks. These peaks are at approximately 100 for the non-induced spheroids, 200 for the induced spheroids and approximately 150-170 for the induced spheroids treated with Vemurafenib. Notably, the induced spheroids (b) with and (c) without Vemurafenib treatment appear to be distinguishable when observing the nuclear intensity distributions as they are skewed differently, unlike with the mean nuclear intensity plots, see Figure 4.28, bottom left panel. This indicates that, although the mean signal plots are easier to interpret, there is potentially less information available.

Mean nuclear size

Following the previous examples, the results for nuclear size (measured in volume) from the *ERBB2WT* and *ERBB2D16* combination and the three-oncogene combination spheroids are examined, with the relevant figures shown in Appendix A.4.3.

ERBB2WT and *ERBB2D16* combination spheroids

In the *ERBB2WT* and *ERBB2D16* combination spheroids, both *ERBB2WT* and *ERBB2D16* nuclei appear to grow larger on average by ~120 h when spheroids are induced in live plate 4, but this disagrees with the results from live plate 6 where no obvious difference is observed, see Figure A4.6 in appendix.

Three-oncogene combination spheroids

For the three-oncogene spheroids in live plate 4, at the end of the experiment the volume of *ERBB2WT* cell nuclei had large errors associated with them, making any difference between the treatments unlikely to be statistically significant, and there was also no clear difference in live plate 6. In live plate 4, the nuclear volume of *ERBB2D16* nuclei did not vary across treatments throughout the experiment for both experiments, and the induced *BRAF V600E* cells (both with and without Vemurafenib) had nuclei which gradually became on average larger than in the non-induced cells. In live plate 6, the *BRAF V600E* nuclei in the induced case (both with and without Vemurafenib) were also larger than in the non-induced case for most of the experiment, but the difference appeared to disappear at the end of the experiment, see Figure A4.5 in the appendix (and Figure 4.31(d) for live plate 6).

Further analysis of *BRAF V600E* nuclei

It was found that in both live plates 4 and 6 in some cell line combinations including *BRAF V600E* that the *BRAF V600E* cell nuclei become larger when induced, see Figure 4.31, and therefore I investigated the nuclear volume of *BRAF V600E* nuclei here in more depth. The increase in *BRAF V600E* cell nuclear volume (when induced) was most evident in live plate 4, specifically in the *BRAF V600E* only spheroids and *BRAF V600E* and *ERBB2WT* spheroids and least evident in the *BRAF V600E* and *ERBB2D16* combination spheroids, which may suggest that the *ERBB2D16* cell line resulted in selective pressure towards smaller *BRAF V600E* nuclei. In live plate 6, the difference in average nuclear volume between induced and non-induced *BRAF V600E* nuclei was generally not as noticeable as in live plate 4, but still observed in most combinations that include *BRAF V600E*; the difference was more ambiguous in the three-oncogene combination spheroids.

The dataset presented here is complex, however, some trends can be seen in the *BRAF V600E* cell line population. In both live plates 4 and 6, there is evidence to suggest that the induced *BRAF V600E* results in enlarged nuclei. In live plate 6, this is accompanied by brighter nuclei, and indeed, this is reflected in the image data for live plate 6, with an example *BRAF V600E* spheroid shown in Figure 4.32.

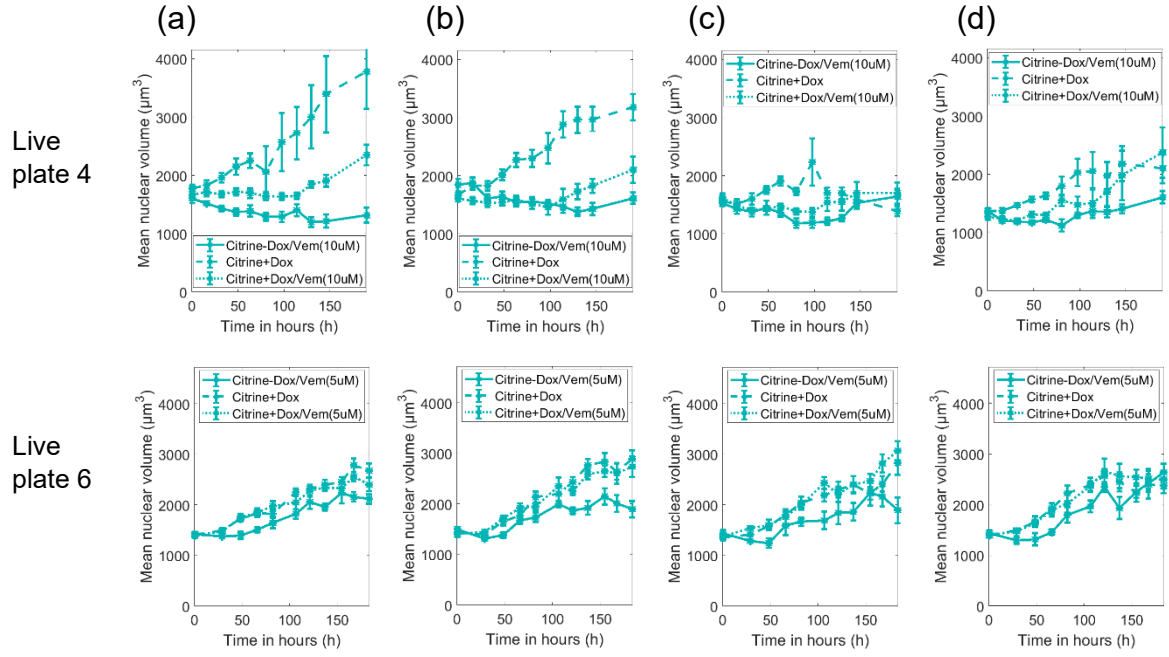


Figure 4.31 – *BRAF V600E* cell line average nuclear volume for all cell line combinations containing the *BRAF V600E* cell line in live plate 4 (above) and 6 (below): (a) single combination spheroids, (b) *BRAF V600E* and *ERBB2WT*, (c) *BRAF V600E* and *ERBB2D16* and (d) three-oncogene spheroids. The three different treatments are plotted on the same axis: non-induced with Vemurafenib (solid line), induced (dashed line), and induced with Vemurafenib (dotted line).

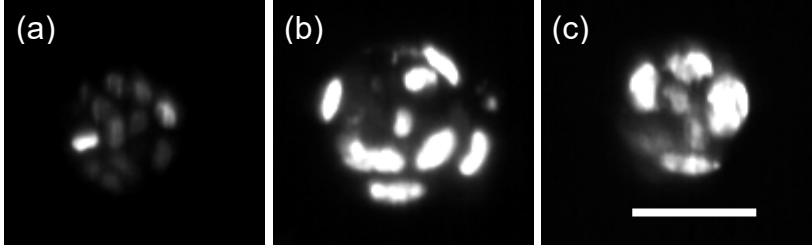


Figure 4.32 – Comparison of example *BRAF V600E*-only spheroids in live plate 6, x-y slice of volume taken near equator: (a) non-induced (M6), (b) induced (M7), (c) induced with Vemurafenib (M8). The induced spheroids contain brighter nuclei and those with different morphology. Scale bar 60 μm , Citrine channel.

Distribution of nuclear size

Similar to what was done in the analysis of the distribution of nuclear signal, the nuclear volumes of *BRAF V600E* cells are investigated further using the nuclear volume histograms. As shown in the previous section, in live plate 6 for the *BRAF V600E* only spheroids, the nuclear volume is slightly higher by the end of the experiment when induced. This data is represented for the full distribution of nuclear volumes, rather than just the average, in Figure 4.33. At the final timepoint, the nuclear volume distribution for non-induced condition (Figure 4.33(a), right) is peaked at approximately 1500 μm^3 , while for the induced condition (Figure 4.33(b), right) it is peaked at approximately 2400 μm^3 , with potentially a second peak at $\sim 500 \mu\text{m}^3$, and the distribution is slightly broader than the non-induced histogram. This difference is not as clearly reflected when the histogram is reduced to a single value of the mean (2400 μm^3 versus 2700 μm^3) or median (1800 μm^3 versus 2200 μm^3). The second potential peak in the induced case could just be due to noise, but the same dip is

observed in Figure 4.33(c), right. If confirmed, it could indicate the emergence of a subpopulation, or clonal population that arises due to an evolutionary advantage. It may also be dead cells which have shrunk but still detected by the segmentation.

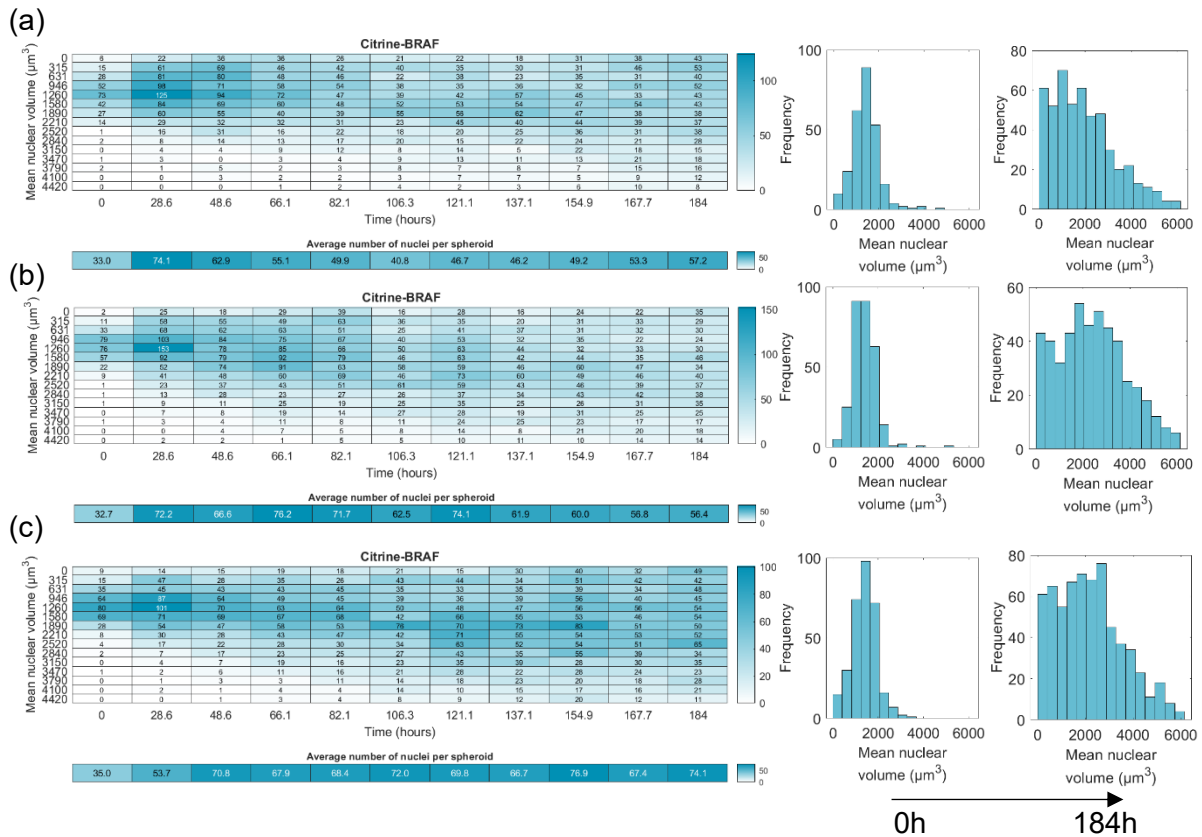


Figure 4.33 – 2D heatmaps to illustrate nuclear volume distribution against time for nuclei from spheroids of same cell line combination and treatment. The results are shown for (a) non-induced spheroids of the *BRAF V600E*-Citrine cell line only with Vemurafenib, (b) induced spheroids and (c) induced with Vemurafenib treatment. Here, the nuclei from each repeat are combined into one single dataset, shown by this 2D histogram/heatmap, and the average number of nuclei for each timepoint that make up this dataset are plotted below each 2D histogram in a 1D heatmap. 1D histograms for the first and last time-points are displayed on the right, and these represent line profiles of the 2D heatmap at $t=0$ h and $t=184$ h.

4.4 Conclusion and future work

4.4.1 Conclusion

Breast cancer-mimicking spheroids comprising combinations of up to four cell lines with inducible expression (using Doxycycline) of up to four unique oncogenes were generated from non-tumorigenic HB2 human breast epithelial cell lines. These spheroids were cultured and imaged in an initial experiment and, due to variability in fluorophore brightness, the spheroid model was then adapted to use combinations of up to three unique oncogenes. The new three-oncogene spheroid model included a common fluorescence channel in all cell lines to serve as a segmentation channel and subsequent live imaging experiments were conducted with these spheroids. Cellpose was re-trained with manually annotated data for 3D image segmentation of nuclei to enable spheroids to be studied on the nucleus/cell-level. Time-resolved analysis on the segmented data was developed to consider various metrics to

compare between non-induced control spheroids, induced spheroids and induced spheroids treated with the *BRAF V600E*-targeting drug, Vemurafenib.

Two live imaging experiments were analysed, one with Vemurafenib solution at 10 μ M (live plate 4) and one with a 5 μ M concentration of Vemurafenib (live plate 6). The proportion of cells from each cell line, the nuclear (fluorescence) signal and nuclear size were compared between the induced and non-induced conditions as well as between experiments where applicable. The effect of the *BRAF V600E*-targeting therapeutic Vemurafenib was also shown and discussed.

When qualitatively assessing repeatability between the two experiments by comparing like-for-like conditions, there was limited agreement: the proportion of *ERBB2WT* cells to *ERBB2D16* cells in this two-oncogene combination followed a similar trend between live plates 4 and 6, but not when comparing nuclear fluorescence intensity and volume. Absolute cell counts were not presented here since this is not robust to parts of the spheroid being out of the FOV (or even splitting into two), but this is an important readout to assess the effect of oncogenes on spheroid growth rate—this is an area of future work.

In both live plates 4 and 6, however, nuclei of the *BRAF V600E* cell line became larger in volume when induced with Dox for most cell line combinations containing the *BRAF V600E*. The exceptions were the *BRAF V600E* and *ERBB2D16* combination spheroids in live plate 4, and the three-oncogene combination spheroids in live plate 6.

In live plate 6, the mean nuclear signal for the *BRAF V600E* cell line nuclei increased with time in spheroids induced with Dox compared to the non-induced spheroids; this was seen in all combinations containing the *BRAF V600E*. There was no such clear trend in live plate 4, but this difference may be explainable by the higher concentration of Vemurafenib used in live plate 4, which caused cell death and shrinkage in the non-induced spheroids, increasing FP concentration and positively skewing the nuclear intensity.

In addition to the average nuclear volume and signal being computed across all nuclei, the distribution of nuclear volume and signal was investigated to determine if additional information could be extracted. In live plate 6, there was no obvious difference in mean nuclear signal in *BRAF V600E* only spheroid nuclei between Vemurafenib-treated and untreated spheroids (both induced). However, there was evidence to suggest that the distribution of nuclear fluorescence was different between Vemurafenib-treated and untreated spheroids by comparing the peak locations in the final timepoint histograms. Similarly, the nuclear volume distribution of *BRAF V600E* only spheroid nuclei in live plate 6 suggested that the differences in the histogram allow conditions to be distinguished more so than with the average nuclear volume plots.

Towards the end of the live plate 4 experiment (beyond 180 h), unwanted fluorescence originated from presumed dead cells in the iRFP670 channel, which interfered with nuclear assignment (false positives) and signal measurements. This was particularly evident in live

plate 4, where the 10 μM concentration of Vemurafenib was cytotoxic to all cells, and this effect should be minimised in later experiments by the reduced Vemurafenib concentration (live plate 6). Autofluorescence originating from the nuclei in dead cells was not observed in the mRFP segmentation channel, so with proper segmentation, these should be rejected—this motivates further improvement to the segmentation. Another solution would be to choose a brighter FP than iRFP670 to increase the FP signal to background ratio, making it easier to select an appropriate threshold for this channel.

The results presented in this chapter represent, to our knowledge, the first attempt to use HCS to study the competition of up to three different genetically altered breast cell lines in >100 spheroids in parallel, at the cellular level and in time-lapse. The imaging speed of the OPM system is exploited to image 180 volumes within 150 minutes at 8-hour intervals over a period of up to 8 days. In HCS systems that use confocal microscopes (as is the industry standard), this is likely to be unviable due to photodamage. By imaging the different spheroids formed of different mixtures of the same cell lines on the same plate, many potential sources of systematic error and bias in the cell biology and sample preparation are avoided. These technical repeats can then be used to uncover trends through statistical analysis. The work presented in this chapter lays important groundwork to allow future studies comparing the competition of different cell lines expressing different oncogenes within 3D spheroids.

4.4.2 Future work

To provide more evidence to determine whether the results are repeatable, more data from separate experiments are required, and this would be the next step in the future for this project. Although repeat conditions provide up to 10 technical replicates per plate, additional biological repeats are required (and planned) to confirm that the results are real biological phenomena. The aim would be to repeat the latest iteration of the experiment (with 5 μM Vemurafenib concentration) and see if this agrees with live plate 6.

A readout of interest is the shedding of *BRAF V600E*-overexpressing cells, and a future goal would be to derive a method to identify a central connected region i.e., the spheroid, and to identify cells that have detached from the main spheroid. An additional readout of interest is clonality: the quality of cells being derived from parent cells. Clonality may be identified by performing cluster analysis of nuclei with similar brightness, i.e. they have the same number of oncogene copies and are likely to be clones. The 3D dataset would allow this type of analysis, and this could be achieved by representing the dataset as a graph.

Nuclear morphology is another potential readout and can be calculated from the 3D nuclear segmentations, with differences in morphology being apparent in Figure 4.32. The more detailed the shape analysis of nuclei, however, the higher confidence required in segmentation, and further manual segmentation and retraining of the Cellpose model may achieve this, as well as further investigation into alternative segmentation models.

Overall, this is a large dataset with many different readouts from many different conditions and indeed only a subset of the data is presented here. Care must be taken in summarising this data in a non-biased way, and comparisons between experiments and conditions are currently qualitative. Dimensionality reduction techniques such as PCA may be a method to compare conditions more easily—and therefore more easily avoiding bias—and statistical tests may also be derived to perform comparisons quantitatively. To date, the analysis is preliminary, and the trends presented here are used to indicate potential readouts of interest, rather than make conclusions; in the future, this analysis would aim to be more quantified and robust.

5 Modelling OPM and dOPM light-collection efficiency

5.1 Introduction

The light collection efficiency of a fluorescence microscope is an important factor in biological imaging, as higher light collection efficiency allows the use of lower excitation intensities for the same detected signal. In this chapter, the light-collection efficiency of OPM and dOPM is discussed and a vectorial 3D ray-tracing model is introduced and used to calculate theoretical light collection efficiency values for different fluorescence emission patterns and optical setups.

One of the motivations for developing a vectorial ray-tracing approach—which calculates electric fields and therefore the light polarisation—was because of the potential polarisation-dependent effects on collection efficiency in dOPM. Due to the folded configuration of dOPM which employs the use of a polarising beamsplitter to out-couple the fluorescence, the system is sensitive to polarisation-changing depolarisation effects such as those at the folding (prism) mirror.

The ray-tracing simulation calculates a series of sets of 3×3 Jones matrices to trace a cone of rays through the system as wavevectors, and then this same sequence of Jones matrices is applied to the initial electric field vectors to propagate them through the system. Jones matrices are used to model lenses, linear polarisers or polarising beamsplitters, waveplates and protected (coated) metallic mirrors. The rays originate from a fluorescent source modelled as an ensemble of dipoles which can either be static or are free to tumble on a timescale much faster than the fluorescence lifetime.

To start with, the geometric approach to calculating light collection efficiency will be presented, which only considers the proportion of the light cone collected by the first objective that is collected by the entire system. This method will be referred to as the Geometric Method.

5.1.1 Collection efficiency of OPM (Geometric Method)

In OPM the light collection efficiency is heavily dependent on the relative tilt between O2 and O3, resulting in occlusion of the light cone emitted from O2, and the effective light cone is given by the intersection of the two cones on a unit sphere as shown in Figure 5.1. When considering the angles involved in OPM, one can derive the full collection angle Φ_{em} from the intersection of the cones in the $y = 0$ plane, where y is the vertical direction in lab coordinates. This full collection angle is given by

$$\Phi_{\text{em}} = \min(\alpha_{\text{O1,2}} - \theta_{\text{OPM}}, \alpha_{\text{O3}}) + \min(\alpha_{\text{O3}}, \alpha_{\text{O1,2}} + \theta_{\text{OPM}}) \quad 5.1$$

where α_{O3} is the half acceptance angles for O3 and $\alpha_{\text{O1,2}}$ is the limiting half acceptance angle of O1 and O2—these half angles are calculated from the NA of the objective and are

equal to $\text{asin}(\text{NA}/n)$, where n is the refractive index at the sample/immersion medium. In the configuration shown in Figure 5.1, $\alpha_{O1,2} + \theta_{\text{OPM}} > \alpha_{O3}$ and $\alpha_{O1,2} - \theta_{\text{OPM}} < \alpha_{O3}$ (the latter is the condition for an asymmetric final pupil due to clipping) and the equation then reduces to

$$\Phi_{\text{em}} = \alpha_{O1,2} + \alpha_{O3} - \theta_{\text{OPM}} \quad 5.2$$

It is important to note that Φ_{em} does not describe a collection cone symmetric about the optical axis of O3, see Figure 5.1(b); the largest possible symmetric collection cone is given by

$$\varphi_{\text{em}} = \min(\alpha_{O1,2} - \theta_{\text{OPM}}, \alpha_{O3}) \quad 5.3$$

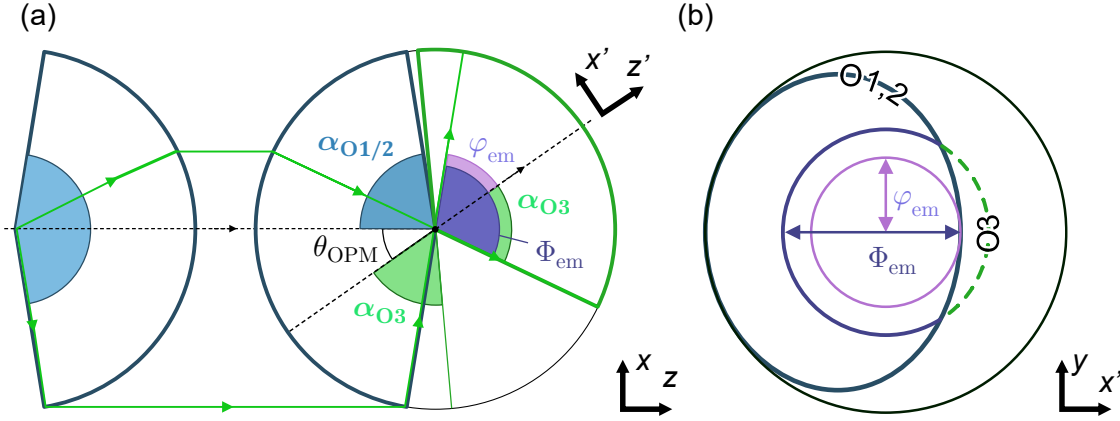


Figure 5.1 – (a) The geometry of the effective collection in OPM, as viewed from above, specifically showing the collection of the light cone from O2 by O3, with O3 tilted by the OPM angle and its optical axis aligned with z' . The green arrowed lines show the limiting rays in the $y=0$ plane that are accepted by O3 and define the effective collection full angle Φ_{em} (green) and circular collection half angle φ_{em} (pale purple). $\alpha_{O1,2}$, limiting collection half angle of O1 and O2 (pale blue); α_{O3} , collection half angle of O3 (dark purple). The bold grey-blue lines indicate the limiting collection cone of O1 and O2, and the bold green lines are the collection cone of O3. (b) The limiting collection cone of O1 and O2 and collection the cone of O3 projected onto a unit sphere as viewed along z' . The effective collection solid angle is found by the intersection of these cones shown by the green line and the symmetric collection solid angle is shown by the blue line.

In previous literature, namely in Vincent Maioli's thesis [86] and the 2015 OPM publication [126], the light collection of OPM systems is calculated geometrically by calculating the effective collection solid angle of the system. This calculation is realised numerically in MATLAB (written by Prof. Chris Dunsby) by calculating the intersection of the limiting collection cone of O1 and O2, and the rotated collection cone of O3. Specifically, points are sampled in a circle defined on the surface of a sphere by the **scircle1** function, and the area intersection is calculated with the **polybool** function. When this effective collection solid angle is evaluated relative to the collection solid angle of O1, it is known as the geometric relative collection efficiency (GRCE) and is parametrised in Vincent' Maioli's thesis and the 2015 OPM paper as t_{geom} . For the combination of objectives used in the OPM system presented in this thesis and in literature [63], [86], [126], the GRCE (t_{geom}) is 0.328 when the OPM angle is 35° .

The geometric collection efficiency (CE) is also defined which evaluates the effective solid angle relative to the collection solid angle of a hemisphere (2π) and this therefore accounts for the collection of O1. The different metrics for collection efficiency and their definitions can be found summarised in Table 5.1.

Table 5.1 - Definitions for collection efficiency metrics calculated using the MATLAB script (Geometric Method) and by using the ray-tracing simulation.

Metrics	Equation	Description
Geometric Method metrics (OPM)		Based on collection cone intersection code
Geometric relative collection efficiency (GRCE)	$\frac{\Omega_{\text{exit}}}{\Omega_{\text{O1}}}$	Intersection of the limiting O1/O2 collection cone and O3 collection cones solid angle (Ω_{exit}), divided by O1 collection solid angle (Ω_{O1}).
Geometric collection efficiency (GCE)	$\frac{\Omega_{\text{exit}}}{2\pi}$	See GRCE, but the pupil intersection is divided by full hemisphere collection solid angle (2π).
Geometric Method metrics (dOPM)		
Geometric relative collection efficiency (GRCE)	$\frac{\Omega_{\text{exit}}}{\Omega_{\text{O1}}} \cdot t_{\text{PBS}}$	GRCE for OPM, multiplied by PBS transmission (t_{PBS}). The PBS transmission is found by calculating emission intensity parallel and perpendicular to PBS transmission axis.
Geometric collection efficiency (GCE)	$\frac{\Omega_{\text{exit}}}{2\pi} \cdot t_{\text{PBS}}$	GCE for OPM, multiplied by transmission through PBS (t_{PBS}).
Metrics from ray-tracing simulation (OPM and dOPM)		
Relative collection efficiency (RCE)	$\frac{I_{\text{out}}}{I_{\text{in}}}$	Total/output energy collected by system divided by energy collected by O1.
Collection efficiency (CE)	$\frac{I_{\text{out}}}{I_{\text{emitted}}}$	Total/output energy collected by system divided by energy emitted by source in a hemisphere (2π solid angle).
Emission efficiency (EE)	$\frac{I_{\text{in}}}{I_{\text{emitted}}} \frac{2\pi}{\Omega_{\text{O1}}}$	Ratio of energy per unit solid angle collected by O1 ($I_{\text{in}}/\Omega_{\text{O1}}$) to energy per unit solid emitted into 2π steradians ($I_{\text{emitted}}/2\pi$).

The GRCE can also be compared to experimental measurements of the relative collection efficiency (RCE), but first, t_{geom} must be multiplied by the transmission efficiency of the additional OPM optics of microscope 2 (O2, TL2) and microscope 3 (O3, TL3), t_{optics} , to give the total theoretical relative collection efficiency. This total relative collection efficiency is therefore given by

$$t_{\text{total}} = t_{\text{geom}} \cdot t_{\text{optics}}. \quad 5.4$$

The RCE was measured experimentally by Vincent Maioli and presented in his thesis and in literature, and the results of this are shown in Figure 5.2 [63]. This was achieved by using a single fluorescent bead and comparing signal measurements with epifluorescence (using a camera on the side port of the microscope) to the signal measured by the OPM camera. To compare experiment to theory, Vincent Maioli determined t_{optics} manually for his system to be 0.7 by finding the value giving the best fit of t_{total} to the data. Although this can be calculated based on manufacturers' stated transmissions for each of the optical elements combined (O1, TL1, TL2, O2, O3 and TL3), this yielded a t_{optics} value of 0.8, indicating that there may be additional losses in the system.

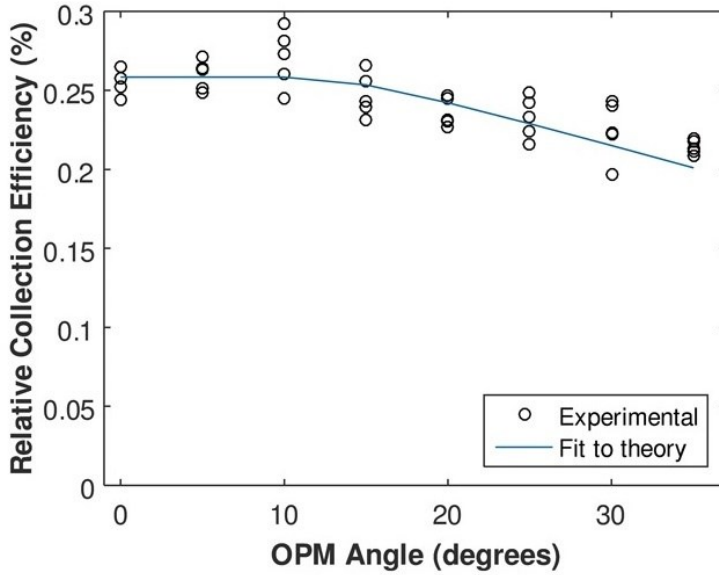


Figure 5.2 – Experimental measurements of collection efficiency from a single bead for different OPM angles, together with theoretical collection efficiency which is scaled by t_{optics} . The value of t_{optics} is calculated for the best (visual) fit to the experimental data. Reproduced with permission from Maioli et al. [63].

The MATLAB code used to calculate the GRCE, however, only considers the geometry of the effective collection to describe the efficiency of the system and does not consider the spatial distribution of the intensity emitted by the source (it assumes that the fluorescence signal is distributed uniformly on the spherical surface of the light cone). Fluorescent samples can be modelled as an ensemble of emitting dipoles that are selectively excited under polarised excitation, resulting in a non-uniform electric field (and intensity) due to the dipole radiation pattern. This non-uniformity means that the energy collected is not

necessarily proportional to the collection solid angle. The concept of this non-uniform electric field distribution is introduced in Section 5.1.2.

The intensity distribution is important since the light cone emitted by O2 is not fully collected by O3 and collected at an angle; therefore, only a sub-region of the distribution is sampled, and it is sampled asymmetrically. As a result, there is motivation to calculate the collection efficiency by considering not only the geometry of the collection, but the intensity distribution and its propagation through the system; this is achieved through the development of the vectorial ray-tracing simulation presented in Section 5.2, which considers the geometry in 3D and the vectorial nature of the electric field (to account for depolarisation in dOPM), in other words, the polarisation.

Note on geometric collection efficiency nomenclature

The MATLAB code used to calculate the collection efficiency in OPM is also used in the calculation of the collection efficiency for dOPM systems (see Section 5.1.3). As discussed in Section 1.5.4, the PBS used in dOPM results in additional transmission losses which depend on the polarisation of the light emitted by the sample. For dOPM, instead of including these transmission losses in t_{optics} , the GRCE and GCE are calculated by multiplying the expected transmission of the PBS with the results from the collection cone intersection method. For simplicity (and to retain the form of Equation 5.4), the terms “GRCE” and “GCE” are still used for these PBS transmission-scaled dOPM collection efficiency metrics. Henceforth, the variable t_{geom} (which the GRCE for OPM is equal to) will not be used and instead the “GRCE” is referred to.

In the following section, the concept of fluorescence anisotropy will be introduced. The effect of different excitation light polarisations on the emission polarisation states will be explained and formulated mathematically; this is important in calculating the PBS transmission and thus the dOPM collection efficiency.

5.1.2 Fluorescence anisotropy

On-axis case

An important quantity which quantifies the probability of fluorophore excitation is the transition dipole moment (TDM) vector $\vec{\mu}$. The probability of a given electronic transition between two quantum states, i.e., ground state and excited state, is proportional to $|\vec{\mu}|^2$ [127]. The TDM is an intrinsic property of the fluorophore which defines the preferred axis of excitation relative to the structure of the molecule, and when considering the light-matter interactions, the transition rate (proportional to the probability of photon absorption) in the presence of the electric field $\vec{E}$ of the electromagnetic wave is proportional to $|\vec{\mu}_{lm}|^2$, given by [128]

$$|\vec{\mu}_{lm}|^2 = |\vec{\mu} \cdot \vec{E}|^2. \quad 5.5$$

Assuming a one-to-one relationship between photon absorption and emission, the intensity of fluorescence emission in the presence of an excitation electric field $\vec{E}$ is also proportional to

$|\vec{\mu}_{lm}|^2$. When the system transitions from the excited to the relaxed state, the radiation is modelled such that the emission TDM axis—which can differ from that of the absorption TDM by a fixed angle—induces dipole oscillations along the emission TDM axis, resulting in a radiation pattern of an oscillating dipole [84]. In this section, the excitation and emission TDM will be referred to as the excitation and emission dipoles for simplicity and to match the wording in literature.

In a fluorescent sample consisting of fluorophores with their emission modelled as radiating dipoles that are excited by polarised light, the probability distribution of excited fluorophores is proportional to $\cos^2 \theta_{\text{ex}}$, where θ_{ex} is the angle between an excitation dipole and the polarisation of the excitation light (the direction of the electric field vector $\vec{E}$). The phenomenon of the fluorophore excitation probability being higher if the excitation dipole is aligned with the polarisation of excitation light is known as photoselection. For typical fluorophores, the emission dipole is reasonably parallel to the excitation dipole, so if the fluorophores' rotational motion during the excited state is sufficiently small then the emission can also be partially polarised in the same orientation as the excitation polarisation.

The degree of polarisation is quantified by the fluorescence anisotropy r , and is given by

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}, \quad 5.6$$

where $I_{\parallel}$ is the fluorescence intensity emitted parallel to the polarisation of the excitation and $I_{\perp}$ is the fluorescence intensity emitted perpendicular to the polarisation of the excitation. The anisotropy varies between 1 for light fully polarised in the parallel direction, and $-\frac{1}{2}$ for light fully polarised in the perpendicular direction. The denominator gives the total intensity; the factor of 2 in the denominator is due to the two perpendicular directions x and y which can be treated as having the same contribution because of the rotational symmetry in the case of z -polarised excitation, see the setup in Figure 5.3. In this section, the coordinate system used by Lakowicz will be adopted, shown in Figure 5.3, where the illumination axis is x , the detection axis is y and the excitation polarisation axis is z . It is important to note that this is different from the coordinate system used in the modelling of OPM and dOPM presented here, where the detection axis is chosen to be in the z direction.

A sample of randomly and uniformly oriented fluorophores is considered, and the orientation of each is described by polar coordinates (θ, ϕ) , with polar angle θ measured from the z -axis and azimuthal angle ϕ measured from the y -axis as shown in Figure 5.4. For a distribution with fluorophores uniformly spaced in solid angle, the distribution of θ should not be uniform, but instead have $\sin \theta$ dependence due to the solid angle being proportional to $\sin \theta$ [129]. If θ is naively assumed to be uniform, then the distribution of dipoles become concentrated at the poles ($\theta = 0, \theta = \pi$) due to the decreasing size of solid angle of elements near the poles [130].

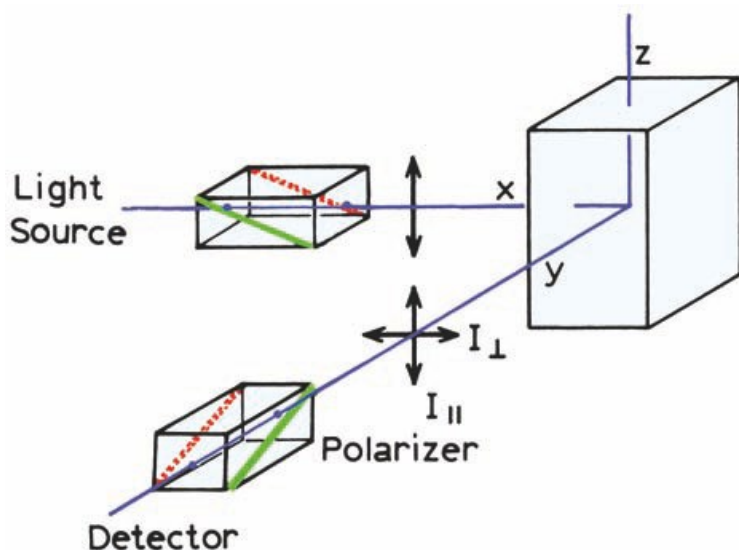


Figure 5.3 - Illustration of the intensities involved in measuring fluorescence anisotropy with a z-polarised light source. Reproduced with permission from Principles of Fluorescence Spectroscopy (Joseph R. Lakowicz, 3rd edition, 2006) [84].

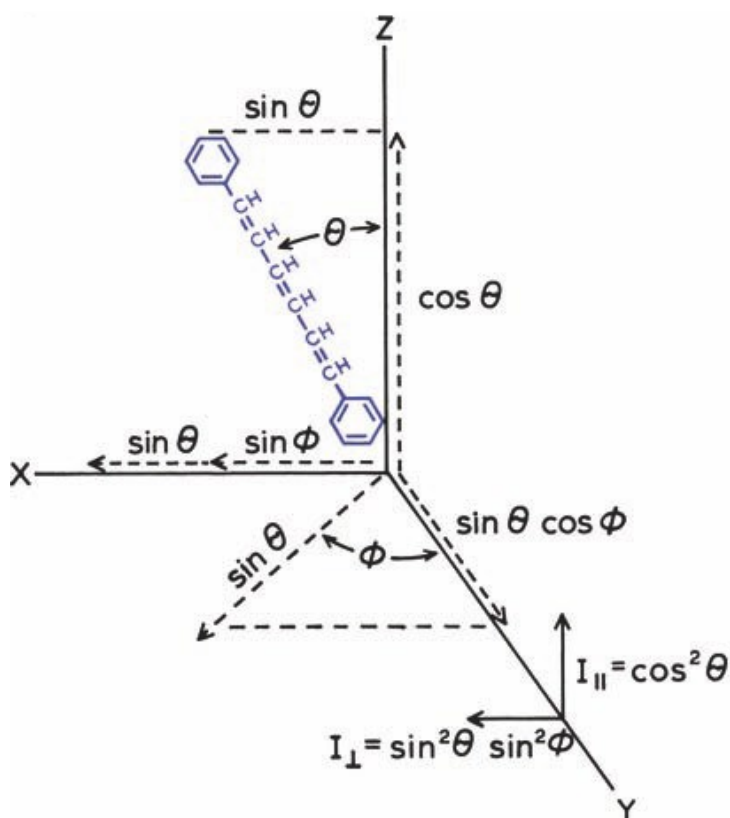


Figure 5.4 – Relative fluorescence intensities in the directions parallel and perpendicular to the excitation polarisation. Intensities are calculated by considering the probability of excitation of a given fluorophore, and this depends on the angle, θ_{ex} , between the fluorophore's excitation dipole (expressed by polar coordinates θ and ϕ) and the excitation polarisation—here, with z-polarised excitation, $\theta_{\text{ex}} = \theta$. For this fluorophore, assuming that the excitation and emission dipoles are parallel, the intensities in the parallel and perpendicular directions are given by the projection of the dipole orientation onto z and x respectively. Reproduced with permission, from Principles of Fluorescence Spectroscopy (Joseph R. Lakowicz, 3rd edition, 2006) [84].

Following the case in Lakowicz, which defines the excitation polarisation axis as z and propagation axis as y , the probability of excitation for an excitation transition dipole moment oriented at (θ, ϕ) is proportional to

$$|\vec{\mu} \cdot \vec{E}|^2 = |\vec{\mu}|^2 |\vec{E}|^2 \cos^2 \theta \quad 5.7$$

And, when combining this with the distribution of θ for uniformly oriented fluorophores, the probability density function (PDF) of angle of the fluorophore (excitation dipole) becomes

$$f(\theta) = \frac{1}{k} \cos^2 \theta \sin \theta d\theta, \quad 5.8$$

where the constant k is necessary for the PDF to integrate to 1 within the bounds of the PDF, as is given by

$$k = \int_0^{\pi/2} \cos^2 \theta \sin \theta d\theta. \quad 5.9$$

For a single fluorophore, the resulting intensity in the parallel and perpendicular polarisation directions can be calculated by projecting the dipole orientation onto the cartesian coordinates z and x , see Figure 5.4. These intensities are given by

$$I_{d,\parallel}(\theta, \phi) = \cos^2 \theta \quad 5.10$$

$$I_{d,\perp}(\theta, \phi) = \sin^2 \theta \sin^2 \phi \quad 5.11$$

when assuming the emission dipole is parallel to the excitation dipole and the fluorophore emits with an intensity of unity.

Finally, $I_{\parallel}$ and $I_{\perp}$ can be calculated for the entire distribution by computing the integral of the product of the intensity expressions (Equations 5.10 and 5.11) and the PDF for the excitation dipole angle from the z -axis, θ .

As a note to the reader, the derivation for the steady-state anisotropy by Lakowicz first considers the case of a general function $f(\theta)$ and arrives at general expressions for $I_{\parallel}$ and $I_{\perp}$ and the anisotropy r_0 . Although not explicitly stated in the text, that derivation uses the definition of the expected value of a function of the random variable θ given the PDF $f(\theta)$ [131].

Here, instead, the integrals are calculated directly with the expression of $f(\theta)$ representing photoselection under z -polarised excitation (Equation 5.8), obtaining

$$I_{\parallel} = \frac{1}{k} \int_0^{\pi} \int_0^{\pi/2} \cos^4 \theta \sin \theta d\theta d\phi = \frac{2\pi}{5k} \quad 5.12$$

$$I_{\perp} = \frac{1}{k} \int_0^{\pi} \int_0^{\pi/2} \cos^2 \theta \sin^3 \theta \sin^2 \phi d\theta d\phi = \frac{2\pi}{15k} \quad 5.13$$

showing that $I_{\parallel} = 3I_{\perp}$, corresponding to a steady-state anisotropy $r_0 = 0.4$. This is the maximum possible anisotropy due to photoselection with a uniform distribution of static fluorophore orientations; in practice, a number of factors can contribute to depolarisation, such that the fluorescence anisotropy r is less than r_0 .

The first cause of depolarisation is if the excitation and emission dipoles of the fluorophore are not parallel; in other words, if the fluorophore emits along a different axis than it is excited. This type of depolarisation is dominant if the fluorophore solution is viscous such that it does not rotate rapidly (but dilute so that it does not transfer energy readily). In this case, the maximum steady-state anisotropy under photoselection of 0.4 is shown to be scaled by an expression with a $\cos^2 \beta$ dependence,

$$r_{\text{static}} = r_0 \left(\frac{3 \cos^2 \beta - 1}{2} \right). \quad 5.14$$

This dependence can be understood when considering a fixed sample of fluorophores aligned with the polarisation axis (i.e. $r_0 = 1$). For an angle of β between the excitation and emission dipoles, the angle between the emission dipole and the $I_{\parallel}$ (z) axis is also β , and so the intensity projected onto the $I_{\parallel}$ axis is proportional to $\cos^2 \beta$.

In addition to non-parallel excitation and emission dipoles, there is depolarisation due to rotational Brownian motion. This is quantified by the rotational correlation time of the fluorophore, θ_c , which describes average time for a molecule to rotate 1 radian, and the steady-state anisotropy due to this source of depolarisation is given by

$$r = r_{\text{static}} / (1 + \tau / \theta_c) \quad 5.15$$

which is known as the Perrin equation [84]. Here, τ is the fluorescence lifetime, which is the average time for the intensity of a fluorescence decay to reduce by $1/e$. From this we see that for rapidly tumbling dipoles with $\theta_c \ll \tau$ the steady-state anisotropy tends to zero, which represents an ensemble of randomly oriented excitation dipoles and unpolarised emission, while for static fluorophores, where $\theta_c \gg \tau$, there is the steady state condition $r = r_{\text{static}}$. For small organic dyes, the rotational correlation time is typically much shorter than for fluorescent proteins where the rotational correlation time is longer than τ . In addition to molecular weight, the rotational correlation time increases for more viscous solutions.

It is important to note that the steady-state anisotropy is not the same as the time-resolved, instantaneous anisotropy (which is a function of time), but rather the anisotropy that results from the integration of the anisotropy emission over time.

Non-zero collection angle case

In these anisotropy calculations, however, the electric field is only measured exactly along the optical axis (y -axis, see Figure 5.4) while microscopy systems will collect light over a range of angles to the optical axis. The electric field due to the dipole emission varies in space (as is seen later according to Equation 5.35). We refer to the case described so far

(above) as on-axis detection, corresponding to a detection NA of 0. Below we consider the $\text{NA} > 0$ case, since the detection NA is necessarily greater than 0 in a microscope system.

The $\text{NA} > 0$ case is considered by Axelrod, who derived expressions for the fluorescence polarisation components parallel and perpendicular to the excitation polarisation as a function of the NA of the collection objective for a single dipole [132]. This general result can be used to calculate the steady state fluorescence anisotropy for a single dipole of a given orientation or it can be integrated over a continuous distribution of dipole orientations to give the fluorescence anisotropy of a multiple dipole source.

In the formulation here, some terms included in the Axelrod publication are neglected, such as the source size (denoted by s in the literature) and the angle of the excitation relative to the emission dipole (denoted by primed angles α', β'), since here we assume that they are parallel. Furthermore, the angles β and α in the Axelrod paper are the same as θ and ϕ respectively (defined in Figure 5.4) and relabelled as such in order to be consistent with the labels used by Lakowicz, along with Cartesian coordinates. The Cartesian coordinates that describe a dipole orientation are expressed in terms of the polar coordinates θ and ϕ by

$$\begin{aligned}x_i &= \sin \theta \sin \phi \\y_i &= \sin \theta \cos \phi \\z_i &= \cos \theta.\end{aligned}\tag{5.16}$$

The intensities emitted by a dipole in the parallel (z) and perpendicular (x) directions can be expressed in terms of K factors and the dipole orientations in Cartesian coordinates (x_i, y_i, z_i) . These intensities are given by

$$I_{i,\parallel}(x_i, y_i, z_i) = K_a y_i^2 + K_b x_i^2 + K_c z_i^2 \tag{5.17}$$

$$I_{i,\perp}(x_i, y_i, z_i) = K_a y_i^2 + K_c x_i^2 + K_b z_i^2 \tag{5.18}$$

where $x_i^2 + y_i^2 + z_i^2 = 1$ and the subscript i denotes the i^{th} dipole. For a given collection half angle σ_0 , such that $\text{NA} = n \sin \sigma_0$, the K factors are defined as

$$\begin{aligned}K_a &= (1/3)(2 - 3 \cos \sigma_0 + \cos^3 \sigma_0) \\K_b &= (1/12)(1 - 3 \cos \sigma_0 + 3 \cos^2 \sigma_0 - \cos^3 \sigma_0) \\K_c &= (1/4)(5 - 3 \cos \sigma_0 - \cos^2 \sigma_0 - \cos^3 \sigma_0).\end{aligned}\tag{5.19}$$

The total intensity of the parallel and perpendicular polarised light emitted by a population of dipoles is calculated by integrating the contributions from the individual dipoles scaled by the distribution (or weighting) function $m(\Omega, \mu)$, with Ω parametrising the angle of the dipole, and μ is a parameter to generalise all other constant parameters and is ignored in the integration. The total fluorescence intensity for parallel and perpendicular polarisations is therefore given by

$$I_{\parallel,\perp} = \int_{\Omega} m(\Omega, \mu) I_{d,\parallel,\perp} d\Omega \tag{5.20}$$

and the element $d\Omega$ can be considered the solid angle element and given by

$$d\Omega = (1/8\pi) \sin \theta d\theta d\phi.$$

Case of photoselection with p-polarised excitation

In the case of photoselection by excitation light polarised along the z-direction (x_3 in the literature), $m(\Omega, \mu) = \cos^2 \theta$ as before in the Lakowicz case. Therefore, the final integral is

$$I_{\parallel, \perp} = (1/8\pi) \int_0^{2\pi} \int_0^{\pi/2} I_{d, \parallel, \perp} \cos^2 \theta \sin \theta d\theta d\phi \quad 5.21$$

remembering that the $\sin \theta$ factor accounts for the smaller solid angle elements near the poles of the sphere over which the dipole orientations are integrated; without the $\sin \theta$, the integration is biased or oversampled towards the poles.

This integral was evaluated using Mathematica, and the result was found to be

$$I_{\parallel} = \frac{2\pi}{15} (K_b + 3K_c + K_a) \quad 5.22$$

$$I_{\perp} = \frac{2\pi}{15} (3K_b + K_c + K_a) \quad 5.23$$

and these expressions of the relative intensity of the parallel and perpendicular components can be used to derive the steady-state anisotropy using Equation 5.4, or calculate the PBS transmission for dOPM as will be shown in Section 5.1.3.

When considering the setup of OPM and dOPM, the excitation scheme here in the z-direction corresponds to light-sheets polarised in-plane, which we call p-polarised, but there is also interest in calculating the relative intensity of the components with excitation polarised out of the plane of the light-sheet, which we will refer to as s-polarised.

Case of photoselection with s-polarised excitation

The probability function $m(\Omega, \mu)$ used to model photoselection is calculated by the square of the dot product of the excitation polarisation vector with the dipole orientation vector (x_i, y_i, z_i) , see Equation 5.5. For s-polarised light-sheets, this is given by

$$m(\Omega, \mu) = (\sin \theta \sin \phi \sin \theta_{OPM} + \sin \theta \cos \phi \cos \theta_{OPM})^2 \quad 5.24$$

and then computing the integral in Equation 5.20 with Mathematica gives

$$I_{\parallel} = \frac{2\pi}{15} [2(K_a + K_b) + K_c + (K_a - K_b) \cos(2\theta_{OPM})] \quad 5.25$$

$$I_{\perp} = \frac{2\pi}{15} [2(K_a + K_c) + K_b + (K_a - K_c) \cos(2\theta_{OPM})]. \quad 5.26$$

In later sections, a fluorescent sample is modelled computationally by an ensemble of dipoles as part of the OPM/dOPM ray-tracing simulation, and at this time, we consider the static fluorophore and rapid tumbling cases with the excitation and emission dipoles parallel. The ray-tracing simulation code used for OPM/dOPM systems can be easily adapted to simulate a test optical system to measure the anisotropy (presented in the Section 5.3.1) and compare it to the theoretical anisotropy derived here. This allows the ray-tracing simulation results to

be verified by theory, and this can be done for both single dipoles sources and ensemble dipole sources with photoselection, and for different collection NAs.

In the following section, the relative intensities of the parallel and perpendicular components are used to calculate the transmission through the polarising beamsplitter (PBS) in dOPM for p-polarisation and s-polarisation. The transmission then is used in the GRCE and GCE calculations for dOPM.

5.1.3 Collection efficiency of dOPM

Dual-view OPM (dOPM), introduced in Section 1.5.4, is a variant of OPM which sequentially acquires two orthogonal views which can be fused to yield higher and more isotropic resolution than standard OPM [83]. A folded setup is used in dOPM, see Figure 5.5, which uses two prism mirrors mounted on a custom holder in the refocusing space, and these mirrors are tilted by half the OPM angle from the horizontal in order to rotate the tilted plane of fluorescence so that it can be imaged onto a camera—this is instead of having a tilted detection microscope (O3-TL3) such as in OPM. This results in a higher geometric collection efficiency than OPM, which is constrained by the physical requirement to have mechanical clearance between O2 and O3; O3 must have a relatively high working distance which is associated with lower NA. In dOPM, the prism mirror can be placed closer to O2 and therefore the NA of the objective that acts as both O2 and O3 can have an NA as high as 0.95 (in air). This folded method of single-objective light-sheet was demonstrated in APOM [133] and obSTORM [80], introduced in Section 1.4.5 and 1.5.3 respectively.

As explained previously and in Chapter 1, the PBS in dOPM results in transmission losses that depend on the polarisation state of the emission light. If the emission is unpolarised the transmission through the PBS is 50%, however, the partial polarisation caused by polarised excitation allows this to be increased if the PBS is oriented correctly. Previously, the ‘geometric’ collection efficiency (GRCE) that considers the intersection of the O1, O2 and O3 pupils was introduced for OPM.

Like what is done for OPM, the GRCE for dOPM is calculated by finding the solid angle intersection of the collection cones of O1, O2 and a rotated instance of O2 to model the reflection of the light cone by the prism mirror. The resulting collection efficiency metrics are then multiplied by the transmission of the PBS. As will be described in this section, this is achieved by calculating the integrated intensity collected by O1 for the polarisation component parallel and perpendicular to the PBS transmission axis according to first Lakowicz’s and then Axelrod’s theory. The exact expression for the dOPM GRCE (and GCE) is shown in Table 5.1.

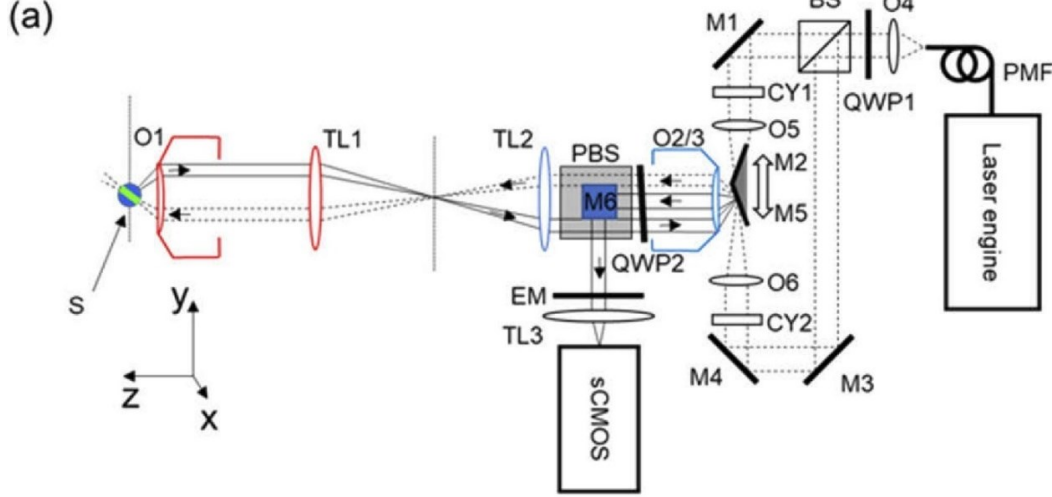


Figure 5.5 – dOPM setup shown for a single view, the view determined by the prism mirror pair (M2 and M5) position. O – objective, TL – tube lens, PBS – polarising beamsplitter, EM – emission filter, QWP – quarter waveplate, M – mirror, CY – cylindrical lens, BS – beamsplitter, PMF – polarisation maintaining fibre. Reproduced with permission from [83].

GRCE calculation for static fluorophores under photoselection using Lakowicz’s low-NA assumption

As shown in Section 5.1.1, the GRCE was calculated for the OPM system used throughout the PhD (presented in Chapter 2) to be 0.328. As an example, consider a dOPM system configured with the same OPM angle (35°), same O1 (1.2NA water), same O2 (0.95NA air), which means that O3 also has an NA of 0.95. To calculate the GRCE for dOPM, the equivalent GRCE for OPM is scaled according to the loss at the PBS, and for a 50% loss in the case of unpolarised light this yields a GRCE of 0.391.

In both the OPM and dOPM setups in our labs, the light-sheet is polarised in the plane of the light-sheet (y , or p -polarisation), resulting in photoselection and thus partial polarisation of the emission light. When using the theory from Lakowicz, which assumes low-NA (on-axis) detection, the (maximum) steady-state fluorescence anisotropy $r_0 = 0.4$. This corresponds to a ratio of parallel-polarised to perpendicular-polarised intensity of 3:1, or $I_{\parallel} = 3I_{\perp}$ and is the assumption used in the original dOPM paper calculations [83]. In this configuration of dOPM, only the parallel component of the intensity is transmitted by the PBS. The fraction of intensity transmitted by the PBS is given by

$$t_{\text{PBS},p} = I_{\parallel} / (I_{\parallel} + I_{\perp}) \quad 5.27$$

and the dOPM GRCE is then equal to product of $t_{\text{PBS},p}$ and the OPM-equivalent GRCE. In this case, this corresponds to a 25% loss rather than a 50% loss at the PBS, which results in a GRCE of 0.586.

Since the limiting NA of this system is set by objective O1 which has an NA of 1.2 (and $n = 1.33$), however, the approximation that $\text{NA} \approx 0$ is not valid and overestimates the value of $t_{\text{PBS},p}$ and the collection efficiency.

GRCE calculation for static fluorophores under photoselection using Axelrod method

In order to provide a better estimate of the dOPM GRCE for the $\text{NA} > 0$ case, the relative intensities for each polarisation state $I_{\parallel}$ and $I_{\perp}$ after O1 and O2 are calculated using Equations 5.22–5.26 that were derived in Section 5.1.2. When calculating these equations, the limiting value (the lowest) of the collection half angle σ_0 for O1 and O2 is used.

As with the low-NA case, these intensities are used to calculate the transmission factor for p-polarised excitation $t_{\text{PBS},p}$ with Equation 5.27. The fraction of light collected by O3 is then calculated using the same geometric intersection of the collection cones of light, also in the same way as before.

For p-polarised excitation, $I_{\parallel}$ and $I_{\perp}$ is given by Equations 5.22 and 5.23, and for our dOPM system, $t_{\text{PBS},p}$ evaluates to 0.710 corresponding to $r_{\text{static}} = 0.326$. This results in a GRCE of 0.555 for the previously described dOPM system with an OPM angle of 35° . In the low NA-case shown above, $t_{\text{PBS},p}$ would equal 0.75 and $r_{\text{static}} = 0.4$.

In addition to considering p-polarised excitation, the s-polarised excitation case is considered where the polarisation of the light sheet is perpendicular to the light sheet plane. The relative intensities of $I_{\parallel}$ and $I_{\perp}$ under s-polarised excitation are now found using Equations 5.25 and 5.26 and the PBS is oriented along the perpendicular direction instead (x-axis in Figure 5.4), therefore the transmission factor for s-polarisation $t_{\text{PBS},s}$ is

$$t_{\text{PBS},s} = I_{\perp} / (I_{\parallel} + I_{\perp}). \quad 5.28$$

For the same dOPM system with an OPM angle of 35° , this corresponds to a $t_{\text{PBS},s}$ value of 0.578 and GRCE of 0.452. Note that the anisotropy is not calculated here since the excitation does not provide the symmetry necessary to assume the x and y intensities are equal, as assumed in Equation 5.6. These dOPM GRCE calculations were also made for OPM angles of 45° and the results are presented in Table 5.5 along with the results from the ray-tracing simulation, which will be described in subsequent sections.

The calculated values for GRCE and GCE for dOPM exclude the transmission of the optics (t_{optics}) and reflection losses at the tilted mirror. The expected transmission losses due to the optics t_{optics} may be estimated using the manufacturer's specifications of the transmission factors of the optical elements, however it can be a challenge to account for all contributions to the total loss.

For example, in measurements made by Hugh Sparks of a dOPM system with a $40\times$ 1.15NA/W objective for O1 and 0.75NA objective for O2/3, calculations of t_{optics} were

shown to be an underestimate [83]. Indeed, for this dOPM system, the experimentally measured RCE was 0.067 [83], while the dOPM GRCE scaled by the calculated t_{optics} was 0.11. O2/3 was a coverslip corrected objective, and one potential cause of the discrepancy is higher reflection losses from the uncoated coverslip with non-normal incident angles than the reported normal incidence. It is worth noting that current dOPM systems instead use a metallurgical objective lens for O2/3, eliminating the need for a coverslip, but there remain discrepancies in the calculated and measured collection efficiency; this is briefly discussed later in this chapter in Section 5.3.4.

It is important to remember that although the Axelrod calculations correctly consider the effect of the (limiting) NA of O1 and O2 on the transmission of the PBS, the collection efficiency is still calculated using the geometric intersection of the collection cones. This calculation would only be correct when the fluorescence signal is distributed uniformly over the effective pupil after O1 and O2, e.g. in the case of low fluorescence anisotropy. In the case of non-zero fluorescence anisotropy, the fluorescence signal is not uniformly distributed over the pupil and so losses due to the clipping caused by O3 cannot be calculated correctly by this method. In addition, the system consisting of the PBS, QWP and mirror used to outcouple the fluorescence is sensitive to polarisation changes at the mirror, and the method described here does not consider this.

Therefore, I set out to perform a more complete calculation using vectorial ray tracing that can also include the electric field distribution of the emission, its propagation through the system, and the polarisation changes due to the mirror. I therefore hoped to obtain a more accurate calculation for the collection efficiencies of dOPM that can be compared with experimental measurements. In particular, I aim to show through ray-tracing simulations the exact effect of excitation light polarisation on the collection efficiency—namely to verify the hypothesis that (p-)polarised light-sheets increase the collection efficiency in dOPM compared to the unpolarised case if the PBS orientation is chosen favourably.

5.1.4 Complex reflection coefficients and ellipsometry

Through experimental measurements by Hugh Sparks and Liuba Dvinskikh, it was realised that the mirror used at the focus of O2/3 in dOPM introduces an angle-dependent retardance, which can be parameterised by a complex reflectance. This then reduces the degree of the desired polarisation which is reflected by the PBS and thus decreases the collection efficiency. There is therefore motivation to model the protected silver mirrors used in the real system, and this involves modelling the multiple Fresnel reflections and including the inference due to the thin film that forms the protective coating. The polarisation-dependent performance is sensitive to the film thickness, but the thickness is not known due to the limited information provided by the manufacturer. Therefore, it is pertinent to find the thickness more accurately.

Ellipsometry is a tool that is used to measure a material's reflective properties using the polarisation change of light when reflected. Specifically, the ellipsometry quantities Ψ and Δ

are directly measured, and these describe the relative phase changes (retardance) and intensity changes and between the p and s components—the polarisation components parallel and perpendicular to the reflection plane respectively. Formally, the quantity Ψ is usually defined as

$$\tan(\Psi) = \frac{|\tilde{r}_p|}{|\tilde{r}_s|} \quad 5.29$$

where $\tilde{r}_p$ and $\tilde{r}_s$ are the complex reflection coefficients for p and s reflection components, respectively, and Δ is defined as

$$\Delta = \delta_p - \delta_s \quad 5.30$$

where δ_p and δ_s are the phase differences introduced on reflection for p and s components [134]. The quantity ρ , which is given by the ratio of the complex reflection coefficients, can be written in a form to combine Δ and Ψ , as

$$\rho = \frac{\tilde{r}_p}{\tilde{r}_s} = \tan(\Psi)e^{i\Delta}. \quad 5.31$$

By measuring Ψ and Δ as a function of wavelength, information about the structure of materials can be found through model fitting, which is described in more detail below.

The total complex reflection in air from a bulk material (substrate) with single thin coating layer is given by the Airy formula [135]

$$\tilde{r}_i = \frac{r_{i,12} + r_{i,23}e^{-i2\beta}}{1 + r_{i,12}r_{i,23}e^{-i2\beta}}, \quad i = p, s \quad 5.32$$

where $r_{i,12}$ and $r_{i,23}$ are the standard Fresnel reflections from interfaces between medium 1 and 2 and between 2 and 3 respectively; medium 1 corresponds to air, 2 the thin film material and material 3 is the substrate. For a protected silver mirror, for example, the substrate is silver and the coating may be a Silicon Oxide. The quantity β is film phase depth (optical path distance within the thin film), given by

$$\beta = \frac{2\pi}{\lambda} \tilde{n}_2 d \cos \phi_2 \quad 5.33$$

where $\tilde{n}_2$ is the (complex) refractive index of medium 2, d is the thickness of the film and ϕ_2 is the angle of the ray within the film with respect to the surface normal.

Equation 5.32 has been widely documented in ellipsometry literature, albeit with varying forms and conventions [136], [137], [138]. Here, we use the common convention that the phase component of the electric field is $e^{i(\omega t + kd)}$, and in this case complex refractive index is given by $\tilde{n} = n - i\kappa$, where κ , the absorption coefficient and $\kappa > 0$ corresponds to absorption [134].

In the case of the protected silver mirror with unknown film thickness d (but known refractive index), Equation 5.32 can be used to find the (unknown) film thickness d by

calculating theoretical values of Δ and Ψ and fitting these to the experimentally measured values obtained with ellipsometry.

A Woollam VASE spectroscopic ellipsometer was used here to measure Δ and Ψ for an SiO_2 -protected silver mirror (Thorlabs) for a range of wavelengths, generating curves which the model is fit to. Within the Woollam software, the user uses the model builder to specify the layers within the reflective sample to be measured—namely the refractive indices and any known thicknesses—and the free parameters are found by the model fitting procedure. The model builder in Woollam is capable of modelling multilayer materials by summing Fresnel reflections, but for our case of a mirror with a single thin film coating, this reduces to Equation 5.32.

5.2 Method

As mentioned in the Section 1.5.3, obSTORM is a folded implementation of OPM that is similar to the subsequently demonstrated dOPM configuration [80]. The authors of the obSTORM work developed a method to evaluate vectorial diffraction integrals to describe the spatial resolution and collection efficiency of their system. To achieve this, the obSTORM authors (Kim et al.) employed 3×3 Jones matrices to model the optical elements of the system, and both the wavevectors and electric field vectors were calculated through matrix multiplication in their approach [139]. We describe this as a vectorial ray-tracing approach, with the wavevectors representing rays. This method treats each lens as a thin lens with a spherically refracting surface which is described here as the lens having a spherical and flat principal surface, and the electric field distribution is evaluated on the final principal surface. The electric field and wavevectors are then used to calculate the vectorial diffraction from the exit pupil of the system to evaluate the on-axis PSF.

The dOPM system has the same folded configuration as the obSTORM system and may be modelled using the same steps; however, in the method used to model obSTORM, the calculations are simplified by modelling the mirror as being normal to the optical axis, and therefore the pupil clipping is calculated *a priori* (and used to define the integral limits in the integral over the pupil). The main consequence of neglecting the mirror tilt is that the incidence angles of reflection change, and it is likely that the complex, polarisation-dependent reflection coefficients have a strong dependence on the reflection angle. The system used by both dOPM and obSTORM to outcouple the fluorescence from the folded system is sensitive to polarisation changes at the tilted mirror, since it relies on the 90° rotation of the polarisation transmitted by the PBS.

This then motivated me to adapt the obSTORM approach to create a vectorial ray-tracing simulation (in Python) that models the mirror tilt. My ray-tracing simulation also calculates the complex reflections from metallic mirror with a thin oxide film coating, while the obSTORM model only calculates Fresnel reflections from a single surface. Furthermore, the effect of photoselection is considered by modelling the fluorophore source as an ensemble of dipoles whose intensity scales depending on their alignment with the excitation polarisation.

The fluorophores are considered in either the static case ($r = r_{\text{static}}$) or the free/rapid tumbling case ($r = 0$) where the timescale of fluorophore rotation is much faster than the fluorescence lifetime—this was discussed in Section 5.1.2. The code was written so that a custom microscope setup can be simulated with different combinations of customisable lens NAs and focal lengths, waveplates, polarisers and mirrors, and the excitation polarisation can be set to represent different illumination schemes. The Python code for the ray-tracing simulation is found on GitHub (see **OPMsim** repository links on page 9).

The ray-tracing simulation presented here does not yet evaluate the ideal on-axis resolution performance by calculating the PSF by a Debye-Wolf integral, as is done in the characterisation of obSTORM, but it may be possible to achieve this with numerical integration and is an area of future work.

During the development of my ray-tracing simulation, a similar simulation, also based on the vectorial ray-tracing method used for obSTORM, was demonstrated for modelling an OPM system with the custom glass-tipped objective (the “snouty” lens) by Sommernes et al. [140]. The main focus of the work by Sommernes et al. is evaluating the effect of reflections at the surface of O3 on collection efficiency and resolution (using the PSF and OTF) for specially constructed high NA snouty-style O3 objectives. The Sommernes et al. paper considers three cases in their simulation: glass-tipped solid immersion O3 with an anti-reflection coating, water immersion O3 with a coverslip, and a theoretical 1.0NA air objective (not physical). This simulation calculates the light transmission efficiency relative to the perfectly transmitting 1.0NA air O3, in addition to calculating the 3D PSF using the Debye-Wolf diffraction integral, similar to how the PSF is evaluated in the obSTORM paper [80]. In the future, the ray-tracing simulation presented in this chapter may be extended to calculate the reflection losses at a similar glass-tipped O3 to validate my results with the results published by Sommernes et al.

The ray-tracing simulation presented in this chapter consists of three main steps: generating the fluorophore source (modelled as dipoles) and the initial rays from the source, calculating the transfer matrix to trace the rays, and evaluating the intensity distribution on a ‘detector’ surface using the transfer matrix i.e. the final principal surface. Each of these steps is explained in the following sections, starting with the source generation. Example iPython Notebooks used to construct and run these ray-tracing simulations are available on GitHub (see **OPMsim** links).

5.2.1 Dipole point source

Dipole generation

The first step of the ray-tracing simulation is to generate the orientations of the fluorophore excitation/emission dipoles that constitute the point source. The polar angle α_d and azimuthal angle ϕ_d parameterise the orientation of the dipole, with α_d defining a cone centred along the optical axis and ϕ_d the location on the circumference of this cone, as shown in Figure 5.6(b). It should be noted that this coordinate system is slightly different to

that shown in Section 5.1.2, with the azimuthal angle defined similar to in a cylindrical coordinate system. The coordinate system is defined such that dipole orientation vector is confined to the surface of a unit sphere.

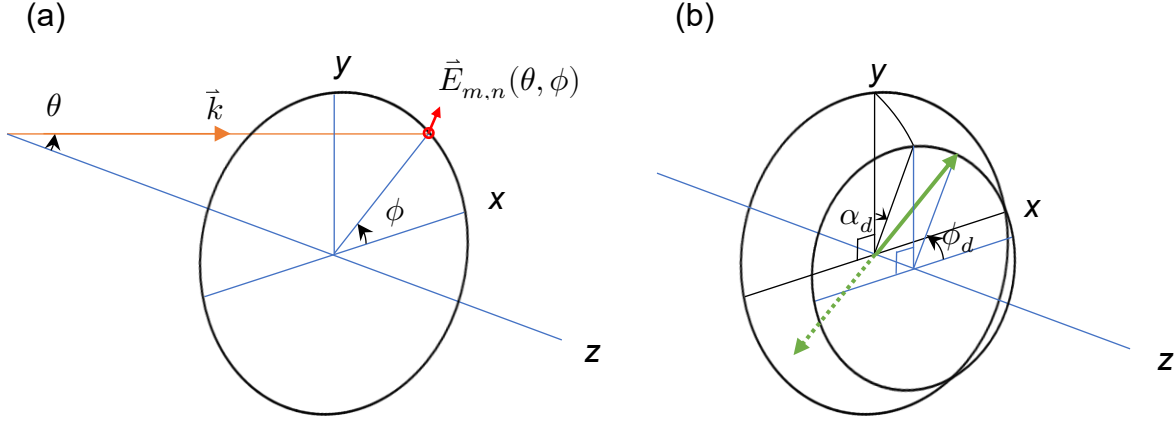


Figure 5.6 – (a) Polar angle definitions for rays (orange) in ray tracing with the electric field perpendicular to the direction of propagation, and (b) polar angle definitions for dipole (green) orientation.

The fluorophore orientations in a general fluorescent sample follow a random and uniform distribution, and to simulate this, dipole orientations are generated to be uniformly spaced in solid angle. This is effectively the problem of generating uniformly distributed points on a (unit) sphere. The approach used here involved segmenting the sphere into rings (or collars) and was based on a pre-existing method in literature [141], [142].

Firstly, a list of polar angles, θ_{collar} , is generated which defines the edges of the collars which build up a sphere. These collars are then segmented azimuthally into equally sized solid angle/area elements and within each element a point is defined in the mid-point to represent a single dipole orientation. The number of segments per collar varies so that the solid angle of each element is equal i.e. so the point density is uniform.

The effect of photoselection is modelled by applying the probability of absorption in the form of an intensity scaling evaluated for each dipole. In the ray-tracing simulation, the excitation polarisation is generalised beyond considering only polarisation along x, as was the case in Section 5.1.2 (where it is labelled as z). The excitation polarisation vector uses the same coordinate system as the dipole orientation and is described by (α_{ex}, ϕ_{ex}) .

As shown previously, the photoselection probability distribution function is given by the square of the dot product of (excitation) dipole vector and the electric field vector, which we write here as $\cos^2\delta$, where δ is the angle between the excitation polarisation and (excitation) dipole orientation.

The expression for $\cos^2\delta$ is therefore obtained by calculating the dot product of the dipole orientation excitation polarisation unit vectors expressed in Cartesian coordinates, and this is given by

$$\cos^2\delta = (\cos\alpha_{\text{ex}}\cos\phi_{\text{ex}}\cos\alpha_d\cos\phi_d + \cos\alpha_{\text{ex}}\sin\phi_{\text{ex}}\cos\alpha_d\sin\phi_d + \sin\alpha_{\text{ex}}\sin\alpha_d)^2. \quad 5.34$$

In this work, the dipoles are modelled to either be static or are free to tumble on a timescale much faster than the fluorescence lifetime, and in the latter case, the photoselection is not applied.

Ray generation

A cone of rays originating from the dipole source is generated up to the acceptance angle of the collection objective in the system (e.g., O1 in OPM) and this angle is given by $\theta_{\text{max}} = \text{asin}(\text{NA})$, where NA is the numerical aperture of the collection objective. Each of these rays has associated electric field vectors for each dipole in the source,

For the electric fields to be evenly sampled, the ray directions are uniformly spaced (in solid angle) and this is again a problem of finding uniformly spaced points on the surface of a sphere; the same collar-based method used for generating the dipole orientations is adapted to achieve this—see above. In this method, the surface of the sphere is divided into equally spaced (in polar angle θ) rings/collars, and each collar is divided further into elements in the azimuthal angle ϕ such that each element has the same solid angle. The mid-point of each element represents a single ray.

Since only the rays collected by O1 are of interest, for a given NA, the final collar angles θ are calculated by first generating collars up to a maximum angle of 90° (NA=1), and then collars whose outside edge are associated with a polar angle greater than θ_{max} are removed. To match the maximum collar angle more accurately to θ_{max} (set by the NA), the array of collar angles θ is scaled slightly so that the maximum collar angle equals θ_{max} since the quantisation of the collars may result in a mismatch.

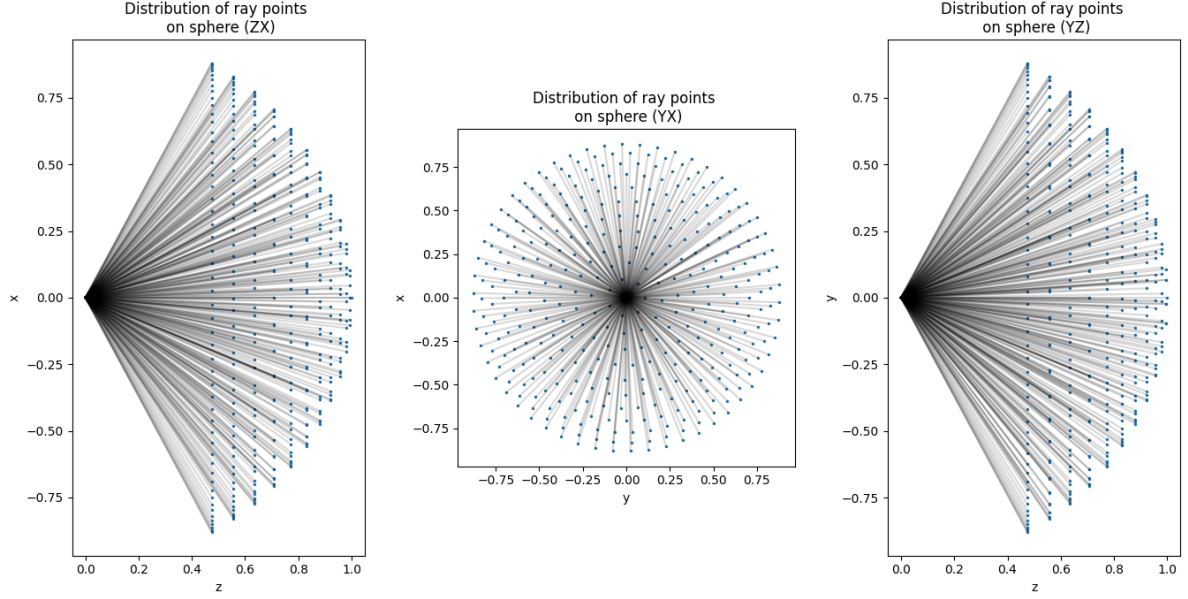


Figure 5.7 – Cone of uniformly spaced rays generated from the source propagated onto the spherical first principal surface of O1.

Once each ray direction is calculated, the rays are propagated a distance equal to the front focal length of the objective O1 to the first principal surface of the O1. On this surface, the initial electric field is calculated for each ray and from each dipole using the equation [143]

$$\vec{E}_{m,n} = \vec{k}_n \times \vec{p}_m \times \vec{k}_n \quad 5.35$$

where $\vec{k}_n$ is the wavevector of the n^{th} ray and $\vec{p}_m$ is the (emission) dipole moment of the m^{th} dipole. The initial energy on this first surface is also computed by considering the incoherent sum of intensity contributions from each dipole

$$I_{in} = \sum_{m,n}^{n_{\text{dipoles}}, n_{\text{rays}}} |\vec{E}_{m,n}|^2. \quad 5.36$$

This quantity is used when calculating the RCE, as described in later sections.

5.2.2 Ray tracing and calculation of electric field

In the ray-tracing simulation code, Jones matrices $\mathbf{M}_{n,i}$ representing each i^{th} optical element are constructed for each ray, and the matrix product is calculated to obtain the total transfer matrix $\mathbf{M}_n^{\text{total}}$ for a ray n , that is

$$\mathbf{M}_n^{\text{total}} = \prod_i \mathbf{M}_{n,i}. \quad 5.37$$

The total transfer matrix for each ray n , $\mathbf{M}_n^{\text{total}}$, is updated sequentially for each optical element at a time, and simultaneously, the rays' wavevectors $\vec{k}_n$ are traced by the same operations represented by the matrices. The individual Jones matrices are calculated for each ray separately (one optical element at a time) since many of the operations depend on ray angle.

The vectorial electric field for each dipole m and ray n is represented as a 3D matrix, $\vec{E}_{m,n}$, and $\mathbf{M}_n^{\text{total}}$ is applied to $\vec{E}_{m,n}$ to calculate the final electric field traced through the system for all rays and dipoles, formally:

$$\vec{E}_{m,n}^{\text{out}} = \mathbf{M}_n^{\text{total}} \vec{E}_{m,n}. \quad 5.38$$

The advantage of this method is that it allows the calculation of the final electric field $\vec{E}_{m,n}^{\text{out}}$ to be fully vectorised. The ray-tracing method calculates the Jones matrices $\mathbf{M}_{n,i}$ depending on the current ray (e.g. angle) itself, and $\mathbf{M}_n^{\text{total}}$ must be calculated progressively as described, but by pre-computing $\mathbf{M}_n^{\text{total}}$, the computation $\mathbf{M}_n^{\text{total}} \vec{E}_{m,n}$ is vectorised and can utilise CPU SIMD/vector instruction sets.

After the initial electric fields are propagated through the system, the intensity is evaluated on the final surface of the optical system—e.g., the second principal plane of the final lens in the system—in a similar way to how it is evaluated on the first surface in Equation 5.36. The surface may be flat or spherical, and in the latter case, before calculating the intensity, the coordinate system is transformed to be local to the spherical surface; this is elaborated upon in Appendix A.5.1.

The intensity is calculated separately for the x and y components of the electric field, I_x and I_y respectively, with each a sum over all ray and dipole combinations. These separate intensity components allow the contributions of each polarisation to the total intensity to be visualised, see Section 5.3.2, and can be used to calculate the fluorescence anisotropy after the light is propagated through the system. The x and y intensity components are summed to obtain the total output intensity, and the full calculation is:

$$I_{\text{out}} = I_x + I_y = \sum_{m,n}^{n_{\text{dipoles}}, n_{\text{rays}}} \delta_n |\vec{E}_{m,n}^{\text{out}} \cdot \hat{x}|^2 + \sum_{m,n}^{n_{\text{dipoles}}, n_{\text{rays}}} \delta_n |\vec{E}_{m,n}^{\text{out}} \cdot \hat{y}|^2 \quad 5.39$$

where $\delta_n = \{0,1\}$, equalling zero if the ray n is lost or occluded due a limiting pupil in the system—this is determined during the ray tracing. The total output intensity is used to calculate the RCE, which is equal to $I_{\text{out}}/I_{\text{in}}$.

The optical element matrices and the final transfer matrix $\mathbf{M}_n^{\text{total}}$ are in the form of 3D Jones matrices, and although this formulation does not directly model unpolarised light such as is achieved with Mueller matrices, it instead provides a microscopic description of the system by considering many separate dipole orientations that are summed incoherently. The Jones matrices for the optical elements here are based on the vectorial ray tracing tutorial by Kim et al. [139] and are presented in the next section along necessary coordinate transforms and extensions to the existing literature, namely implementing the reflection by a tilted mirror (with a thin film coating) at the focus of O2/3.

5.2.3 3D Jones Matrices

Lens model based on sine condition

A common method to model a high numerical aperture lens is to consider the sine condition [60], [144]. Effectively, the objective lens is modelled as a device that converts an incident spherical wavefront into a plane wavefront or vice versa, or alternatively, we describe the lens as having a spherical and flat principal plane. Correspondingly, rays from the object at the front focal point of the objective are collimated after the objective with front focal length f , and the ray height being given by $f \sin \theta$ when the ray angle to the optical axis is θ .

The sine lens matrix applies a rotation to the electric field in the meridional (x_m, z) plane. Therefore, the electric field must first be transformed into the meridional coordinates (x_m, y_m, z) by applying a matrix $\mathcal{R}_z(\phi)$ to transform from (x, y, z) to (x_m, y_m, z) . Here, ϕ is the angle of the meridional plane with respect to the x-axis and is the same as the ray azimuthal angle (also ϕ). The lens refraction matrix $\mathcal{L}(\theta_{\text{lens}})$ is then used to apply a rotation to the electric field about the y_m -axis. The inverse transform $\mathcal{R}_z(-\phi) = -\mathcal{R}_z(\phi)$ also exists and is used to transform the coordinate system back from the meridional coordinates (x_m, y_m, z) to the lab coordinates (x, y, z) . See Figure 5.8 for the Jones matrices $\mathcal{L}(\theta_{\text{lens}})$ and $\mathcal{R}_z(\phi)$ and the geometries involved.

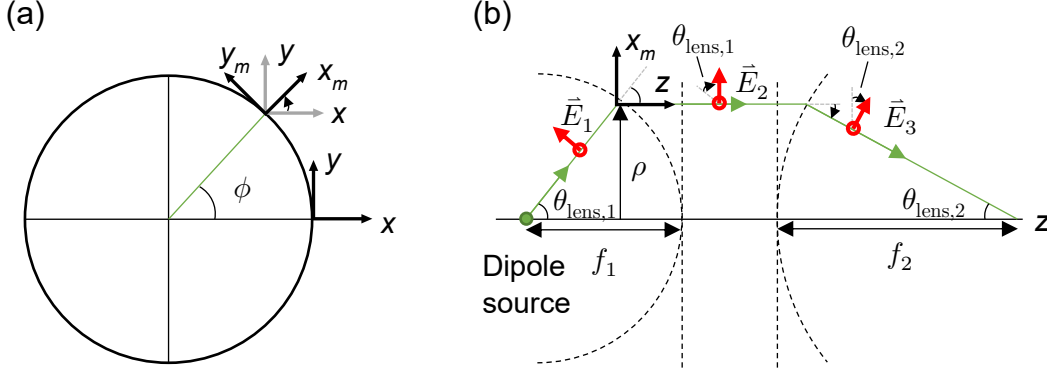
The angle of ray rotation caused by refraction at the lens is given by

$$\theta_{\text{lens}} = \arccos(\rho/f) \quad 5.40$$

with ρ the ray height and f the focal length of the lens, which can be the front focal length (FFL) or back focal length (BFL) depending on whether the light is being collimated (use FFL) or focused (use BFL); this can be understood by consulting Figure 5.8. The front and back positions which define the FFL and BFL are defined by the direction of the rays, i.e. the rays encounter the front before the back.

The ray height is updated in the code by propagating the ray, which has an angle θ_{lens} to the optical axis, in meridional space to the curved principal surface of the lens by the FFL distance or from the curved principal surface by the BFL—when rays are collimated there is no need to perform this propagation. When the ray crosses the optical axis the ray height becomes negative, the sign of θ_{lens} therefore changes and this reverses the rotation due to $\mathcal{L}(\theta_{\text{lens}})$ as expected.

For lenses where the incident rays have a non-zero polar angle θ , the maximum permitted angle is given by the NA of the lens by $\theta_{\text{lens}}^{\text{max}} = \arcsin(\frac{\text{NA}}{n})$, and if $\theta > \theta_{\text{lens}}^{\text{max}}$ the ray mask is updated so that $\delta_n = 0$; in the case of parallel incident rays ($\theta = 0$), this is enforced by $\rho > f$.



$$\mathcal{R}_z(\phi) = \begin{bmatrix} \cos \phi & \sin \phi & 0 \\ -\sin \phi & \cos \phi & 0 \\ 0 & 0 & 1 \end{bmatrix} \quad 5.41$$

$$\mathcal{L}(\theta_{lens}) = \begin{bmatrix} \cos \theta_{lens} & 0 & \sin \theta_{lens} \\ 0 & 1 & 0 \\ -\sin \theta_{lens} & 0 & \cos \theta_{lens} \end{bmatrix} \quad 5.42$$

Figure 5.8 – (a) Meridional transform as viewed along the optical axis, z . (b) Tracing of an example ray in the meridional plane for a system with two sine lenses with focal lengths f_1 and f_2 , with their principal surfaces shown by dashed lines. In this case, f_1 is a FFL and f_2 is a BFL.

Rotated objective lens

In OPM, the objective lens O3 is rotated relative to objective lens O2, and this can be simulated by rotating the cone of rays incident on O3. This is realised by applying the rotation matrix $\mathcal{L}_y(\theta_{OPM})$ in the lab frame (x, y, z) , which is given by

$$\mathcal{L}_y(\theta_{OPM}) = \begin{bmatrix} \cos \theta_{OPM} & 0 & \sin \theta_{OPM} \\ 0 & 1 & 0 \\ -\sin \theta_{OPM} & 0 & \cos \theta_{OPM} \end{bmatrix}. \quad 5.43$$

Linear polariser

The linear polariser only allows light of a particular polarisation to pass through. In the ray-tracing simulation, this orientation is described by the azimuthal angle ψ which is defined from $+x$ in (and constrained to) the x-y plane in lab coordinates. The associated Jones matrix is given by

$$\mathcal{P}(\phi) = \begin{bmatrix} \sin^2 \psi & \sin \psi \cos \psi & 0 \\ \sin \psi \cos \psi & \cos^2 \psi & 0 \\ 0 & 0 & 1 \end{bmatrix}. \quad 5.44$$

The linear polariser is used to model the PBS—the transmission through the PBS as one linear polariser and reflection as another with crossed polarisation.

Waveplate

A waveplate changes the polarisation of an input light wave using birefringence to apply a phase retardation that is different in the two orthogonal axes, named the slow and fast axes. The general waveplate is implemented as a Jones matrix with

$$\mathcal{W}(\delta, \psi) = \begin{bmatrix} \cos(\delta/2) + i \cos(2\psi) \sin(\delta/2) & i \sin(2\psi) \sin(\delta/2) & 0 \\ i \sin(2\psi) \sin(\delta/2) & \cos(\delta/2) - i \cos(2\psi) \sin(\delta/2) & 0 \\ 0 & 0 & 1 \end{bmatrix} \quad 5.45$$

where δ is the retardance or phase difference induced by the waveplate between the polarisation components in the fast and slow axes, and ψ is the angle that defines the azimuthal angle of the fast axis from $+x$ in the x-y plane.

Tilted mirror

The tilted mirror used in dOPM at the focus of O2/3 to replace the need for a tilted O3 is also modelled in this ray-tracing simulation. In the original literature in which the obSTORM system is modelled, this is realised by modelling a flat mirror with reflection coefficients r_p and r_s calculated by Fresnel equations. With a flat mirror, this makes it relatively trivial to perform a p/s component decomposition, so the reflection coefficients can be calculated and applied to the electric field all in the meridional space. In the obSTORM paper (in the supplementary information [80]) the effect of tilting the cone of rays is modelled by manually calculating the exit pupil boundaries and restricting the integration surface to this region.

In the ray-tracing simulation, the aim was to model this fully and consider any effect on the electric field due to the mirror tilt. It is easy to see that this results in the rays having different angles of incidence and therefore reflection coefficients, and this is predicted to change the relative powers in p and s waves and thus overall changes in polarisation.

The method developed here considers the reflection plane for a ray with wavevector $\hat{\mathbf{k}}$, mirror normal $\hat{\mathbf{N}}$. In this plane, the p and s components can be defined as the component in (or parallel) the plane of reflection and perpendicular to the plane of reflection, respectively. The p, s basis vectors are derived by

$$\begin{aligned} \hat{\mathbf{s}} &= \hat{\mathbf{k}} \times \hat{\mathbf{N}}, \\ \hat{\mathbf{p}} &= \hat{\mathbf{k}} \times \hat{\mathbf{s}} \end{aligned} \quad 5.46$$

which is then used to describe $\vec{\mathbf{E}}$ and $\vec{\mathbf{k}}$ in the basis described by $\hat{\mathbf{p}}$, $\hat{\mathbf{s}}$ and $\hat{\mathbf{k}}$, that is, to transform from the lab coordinate space (x, y, z) to the coordinates (x_p, y_s, z_k) ; these vectors are illustrated in Figure 5.9 below.

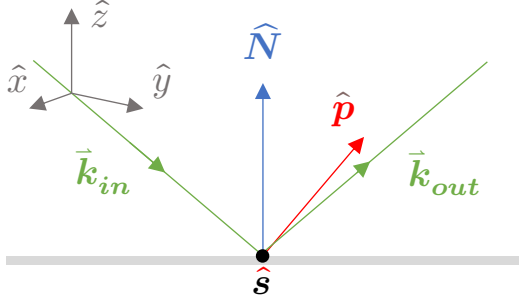


Figure 5.9 – Coordinates and vectors involved in ray reflection from a mirror surface with normal $\hat{\mathbf{N}}$. There is a basis change for $\vec{\mathbf{E}}$ and $\vec{\mathbf{k}}$ in order to apply the Fresnel coefficients in the plane of reflection, from (x, y, z) to (x_p, y_s, z_k) , the latter corresponding to basis vectors $\hat{\mathbf{p}}$, $\hat{\mathbf{s}}$ and $\hat{\mathbf{k}}$ respectively.

The transformation from lab basis $(\hat{\mathbf{x}}, \hat{\mathbf{y}}, \hat{\mathbf{z}})$ to the reflection basis $(\hat{\mathbf{p}}, \hat{\mathbf{s}}, \hat{\mathbf{k}})$ is achieved by projecting the x, y and z components onto the new basis. This is formalised by a change of basis matrix

$$\mathbf{M}_{ps} = \begin{bmatrix} p_x & p_y & p_z \\ s_x & s_y & s_z \\ k_x & k_y & k_z \end{bmatrix} \quad 5.47$$

where the subscript denotes the component of the $\hat{\mathbf{p}}$, $\hat{\mathbf{s}}$ and $\hat{\mathbf{k}}$ vector in the basis (x, y, z) ; e.g. p_x is the x component of $\hat{\mathbf{p}}$.

In this basis, the Fresnel coefficients r_p and r_s can be applied directly by a diagonal matrix

$$\mathcal{F}_R = \begin{bmatrix} r_p & 0 & 0 \\ 0 & r_s & 0 \\ 0 & 0 & 1 \end{bmatrix} \quad 5.48$$

and these components can be calculated for a protected mirror using Equation 5.32 as described in the Ellipsometry section (Section 5.1.4). Alternatively, in the case of a perfectly reflecting mirror this is replaced by the identity matrix.

The final matrix is the Householder matrix [145], [146] and it implements a reflection of a ray (and electric field) according to an arbitrary plane with normal $\hat{\mathbf{N}} = (N_x, N_y, N_z)$ and is given by

$$\mathcal{H}_{xyz} = 1 - 2\hat{\mathbf{N}}\hat{\mathbf{N}}^T = \begin{bmatrix} 1 - 2N_x^2 & -2N_xN_y & -2N_xN_z \\ -2N_yN_x & 1 - 2N_y^2 & -2N_yN_z \\ -2N_zN_x & -2N_zN_y & 1 - 2N_z^2 \end{bmatrix} \quad 5.49$$

Where $\hat{\mathbf{N}}\hat{\mathbf{N}}^T$ is the outer product of $\hat{\mathbf{N}}$ and its transpose $\hat{\mathbf{N}}^T$. The Householder matrix is applied after the Fresnel coefficients and in lab coordinates, and therefore, the final combination of matrices which calculates the electric field after a (Fresnel) reflection from a mirror is

$$\mathcal{R}_M = \mathcal{H}_{xyz} \mathbf{M}_{ps}^{-1} \mathcal{F}_R \mathbf{M}_{ps}. \quad 5.50$$

5.2.4 Collection efficiency quantities

Three quantities are defined for measuring the collection efficiency of each simulated configuration, and these are the relative collection efficiency (RCE), collection efficiency (CE) and emission efficiency (EE). The RCE and CE, which are calculated from the ray-tracing simulation output, are analogous to the GRCE and GCE that are calculated using the intersection of the objective collection cones (Geometric Method)—these will be compared in future sections. The RCE and CE are calculated directly using the summed intensities, which we refer to as energy, and the equations for these are in Table 5.1.

The EE is defined by the ratio of the energy per steradian emitted by the sample into the collection solid angle of O1, to the energy per steradian emitted into a hemisphere (2π steradians). For this reason, as the NA of O1 approaches 1 (collection in a hemisphere), the EE also approaches 1. The EE therefore describes how much the energy emitted by the sample is concentrated within the collection cone of O1 and is, for instance, higher if the energy is concentrated along the detection axis (z), and this becomes more significant for smaller NAs. The energy distribution emitted by a dipole source has two-fold symmetry, so collection in a hemisphere fully describes the intensity distribution of the sample and the collected energy in a hemisphere is always half the total energy (emitted in a sphere).

This effect of the intensity distribution on the EE is depicted in Figure 5.10, illustrating three different distributions each resulting in different values of the EE.

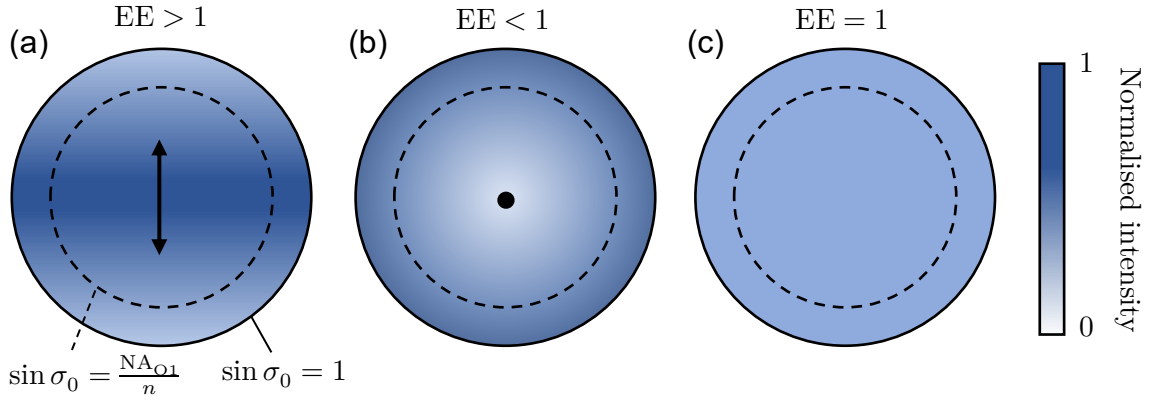


Figure 5.10 – Illustration of intensity distribution in O1 pupil on the emission efficiency (EE). O1 pupil is viewed along the detection axis, and the dashed circle indicates the collection of O1 while the solid circle indicates collection in a hemisphere. The NA of O1 is NA_{O1} , the refractive index of the sample medium is n and collection half angle is σ_0 . (a) Illustrative intensity distribution with excitation perpendicular to the detection axis, shown by arrow (i.e. p-polarised excitation). It can be seen that the horizontal band of intensity means that the average intensity in the inner circle (detection of O1) is greater than in the full hemisphere, resulting in $EE > 1$. (b) Illustrative intensity distribution with $EE < 1$, here depicting excitation along the detection axis (shown by dot/arrow pointing out of page). (c) Uniform intensity distribution in O1 resulting in $EE = 1$.

Since the energy emitted by a radiating dipole is highest in the direction perpendicular to the dipole orientation (and zero in the direction along the dipole), under p-polarised excitation it is expected that $EE > 1$. This is because p-polarised excitation selectively excites dipoles aligned with the y-axis, which is perpendicular to the detection axis, z.

Conversely, for s-polarisation, the excitation is partially along the optical axis of O1 which results in less energy being concentrated along the detection axis and $EE < 1$. For uniformly distributed intensity in the O1 pupil such as if the fluorophores are totally depolarised, the EE is 1.

The CE defines the ratio of the output energy and the total energy emitted in a hemisphere, and therefore implicitly combines the EE and the RCE and allows each system configuration to be directly compared in terms of absolute energy collection. The CE is therefore more complete in characterising the collection efficiency of a setup, but is difficult to compare to experimental measurements which are of the RCE; the experimental measurements involve measuring the collection of the whole system and comparing it to the collection of just microscope 1 (O1 and TL1) with, for example, epifluorescence.

It is important to note that experimental measurements of the RCE implicitly include the transmission losses (characterised by t_{optics}), while the RCE in the ray-tracing simulations does not include this. Just as with the GRCE, to directly compare the ray-tracing simulation RCE to experimental measurements, it must be multiplied by t_{optics} .

5.2.5 Calculating mirror film thickness for dOPM ray-tracing simulation

The mirror currently in use on the dOPM system is an Edmund Optics silver prism mirror with a (presumed) SiO protective coating, but ellipsometry measurements failed to find a fit with the data when using SiO in the model. Therefore, to obtain the reflection coefficients $\tilde{r}_p$ and $\tilde{r}_s$, it may be necessary to directly measure them directly for different angles using ellipsometry, but this is limited to measuring angle of incidences of 15° – 90° . At time of writing, preliminary work has been carried out using the Woollam VASE ellipsometer to measure the polarisation-changing reflective properties (Δ and Ψ) of a silver mirror protected with SiO₂ (Thorlabs), and this is the mirror that is currently simulated.

Using Equation 5.32, MATLAB code was written to calculate reflection coefficients for a protected silver mirror, with inputs being wavelength range, film and substrate refractive indices and film thickness.

Experimental data was acquired using the Woollam VASE. A spectroscopic ellipsometry scan was run at 70° , chosen because of its approximate proximity to the pseudo-Brewster angle which makes the features easier to fit, and the film thickness was found to be 161 nm by fitting a model to the experimental data within the Woollam software; the model consisted of an optically thick (1 mm) Ag layer and single SiO₂ layer. The thickness was used as an input for the MATLAB code to derive theoretical values of Δ and Ψ which were then compared to those calculated by the Woollam software model fitting software for verification; these two models and the experimental data are shown in Figure 5.11. There is a common convention in ellipsometry where the positive coordinate direction for the reflected p component of the E-field ($\hat{\mathbf{E}}_p$) is defined anti-parallel to the incident positive coordinate direction to preserve the handedness of the coordinate system ($\hat{\mathbf{E}}_p, \hat{\mathbf{E}}_s, \hat{\mathbf{k}}$) [134].

Under this definition, $r_p = -r_s$ at normal incidence while physically, the reflections coefficients for the p and s components should be identical due to symmetry. The formulation used in this chapter, see Equation 5.32, instead uses the convention that $r_p = r_s$ at normal incidence.

These two definitions result in a 180° shift in the relative phase difference Δ , and we believe that Woollam use the former definition where $r_p = -r_s$ at normal incidence. Therefore, to compare the model derived from Equation 5.32 to the experimental data and model fit obtained from the Woollam ellipsometer, we define $\Delta' = \Delta - 180^\circ$ and plot this instead, where Δ' is the relative phase difference obtained from Equation 5.32. The model I derived appears to agree with and is verified by the Woollam model, and both models fit reasonably with the experimental data. After finding that Equation 5.32 and the Woollam models are equivalent for this SiO_2 -protected silver mirror, the equation is used in the Python ray-tracing simulation to calculate the complex reflection coefficients used in the Fresnel matrix $\mathcal{F}_R$. Due to the potential confusion caused by the different definitions/conventions used here, the complex reflection coefficients were verified further using FilmStar [147] and they matched those obtained from our model.

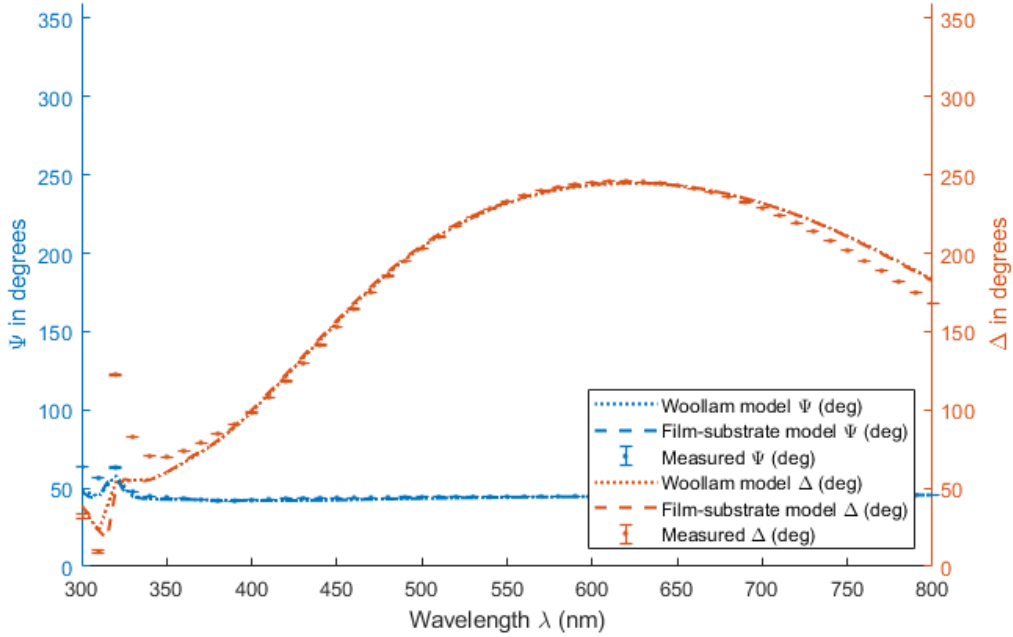


Figure 5.11 - Ellipsometry data (points) from spectroscopic scan of Thorlabs protected silver mirror and model fits. Ellipsometry quantities Ψ and Δ were obtained through measurement. A fit to the data assuming a single SiO_2 layer of 161 nm thickness was performed using the Woollam fitting software. The MATLAB-based film-substrate-air model is also plotted (short dashes) so that it can be compared to the model fit from Woollam (long dashes), and these appear to agree.

5.2.6 Ray tracing simulation setups

The ray-tracing simulation was implemented using Python and structured and written in a way that allows different systems to be quickly set up and compared by a user relatively unfamiliar with python using, for example, a Jupyter notebook. At the user level, optical element objects are initialised and combined into a list, a dipole source object is initialised

which allows the dipole orientations to be specified, and the excitation polarisation is supplied to the dipole source for photoselection. These optical element objects implement the Jones matrices presented in the last chapter, while the dipole source object and its methods implement the dipole generation and ray generation, which includes the generation of the wavevectors and electric fields, $\vec{k}$ and $\vec{E}_{m,n}$. The whole process is illustrated in the pseudocode flow chart in Figure 5.12, which describes the vectorial ray tracing for a system with general inputs. For OPM and dOPM setups in particular, the following section describes their setup using the same pseudo code syntax.

Model of OPM setup

The model of the OPM system consists of the SineLens objects to model the objective lenses and tube lenses, and a y-rotation equal to the OPM angle is supplied to the SineLens that models O3. The list of elements as supplied to the ray-tracing simulation code is shown in Table 5.2.

Table 5.2 – Elements used in ray-tracing simulation of OPM system. For SineLens objects, f is focal length of the lens on the side of the lens where rays are converging/diverging (not collimated).

Type	Element	Parameters			
SineLens	O1	NA=1.2	f=180/60 mm	n=1.33	
SineLens	TL1	NA=1	f=180 mm	n=1	
SineLens	TL2	NA=1	f=162 mm	n=1	
SineLens	O2	NA=0.95	f=180/60 mm	n=1	
SineLens	O3	NA=0.6	f=200/40 mm	n=1	y-axis rotation = θ_{OPM}

In OPM and dOPM setups, the light-sheet is polarised either in-plane of the illumination sheet, which we call p-polarisation, or perpendicular to the illumination sheet, s-polarisation; these correspond to polarisation in the y-axis, or $\hat{y}$ direction, and in the $\cos \theta_{\text{OPM}} \hat{x} + \sin \theta_{\text{OPM}} \hat{z}$ direction respectively. In the polar coordinates $(\alpha_{\text{ex}}, \phi_{\text{ex}})$ the excitation polarisation directions are $(\pi/2, 0)$ and $(0, \theta_{\text{OPM}})$ respectively. The p-polarised and s-polarised illumination polarisations are simulated for the static fluorophore case, and the rapidly tumbling fluorophore case is also simulated. In the rapid tumbling fluorophore case, the emission is totally depolarised and fully independent of the polarisation of the excitation light and is modelled as a uniform dipole distribution without photoselection. The rapid tumbling fluorophore case is therefore equivalent to unpolarised excitation. The excitation polarisation directions and the resulting energy scaling of each dipole due to photoselection are illustrated in Figure 5.13.

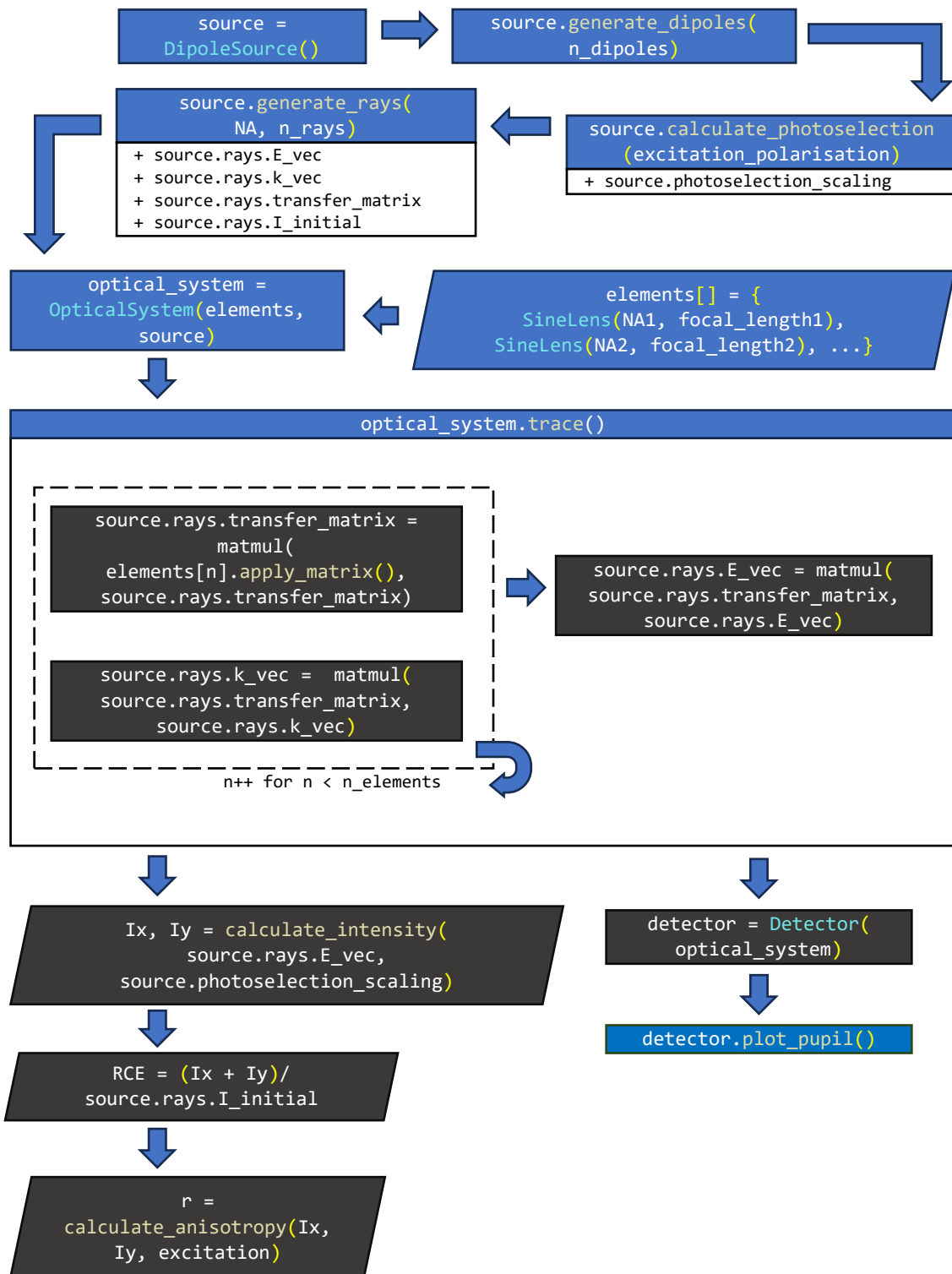


Figure 5.12 – Pseudocode flowchart of vectorial ray-tracing simulation. Blue boxes indicate code that is called by the user, while grey boxes are executed within the code. Plus signs indicate creation of new (in the Python parlance) attributes within a method.

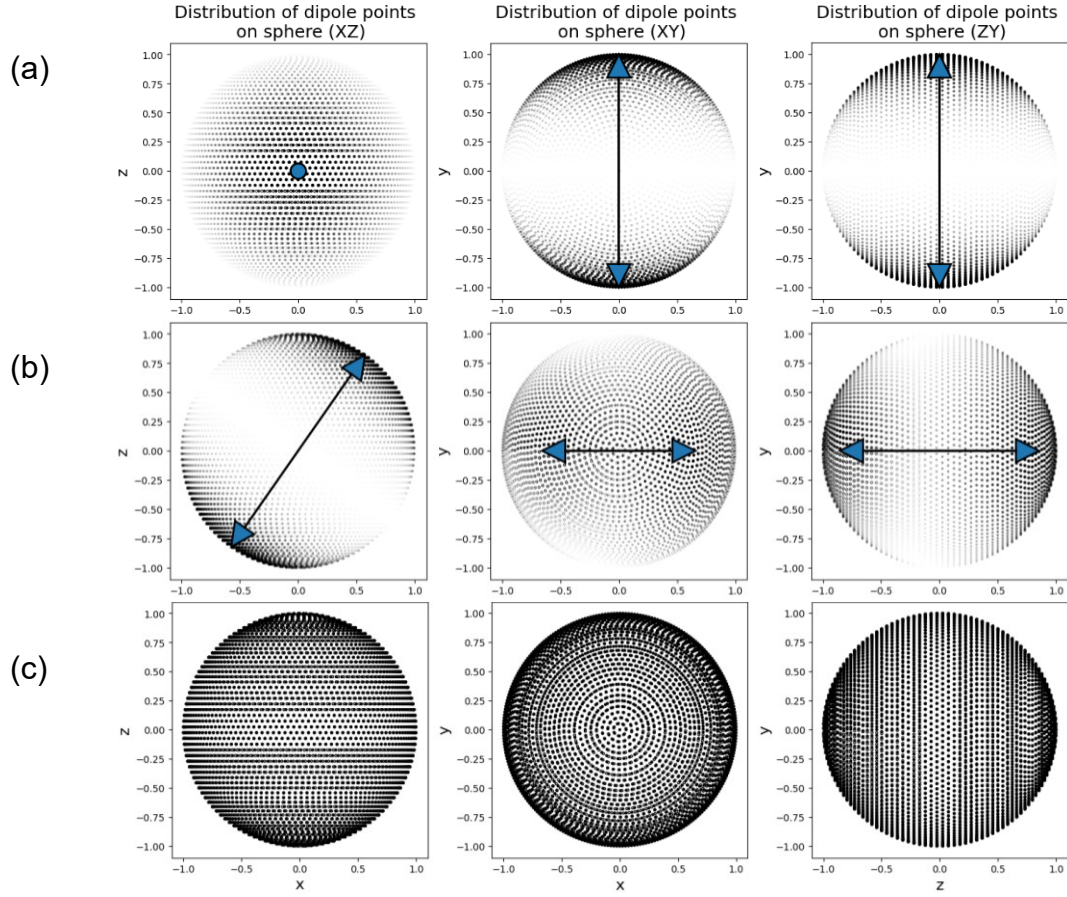


Figure 5.13 – Scatter plot in X-Z, X-Y, and Y-Z views of 5000 uniformly spaced points on a sphere representing dipole orientations which each act as emitters in the ray-tracing simulation. The polarised excitation is indicated by the blue-tipped arrow, and the resulting photoselection which scales the intensities by $\cos^2 \Delta$ is illustrated by the opacity. (a) p-polarised excitation, polarised in light sheet plane (along y); (b) s-polarised excitation, polarised out of light sheet plane and (c) unpolarised excitation.

Model of dOPM setup

The model of the dOPM system setup, in addition to using the SineLens objects, includes two crossed linear polarisers (LP) to model the transmission and reflection of a polarising beamsplitter (PBS), quarter wave plates (QWP), and a plane mirror tilted by half the OPM angle. The double pass of the QWP and O2 are modelled by additional instances in the elements list. The Thorlabs mirror is simulated here, and the thin film thickness d obtained from ellipsometry and refractive index data is used to calculate the complex reflectance coefficients in the Fresnel matrix. These elements are listed in order in Table 5.3.

As in the model of the OPM system, dOPM was simulated for p- and s- excitation polarisations and static fluorophores, in addition to depolarised emission for modelling rapidly tumbling fluorophores.

Table 5.3 – Elements used in ray-tracing simulation of dOPM system with the Thorlabs protected silver mirror. For SineLens objects, f is the rear focal length of the lens.

Type	Element	Parameters			
SineLens	O1	NA = 1.2	$f = 180/60$ mm	$n = 1.33$	
SineLens	TL1	NA = 1	$f = 180$ mm	$n = 1$	
SineLens	TL2	NA = 1	$f = 162$ mm	$n = 1$	
LinearPolariser	LP (PBS transmission)	$\Psi = \pi/2$			
WavePlate	QWP (first pass)	$\delta = \pi/2$	$\Psi = \pi/4$		
SineLens	O2	NA = 0.95	$f = 180/60$ mm	$n = 1$	
FlatMirror	Mirror	Film = SiO ₂	Substrate = Ag	$d = 161$ nm	Mirror angle = $\theta_{OPM}/2$
SineLens	O2 (second pass)	NA = 0.95	$f = 180/60$ mm	$n = 1$	
WavePlate	QWP (second pass)	$\delta = \pi/2$	$\Psi = \pi/4$		
LinearPolariser	LP (PBS reflection)	$\Psi = 0$			

5.3 Results

5.3.1 Verifying ray-tracing simulation against theoretical anisotropy

The vectorial ray-tracing simulation that I developed was first set up to calculate anisotropy and compare it to Axelrod’s theory for several cases, namely for different dipole orientations and for different NA detection. The results from Axelrod et al. [132], see Section 5.1.2, give the parallel and perpendicular intensity components—and therefore anisotropy—for a given excitation/emission dipole orientation and the collection NA of the detector.

As a first step, my code was used to calculate anisotropy values for a single SineLens (air) objective with a single, static dipole oriented along the x-axis against collection NA; this showed agreement with theory to within 10^{-4} . This data is not presented here.

Then a full dOPM-like system with identical objective lenses and (perfect) mirror oriented at 0° (no tilt), see Table 5.4, was then used to verify the full vectorial ray-tracing simulation against Axelrod’s theory. Theoretically, this configuration with no tilt and a perfect mirror is correctly modelled by Axelrod’s theory. The purpose of this more complex configuration was to verify the ray-tracing simulation results in the presence of additional elements, particularly the folding mirror.

The results for anisotropy against collection NA (realised by changing NA_{O1}) for a single, static x-oriented dipole from this dOPM-like setup were also found to agree with theory to within 10^{-4} , see Figure 5.14. The anisotropy was also measured for a multiple dipole source with photoselection (y-polarised excitation) against collection NA, and this also agreed with theory to within 10^{-4} as shown in Figure 5.15.

Table 5.4 – Elements used in dOPM-like simulated optical setup to test against Axelrod-derived theoretical fluorescence anisotropy.

Type	Element	Parameters		
SineLens	O1	$NA = NA_{O1}$	$f = 180/60$ mm	$n = 1$
SineLens	TL1	$NA = 1$	$f = 180$ mm	$n = 1$
SineLens	TL2	$NA = 1$	$f = 180$ mm	$n = 1$
SineLens	O2	$NA = 1$	$f = 180/60$	$n = 1$
FlatMirror	Mirror	$r_p = r_s = 1$	Mirror angle = 0	
SineLens	O2	$NA = 1$	$f = 180/60$ mm	$n = 1$

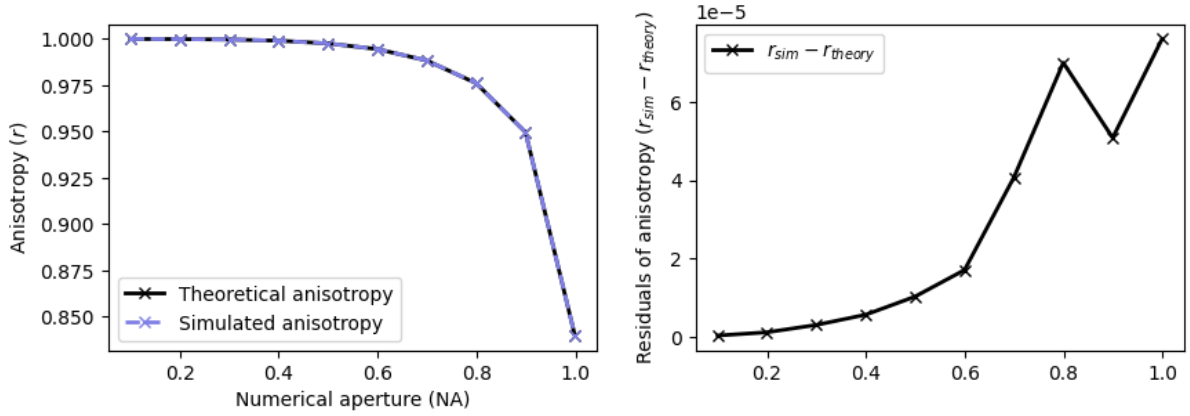


Figure 5.14 – Steady-state anisotropy against NA (here for sample refractive index $n=1$ and air objective) calculated by the ray-tracing simulation of dOPM ($\theta_{OPM} = 0$) with a perfect mirror and comparison to theoretical expressions from Axelrod [132] for a single dipole oriented along the x-axis, see lefthand panel. The number of rays used for the simulation was 7000. The righthand panel shows the difference between the ray-tracing simulation and theoretical expression. Note the vertical axis scale of 10^{-5} .

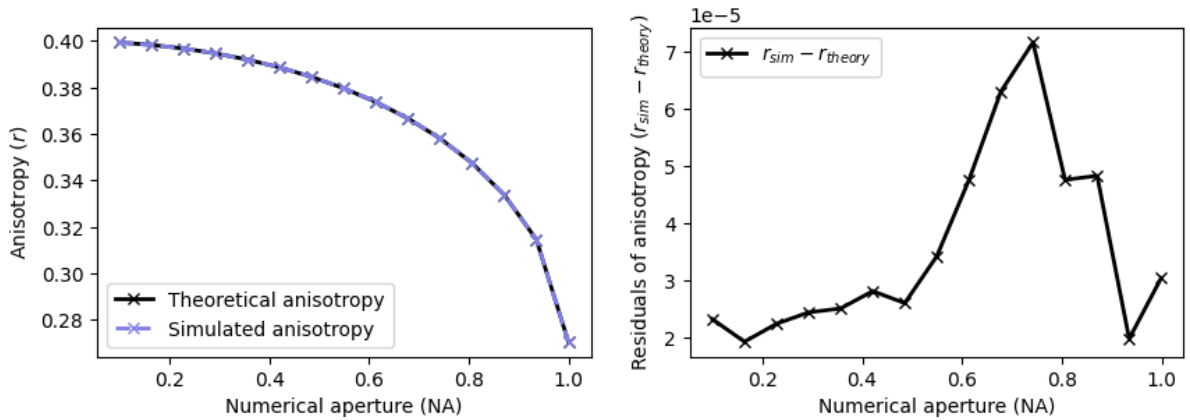


Figure 5.15 – Steady-state anisotropy against NA calculated by the ray-tracing simulation of dOPM ($\theta_{OPM} = 0$) with a perfect mirror and compared to theory for a dipole source with photoselection from y-polarised excitation, see lefthand panel. The righthand panel shows the difference between the ray-tracing simulation and theoretical expression. The number of rays was 7000 and the number of dipoles was 2500.

Finally, the dOPM-like system was used to model the steady-state anisotropy for single static dipoles at a range of different orientations where the NA of the system was 1 (O1

1.0NA/air), see Figure 5.16; a good visual match between the ray-tracing simulation and theory was obtained.

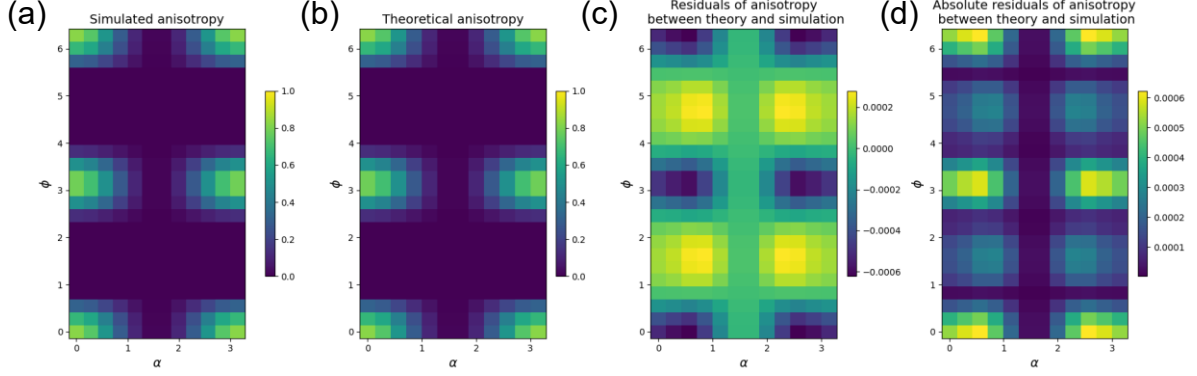


Figure 5.16 – (a) Steady-state fluorescence anisotropy for a range of emission dipole orientations (ϕ , α) represented by a heatmap, calculated by the ray-tracing simulation of dOPM ($\theta_{OPM} = 0$) with a perfect mirror and (b) compared to Axelrod’s theory. (c) The residuals (simulated minus theory) and the (d) absolute value of the residuals of the fluorescence anisotropy. Excitation axis (or $I_{\parallel}$ axis) was defined along the y-axis. The number of rays was 7000.

In Figure 5.14, the difference in anisotropy between the ray-tracing simulation and theory appears to increase at larger NA—this is perhaps because the collection solid angle element $d\Omega$ is proportional to $\sin \theta$, meaning that more energy is collected at these higher angles. For this reason, any constant error originating from the approximation of $d\Omega$ being a flat surface containing a spatially uniform electric field (these elements $d\Omega$ are associated with a ray and a single electric field vector for each dipole) is multiplied by a factor of $\sin \theta$. In other words, when rings are evenly spaced in polar angle by $d\theta$, the area of the outer rings is larger, and hence more energy is collected, and the numerical error is expected to be proportional to this. In Figure 5.16, however, the error initially increases but then decreases beyond an NA of ~ 0.8 , and the reason for this is unclear.

Since it was shown that the results from the ray-tracing simulation of a dOPM-like system with a 0° perfectly reflecting mirror closely followed the Axelrod anisotropy theory, there was confidence in calculating the RCE for OPM and dOPM systems, and these are presented in the next sections.

5.3.2 Ray-tracing simulation output for OPM and dOPM at 35° and 45°

The OPM system ray-tracing simulation outlined in the Methods section was simulated with an OPM angle of 35° and 45° , with 35° being the standard angle for the current OPM and dOPM setups. The following section presents the RCE and the intensity distributions evaluated in the (flat) exit pupil of the 35° OPM and dOPM systems, this pupil being viewed along the optical axis of O3. The view of the incident field on the collection objective, O1, is also shown to explain the effect of the intensity distribution on the collection efficiency. These figures for OPM and dOPM systems with an OPM angle of 45° are shown in the Appendix A.5.3 since the qualitative results are similar to those with an OPM angle of 35° .

Following this, the collection efficiency metrics (introduced in Section 5.1.1) obtained using the ray-tracing simulation for both 35° and 45° OPM and dOPM setups are summarised fully in Section 5.3.3. For OPM and dOPM and for each OPM angle, the three conditions described in Section 5.2.6 are considered: p-polarised excitation, s-polarised excitation, and depolarised emission—the rapidly tumbling fluorophore case. The latter case is also equivalent to excitation with unpolarised light. In all cases, an ensemble of uniformly orientated dipoles is used to model an ensemble of randomly oriented fluorophores.

The collection efficiency results from the ray-tracing simulation are then compared to results obtained using the collection cone intersection method (with MATLAB) as described in Section 5.1.1 for OPM and in Section 5.1.3 for dOPM; these are collectively referred to as the Geometric Method. As described previously, in dOPM, the GRCE and GCE are first calculated from the intersection of the collection cones and then multiplied by the transmission factor due to the polarising beamsplitter (PBS). It is important to remember that using the intersection of the collection cones to calculate the transmission, as is done in the Geometric Method, does not account for how the intensity varies over the pupil.

OPM at 35°

The intensity in the exit pupil of a simulated OPM system (described in Section 5.26) with an OPM angle of 35° is shown in Figure 5.17 below, with Figure 5.17(a) the p-polarised excitation case, Figure 5.17(b) the s-polarised excitation case and Figure 5.17(c) the depolarised emission case. In these ray-tracing simulation results for OPM, the RCE is 0.3504 for p-polarised excitation, 0.3293 for depolarised emission and 0.2483 for s-polarised excitation.

In the Geometric Method, the intensity distribution is not factored into the collection efficiency calculations, and hence there is no difference between the p-polarised excitation, s-polarised excitation and depolarised emission cases. The ray-tracing simulations indicate the differences between the three cases, showing that the collection efficiency with p-polarised excitation is higher than with depolarised emission and while with s-polarised excitation it is lower. The comparisons of collection efficiency between the excitation polarisations and depolarised emission for all simulated OPM and dOPM systems are made in more detail in Section 5.3.3.

In the exit pupil (total) intensity diagram for p-polarised excitation, see Figure 5.17, the intensity is distributed more evenly than in the s-polarised excitation case, where the energy is strongly distributed at the edges of the pupil. The effect of this on the collection efficiency is illustrated more explicitly by Figure 5.18, which shows the intensity at the first principal plane of O1 (three leftmost plots) under p-polarised (a) and s-polarised excitation (b), along with the effective collection of the system (three rightmost plots) relative to O1 and in O1 space. In OPM, the intensity distribution at O1 is ideally reproduced (but mirrored) at O2 and collected, or sampled, by O3 at the OPM angle. This collection by O3, or more specifically, the effective collection solid angle of the microscope compared to that of O1 is

indicated by the dashed line on the pupil diagram. It can then be seen that for s-polarised excitation, the (total) intensity is highest in regions that are outside of the effective collection region, while for p-polarised excitation the intensity is highest within the effective collection region. The collection efficiency is thus comparatively lower with s-polarised excitation than with depolarised emission where the intensity distributed evenly, and with p-polarised excitation it is higher than with depolarised emission—this is confirmed by the RCE values for the three cases.

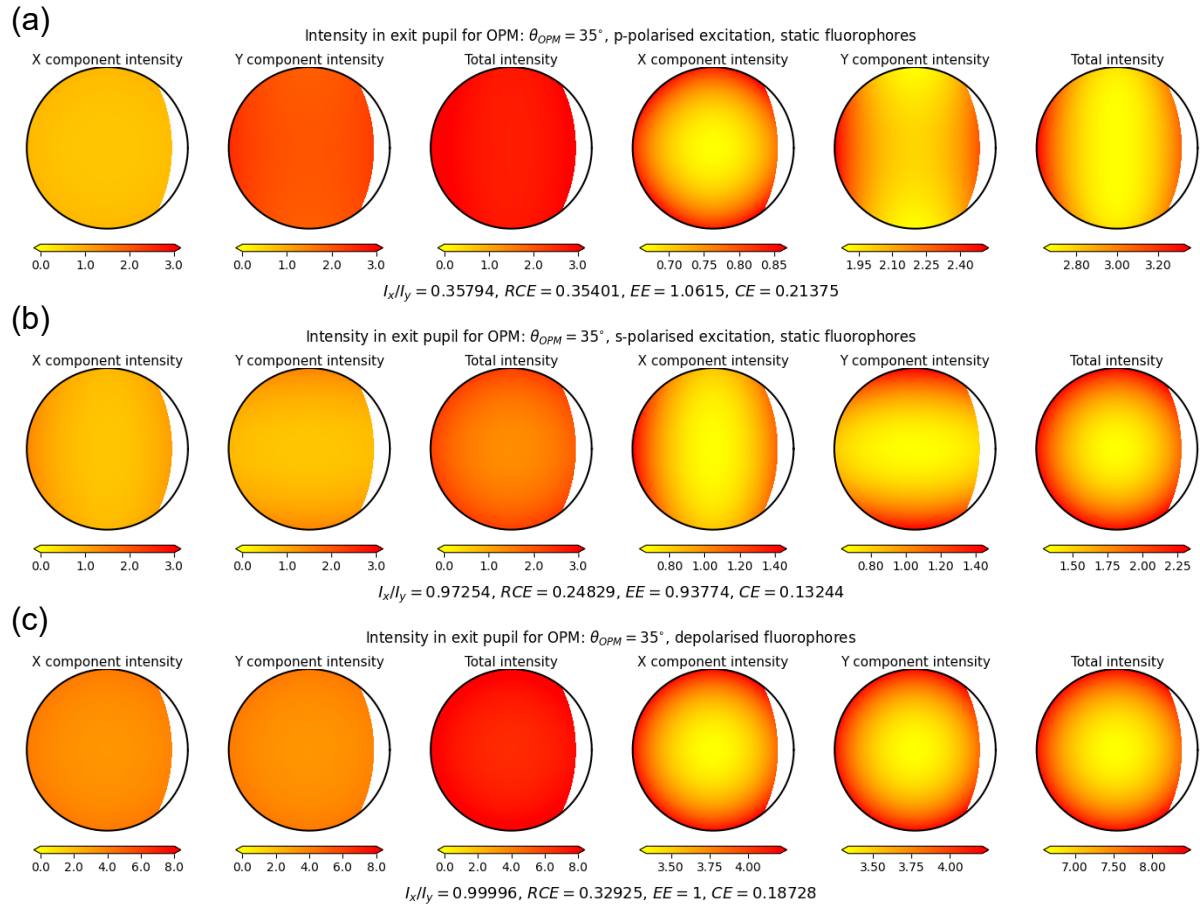


Figure 5.17 – Exit pupil intensity distributions (flat wavefront) for OPM system with $\theta_{OPM} = 35^\circ$, with the black circle marking the O3 pupil. (a) Intensity distributions for static, randomly oriented fluorophores with p-polarised excitation, (b) for s-polarised excitation of static randomly oriented fluorophores and (c) for depolarised emission, the rapid tumbling fluorophore case. In each row, the three lefthand three plots have a common scale, whereas the three righthand plots are autoscaled to show the variation in intensity more clearly. Simulation generated with 10,000 rays and 5,000 dipoles.

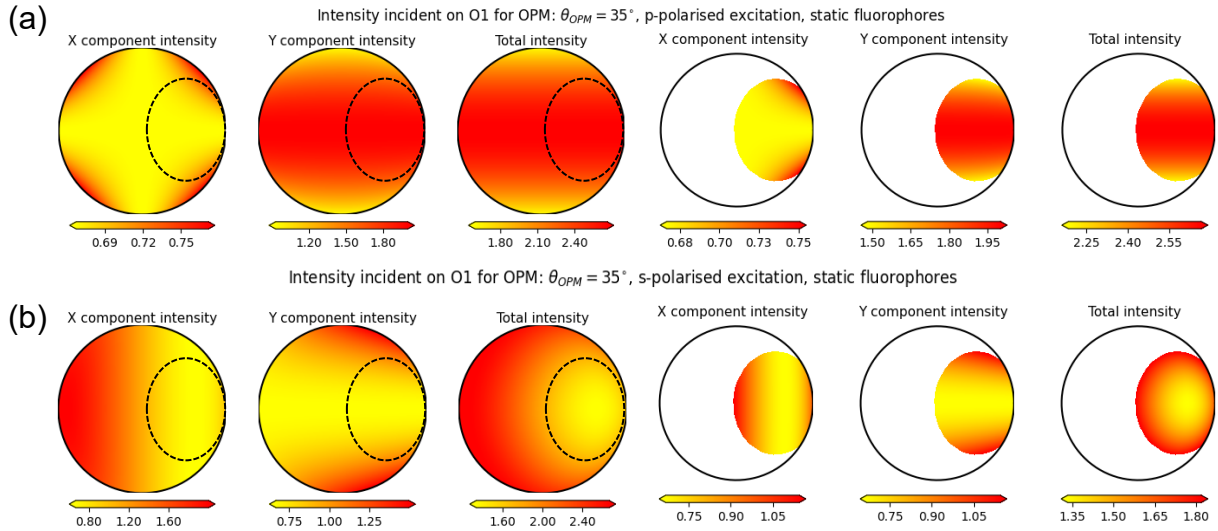


Figure 5.18 – Intensity distributions (curved wavefront) incident on the first principal surface of O1 in an OPM system with $\theta_{\text{OPM}} = 35^\circ$, for (a) p-polarised and (b) s-polarised excitation of static randomly oriented fluorophores. The right three plots show the intensity incident on O1 only for the effective collection region, i.e. the energy in the white region is lost due to O2-O3 pupil clipping. In the left three plots, the dashed oval region denotes the effective collection of the system in order to illustrate if the intensity is distributed favourably within the collection of the system.

In addition to a lower RCE, the light emitted under s-polarised excitation is collected inefficiently by O1, i.e., the EE is less than 1 and the CE further reduced relative to p-polarised excitation—explained further in Section 5.3.3. This is illustrated by Figure 5.19, which shows the intensity distribution with p-polarised and s-polarised excitation for a hemispherical collection cone, with the collection cone of O1 marked as a dashed line. This shows that for p-polarised excitation, the intensity is distributed in a horizontal band on the hemispherical surface with little energy at the top and bottom edges, resulting in $\text{EE} > 1$, while for s-polarised excitation, the intensity is highest at the edges of the pupil, notably the left-hand side—beyond the collection cone of O1 (dashed line)—resulting in $\text{EE} < 1$.

This can also be explained theoretically by the fact that the radiation emitted by dipoles (which are used to model the fluorophores) has a higher intensity in the direction perpendicular to the dipole moment, and that dipoles that are aligned with the polarisation excitation are preferentially excited. Therefore, the most efficient excitation polarisation is perpendicular to the optical/detection axis, i.e. p-polarization, while the least favourable polarisation is along the optical axis, and s-polarisation has a significant component of $\cos(\theta_{\text{OPM}})$ along the detection axis. The EE and the CE values for this setup and each excitation are shown in Figure 5.17.

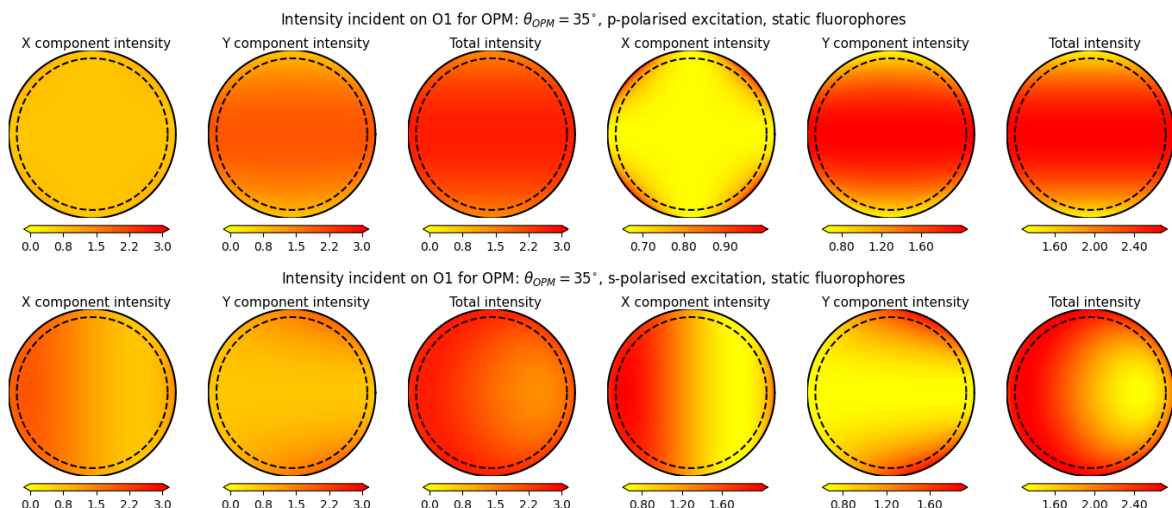


Figure 5.19 – Intensity distribution emitted by dipole source into a hemisphere or 90° collection half angle for ray-tracing simulation of OPM system, viewed along the optical/detection axis. (a) intensity distribution under p-polarised excitation and (b) s-polarised excitation. Depolarised emission results in a uniform intensity distribution. Dashed circles indicate the collection of O1, which has a collection half angle less than 90° .

We note that the collection efficiency is also found to be lower with s-polarised than with p-polarised excitation in the Sommerne et al., publication (with p- and s- defined in the opposite way to here). However, the Sommerne paper evaluates the collection efficiency relative to the transmission of a system where O3 has an NA of 1.0 (and is perfectly transmitting), rather than relative to the collection of O1 like in the RCE. Indeed, the focus of this paper is to specifically evaluate the losses due to Fresnel reflections for different O3 media interfaces at the O3 surface for p- and s-polarised excitation. For this reason, the results from this paper are not currently comparable to the results found in this chapter. Since the code is freely available, however, it may be adapted in the future to calculate the same RCE and CE measurements for comparison.

dOPM at 35°

The dOPM system was simulated for an OPM angle of both 35° and 45° which included a silver mirror with a 161 nm thick SiO_2 coating to model the Thorlabs mirror measured by the ellipsometer (see Section 5.1.4). The ray-tracing simulation was also run for a perfect or ideal mirror, which is implemented by a mirror satisfying $r_p = r_s = 1$ (an identity Fresnel matrix). Here, the intensity plots for the dOPM setup with an OPM angle of 35° are presented with the modelled Thorlabs mirror. In the ray-tracing simulation of this 35° dOPM setup, the RCE is 0.5290 for p-polarised excitation, 0.3675 for depolarised emission and 0.3711 for s-polarised excitation.

The intensity in the exit pupil of the simulated dOPM system is shown in Figure 5.20 below, with Figure 5.20(a) the p-polarised excitation case, Figure 5.20(b) the s-polarised excitation case and Figure 5.20(c) the depolarised emission case. The intensity plots for systems with a perfect mirror and with an OPM angle of 45° plots are found in the Appendix A.5.3.

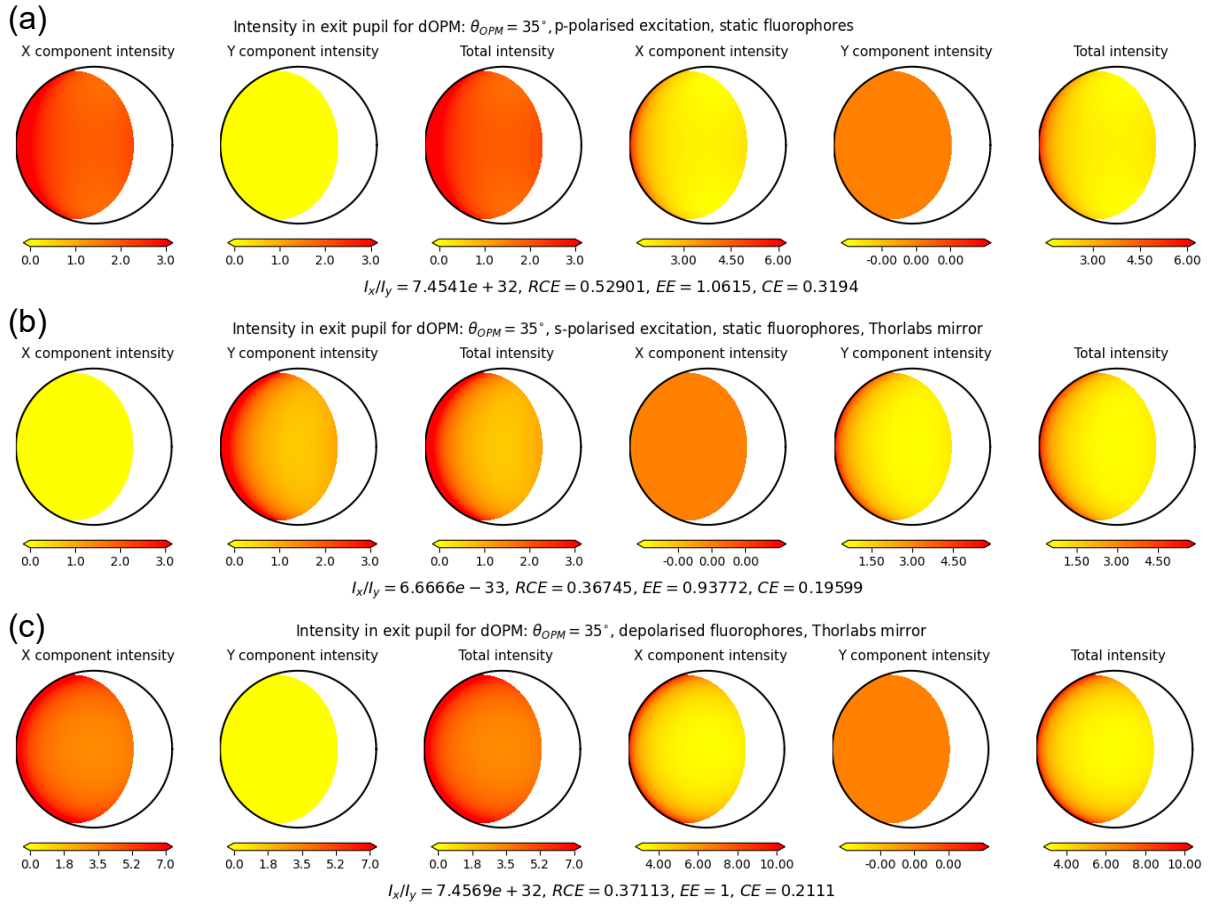


Figure 5.20 – Intensity distributions (flat wavefront) within the exit pupil (black circle) for dOPM system with $\theta_{OPM} = 35^\circ$ and a SiO_2 -coated silver mirror to model the Thorlabs mirror. The intensity distribution is evaluated after the reflection from the PBS after O2/3. (a) Intensity distributions for p-polarised excitation with static randomly oriented fluorophores, (b) for s-polarised excitation with static randomly oriented fluorophores and (c) for depolarised fluorophores, the rapid tumbling fluorophore case. In the case of p-polarised excitation, the PBS is oriented so that the energy is recorded in the x-component of the intensity. In the case of s-polarised excitation, it is recorded in the y-component. In the depolarised case, it is recorded in the x-component. In each row, the three lefthand three plots have a common scale, whereas the three righthand plots are autoscaled to more clearly show the variation in intensity. Simulation generated using 10,000 rays and 5,000 dipoles.

This dOPM system has a higher effective collection solid angle (and therefore NA) than the equivalent OPM system due to the NA of O2/3 being 0.95 compared to the 0.6 of O3 used in OPM, and less of the pupil is clipped because of this. The Geometric Method quantifies the effective collection solid angle explicitly by considering the geometric intersection of collection cones, while in the ray-tracing simulation this information can be found by comparing the number of rays that are traced through the system without being lost relative to the number of rays initially generated. The increased effective collection solid angle can also be visualised by Figure 5.21, which shows the intensity distribution at the first principal surface of O1 (the same O1 as in OPM) alongside the effective collection solid angle of the system as a result of the pupil clipping. The result of this is that, notwithstanding the transmission losses at the PBS, the collection efficiency of the dOPM system is higher than that of the equivalent OPM system in the simulated results—even with fully depolarised emission (50% loss at the PBS). This was shown in the Geometric Method calculations of

the collection efficiency for dOPM (GRCE, GCE), which also include the transmission losses at the PBS, and this is supported here by RCE from the ray-tracing simulations.

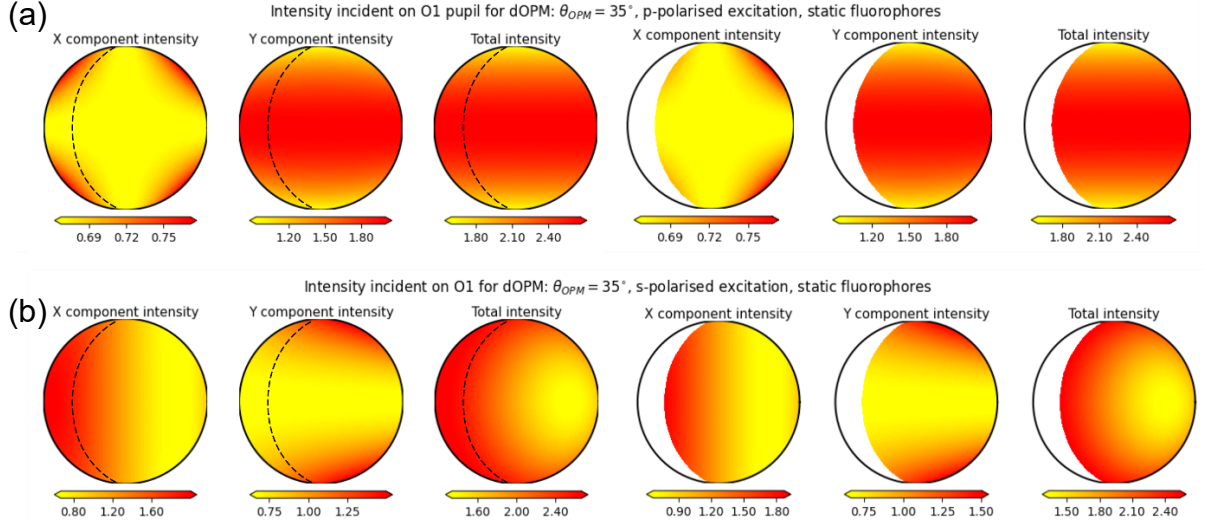


Figure 5.21 – Intensity distributions (curved wavefront) incident on the first principal surface of O1 in a dOPM system with $\theta_{OPM} = 35^\circ$, for (a) p-polarised and (b) s-polarised excitation of static randomly oriented fluorophores. The right three plots show the intensity incident on O1 only for the region that is collected by the entire system, i.e. the energy in the white region is lost due to O2/3 pupil clipping. In the left three plots, the dashed oval region denotes the region that is collected by the entire system in order to illustrate if the intensity is distributed favourably within the collection of the system.

It was seen in the previous section with the OPM system ray-tracing simulations that the collection efficiency depends on the intensity distribution in the pupil and hence depends on the excitation polarisation. Namely, for s-polarised excitation, the collection efficiency is lower than with p-polarised excitation and the same effect is observed here in dOPM. This can be again understood by observing the intensity distribution alongside the effective collection of the system compared to the collection of O1 (marked by the dashed line), see Figure 5.21. In dOPM the light is collected by O1 and then linearly polarised after transmission through the PBS, meaning that the collection of O2/3 (after the light cone is rotated by the mirror) only contains the energy from one of the polarisation components collected by O1: for p-polarised excitation this is along the y-axis and for s-polarised excitation this is along the x-axis. For this reason, when interpreting the plots shown in Figure 5.21, the y and x component intensities should only be considered for p- and s-polarised excitation respectively rather than the total intensity as in OPM.

Measuring the excitation polarisation's effect on the RCE and CE due to the spatial intensity distribution alone is more difficult than with OPM, since in dOPM it must also be decoupled from the transmission of the PBS. This is addressed in Section 5.3.3, where the RCE and CE are compared to the Geometric Method results (GRCE and GCE) as these do not include the effect of the intensity distribution except for the initial outside of the PBS transmission calculations.

5.3.3 Discussion and comparison to Geometric Methods

The collection efficiency metrics in Table 5.1 for each of the OPM and dOPM systems described were calculated using the ray tracing, and these are listed in Table 5.5 below along with those calculated using the Geometric Method.

The RCE and CE results from the ray-tracing simulation and Geometric Method are illustrated by a bar graph in Figure 5.22. This includes results for OPM, dOPM with a perfectly reflecting mirror and dOPM with a modelled SiO_2 -coated silver mirror, and in each case for OPM angles of 35° and 45° . For each of these systems, the results for the p-polarised and s-polarised excitation schemes and depolarised emission case are presented and, where applicable, this data is compared to the Geometric Method calculations of the GRCE and GCE in Figure 5.23. The raw data for the percentage differences between simulated and Geometric Method results can be found in Table A5.1.

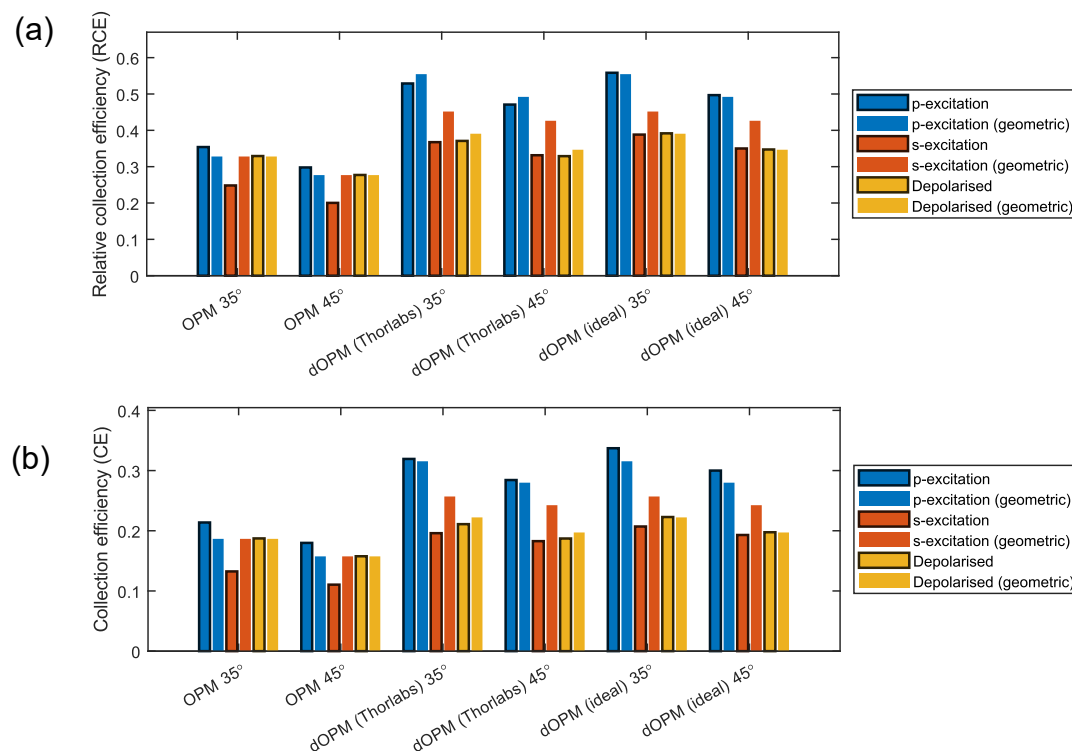


Figure 5.22 – (a) Relative collection efficiency (RCE) and (b) collection efficiency (CE) for the ray-tracing simulation for p-polarised and s-polarised excitation with static, randomly oriented fluorophores, and depolarised emission (rapidly tumbling fluorophores). Results from the ray-tracing simulations are compared to the Geometric Method values, and the dOPM ray-tracing simulation with the modelled Thorlabs mirror is also compared to the Geometric Method values for dOPM which assumes a perfect mirror.

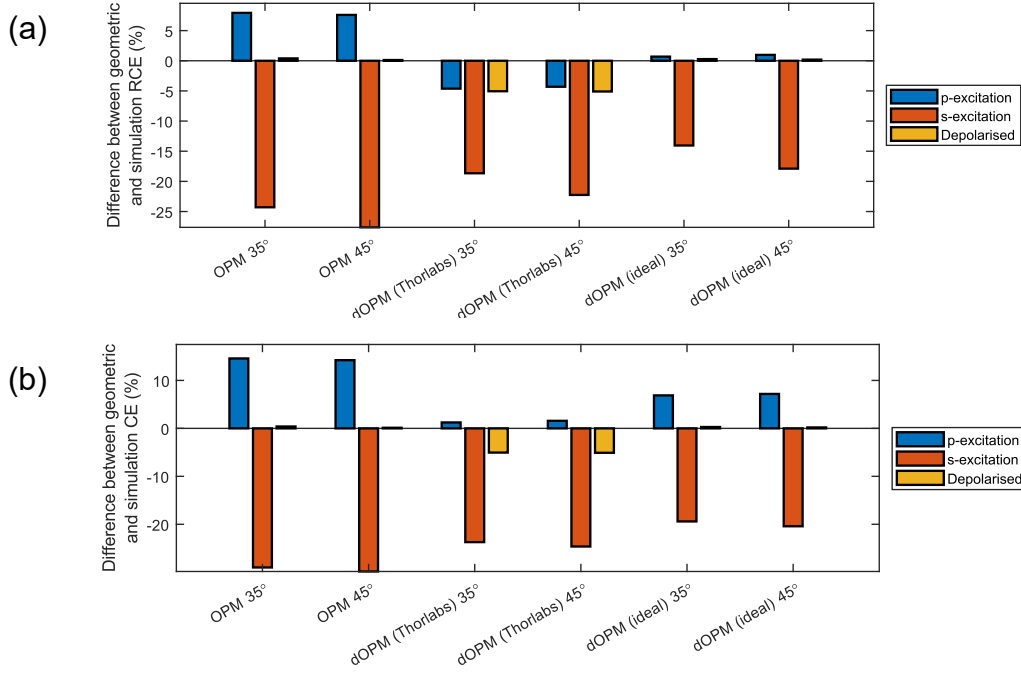


Figure 5.23 – Collection efficiency calculated by Geometric Method compared to collection efficiency calculated by ray-tracing simulation. (a) Percentage difference in relative collection efficiency (RCE) between Geometric Method and ray-tracing simulation, and (b) percentage difference in collection efficiency (CE). Positive percentage difference indicates higher values in ray-tracing simulation than Geometric Method.

In the results from the Geometric Method, for OPM there is no difference in the collection efficiency between the excitation polarisations, or depolarised emission—it assumes uniformly distributed intensity on the first (spherical) principal surface of O1. We can therefore first compare the ray-tracing simulation results with depolarised emission to the Geometric Method to verify the results of the ray-tracing simulation—we see that the depolarised emission RCE and CE is within 0.4% of the Geometric Method.

Qualitatively, the trends in RCE and CE values from the ray-tracing simulation that were presented in the previous section for an OPM angle of 35° are similar for 45°, for both OPM and dOPM systems. For OPM, with OPM angles of both 35° and 45°, p-polarised excitation results in higher collection efficiency than with s-polarised excitation or depolarised emission. These differences are visualised by Figure 5.24, and the raw percentage differences between p-polarised and s-polarised collection efficiencies that were calculated for these figures are found in Table A5.2.

The RCE is seen to be 29.9% and 32.7% higher in OPM with p-polarised excitation compared to s-polarisation excitation for an OPM angle of 35° and 45° respectively. The CE shows a 38–39% increase for p-polarised over s-polarised excitation, and this higher increase is explained by the intensity distribution with s-polarised excitation being distributed in such a way that it is less efficiently collected by O1—quantified by the EE, see Section 5.2.4. This effect is most visibly illustrated in Figure 5.25, which plots the EE for each simulation setup and for p-polarised excitation, s-polarised excitation and depolarised emission. The p-polarised excitation yields identical EE in all cases, while because the s-polarised excitation

axis depends on the light sheet angle the EE is lower for lower OPM angles. As alluded to in Section 5.3.2, this is because the polarisation axis component along the optical axis is equal to $\cos(\theta_{\text{OPM}})$, with excitation along the optical/detection axis being the least efficient for collection. This also affects the CE, and it is observed in Figure 5.24 that although the RCE is further reduced under s-polarised excitation with an 45° OPM angle (relative to p-polarised), the CE is ‘compensated’ by the increased EE.

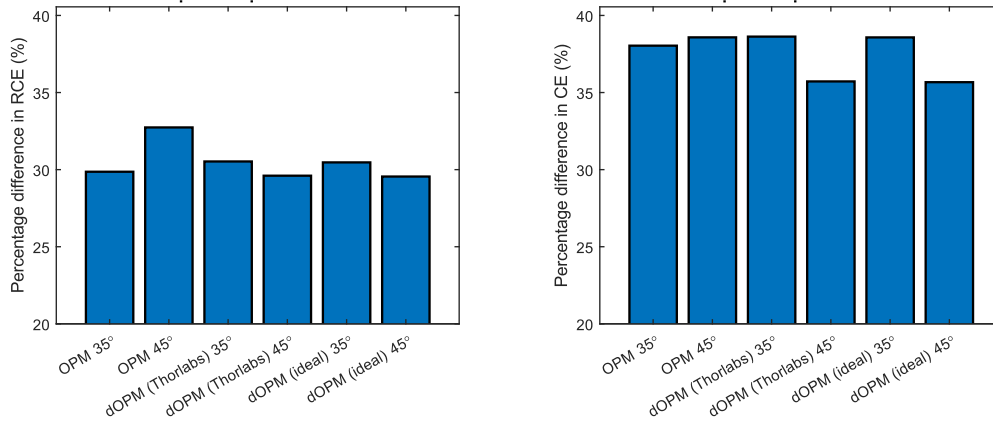


Figure 5.24 – Percentage difference in (a) RCE and (b) CE between p-polarised and s-polarised excitation (ray-tracing simulation results).

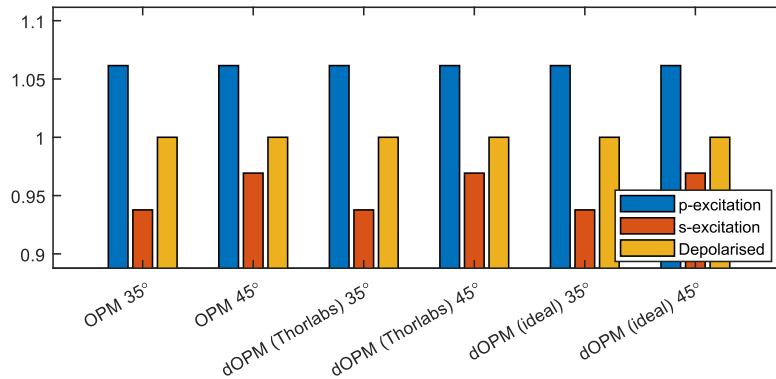


Figure 5.25 – Emission efficiency (EE) according to the ray-tracing simulations of OPM and dOPM, for the different excitation schemes (p-polarised and s-polarised) of static randomly oriented fluorophores, and depolarised (rapid tumbling) fluorophores. The EE is identical for the Thorlabs mirror and perfectly reflecting mirror since it is defined only by the excitation/depolarisation and the collection solid angle of O1.

As with OPM, the Geometric Method calculations for dOPM should be the same as those from the ray-tracing simulation with perfectly reflecting mirror with depolarised emission. We see that the depolarised emission RCE and CE is within 0.3% of the GRCE and GCE of the Geometric Method.

For dOPM systems (now with the Thorlabs mirror) as well, the simulation showed that p-polarised excitation resulted in a higher collection efficiency compared to both s-polarised excitation and depolarised emission for OPM angles of 35° and 45° , see Figure 5.22. The RCE in dOPM systems is 30.5% and 29.6% higher for p-polarised excitation than for s-polarised excitation for OPM angles of 35° and 45° respectively; the CE is 38.6% and 35.7%

higher for p-polarised excitation compared to s-polarised excitation. It is observed that, like in OPM, since the CE accounts for the fraction of energy emitted by the source that is collected by O1 (i.e. the EE, see Figure 5.25), there is a greater difference between the CE values for p-polarised excitation and s-polarised excitation (and depolarised emission) compared to with the RCE.

It is worth noting that although the Geometric Method predicts that the GRCE with s-polarised excitation scheme is 15.6% and 23.0% higher than with depolarised emission for OPM angles of 35° and 45° respectively, the ray-tracing simulation finds the RCE actually be slightly lower with s-polarised excitation by 0.9% and 0.8%. The Geometric Method predicts the GCE to be 13.5% and 18.7% higher for s-polarised for OPM angles of 35° and 45° respectively, while the ray-tracing simulation finds the CE to be 7.1% and 2.3% lower. This is explained by the aforementioned effect of the intensity distribution being distributed at the edges of the pupil under s-polarised excitation, reducing the RCE. Additionally, the reduced EE under s-polarised excitation further reduces the CE.

When comparing the results with the Thorlabs SiO₂-coated silver mirror to those with the perfectly reflecting mirror, the Thorlabs mirror reduces the RCE and CE for the cases of p-polarised excitation, s-polarised excitation and depolarised emission by similar quantities. It was shown in the previous section that the losses from the mirror setup were 5.6-5.7% lower than those of the perfectly reflecting mirror setup for an OPM angle of 35°; this was also the case for an OPM angle of 45°. Indeed, there is little variation in the effect of the mirror between the p-polarised excitation, s-polarised excitation and depolarised emission.

Table 5.5 – (Top) Values for collection efficiency metrics RCE, EE and CE for each OPM and dOPM setup obtained using the ray-tracing simulation to 4 decimal places. (Bottom) The geometric collection efficiency metrics (GRCE, and GCE) obtained using the collection cone intersection MATLAB code and the fluorescence anisotropy from Axelrod to calculate the loss at the LP.

Vectorial ray-tracing simulation									
	RCE			EE			CE		
	p	s	Depol.	p	s	Depol.	p	s	Depol.
OPM 35°	0.3540	0.2483	0.3293	1.0615	0.9377	1.0000	0.2138	0.1324	0.1873
OPM 45°	0.2979	0.2004	0.2772	1.0615	0.9693	1.0000	0.1798	0.1105	0.1577
dOPM 35° Thorlabs mirror	0.5289	0.3675	0.3709	1.0615	0.9377	1.0000	0.3193	0.1960	0.2110
dOPM 45° Thorlabs mirror	0.4709	0.3315	0.3290	1.0615	0.9693	1.0000	0.2843	0.1827	0.1871
dOPM 35° perfect mirror	0.5584	0.3883	0.3917	1.0615	0.9377	1.0000	0.3371	0.2071	0.2228
dOPM 45° perfect mirror	0.4969	0.3500	0.3473	1.0615	0.9693	1.0000	0.3000	0.1930	0.1975
Geometric collection efficiencies (MATLAB and Axelrod et al.)									
	GRCE			EE			GCE		
	p	s	Depol.				p	s	Depol.
OPM 35°	0.3280	0.3280	0.3280				0.1866	0.1866	0.1866
OPM 45°	0.2768	0.2768	0.2768				0.1575	0.1575	0.1575
dOPM 35° perfect mirror	0.5546	0.4517	0.3907				0.3154	0.2569	0.2222
dOPM 45° perfect mirror	0.4921	0.4263	0.3466				0.2799	0.2425	0.1972

5.3.4 Comparison to experiment

Recently, Hugh Sparks has made experimental RCE measurements for a similar dOPM system to the one that is modelled here using fixed fluorescence bead samples with p-polarised and unpolarised LED excitation. In this section, these new results are briefly addressed. In the experimental system here, an Edmund Optics silver mirror with an unknown coating (presumed SiO) is used rather than the SiO₂-coated silver (Thorlabs) mirror that was modelled in the ray-tracing simulations. We are yet to have a successful model fit when using the Woollam ellipsometer to measure the film thickness when assuming

a film material of SiO or SiO₂; this suggests that the coating is not simply SiO or SiO₂. The results of the ray-tracing simulation and the experimental data may not be directly comparable because the mirrors are different, but they may give an insight into the accuracy of the ray-tracing simulation.

Measurements of the RCE were taken for OPM angle of 0°, 35° and 45°, and these are compared to the ray-tracing simulation results for both the SiO₂-coated silver mirror and a theoretical perfect mirror in Table 5.6.

Table 5.6 – (Preliminary) experimental measurements of RCE and RCE values from ray-tracing simulation for dOPM with OPM angles of 0°, 35° and 45° with p-polarised excitation and unpolarised excitation (or, equivalently, depolarised emission). Measurements made by Hugh Sparks.

OPM angle	Excitation	Measured RCE	Ray-tracing simulation RCE	
			SiO ₂ -coated silver mirror	Perfect mirror
0°	unpolarised	0.240	0.473	0.500
0°	p-polarised	0.300	0.671	0.710
35°	unpolarised	0.180	0.371	0.392
35°	p-polarised	0.229	0.529	0.558
45°	unpolarised	0.150	0.329	0.347
45°	p-polarised	0.197	0.471	0.497

The measured RCEs from p-polarised excitation and unpolarised excitation are presented relative to each other as R_{RCE} , shown in Table 5.7. This is done to cancel out the constant transmission factor t_{optics} that is not included in the simulated RCE but implicitly present in the experimentally measured RCE. If the polarisation-dependant performance is accurately modelled by simulation, R_{RCE} (p-polarised RCE divided by unpolarised RCE) the measured and simulated values of R_{RCE} should be the same.

Table 5.7 – Ratio of RCE under p-polarised excitation to unpolarised excitation/emission. Values obtained from simulated dOPM and experimental measurements of dOPM.

OPM angle	p-polarised RCE/unpolarised RCE (R_{RCE})		
	Measured SiO-coated silver mirror	Ray tracing simulation SiO ₂ -coated silver mirror	Ray tracing simulation perfect mirror
0°	1.250	1.419	1.420
35°	1.272	1.426	1.423
45°	1.313	1.432	1.432

We see that the RCE ratio between p-polarised and unpolarised excitation (R_{RCE}) is lower experimentally than in simulation, suggesting perhaps that there is additional depolarisation that occurs in the system that the simulation fails to model, or that the depolarisation/change to the polarisation due to the Edmund Optics SiO-coated silver mirror is not modelled correctly by the simulated SiO₂-coated silver mirror.

The ratio of the measured RCE and simulated RCE is also presented in Table 5.8. If the simulation accurately characterises the polarisation performance of dOPM then this ratio should be constant with OPM angle and equal to t_{optics} , the transmission losses of the system, namely due to the lenses and polarising beamsplitter (PBS). The transmission value for O2/3 reported by manufacturers is 0.9, and the total calculated transmission from the double pass is therefore 0.81. Transmission measurements of the PBS transmission were made by Freya Xu, who found that for a 630 nm laser the transmission was approximately 0.8 (double pass—transmission and reflection). Measurements of the reflection and transmission separately for a 520 nm laser suggest that the double pass measurements may be lower at this wavelength, perhaps approximately 0.7. The experimental RCE measurements were made using a GFP emission filter which is centred at around 525 nm, and so the approximate value of 0.7 is used and t_{optics} may be estimated to be 0.6. Here, a value of 0.47 ± 0.01 was measured for t_{optics} , see Table 5.8, and the source of this discrepancy should be investigated.

Table 5.8 – Experimentally Measured RCE divided by ray-tracing simulation RCE for dOPM, which equals t_{optics} . Results from simulation with both SiO₂-coated mirror and the perfectly reflecting mirror are presented. For the simulated data with the SiO₂-coated mirror, the average value of t_{optics} was computed across the different angles for each excitation scheme and then averaged over the excitation schemes—the combined average. The error is standard error.

	Measured RCE/ray-tracing simulation RCE (t_{optics})					
	SiO ₂ -coated silver mirror			Perfect mirror		
OPM angle	Unpolarised	p-polarised	Combined average t_{optics}	Unpolarised	p-polarised	
0°	0.5074	0.480	0.47 ± 0.01	0.447	0.423	
35°	0.485	0.459		0.433	0.410	
45°	0.456	0.432		0.418	0.396	
Average t_{optics}	0.48 ± 0.01	0.46 ± 0.01				

The data in Table 5.8 is then visualised in Figure 5.26 which illustrates that the ratio of measured RCE to simulated RCE (t_{optics}) also appears to decrease with angle for both p-polarised and unpolarised excitation. This suggests that transmission losses may increase at higher OPM angles, which is to be expected as reflections tend to increase at higher angles.

We also note that, as well as showing the angle dependence of t_{optics} , Figure 5.26 shows a dependence on the excitation polarisation—there is an offset between the p-polarised and unpolarised points. This is the same effect shown by the discrepancy in R_{RCE} between the ray-tracing simulation and experiment, which indicates the simulation not fully characterising depolarisation or polarisation dependent effects.

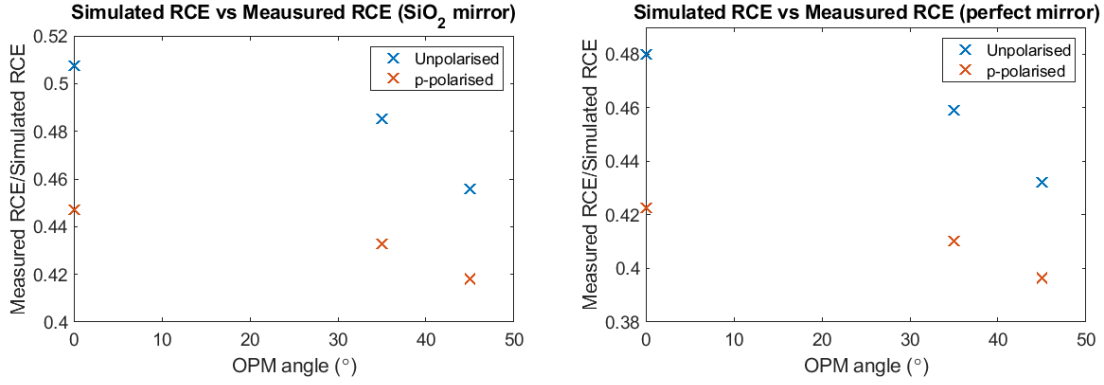


Figure 5.26 – Ratio of experimentally measured RCE to RCE obtained through ray-tracing simulation of dOPM against OPM angle (t_{optics}), according to data in Table 5.8. Results for unpolarised emission (realised experimentally through unpolarised excitation of beads) and p-polarised excitation of static randomly oriented fluorophores are plotted separately.

5.4 Conclusion

In this chapter, two methods were presented to calculate the collection efficiency of OPM and dOPM systems. The first was called the Geometric Method, which uses the geometric clipping of the objective collection cones to calculate the collection efficiency and has been used in previous OPM literature [63], [83], [86], [126]. The Geometric Method considers the tilt of O3 relative to O2, but assumes that the energy emitted by the sample is distributed uniformly over the pupil of O1.

In dOPM there are additional losses from the transmission of the PBS used to outcouple fluorescence, although the losses are reduced if the sample emission is partially polarised (such as with polarised excitation and fluorescence from sample with high steady-state anisotropy). The transmission of the PBS was incorporated into the Geometric Method by using the results of Axelrod to calculate the polarisation state of light after O1. However, the Geometric Method does not model the variation in fluorescence signal over the pupil of O2 and therefore how much of this energy is collected by O3. The Geometric Method is similar to the approach used to calculate the dOPM collection efficiency seen in the original dOPM paper [83], but here the only the polarisation state detected on the axis of O1 is considered for the PBS transmission.

Two metrics were used to quantify the collection efficiency using the Geometric Method: the geometric relative collection efficiency (GRCE) and the geometric collection efficiency (GCE). The GRCE is the collection efficiency relative to the primary objective O1, while the GCE is relative to hemispherical collection.

The second method used to calculate the collection efficiency was the ray-tracing method. The ray-tracing method was an extension of the existing method by Kim et al. which uses the Jones matrix formalism to calculate vectorial diffraction integrals for optical systems and provides a method to account for the electric field distribution. The existing ray tracing method has been applied to obSTORM, which is a system with the same geometry as dOPM. A significant limitation of the existing method is that it does not include the effect

of the mirror tilt on the electric field and only models a single-surface Fresnel reflection for the mirror placed at the focus of O2/3. The latter is important because the reflection by the PBS is sensitive to polarisation changes at the mirror.

The ray-tracing simulation presented here for OPM and dOPM overcomes the limitations of previous approaches by extending the existing simulation to model a tilted mirror surface, calculating complex reflection coefficients for metallic mirrors with a thin film oxide coating, and modelling the effect of polarised excitation on a source of static, uniformly oriented fluorophores.

To calculate the complex reflection coefficients, the oxide film thickness had to be found and this was done for a Thorlabs SiO₂-coated silver mirror using ellipsometry. The collection efficiency metrics—relative collection efficiency (RCE) and collection efficiency (CE)—were obtained for OPM and dOPM systems with OPM angles of 35° and 45° using the ray-tracing simulation and compared to the Geometric Method equivalents. In the ray-tracing simulation, two factors contributed to the CE: the RCE, which considers how much of the initially collected energy is in the output, and how concentrated the emitted intensity is within the collection of O1—parametrised by the emission efficiency (EE). The CE is a more complete measure of collection efficiency, but the RCE is more easily compared to experiment.

Results for the RCE and CE of OPM obtained using the ray-tracing simulation with unpolarised emission (e.g. depolarised from rapidly tumbling fluorophores) agreed with the Geometric Method to within 0.4%, which both assume uniform emission. However, when considering the photoselection of static fluorophores, p-polarised (in light sheet plane) excitation resulted in 29.9–32.7% higher RCE than with s-polarisation (out of light sheet plane). In contrast, the Geometric Method cannot distinguish between p- and s-polarised excitation and depolarised/unpolarised emission for OPM. For an OPM system with an OPM angle of 35°, i.e. the system that is used in this thesis, the RCE was 0.354 for p-polarised excitation, 0.248 for s-polarised excitation, and 0.329 for depolarised emission or unpolarised excitation.

In dOPM, the ray-tracing simulation results supported the theory that p-polarisation light sheets provide higher collection efficiency than s-polarised excitation or depolarised emission due to the PBS transmission. However, although the Geometric Method predicted that the GRCE is higher with s-polarised excitation than with depolarised emission by 15.6–23.0%, the ray-tracing simulation instead found that the RCE for s-polarised excitation was ~1% lower due to the electric field pattern. Similar to what was seen in the OPM simulations, the RCE in the dOPM simulations with p-polarised light sheets was 29.6–30.5% higher than with s-polarised excitation. The depolarised or unpolarised emission results from a ray-tracing simulation of dOPM with a perfectly reflecting mirror agreed to within 0.3% of the Geometric Method calculations for dOPM. When a SiO₂-coated silver mirror was introduced into the ray-tracing simulation, both the RCE and CE dropped by 5.6–5.7% for both OPM

angles and for both polarisation excitations and depolarisation emission. For the dOPM system with a SiO₂-coated silver mirror and an OPM angle of 35°, i.e. the systems that are currently used by the group, the RCE was 0.529 for p-polarised excitation, 0.367 for s-polarised excitation, and 0.371 for depolarised emission or unpolarised excitation.

Limited comparisons to preliminary experimental measurements of dOPM collection efficiency (obtained by Hugh Sparks) were also made. In these comparisons, it appeared that using p-polarised excitation over unpolarised excitation increased the RCE, but less so than expected according to the simulations – this could be due to the partially polarised light emission being further depolarised by optical elements in the real system. Furthermore, the measured and simulated RCE values were used to estimate the transmission of the dOPM optics, t_{optics} , but this was found to be 0.47 ± 0.01 compared to the estimated value of t_{optics} which was approximately 0.6, corresponding to a 22% difference in experiment compared to theory. This suggests that there may be additional losses in addition to those estimated by manufacturer data (and data obtained on PBS transmission), and this should be investigated further.

Recently, another ray tracing-based method has been developed for OPM systems by Sommernes et al. [140] to characterise the collection efficiency and resolution for a 1.0NA glass-tipped, zero working distance ‘snouty’ objective as O3. In this paper, the collection efficiency performance of the glass-tipped ‘snouty’ lens with an anti-reflection coating is compared to using a water immersion O3 with a coverslip, but not in a comparable form to what is presented here.

5.5 Future work

At the time of writing, experimental measurements of the Edmund Optics protected silver mirror used in the current dOPM system are being made. This mirror has a nominally unknown coating material, but the manufacturer has suggested it may be SiO, but when ellipsometry was used the fitting process failed for both SiO and SiO₂. Ellipsometry may be used to directly measure the complex reflection coefficients of the Edmund optics mirror as a function of incidence angle, but the measurable incident angles are limited to between 15° and 90°. In the future, measurements of the Edmund Optics mirror may be able to be incorporated into the ray-tracing simulation to provide a better comparison between experiment and theory. Thorlabs right-angle prism mirrors with silver coating protected by a SiO₂ film are available, however they were not used in the dOPM setup as the coating did not extend close enough to the edges of the mirror, meaning that there was insufficient clearance with O2.

It would also be important to perform measurements of the PBS transmission (using polarised incident light) so this can be incorporated in the model. Preliminary measurements by Freya Xu suggest that this is approximately 80%; further, more direct measurements of the PBS transmission for different wavelengths would be valuable.

The Sommernes et al. paper on simulating OPM with a ‘snouty’ lens uses a similar approach to the ray-tracing simulation presented here, and it would be pertinent to compare my results to theirs. This will require altering my code or their code to calculate the same metrics that are used here to quantify collection efficiency and is another area of future work.

Finally, only the steady state or static fluorophore case and fully depolarised cases are considered for the dipole source. In practice, the degree of depolarisation due to Brownian rotational motion of the fluorophore (or the entire construct it is bound to) may be in between these two extremes and it would be pertinent to include any value of fluorophore steady-state anisotropy into the model. This may be done by a Monte Carlo simulation to randomise the rotation of each dipole according to Brownian motion statistics about a random (but uniformly distributed) axis. This simulation method could be validated by using the simulation results to calculate the fluorescence anisotropy and comparing this to the theoretical anisotropy given by the Perrin equation.

6 Conclusion

In this thesis, I have introduced the technique of oblique plane microscopy (OPM), for which I have developed hardware and software to achieve high-throughput 3D live-cell imaging in four spectral channels, in addition to creating analysis pipelines to explore the rich multi-dimensional datasets which OPM generates. As well as this, I have simulated OPM and dual-view OPM (dOPM) systems with vectorial ray tracing to calculate the theoretical light collection efficiencies for an on-axis, dipole point source.

Here, I summarise the details and findings of each results chapter of the thesis, and how they come together in the development of tools to make OPM more readily applicable to life-sciences research.

6.1 Development of OPM instrumentation

The OPM system presented in this thesis was inherited from Vincent Maioli, whose thesis describes the original optical design and the instrumentation for stage-scanning image acquisition. The optical (and optomechanical) design of the system was updated by Nathan Curry to allow a third camera and a galvo in the illumination path (for angle dithering) to be mounted, and I worked to help build this new system.

The instrumentation for this system was still only capable of imaging 2 spectral channels in a single acquisition sequence, limited by the setup of the digital acquisition (DAQ) device and the LabVIEW code for hardware-triggered acquisition. After reorganising the DAQ outputs, updating the LabVIEW code and writing a MicroManager device adapter for a motorised filter wheel, imaging with up to 4 lasers was possible (now limited by the number of AOTF channels used to control the laser beam powers). Further changes were made to existing acquisition scripts to implement the ability to image with all possible laser/excitation filter combinations, as well as error handling to allow time-lapse acquisitions to run for multiple days.

Alignment procedures for OPM were also presented, including procedures for aligning the primary and secondary microscopes which comprise the refocussing system, and aligning the light sheet with respect to the detection optics.

6.2 Spheroid pre-find in multi-well plates

In high-throughput microscopy with multi-well plates, manually locating samples in each well is a laborious process which limits the throughput of an experiment. An automated process to find the samples before imaging was therefore developed and used in the spheroid experiments presented in Chapter 4. The first stage of the pre-find process detects fluorescence in low-magnification widefield epifluorescence images which contain the entire well of a 384-well plate in the FOV. This had already been implemented into the system I inherited, but the method was adapted to be fully integrated into MicroManager and made to be more robust through automatic z-scanning. The low magnification method is not sufficient for finding the sample lateral position with sufficient accuracy nor the focus (z

position), especially for the high magnification objectives used in OPM. The first step in finding the sample z-position was developing a coverslip autofocus. The autofocus involved using the OPM camera to view the laser scatter from the bottom coverslip surface which appeared as a line, and the height of this line was used in the calculation of the coverslip height. Following the autofocus, an automated 3D spheroid-finding and centring script acquired raw OPM images for each well with $\sim 10\text{ }\mu\text{m}$ slice y-spacing, and the stage position and objective z position were updated to so that the centre of mass of the sample was in the middle of the OPM FOV. The full process positioned spheroid samples in the centre of the OPM FOV with $>90\%$ success rate across an imaging plate with 180 wells, taking approximately 1 hour in total. Previously, this had taken on average more than 3 hours to complete in semi-automated but very labour-intensive process. The coverslip autofocus could also be used during timelapse experiments to account for focus drift, and the methods used to find the centre of mass of the sample in a raw OPM volume (from a previous timepoint) could be used to update stage/objective positions to account for sample drift. Indeed, both were also applied to the experiments presented in Chapter 4.

6.3 Multi-oncogene breast cancer-mimicking spheroid experiment

In a project initially funded by IBIN, I worked with Nina Moderau from the Department of Surgery and Cancer at Imperial College London, imaging spheroids which comprised multiple cell lines each expressing a unique oncogene-fluorescence protein pair. The OPM system with the newly improved pre-find was used to image up to 180 spheroids every 8 hours for at least 8 days in 3D and in 4 spectral channels. In the final iteration of the project, 3 cell lines were used and spheroids comprised different combinations of up to 3 of these cell lines, each with a common segmentation channel (mRFP). Nuclear segmentation was performed using Cellpose [122] – a deep-learning-based algorithm – which allowed a quantitative analysis pipeline to be developed to analyse the progression of each cell line within the spheroids to investigate how the combinations of oncogenes may interact.

As a proof of concept for drug screening, a therapeutic vemurafenib was applied to a subset of the wells/spheroids to observe its efficacy in treating spheroids with different combinations of oncogenes. The metrics observed in the analysis included cell counts, nuclear size and fluorescence intensity as readouts, and in some of these metrics, clear trends were seen between control spheroids, spheroids with active expression of oncogenes, and spheroids treated with vemurafenib. For example, we saw that *BRAF V600E* nuclei tended to be enlarged when oncogene expression is switched on (induced with doxycycline), and in the experiment live plate 6, the intensity of fluorescence in *BRAF V600E* nuclei also increased when induced.

The resulting dataset provides the potential for many parameters to be quantified. In addition to the ones presented, in the future many further possible readouts could be derived from the nuclear segmentations, such as nuclear morphology, or on a larger scale, the spatial distribution and clustering of cells. There are many future avenues for this experiment, and

future repeat experiments with these multi-oncogene spheroids are intended to support the data already collected. To our knowledge, this is the first time that the competition between up to 3 different genetically altered breast cell lines have been followed in 170–180 spheroids in parallel at the individual cellular level over time.

6.4 Vectorial ray tracing model for OPM and dOPM

Finally, the light collection efficiency of OPM and dual-view OPM (dOPM) systems was evaluated using two different methods. The collection efficiency of OPM has been calculated in literature by considering the geometric intersection of the objective lens’ collection cones. This approach can be extended to dOPM by including the transmission of the PBS used to outcouple the fluorescence. The PBS transmission can be greater than 50% for partially polarised light, and indeed it depends on the polarisation of the light emitted by the sample. In the original dOPM paper [83], the intensity of light parallel and perpendicular to the PBS transmission axis was calculated using low NA expressions for fluorescence anisotropy from Lakowicz’s theory [84]. I improved this calculation by using Axelrod’s theory, which considers non-zero NA detection, to calculate the fraction of light passing through the PBS before it reaches the second objective. This method is referred to in this thesis as the Geometric Method, and it showed that the collection efficiency was higher for light sheets polarised in plane (p-polarised) than light sheets polarised out of plane (s-polarised), while both resulted in higher collection efficiency than with unpolarised emission (or depolarised emission due to rapid tumbling of fluorophores). The effect of the intensity distribution over the pupil of the secondary objective on the collection efficiency, however, is not considered in the Geometric Method, nor are any depolarisation effects that would affect the collection efficiency in dOPM.

For these reasons, I developed a vectorial ray tracing simulation for a more complete calculation of the collection efficiency. The ray tracing simulation was based on a previously reported method to calculate vectorial diffraction integrals to evaluate optical system PSFs (Kim et al. [139]), such as for obSTORM [80] – a system with the same geometry as dOPM. The existing method for obSTORM, however, neglects the effect of the mirror tilt on the electric field distribution. The vectorial ray tracing simulation that I developed for (OPM and) dOPM models the effect of the mirror tilt and additionally calculates complex reflection coefficients for metallic mirrors with a thin film metal oxide coating (here, a SiO₂-coated silver mirror) to model depolarisation/phase retardation effects (which we have seen in dOPM systems recently). The collection efficiency was evaluated in two ways: with the relative collection efficiency (RCE) – relative to the collection of the primary objective O1; or the collection efficiency (CE) – relative to the collection in a hemisphere.

The ray-tracing simulations showed that in standard OPM systems, p-polarised (polarisation in-plane) light sheets resulted in 29.9% and 32.7% higher RCE than s-polarised (polarised out-of-plane) light sheets for OPM angles of 35° and 45° respectively. These differences were caused by the fluorescence emission patterns differing between excitation polarisations, while

the Geometric Method did not distinguish between polarisation excitation. For the OPM system that is used in this thesis (with an OPM angle of 35°), the RCE calculated by the ray-tracing simulation was 0.354 for p-polarised excitation, 0.248 for s-polarised excitation, and 0.329 for depolarised emission or unpolarised excitation.

For dOPM (with a SiO_2 -coated silver mirror), ray-tracing simulations calculated that the RCE was 29.6% and 30.5% higher with p-polarised excitation than with s-polarised excitation for OPM angles of 35° and 45° respectively. Although the Geometric Method found that s-polarised excitation should result in the RCE being 15.6% and 23.0% higher, the ray tracing simulations found that the RCE was actually lower than the RCE with depolarised emission by $\sim 1\%$ due to the (unfavourable) variation of fluorescence intensity over the pupil. It was also found that, compared to a perfectly reflecting mirror, the SiO_2 -coated silver mirror reduced the RCE and CE in the ray-tracing simulations by 5.6–5.7% consistently across OPM angles, both excitation polarisations and with depolarised emission. For the dOPM system with a SiO_2 -coated silver mirror and an OPM angle of 35° (the setup used by the group), the RCE was 0.529 for p-polarised excitation, 0.367 for s-polarised excitation, and 0.371 for depolarised emission or unpolarised excitation.

My ray-tracing simulation results for dOPM were briefly compared to experimental results with p-polarised excitation and unpolarised excitation, finding that the results agree qualitatively but the improvement in collection efficiency with p-polarised excitation predicted by the ray-tracing simulation is higher than that obtained in preliminary experimental measurements. Furthermore, the transmission of the dOPM optics was found by comparing simulated RCE values to measured RCE values, and this was 22% lower than the transmission estimated using the manufacturers' data and measured PBS transmission data—this discrepancy should be investigated further.

The mirror modelled by the ray tracing simulation (Thorlabs) is, however, not the same mirror as used in the dOPM system (Edmund Optics). Further work is necessary to either model the Edmund Optics protected silver mirror or take measurements with the Thorlabs SiO_2 -coated silver mirror in the dOPM system.

6.5 Final words

Compared to confocal microscopy, OPM is a gentle, optical sectioning technique that is compatible with high-throughput imaging workflows. OPM can be a valuable tool in biological research, however, it has not yet been widely adopted by those who are not microscopy specialists. By developing automation, such as with spheroid pre-find, I hope that OPM can be more accessible to life scientists, and one of the aims of this thesis is to demonstrate this possibility with our experiments with multi-oncogene-expressing, breast cancer-mimicking spheroids. By its nature, OPM generates multi-TB, multi-dimensional datasets which can be rich in information. This is the philosophy of high-content analysis, and modern computational methods are vital to extract such information. I have shown the potential for machine-learning-enabled data analysis with the cell/nuclei-level analysis of the

progression of these spheroids, yet there is still the potential for more information to be obtained from this type of data. Finally, I have developed a vectorial ray-tracing software package that provides a more comprehensive model of OPM and dOPM systems than previous work for calculating collection efficiency.

It is an exciting time to be involved in microscopy-based research, where automation has become a large focus, and more recently, the popularity of machine learning has exploded allowing unique data analyses. Right now, we are able to obtain more information than ever from optical microscopy of biological experiments, and I feel the techniques presented in this thesis have demonstrated an example of this.

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Appendix

A.2.1 OPM setup on IX71 frame

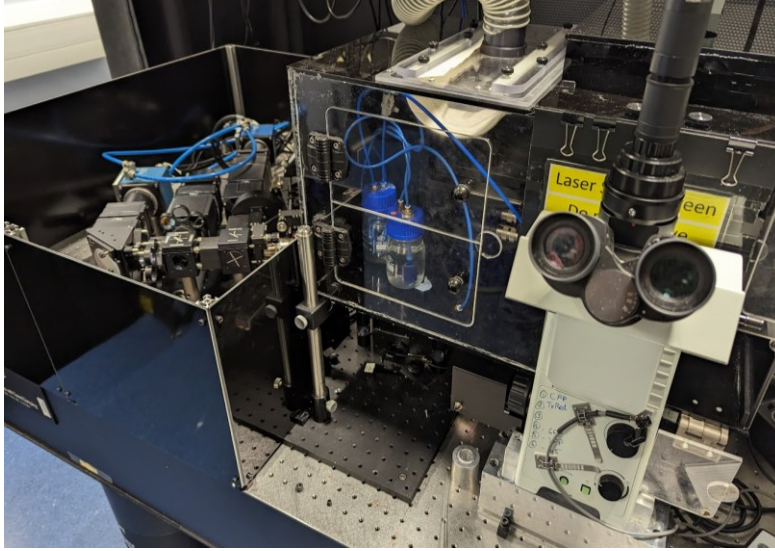


Figure A2.1 – Photo of OPM system. The primary objective (O1) and tube lens (TL1) is the IX71 frame, and the left-hand port of the IX71 couples to the additional OPM optics.

A.4.1 Absolute nuclear count plots

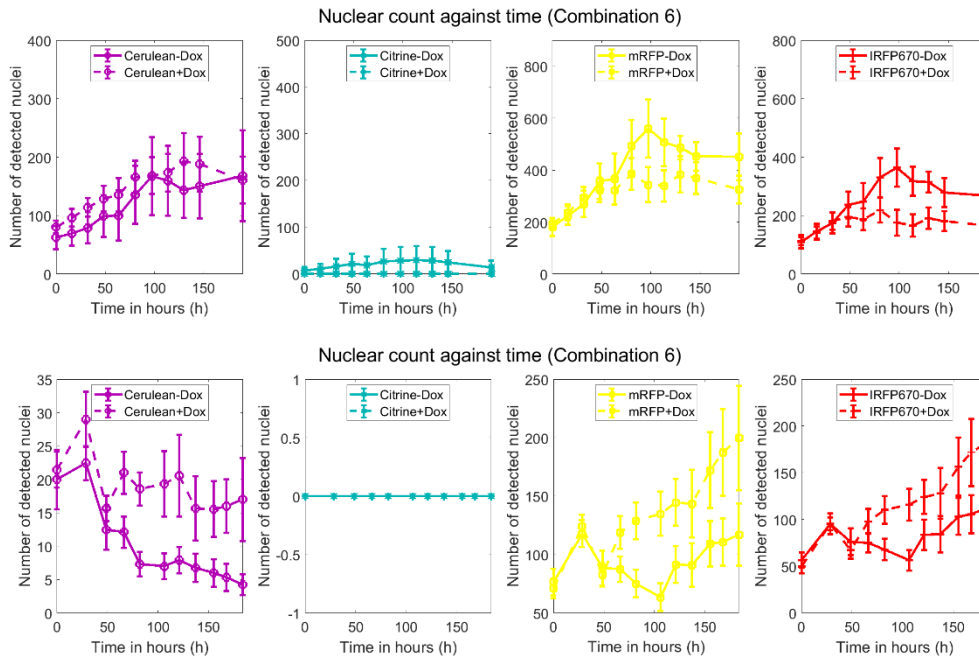


Figure A4.1 – Number of detected nuclei in each cell line population in *ERBB2*^{WT}-Cerulean and *ERBB2*^{D16}-iRFP670 combination spheroids for (a) plate 4 and (b) plate 6, with non-induced (solid line) and induced (dashed line) treatments plotted on the same axis.

A.4.2 Average nuclear signal plots

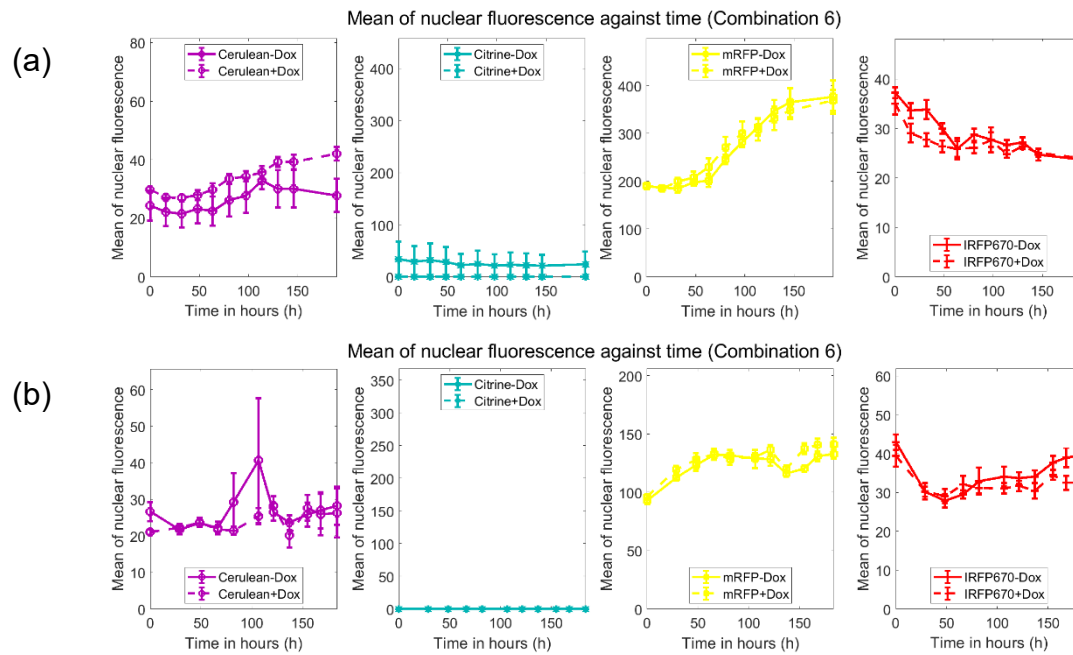


Figure A4.2 – Mean nuclear signal for *ERBB2*WT-Cerulean and *ERBB2*D16-iRFP670 combination spheroids, data from live plate 4 (a) and live plate 6 (b). The three different treatments are plotted on the same axis: non-induced with Vemurafenib (solid line), induced (dashed line), induced with Vemurafenib (dotted line).

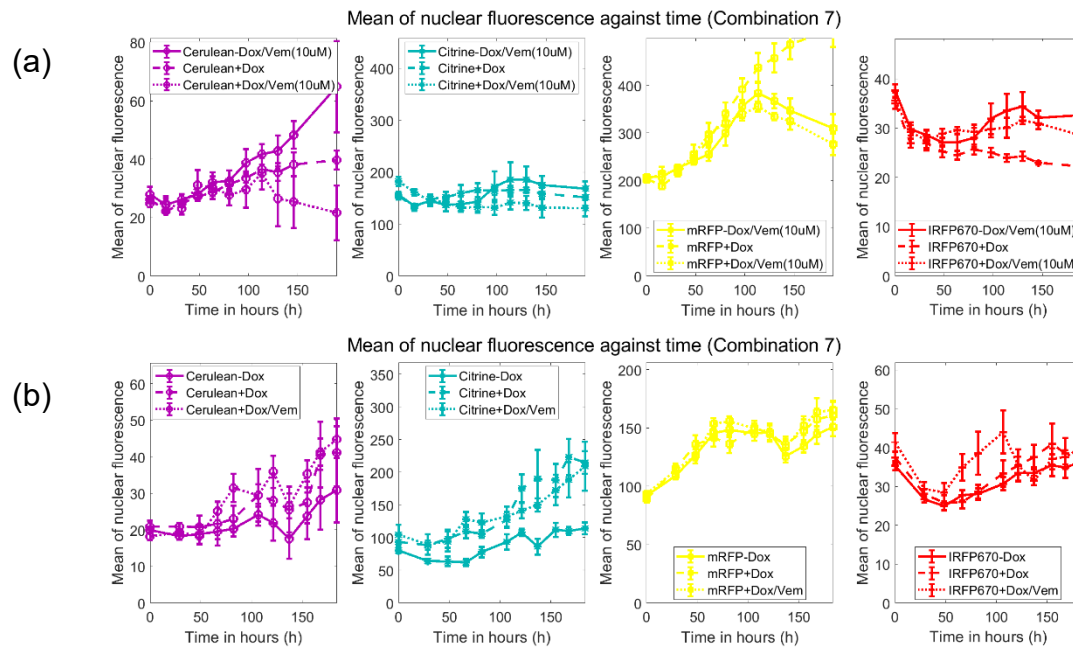


Figure A4.3 – Mean nuclear signal for three-oncogene combination spheroids, data from live plate 4 (a) and live plate 6 (b). The three different treatments are plotted on the same axis: non-induced with Vemurafenib (solid line), induced (dashed line), induced with Vemurafenib (dotted line). *ERBB2*WT-Cerulean, *BRAF* V600E-Citrine and *ERBB2*D16-iRFP670.

A.4.3 Average nuclear size plots

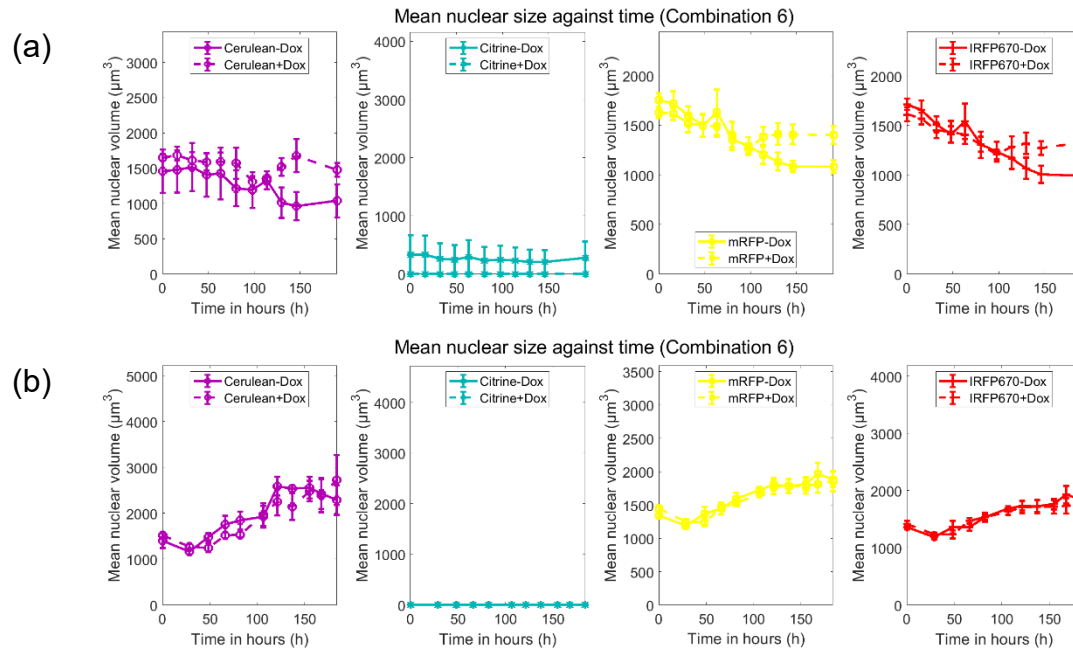


Figure A4.4 – Mean nuclear volume for *ERBB2WT*-Cerulean and *ERBB2D16*-iRFP670 combination spheroids, data from live plate 4 (a) and live plate 6 (b). The three different treatments are plotted on the same axis: non-induced with Vemurafenib (solid line), induced (dashed line), induced with Vemurafenib (dotted line).

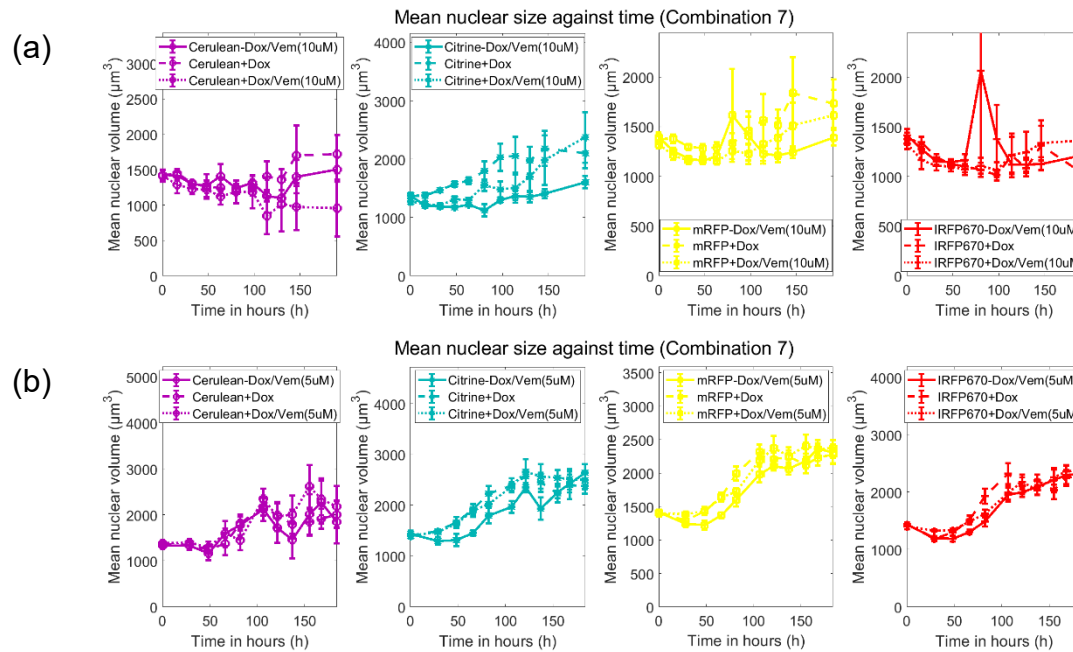


Figure A4.5 – Mean nuclear volume for three-oncogene combination spheroids, data from live plate 4 (a) and live plate 6 (b). The three different treatments are plotted on the same axis: non-induced with Vemurafenib (solid line), induced (dashed line), induced with Vemurafenib (dotted line). *ERBB2WT*-Cerulean, *BRAF V600E*-Citrine and *ERBB2D16*-iRFP670.

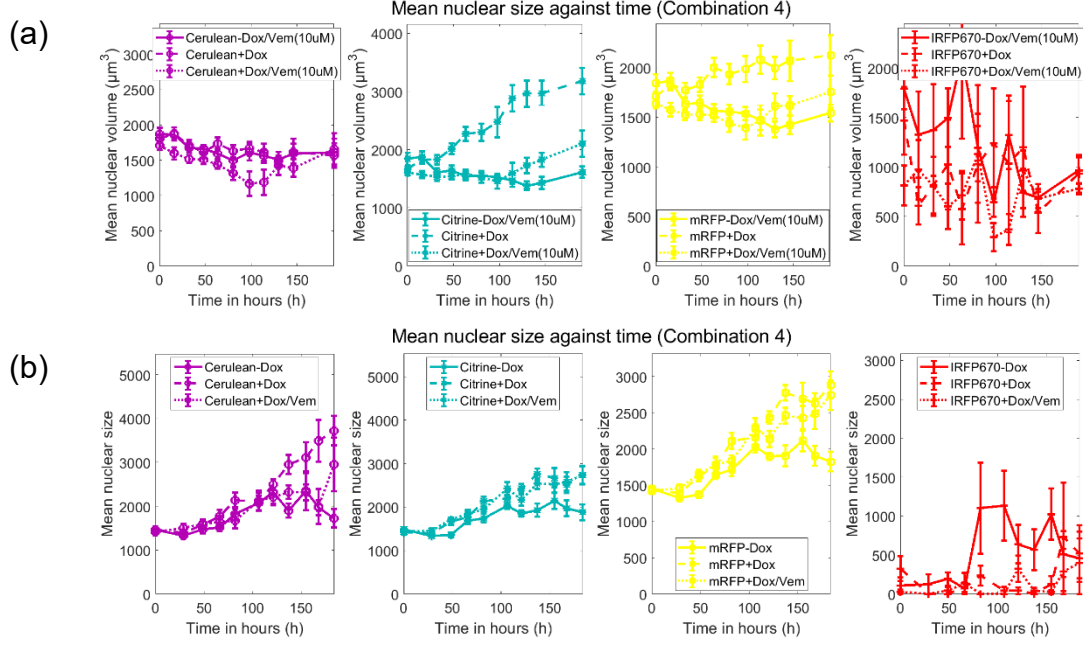


Figure A4.6 – Mean nucleus volume for spheroids with *ERBB2WT* and *BRAF V600E* cell lines under each condition; data from live plate 4 (a) and live plate 6 (b). The three different treatments are plotted on the same axis: non-induced with Vemurafenib (solid line), induced (dashed line), induced with Vemurafenib (dotted line).

A.5.1 Local basis transform for curved surface

In this local basis associated with a spherical surface, the local z is parallel to the surface normal, and the local x and y are tangential to the surface as illustrated below.

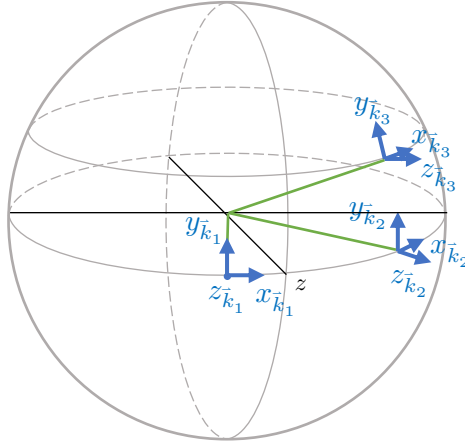


Figure A5.1 – Local basis transformation of wavevector and E-field on spherical surface in order to evaluate intensity on a spherical surface. Each set of local coordinates here is unique to each ray direction.

The necessary coordinate transform from the lab basis to the local basis can be found by first applying the meridional transform and applying the inverse of the meridional rotation, $\mathcal{L}^{-1}(\theta)$, to function as a passive transformation, and then the inverse meridional transform is applied, giving the matrix

$$\mathcal{R}_{local}(\theta, \phi) = \mathcal{R}_z^{-1}(\phi) \mathcal{L}^{-1}(\theta) \mathcal{R}_z(\phi).$$

A.5.2 Integration of parallel and perpendicular fluorescence

To calculate the theoretical collection efficiencies in dOPM, the transmission through the linear polariser is calculated. This requires the (relative) intensities parallel and perpendicular to the polariser transmission axis to be calculated and the results from Axelrod et al. to define integrals for the fluorescence intensity from a dipole ensemble under photoselection. Mathematica code was written to calculate these integrals and is shown below.

Calculate parallel and perpendicular intensity components under photoselection

```
In[1]:= ClearAll[Evaluate[Context[] <> "*"]]

x = Sin[theta] * Sin[phi];
y = Sin[theta] * Cos[phi];
z = Cos[theta];

Ip = Ka * y^2 + Kb * x^2 + Kc * z^2;
Is = Ka * y^2 + Kc * x^2 + Kb * z^2;

mSpol = (Cos[thetaOPM] * Sin[theta] * Cos[phi] + Sin[thetaOPM] * Sin[theta] * Sin[phi])^2;
mPpol = Cos[theta]^2;

p-polarisation

In[9]:= FpP = Integrate[Ip * mPpol * Sin[theta], {phi, 0, 2 Pi}, {theta, 0, Pi / 2}]
FsP = Integrate[Is * mPpol * Sin[theta], {phi, 0, 2 Pi}, {theta, 0, Pi / 2}]

Out[9]=  $\frac{2}{15} (Ka + Kb + 3 Kc) \pi$ 

Out[10]=  $\frac{2}{15} (Ka + 3 Kb + Kc) \pi$ 

s-polarisation

In[11]:= FpS = Integrate[Ip * mSpol * Sin[theta], {phi, 0, 2 Pi}, {theta, 0, Pi / 2}]
FsS = Integrate[Is * mSpol * Sin[theta], {phi, 0, 2 Pi}, {theta, 0, Pi / 2}]

Out[11]=  $\frac{2}{15} \pi (2 (Ka + Kb) + Kc + (Ka - Kb) \cos[2 \theta_{OPM}])$ 

Out[12]=  $\frac{2}{15} \pi (2 Ka + Kb + 2 Kc + (Ka - Kc) \cos[2 \theta_{OPM}])$ 

In[13]:= energyRatioP = (FpP / (FpP + FsP))
energyRatioS = (FsS / (FpS + FsS))

Out[13]= 
$$\frac{2 (Ka + Kb + 3 Kc) \pi}{15 \left( \frac{2}{15} (Ka + 3 Kb + Kc) \pi + \frac{2}{15} (Ka + Kb + 3 Kc) \pi \right)}$$


Out[14]= 
$$\frac{2 \pi (2 Ka + Kb + 2 Kc + (Ka - Kc) \cos[2 \theta_{OPM}])}{15 \left( \frac{2}{15} \pi (2 (Ka + Kb) + Kc + (Ka - Kb) \cos[2 \theta_{OPM}]) + \frac{2}{15} \pi (2 Ka + Kb + 2 Kc + (Ka - Kc) \cos[2 \theta_{OPM}]) \right)}$$

```

Figure A5.2 – Mathematica code for calculating integral to derive parallel and perpendicular polarisation components of intensity collected from dipole ensemble with p-polarised or s-polarised excitation, based on Axelrod theory. The probability distribution $m(\Omega, \mu)$ is calculated by the dot product of excitation polarisation and general vector describing dipole orientation.

A.5.3 Exit pupil intensity plots for OPM and dOPM

A.5.3.1 dOPM at 35° with perfect mirror

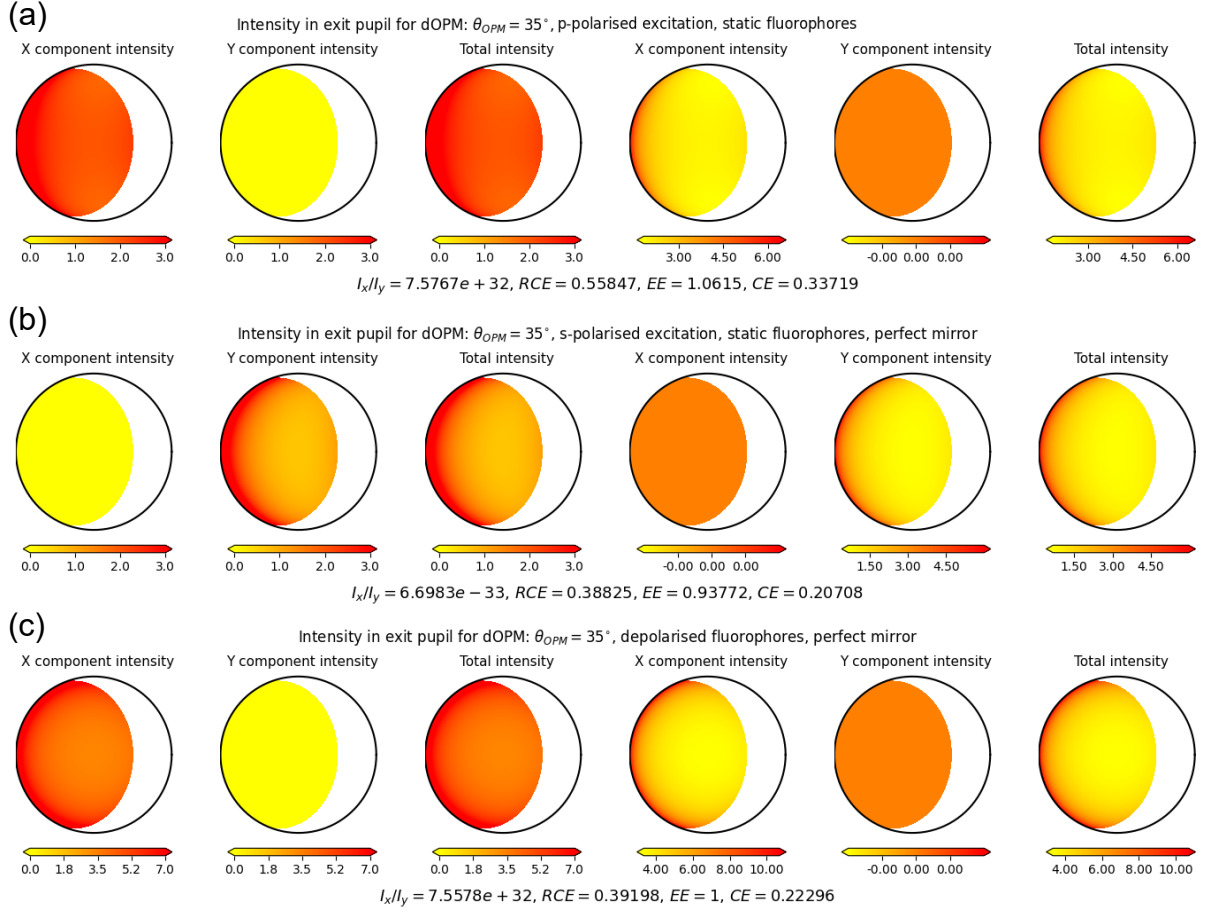


Figure A5.3 – Intensity distributions (flat wavefront) within the exit pupil (black circle) for dOPM system with $\theta_{OPM} = 35^\circ$ and a perfectly reflecting mirror. The intensity distribution is evaluated after the reflection from the PBS after O2/3. (a) Intensity distributions for p-polarised excitation with static randomly oriented fluorophores, (b) for s-polarised excitation with static randomly oriented fluorophores and (c) for depolarised fluorophores, the rapid tumbling fluorophore case. In each row, the three lefthand three plots have a common scale, whereas the three righthand plots are autoscaled to more clearly show the variation in intensity. Simulation generated using 10,000 rays and 5,000 dipoles.

A.5.3.2 OPM at 45°

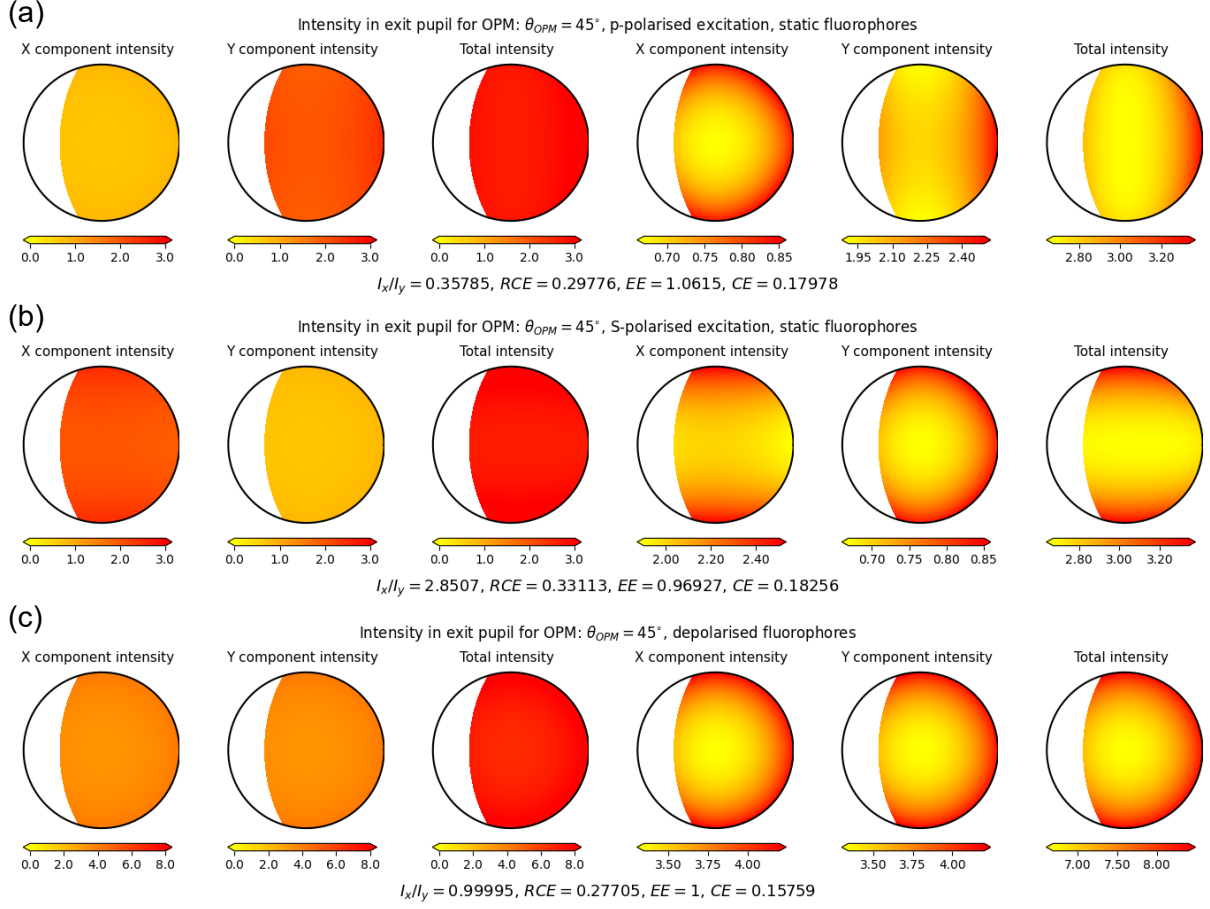


Figure A5.4 – Exit pupil intensity distributions (flat wavefront) for OPM system with $\theta_{OPM} = 45^\circ$, with the black circle marking the O3 pupil. (a) Intensity distributions for static, randomly oriented fluorophores with p-polarised excitation, (b) for s-polarised excitation of static randomly oriented fluorophores and (c) for depolarised emission, the rapid tumbling fluorophore case. In each row, the three lefthand three plots have a common scale, whereas the three righthand plots are autoscaled to show the variation in intensity more clearly. Simulation generated with 10,000 rays and 5,000 dipoles.

A.5.3.3 dOPM at 45° with perfect mirror

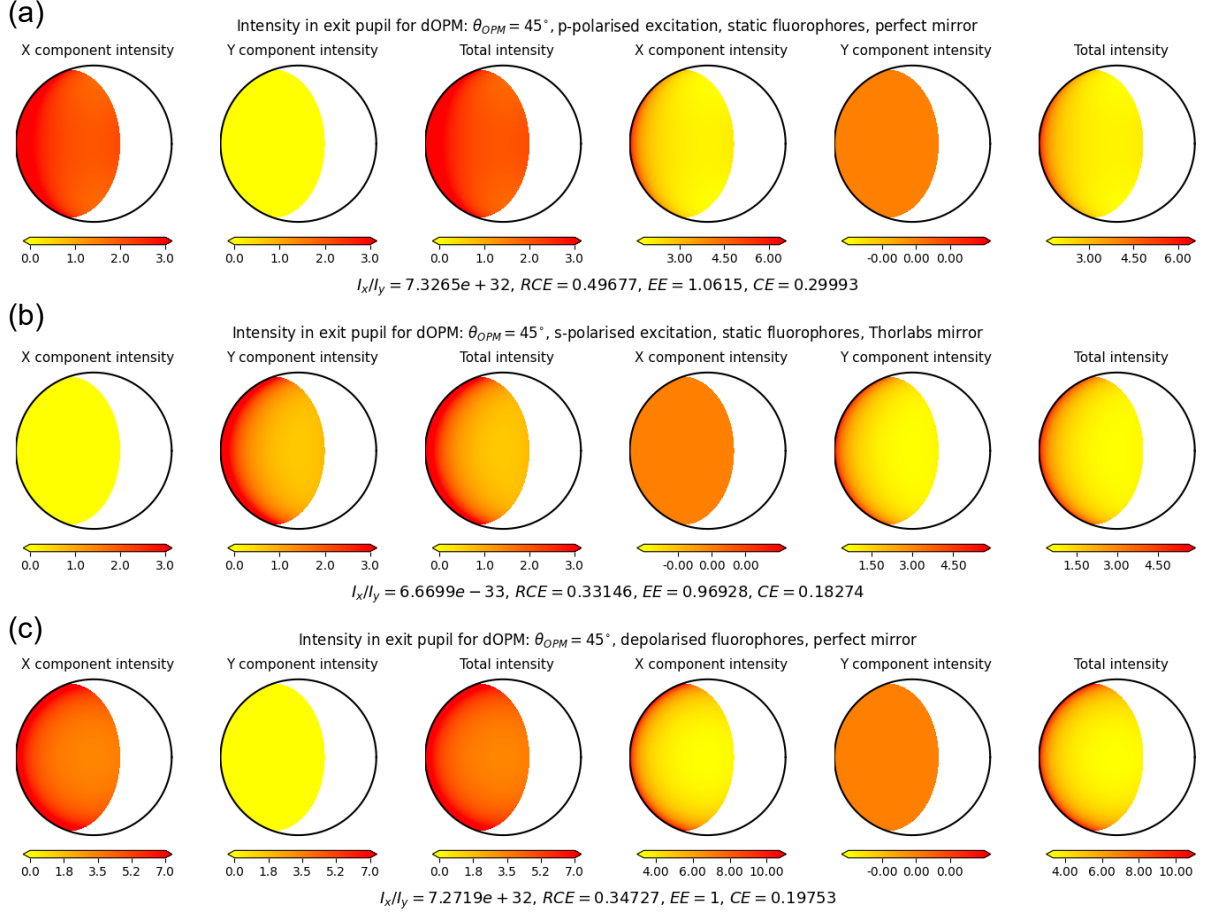


Figure A5.5 – Intensity distributions (flat wavefront) within the exit pupil (black circle) for dOPM system with $\theta_{OPM} = 45^\circ$ and a perfectly reflecting mirror. The intensity distribution is evaluated after the reflection from the PBS after O2/3. (a) Intensity distributions for p-polarised excitation with static randomly oriented fluorophores, (b) for s-polarised excitation with static randomly oriented fluorophores and (c) for depolarised fluorophores, the rapid tumbling fluorophore case. In each row, the three lefthand three plots have a common scale, whereas the three righthand plots are autoscaled to more clearly show the variation in intensity. Simulation generated using 10,000 rays and 5,000 dipoles.

A.5.3.4 dOPM at 45° with Thorlabs mirror

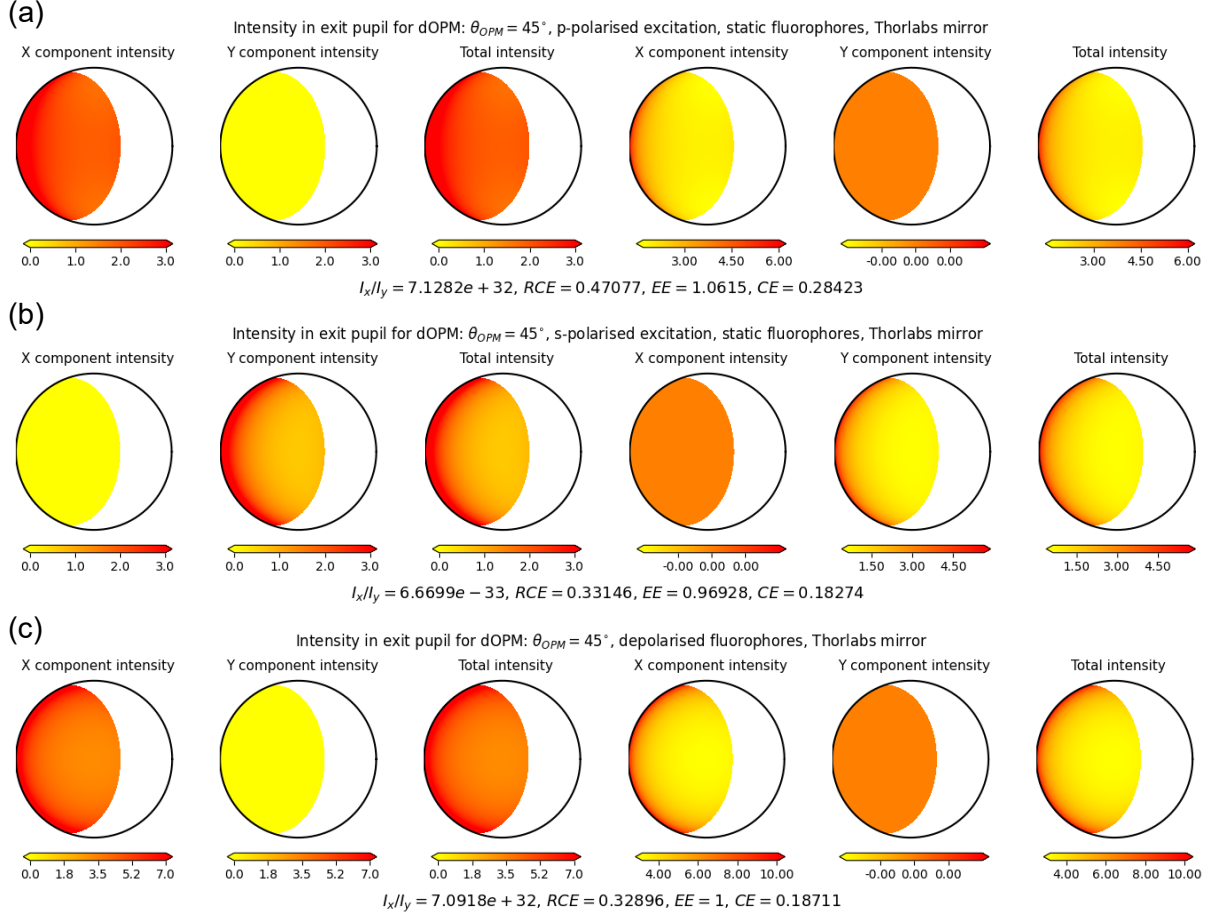


Figure A5.6 – Intensity distributions (flat wavefront) within the exit pupil (black circle) for dOPM system with $\theta_{OPM} = 45^\circ$ and a SiO_2 -coated silver mirror to model the Thorlabs mirror. The intensity distribution is evaluated after the reflection from the PBS after O2/3. (a) Intensity distributions for p-polarised excitation with static randomly oriented fluorophores, (b) for s-polarised excitation with static randomly oriented fluorophores and (c) for depolarised fluorophores, the rapid tumbling fluorophore case. In each row, the three lefthand three plots have a common scale, whereas the three righthand plots are autoscaled to more clearly show the variation in intensity. Simulation generated using 10,000 rays and 5,000 dipoles.

A.5.4 Intensity incident on O1 plots

In addition to the plots of the intensity distribution in the exit pupil, the intensity distribution incident on the primary objective O1 was plotted and masked for rays that are lost during the ray-tracing process. These plots therefore illustrate the effective collection in the space of O1, however, these electric fields are the fields incident on O1—not the final fields.

A.5.4.1 OPM at 35°

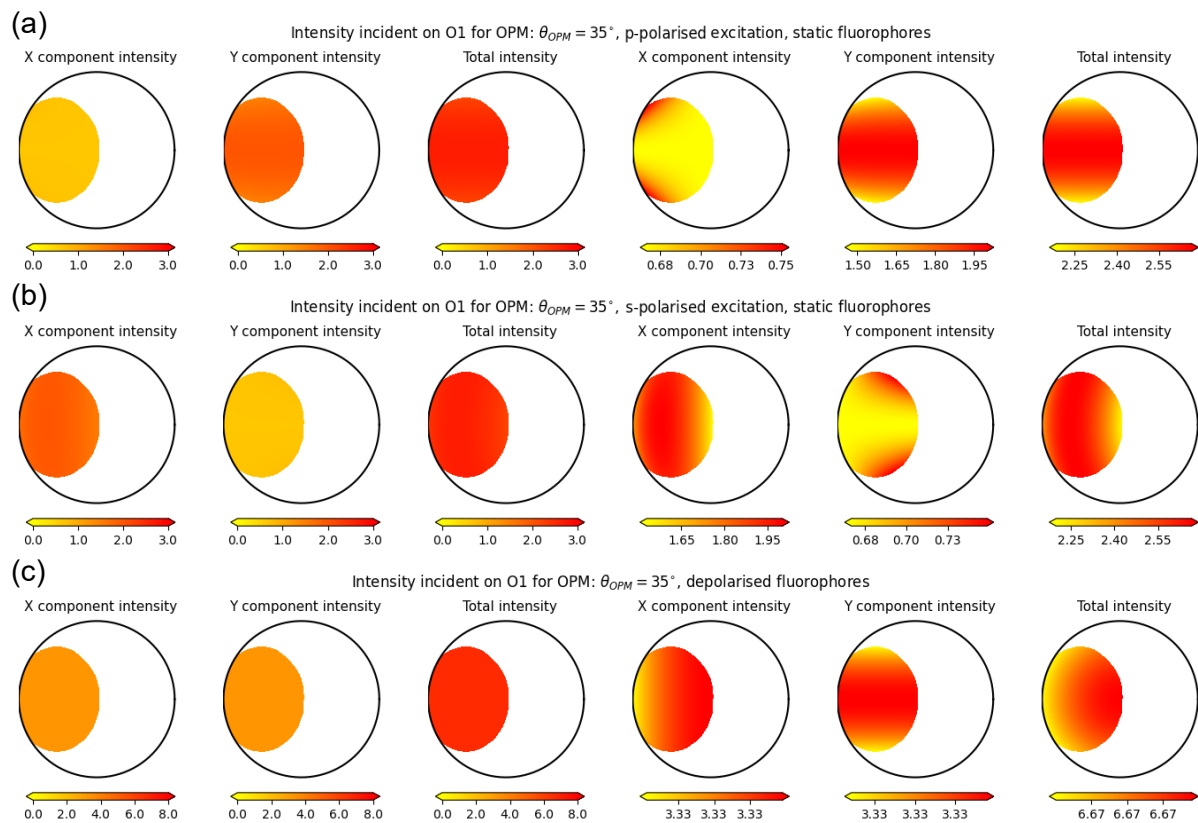


Figure A5.7 – Intensity distributions (curved wavefront) incident on the first principal surface of O1 in the region that is collected by the entire system, for an OPM system with $\theta_{OPM} = 35^\circ$. Intensity distribution plots for (a) p-polarised and (b) s-polarised excitation of static randomly oriented fluorophores, and (c) for depolarised emission. The right three plots use an autoscaled intensity scale.

A.5.4.2 OPM at 45°

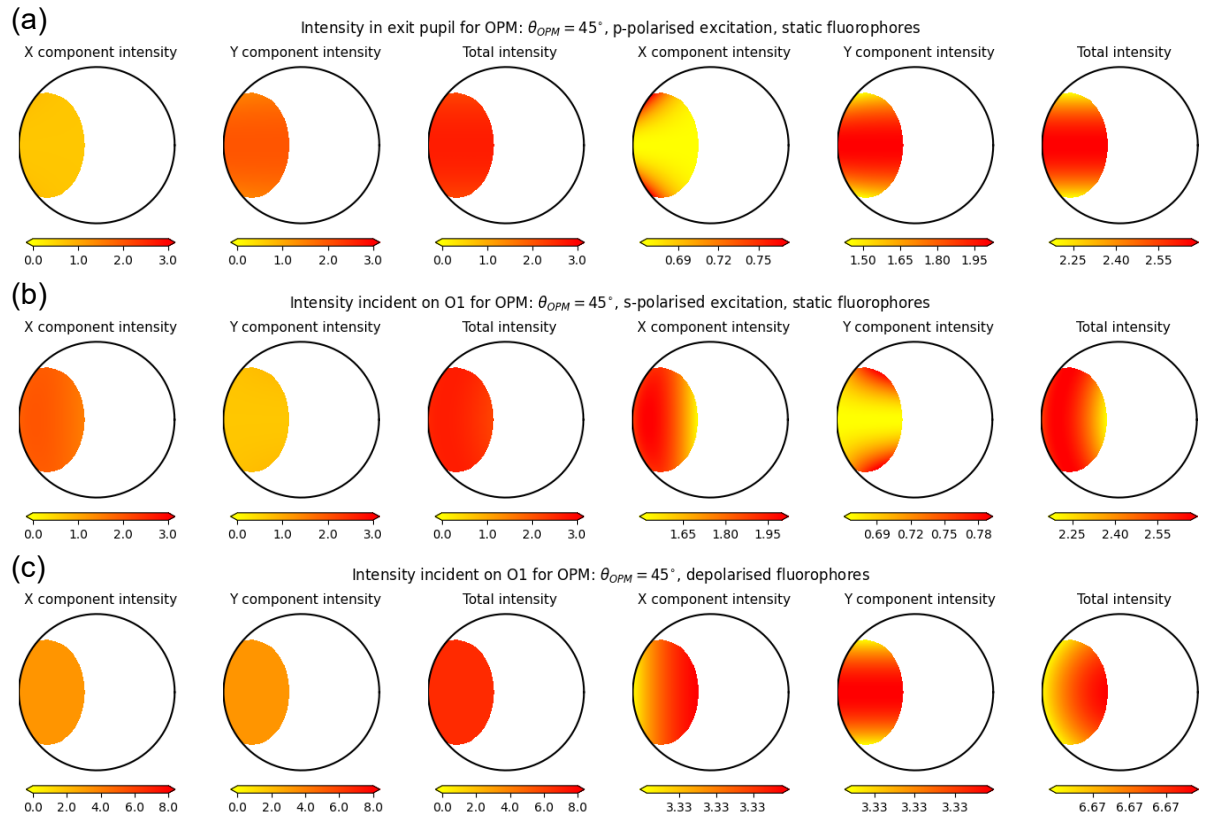


Figure A5.8 – Intensity distributions (curved wavefront) incident on the first principal surface of O1 in the region that is collected by the entire system, for an OPM system with $\theta_{OPM} = 45^\circ$. Intensity distribution plots for (a) p-polarised and (b) s-polarised excitation of static randomly oriented fluorophores, and (c) for depolarised emission. The right three plots use an autoscaled intensity scale.

A.5.4.3 dOPM at 35°

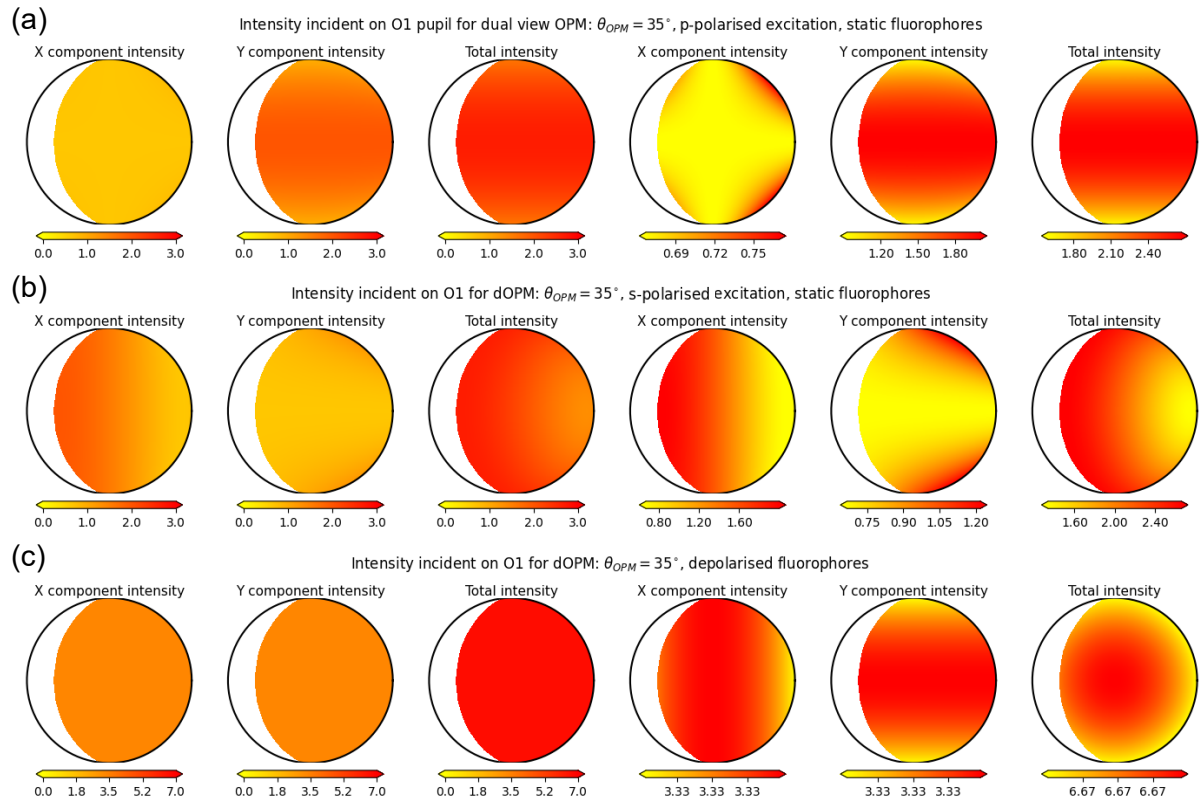


Figure A5.9 – Intensity distributions (curved wavefront) incident on the first principal surface of O1 in the region that is collected by the entire system, for a dOPM system with $\theta_{OPM} = 35^\circ$. Intensity distribution plots for (a) p-polarised and (b) s-polarised excitation of static randomly oriented fluorophores, and (c) for depolarised emission. The right three plots use an autoscaled intensity scale.

A.5.4.4 dOPM at 45°

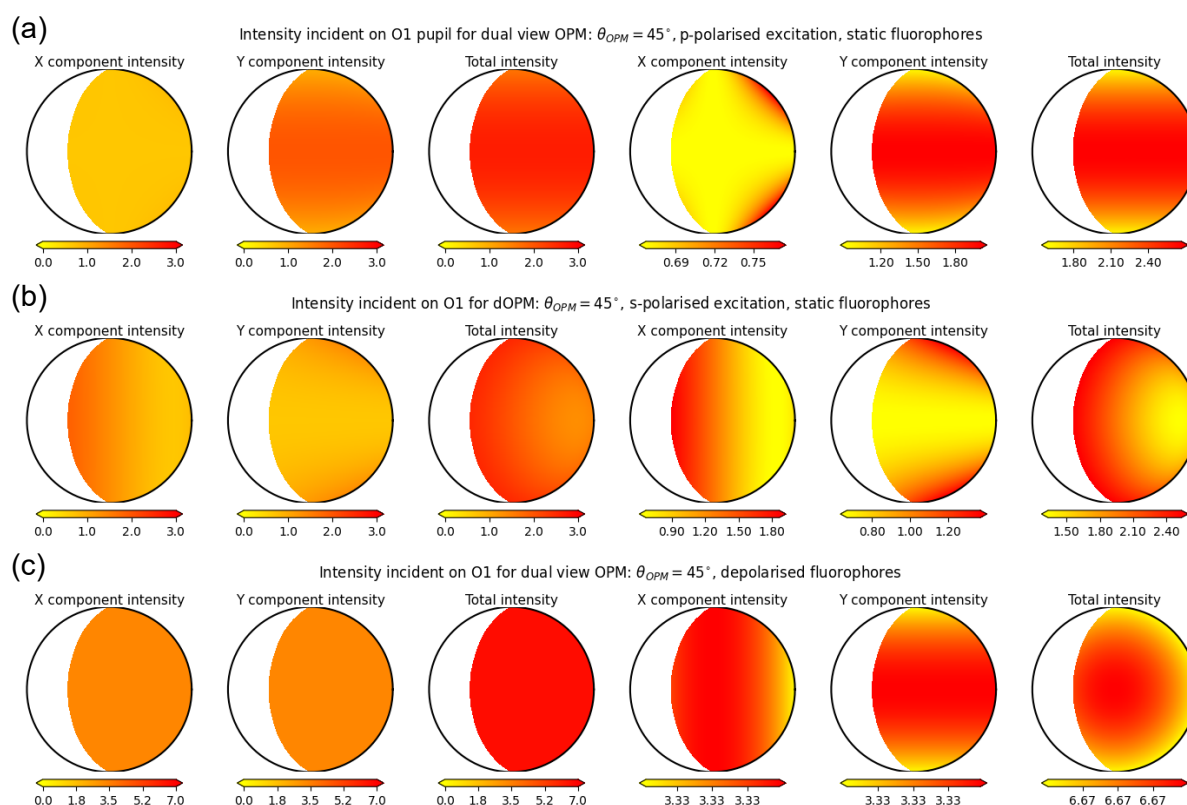


Figure A5.10 – Intensity distributions (curved wavefront) incident on the first principal surface of O1 in the region that is collected by the entire system, for a dOPM system with $\theta_{OPM} = 45^\circ$. Intensity distribution plots for (a) p-polarised and (b) s-polarised excitation of static randomly oriented fluorophores, and (c) for depolarised emission. The right three plots use an autoscaled intensity scale.

A.5.5 Percentage difference tables for RCE and CE

Table A5.1 – Percentage difference in collection efficiency metrics – (G)RCE and (G)CE – between ray-tracing simulation results and Geometric Method results.

	Percentage difference in RCE between ray-tracing simulation and Geometric Method $100\% \cdot (RCE - GRCE)/RCE$			Percentage difference in CE between ray-tracing simulation and Geometric Method $100\% \cdot (CE - GCE)/CE$		
	P	S	Depol.	P	S	Depol.
OPM 35°	7.9%	-24.3%	0.4%	14.6%	-29.0%	0.4%
OPM 45°	7.6%	-27.6%	0.1%	14.2%	-29.8%	0.1%
dOPM 35° Thorlabs mirror	-4.6%	-18.7%	-5.1%	1.2%	-23.7%	-5.0%
dOPM 45° Thorlabs mirror	-4.3%	-22.2%	-5.1%	1.6%	-24.6%	-5.1%
dOPM 35° perfect mirror	0.7%	-14.1%	0.3%	6.9%	-19.4%	0.3%
dOPM 45° perfect mirror	1.0%	-17.9%	0.2%	7.2%	-20.4%	0.2%

Table A5.2 – RCE/CE percentage difference between p-polarised excitation and s-polarised excitation, calculated for each OPM and dOPM ray-tracing simulation setup. RCE_p – RCE with p-polarised excitation; RCE_s – RCE with s-polarised excitation; CE_p – CE with p-polarised excitation; CE_s – CE with s-polarised excitation.

System	RCE percentage difference between p- and s-polarisation $100\% \cdot (RCE_p - RCE_s)/RCE_p$	CE percentage difference between p- and s-polarisation $100\% \cdot (CE_p - CE_s)/CE_p$
OPM 35°	29.9%	38.0%
OPM 45°	32.7%	38.6%
dOPM 35° Thorlabs mirror	30.5%	38.6%
dOPM 45° Thorlabs mirror	29.6%	35.7%
dOPM 35° perfect mirror	30.5%	38.6%
dOPM 45° perfect mirror	29.6%	35.7%

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Expected presentation date	Dec 2024
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Title of new work	Development of oblique plane microscopy techniques and their application to live-cell time-lapse multi-well-plate imaging of cancer spheroids
Institution name	Imperial College London
Expected presentation date	Dec 2024
Portions	Figure 1: Airy beam light-sheet microscopy. (VOL.11 NO.5 page 542)
Requestor Location	Mr. Leo Rowe-Brown Imperial College London Blackett Laboratory South Kensington London, SW7 2BB United Kingdom Attn: Leo Rowe-Brown
Total	0.00 GBP

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Institution name	Imperial College London
Expected presentation date	Dec 2024
Portions	Fig. 1: ASLM. NATURE PROTOCOLS, VOL 17, SEPTEMBER 2022, page 2027
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Institution name	Imperial College London
Expected presentation date	Dec 2024
Portions	Figure 1: HILO microscopy for molecular imaging in cells and three-dimensional imaging of the NPCs. VOL.5 NO.2, FEBRUARY 2008, page 159
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Title of new work	Development of oblique plane microscopy techniques and their application to live-cell time-lapse multi-well-plate imaging of cancer spheroids
Institution name	Imperial College London
Expected presentation date	Dec 2024
Portions	Fig. 1 Imaging principle and cell-scale demonstration of obSTORM (page 854, Nature Methods, VOL 16)
Requestor Location	Mr. Leo Rowe-Brown Imperial College London Blackett Laboratory South Kensington London, SW7 2BB United Kingdom Attn: Leo Rowe-Brown
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Title of new work	Development of oblique plane microscopy techniques and their application to live-cell time-lapse multi-well-plate imaging of cancer spheroids
Institution name	Imperial College London
Expected presentation date	Dec 2024
Portions	Fig. 1: Model architecture. VOL 18, January 2021, 101
Requestor Location	Mr. Leo Rowe-Brown Imperial College London Blackett Laboratory South Kensington London, SW7 2BB United Kingdom Attn: Leo Rowe-Brown
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Portions	p354 Figure 10.1 Schematic diagram for measurement of fluorescence anisotropies. p356 Figure 10.4 Emission intensities for a single fluorophore in a coordinate system.
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