

Using fluorescence lifetime imaging to assess DNA damage response and PARP trapping in ovarian cancer

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Abstract

The activity of poly(ADP-ribose) polymerase inhibitors (PARPi) in ovarian high-grade serous carcinoma (HGSC) depends upon inducing synthetic lethality in homologous recombination-deficient cancer cells, impairing DNA double-strand break repair. Despite initial success, over 50% of patients recur within four years. One proposed mechanism of resistance is reduced PARP trapping, where the PARP1-inhibitor complex fails to remain adequately trapped at DNA damage sites. Traditional methods for assessing PARP trapping rely on bulk biochemical assays with limited throughput, typically performed on fixed cells.

This thesis aimed to develop a novel tool to assay PARP trapping with single-cell resolution in a high-throughput manner. For this, an intramolecular FRET biosensor was developed using CRISPR-Cas9 knock-in technology to dual-label endogenous PARP1 in OVCAR4 cells with EGFP and mCherryFP. The FRET biosensor allows real-time analysis of PARP1-DNA binding dynamics in live cells and was assayed using automated high-content analysis (HCA) with wide-field time-gated fluorescence lifetime imaging microscopy (FLIM).

This approach enabled quantitative FRET measurements across 96-well plates, allowing statistical robustness for the comparison of perturbations due to the relatively high sampling rate. Significant intra-population heterogeneity was detected, which traditional pooled-sample assays, such as chromatin immunoprecipitation, could not resolve. The biosensor was first validated using clinically approved PARPi with varying trapping potencies and subsequently applied to novel models of PARPi resistance. A dose-dependent decrease in fluorescence lifetime was observed upon PARPi treatment, with reduced PARP trapping detected in cells resistant to olaparib and rucaparib.

The biosensor may provide critical insights into resistance mechanisms and enable screening of novel PARPi to enhance therapeutic options. Furthermore, it may be applied to clinical samples in the future, supporting personalized medicine approaches. The ability to assess PARP trapping in live cells with single-cell resolution offers an unprecedented capability for advancing therapeutic strategies and enhancing the efficacy of PARPi treatments for HGSC patients.

Statement of originality

I state that this work is original, except where I have referenced or acknowledged the work of others throughout this thesis.

I used the multibeam multiphoton multiwell plate microscope (M³M) system implemented on the *openFrame* that was developed by members of the biophotonics group within the Physics Department in Imperial College London. The initial concept of the *openFrame* was proposed by Professor Paul French with contributions from other members of the group, the initial design and development was performed by Simon Johnson and Martin Kehoe, members of the Optomechanical Instrumentation workshop within the Light Community of the Department of Physics of Imperial College London. CAD designs of the instrument were made by Dr Frederik Gorlitz, in collaboration with Cairn Research Ltd.

Cell sorting experiments described in chapter 4 were performed with the aid of the LMS NIHR Flow Cytometry Facility, Imperial College London. Rhodopsin targeting vector (EGFP) was kindly gifted by Professor Izumi Oinuma of the Laboratory of Cell and Molecular Biology, University of Hyogo, Japan. AW69_pDonor^{PARP1.TS} and AM70_pgRNA^{PARP1} plasmids were generated and kindly gifted by Dr Manual A.F.V Gonçalves, Leiden University Medical Center, Department of Cell and Chemical Biology, The Netherlands.

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List of abbreviations

Abbreviation	Definition
2D	Two dimensional
3D	Three dimensional
ADP	Adenosine diphosphate
APS	Ammonium persulfate
BER	Base excision repair
BRCA	Breast cancer gene
CRISPR	Clustered regularly interspaced short palindromic repeats
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSB	Double strand break
ECM	Extracellular matrix
EGFP	Enhanced green fluorescent protein
EMT	Epithelial to mesenchymal transition
FBS	Foetal bovine serum
FISH	Fluorescence in situ hybridisation
FLIM	Fluorescence lifetime imaging
FOV	Field of view
FP	Fluorescence protein
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
GOI	Gated optical intensifier
HCA	High content analysis
HGSC	High grade serous carcinoma
HRD	Homologous recombination deficiency
IP	Intraperitoneal
IRF	Instrument response function
NA	Numerical aperture
NAD	Nicotinamide adenine dinucleotide
NHEJ	Non-homologous end joining
PAR	Poly ADP-ribose
PARG	Poly (ADP-ribose) glycohydrolase
PARP	Poly (ADP-ribose) polymerase
PFS	Progression free survival
PLL	Poly-L-lysine
POI	Protein of interest
RNA	Ribonucleic acid
ROI	Region of interest
SNR	Signal-to-noise ratio
SSB	Single strand break
TCGA	The Cancer Genome Atlas Network
TCSPC	Time-correlated single photon counting

1 Introduction

1.1 Ovarian high-grade serous carcinoma

Ovarian cancer ranks as the eighth most prevalent cancer amongst women worldwide and the fifth most common cause of cancer death in women, contributing to around 3.7% of all cancer cases and 4.7% of total cancer deaths in 2020 [1]. Importantly, the disease referred to as ‘ovarian cancer’ encompasses a wide range of tumour types, including some that originate in the fallopian tube. Within epithelial ovarian cancers, high-grade serous carcinoma (HGSC) is both the most common and most lethal subtype [2]. It is now recognised that HGSC originates from serous tubal intraepithelial carcinoma (STIC) lesions, which arise in secretory cells at the fimbrial ends of the fallopian tubes [3]. The high mortality rates are mostly due to late-stage detection coupled with high rates of relapse and, until recently, limited treatment options. Clinically, most patients are diagnosed at a late stage (stage III or IV), due to the lack of screening methods available and ambiguous symptoms, leading to a substantial decrease in the 10-year survival rate—from 55% down to 15%—compared to cases detected early [4]. HGSC is particularly challenging to manage due to early metastatic dissemination via spheroids via ascitic fluid within the peritoneal cavity.

Although ovarian cancer is an incredibly heterogeneous disease, HGSC is characterised by universal *TP53* mutations, with The Cancer Genome Atlas (TCGA) confirming their presence in 96% of patients [5]. This ultimately results in genomic instability, causing widespread copy number abnormalities. Moreover, around 50% of HGSC patients have homologous recombination deficiency (HRD), referring to a state where the normal DNA double-strand break repair via homologous recombination is impaired or dysfunctional. This leads to an increased reliance on alternative DNA repair pathways for cell survival. HRD occurs via several mechanisms, with around 20% of patients having germline or somatic *BRCA1* or *BRCA2* mutation, and mutations or epigenetic silencing of genes such as *RAD51C*, *PALB2*, *ATM* and *ATR* also contributing to the high rate of HRD. Unlike many cancer types, mutations in oncogenes are not common in HGSC; rather the disease is dominated by copy number alterations and genomic structural variations due to the near-ubiquitous genomic instability found in patients.

1.1.1 Standard treatment approaches for HGSC

Until now, the standard approach to treatment has been primary debulking surgery and a combination of platinum and taxane chemotherapy (most often carboplatin and paclitaxel). Despite initial sensitivity to platinum, around 85% of patients relapse and the vast majority of relapsed patients ultimately go on to develop fatal platinum-resistant disease [6]. The carboplatin/paclitaxel regime has remained the standard approach for therapy since the mid-1990s and hence patient outcomes over

that period have not improved significantly. However, efforts towards developing targeted therapies brought about a paradigm shift, due to the pioneering clinical trials such as SOLO1 and SOLO2 for a new class of chemotherapeutic drugs called poly (ADP-ribose) polymerase inhibitors (PARPi). The complexity and heterogeneity of the disease has made the development of pre-clinical models difficult, whether that be patient-derived cell lines or more complex 3D cell cultures such as spheroids and organoids which better recapitulate the primary tumour and ascitic environment.

1.1.2 Models of HGSC used in preclinical and basic research settings

At present, physiologically relevant *in vitro* and *ex vivo* models of ovarian cancer for fundamental and discovery science are lacking. Until now, traditional two-dimensional *in vitro* cell cultures of ovarian cancer cells have been used as the “workhorse” of such research in this area. Despite their ease of use, these models lack the complexity offered by three-dimensional cultures, which have cell-to-cell interactions and can mimic elements of the tumour microenvironment. Hence, there is currently an unmet need to grow 3D culture models routinely and reliably whether that be spheroids, co-cultures, or patient-derived organoids. Those models can be thought of as a continuum of complexity, with spheroids consisting of a 3D aggregation of a single-cell type (**Figure 1**). Co-cultures go one step further, containing multiple cell types, be that fibroblasts or immune cells such as macrophages, in addition to malignant cells. Organoids are 3D structures grown from stem cells or organ-specific progenitor cells. These cells can self-organise, undergoing lineage commitment, allowing them to mimic one or more functions of the tissue or organ [7]. Tissue-derived organoids are generally sourced from adult tissues such as patient surgeries. Thus, organoids encapsulate far more complexity than a spheroid and even more so than a 2D monolayer of cells. Beyond this, the use of animal models (mainly murine) is often used to validate findings in human cell lines, before moving into human clinical trials.

The overarching aim of these different 3D cell culture models is to better recapitulate the biology occurring in the patient’s disease, without having the actual patient in the lab. Thanks to the vast uptake and development of these methods in recent years, standardised protocols for the continuous culturing of spheroids and organoids are now becoming established. Thus, these models are now more accessible to be used routinely in a vast array of experimental protocols. However, it is important to consider the parallel and converging technological advancements that can allow us to extract more valuable information from these complex models. In the context of my project, this refers mainly to the advancements of 3D live imaging microscopy modalities. There are numerous advantages and disadvantages to using 3D cell culture models, as summarised in **Table 1**. In light of this, despite an

uptick in usage of more physiologically relevant models in recent years, most of the knowledge gained on HGSC thus far has been in simple 2D monolayers.

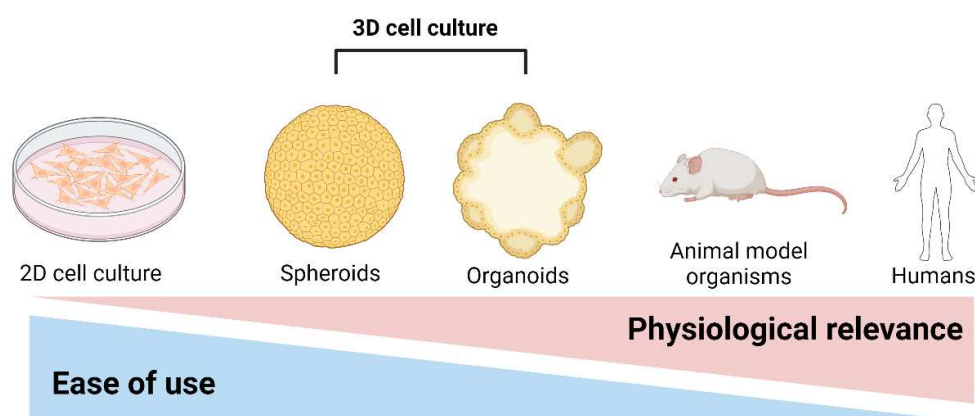


Figure 1: Models used for laboratory research of HGSC. Models range from simple 2D cell culture to complex 3D cell cultures such as spheroids and organoids, and animal models or human experiments. From left to right these are ordered in terms of increasing physiological relevance but decreasing ease of use. Figure created in BioRender.

3D cell culture	2D cell culture
Variable access to oxygen, nutrients, metabolites, and signalling molecules	Equal access to oxygen, nutrients, metabolites and signalling molecules
Allows sampling of the genetic background of a disease cohort via patient derived organoids	Limited components of the tumour microenvironment
Gene expression, topology, and biochemistry better recapitulate <i>in vivo</i> models	Does not mimic the structural or genetic complexity of <i>in vivo</i> tumours
Similar drug resistance profiles as <i>in vivo</i> models	Drug sensitivity can be altered due to continuous culturing on plastic
Time-consuming and expensive, often with complex medium components	Cheaper, more established protocols, accessible to low resource settings
3D assays (such as imaging based assays) are somewhat limited, technology lacking	Most cells in the same cell cycle stage

Table 1: Comparison of 3D and 2D cell culture methods for disease modelling.

The current organoid technology relies on a combination of growth factors and small molecules, along with a Matrigel-based basement membrane mimic, to replicate the specific niche conditions necessary for sustained cell growth. These cultures can be initiated from individual cells obtained from tumour tissue, enabling the investigation of tumour heterogeneity. Organoids allow the assessment of correlations between phenotype and genotype, as well as drug responsiveness, whilst also offering

information on potential patient treatment outcomes [8,9]. Hence, patient-derived organoids are currently viewed as the gold standard for screening of potential therapeutics for a path towards a more personalised medicine approach. As outlined here, 3D models better recapitulate the inter-cellular heterogeneity observed in patient tumours much better than 2D cultures. In this sense, 3D models offer a bridge between traditional 2D culture and *in vivo* mouse models. Although more physiological models of disease are in development, the majority of the basic science research on PARP1 functionality has been carried out in 2D immortalised cell lines.

1.1.3 PARP1 function and activity in DNA damage repair and beyond

Of the myriad of DNA damage repair pathways that cells use to maintain genome integrity in response to double-strand DNA breaks (DSB), homologous recombination (HR) has the lowest error rate. HR relies on a template sister chromatid to perform DNA repair and hence can only occur in the late S and G2 phases of the cell cycle [10]. However, in HRD tumours, there is an increased reliance on alternative repair pathways, including non-homologous end-joining (NHEJ) and mismatch end-joining (MMEJ).

1.2 Introduction to PARP1

PARP1 is best known for its role in sensing single-strand DNA breaks (SSBs) and detecting DSBs as well as a list of other DNA-based structures. Upon detection, PARP1 binds DNA breaks via its N-terminal zinc finger domain (**Figure 2A**). Binding occurs rapidly, within milliseconds of damage occurring, and results in a conformational change of the helical domain that opens the active site of the enzyme, resulting in allosterically active PARP1 (**Figure 2B**). This triggers a rapid spike in the enzymatic activity of PARP1, going from its relatively low basal rate of activity, utilising NAD⁺ to increase its catalytic ability by up to 1000-fold. PARP1 then performs the addition of long, branching negatively charged poly(ADP-ribose) (PAR) polymer chains in a process termed PARylation. This post-translational modification is formed as PARP1 catalyses the transfer of the ADPr unit from NAD⁺, forming long branched chains of PAR covalently bound to specific amino acid sites on PARP1 itself (auto-PARylation), or other target proteins (trans-PARylation) within the vicinity of breaks [11]. PARylation chains are formed within ~15-30 s of DNA break formation, highlighting the sensitivity, speed, and efficiency of PARP1. Notably, of the 18 PARP genes found in the human genome, PARylation activity has been observed in only PARP1-5, and the nature of PAR generated by each of these enzymes varies. Moreover, PARP1 carries out around 90% of total PARylation involved in DNA damage response (DDR) and therefore has been most heavily studied as the key PARP enzyme in these pathways [12].

Following PARylation, the chromatin becomes relaxed at the DNA damage site. But simultaneously, PARylation chains act as a molecular scaffold, or a “cellular Velcro”. This facilitates the recruitment of PAR-binding proteins for downstream DNA repair events, such as XRCC1 and the MRN1 complex, comprising MRE11, RAD50 and NBS1 [13]. Due to the importance of PARylation in cellular stress response signalling, it is a tightly regulated process and is actively reversed by several dePARylating enzymes. Following the repair of DNA damage, these PAR degradation enzymes, in particular poly(ADP-ribose) glycohydrolase (PARG), rapidly degrade PAR chains and cause the dissociation of the DDR complex. The continued auto-PARylation of PARP1 triggers its subsequent release from the DNA, in a process mediated by the interaction of the allosteric helical domain of PARP1 [14].

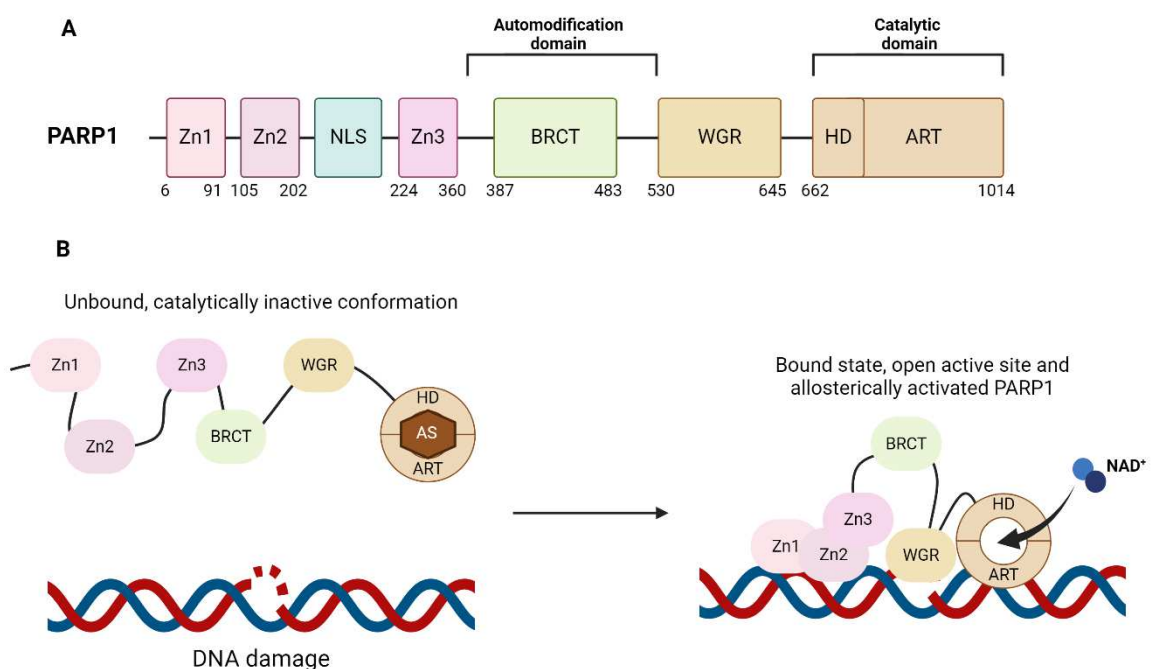


Figure 2: PARP1 protein structure and conformational change upon binding to DNA break sites. **A:** The functional domains of the PARP1 enzyme consists of three zinc finger domains (Zn1-3), an automodification domain that contains a BRCT fold, a Trp-Gly-Arg (WGR) domain and the catalytic domain (CAT), consisting of the autoinhibitory helical domain (HD) and ADP-ribosyl transferase (ART) subdomains. A nuclear localisation signal (NLS) is also present. **B:** As PARP1 binds to sites of DNA breaks it undergoes a conformational change that opens the active site (AS) of the enzyme for its substrate NAD⁺, resulting in allosteric activation. Figure created in BioRender.

1.2.1 Role of PARP1 in DNA single-strand break repair

With regards to its functionality in DNA repair, the role of PARP1 in SSB repair is the most widely studied. PARP1 detects SSBs rapidly, usually within seconds of their creation. Upon sensing a novel break, PARP1 initiates the PARylation of various members of the SSB repair pathway, including X-ray repair cross-complementing protein 1 (XRCC1), a protein that acts as a scaffold of the SSB repair complex. XRCC1 binds immediately, recognising PAR chains and attracting numerous proteins to SSB

sites (**Figure 3A**). Some of the proteins that bind XRCC1 include polynucleotide kinase 3'-phosphatase (PNKP), DNA ligase 3 (LIG3), aprataxin and DNA polymerase β (POLB). PNKP and aprataxin perform end processing of damaged 5' and 3' ends of the SSBs, allowing POLB to repair them through fill-in synthesis before ligation is carried out by LIG3, resulting in a repair DNA sequence.

PARP1 is also involved in the base excision repair (BER) pathway. BER is a DNA repair mechanism deployed when bases are subjected to damage via oxidation, alkylation or deamination (**Figure 3B**). During BER, DNA glycosylases recognise and excise specific damaged bases, forming an abasic site. These sites are processed by apurinic/apyrimidinic endonuclease 1 (APE1). The resulting single gap is filled by POLB and ligated by either LIG1 or LIG3. Although PARP1 is not considered a canonical BER protein, it accelerates BER by binding the SSB intermediates generated by APE1 and rapidly recruits XRCC1, POLB and LIG3. PARP1 is released at the site of an SSB following self-PARylation and poly(ADP-ribose) glycohydrolase (PARG) activity.

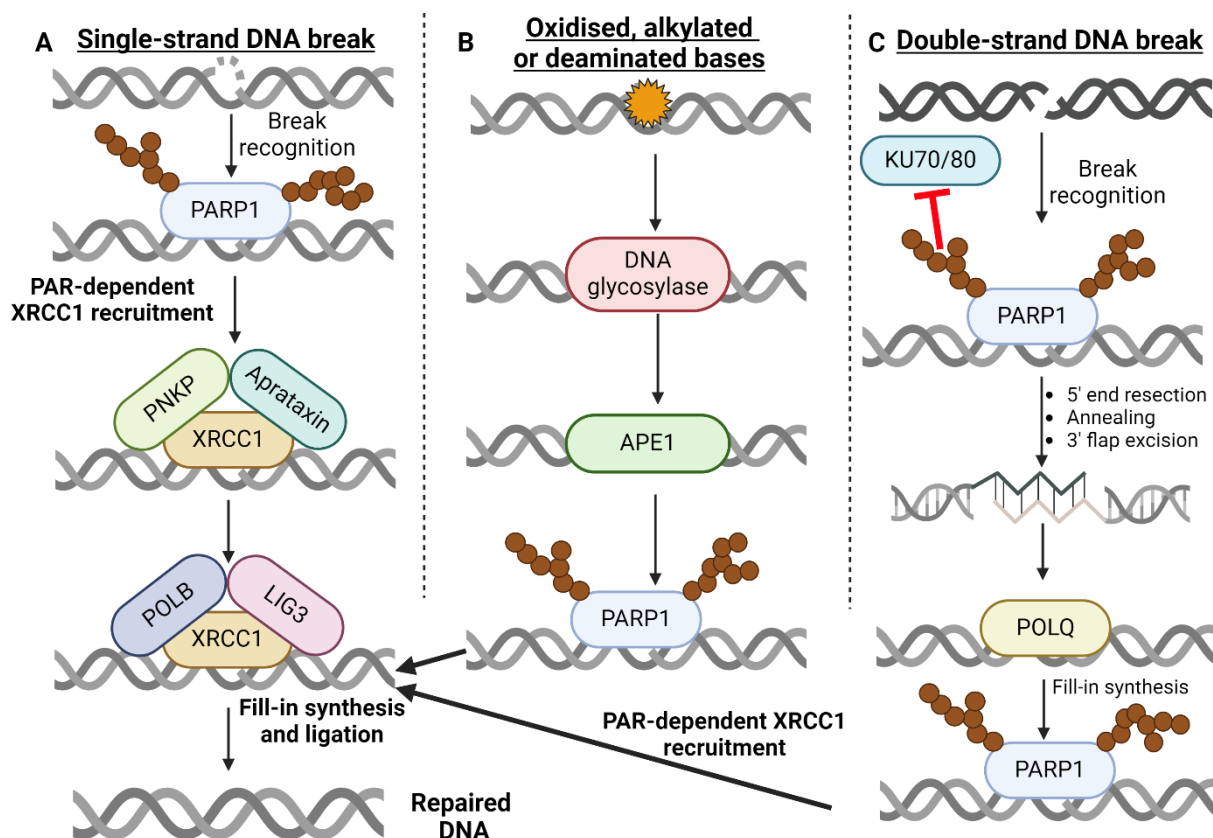


Figure 3: The roles of PARP1 in response to various DNA damage lesions. **A:** PARP1 detects and binds to single-strand DNA breaks, undergoes auto-PARylation and recruits XRCC1 which recruits a series of other repair proteins to perform fill-in synthesis and ligation. **B:** Oxidised, alkylated or deaminated bases are excised by DNA glycosylases and APE1 generates ssDNA breaks that PARP1 binds to repair in an XRCC1-dependent process. **C:** PARP1 detects and binds double-strand breaks, which are subsequently repaired via NHEJ or HR. Figure created in BioRender.

1.2.2 Role of PARP1 in DNA double-strand break repair

DSBs often arise due to exposure to DNA-damaging agents, like ionizing radiation, or internal factors resulting from replication fork collapse. Additionally, an unrepaired SSB at stalled replication forks can lead to DSB formation. DSBs are the most toxic form of DNA lesion as they often give rise to significant chromosomal rearrangements and deletions. PARP1 detects DSBs and triggers one of several dsDNA repair pathways (**Figure 3C**). Most DSBs are successfully repaired throughout the cell cycle by the classical non-homologous end-joining pathway (cNHEJ), directly joining broken ends without the requirement for homology or a sister chromatid. In contrast, HR involves extensive processing of DSBs to generate long 3' single-stranded DNA ends. These ends then find and invade a homologous DNA sequence in the sister chromatid, serving as a template for accurate repair. Therefore, HR operates exclusively in the S and G2 phases of the cell cycle when a homologous DNA sequence is available. PARP1 contributes to both the c-NHEJ and HR pathways.

PARP1's role in HR appears somewhat paradoxical. While the BRCA1 protein is involved in completing the repair of the initial section of a double-strand break (DSB), RAD51 is recruited to the site either through PARP1-dependent mechanisms or DNA damage-induced ubiquitylation. However, inhibiting PARP1 leads to an elevated HR repair rate, as evidenced by increased sister chromatid exchange (SCE) and the formation of RAD51 foci at DSB sites.

Beyond the c-NHEJ pathway, there is growing interest in alternative nonhomologous end-joining (a-NHEJ) pathways. A subset of a-NHEJ mechanisms operate through microhomology sequences at DNA break ends, known as microhomology-mediated end joining (MMEJ). Recent research highlights the significant reliance of a-NHEJ on the DNA polymerase theta (POL θ ; PolQ) function. This process, termed polymerase theta-mediated end-joining (TMEJ), involves initial end resection near short DSB regions with microhomology (ranging from 2 to 20 nucleotides). During MMEJ, PARP1 binds to DSB ends, recruits end resection factors and facilitates repair signalling. Without this initial end resection, a Ku70-Ku80 heterodimer initiates the classical nonhomologous end joining (c-NHEJ) pathway at the DSB site. Subsequently, PARP1, the MRN complex, and the unique DNA polymerase PolQ align complementary micro-homologous sequences, bridging the DSB ends. Endonucleases (such as ERCC1/XPF, FEN1, and others yet unidentified) remove nonhomologous 3' tails, while FEN1 processes 5' flaps. PolQ then fills the gaps in the DNA double helix resulting from microhomology alignment and 3' tail removal. Finally, the DNA ligase III/XRCC1 complex, recruited by the MRN complex, ligates the DSB ends. Notably, PARP1 interacts with DNA ligase III and the scaffold protein XRCC1.

Of the myriad of DNA damage repair pathways that cells use to maintain genome integrity in response to double-strand DNA breaks (DSB), homologous recombination (HR) has the lowest error rate. However, in HRD tumours there is an increased reliance on alternative repair pathways, including NHEJ and TMEJ. This provides the rationale behind the use of PARPi to induce synthetic lethality [15]. By inhibiting PARP1, HRD cells must use non-homologous end joining (NHEJ) or alternative end joining (alt-EJ), both of which are error-prone repair mechanisms and hence increase genomic instability, ultimately leading to cell death (**Figure 4**). However, healthy cells that are homologous recombination proficient can still utilise the HR pathway, so are less susceptible to the cytotoxic effects of PARPi.

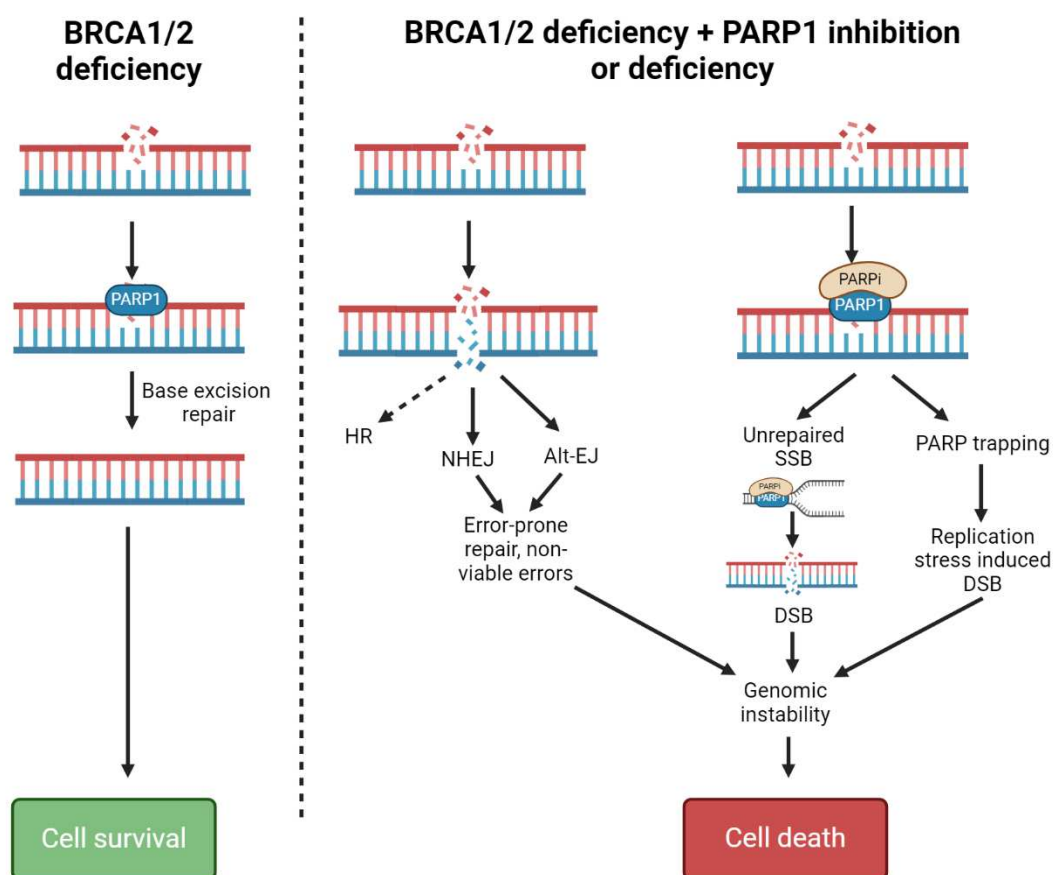


Figure 4: PARPi induce synthetic lethality in BRCA1/2 deficient cells. In BRCA deficient cells homologous recombination repair is non-functional, therefore base excision repair is utilised to repair ssDNA breaks (left). Treating these cells with PARPi results in synthetic lethality as the base excision repair is also inhibited, leaving no functional DNA repair mechanism, leading to cell death. Figure created in BioRender.

Therefore, synthetic lethality with PARP inhibitors represents a targeted therapeutic strategy for treating cancers with HR deficiencies, such as ovarian and breast cancers with *BRCA1/2* mutations, while sparing normal cells. This concept has led to the development and clinical approval of PARP inhibitors as effective treatments for these specific types of cancers.

1.2.3 Clinical development and approval of PARPi

The current generation of PARP1/2 inhibitors (PARPi) are all small molecule compounds that compete with the enzymatic substrate NAD⁺ for the active site of PARP1, suppressing its catalytic activity. PARPi gained huge interest following the discovery that they induce profound toxicity in *BRCA1* and *BRCA2* mutant cancer cells [16,17]. This synergistic killing via a small-molecule drug and a genetic mutation, inherent to the target cells, is a type of synthetic lethality. The earliest human clinical trial was conducted in platinum sensitive relapsed HGSOC patients, demonstrating olaparib improved progression-free survival (PFS) compared to placebo (8.4 vs 4.8 months, HR 0.35), with more pronounced benefits observed in *BRCA* mutant patients (11.2 vs 4.3 months, HR 0.18) [18]. The following SOLO1 and SOLO2 trials showed highly significant improvements in PFS when olaparib was used as maintenance therapy following platinum chemotherapy in *BRCA1/2* mutant patients at first-line and relapsed time-points respectively [19,20]. The importance of this drug class was then amplified further by the subsequent PRIMA, NOVA, ATHENA and ARIEL3 phase III clinical trials that collectively demonstrated that PARPi maintenance therapy can benefit non-*BRCA1/2* mutant patients, especially in those with tumours classified as HRD [21–23]. This ultimately led to the approval of PARPi in the UK, which has revolutionised treatment for the disease, with three PARPi now approved in the UK: niraparib, olaparib and rucaparib. The use of PARPi has now become routine in the clinical setting. Although the approval of PARPi has brought about a paradigm shift in the treatment of HGSC, improving PFS of many patients, within 4 years around 50% of patients become resistant to therapy [24][25]. To begin to understand how resistance to PARPi may occur, the mechanisms of action by which PARPi induce cytotoxicity must first be addressed.

1.2.4 Mechanisms of action of PARPi

1.2.4.1 *Inhibiting SSB repair*

As mentioned, PARP1 plays an essential role in the detection and subsequent repair of SSB. Early work proposed that PARPi may induce synthetic lethality by inhibiting SSB repair, resulting in the accumulation of DNA damage and eventual cell death. However, conflicting evidence exists for this hypothesis, with some studies indicating limited evidence that PARP inhibition directly increases SSBs. Additionally, genetically inhibiting PARP1 is notably less toxic than using PARPi, which would be expected to be similar if the primary mechanism of toxicity were solely through SSB repair inhibition [26]. These observations strongly suggest that PARPi-induced synthetic lethality involves other mechanisms beyond SSB repair reduction.

1.2.4.2 Activation of the NHEJ repair pathway

Another proposed mechanism suggests that PARPi induce excessive non-homologous end joining (NHEJ) activity, leading to heightened genomic instability, resulting in cell death. However, conflicting evidence also surrounds this theory. Initially, it was proposed that PARP inhibitor (PARPi) treatment increases DNA-PK phosphorylation, promoting NHEJ. Additionally, the 53BP1 effector complex, shieldin, is recruited to double-strand breaks (DSBs) and supports NHEJ. Reduced shieldin activity has been associated with PARPi resistance [27].

1.2.4.3 Disrupted processing of Okazaki fragments and replication fork speed

It has been shown that the inhibition or depletion of proteins that regulate replication forks, such as FEN1 and LIG1, results in an accumulation of PARP1, enabling XRCC1-mediated processing. Moreover, PARPi have been shown to enhance the speed of replication fork progression by 1.4-fold, supporting a role for PARP1 in responding to unligated Okazaki fragments [28]. During PARPi treatment, high-speed replication can lead to formation of double-strand breaks (DSBs) and thus may be a mechanism driving PARPi toxicity [29,30]. Following on from these findings, it was recently proposed that this increased speed of replication may cause an accumulation of replication-associated ssDNA gaps [31]. It was postulated that the presence of cytotoxic ssDNA gaps can be attributed to PARP1's involvement in processing Okazaki fragments or reversing stalled replication forks. Consequently, inhibiting PARP activity within these processes leads to the formation of short ssDNA gaps in the DNA sequence. Although this is a more novel theory, there is substantial evidence building. Notably, following PARPi treatment, *BRCA*-deficient tumour cell lines exhibit a significantly higher prevalence of ssDNA gaps compared to *BRCA*-wild type lines. Additionally, PARPi-resistant cell models display fewer ssDNA gaps, highlighting the correlation between PARPi sensitivity and the level of induced ssDNA gaps.

1.2.4.4 Disrupting PARP1-mediated transcription

PARP1 also plays a role in transcription regulation by influencing chromatin structure and histone PARylation. Acting as a transcriptional co-regulator, it directly binds to transcription sites. PARP1 regulates the transcription of a large array of genes, including oncogenes, tumour suppressor genes, and other cancer survival associated genes such as NF- κ B. Consequently, PARP inhibitors (PARPi) may reduce replication by activating tumour suppressor genes [32]. Moreover, a recent study showed that PARPi cause transcription replication conflicts that lead to DSB formation and mediate the synthetic lethality of PARPi [33].

1.2.4.5 Replication fork stalling and physical PARP trapping

PARP1 activation is known to be essential at stalled replication forks, facilitating MRE-11 mediated restarting of replication. If a replication fork encounters a DNA lesion or SSB, DSBs are likely to occur. Consequently, the inhibition of PARP1 in HR-deficient cells may prevent stalled replication forks from

restarting, potentially inducing cancer cell death. This concept gains support from observations that PARP inhibition leads to synthetic lethality in tumours with HR or fork stabilization defects. Furthermore, this mechanism could explain the heightened toxicity observed with PARPi treatment compared to the genetic knockout of PARP1. The physical presence of inhibited PARP likely contributes to this toxicity, and steric disruptions caused by PARP trapping may play a role. Changes in the binding affinity (kD) between DNA and PARP1 may also account for the varying effectiveness of different PARPi.

The complexity of the specific DSB structure resulting from replication forks ultimately determines the repair pathway employed. Generally, non-homologous end joining (NHEJ) repairs simpler DSB break structures, while homologous recombination (HR) is necessary for more intricate or complex ones. These structures are categorised as 1-end, 2-end, and 4-end. When a PARPi binds to this already complex structure, additional steric hindrance occurs, rendering repair highly reliant on HR rather than NHEJ. Consequently, in HR-deficient cells, toxicity increases as reliance on NHEJ contributes to heightened genomic instability.

The preclinical studies that led to the approval of PARPi also uncovered a discrepancy between the inhibitory potency of different PARPi and their cytotoxic potentials. This suggests that another distinct mechanism of action must be at play, beyond the catalytic inhibition of PARP1. Following this, early work showed that PARPi treatment causes an increase in the chromatin-bound fraction of PARP1 following treatment with a range of DNA damaging agents. This effect became commonly referred to as PARP trapping. PARP trapping refers to the persistence of the physical interaction between PARPi-PARP1-DNA complexes. PARP1 becomes “locked” onto damage sites, preventing DNA repair, replication, and transcription, as well as adding to the complexity of the break site, leading to cell death. Hence, PARP trapping has been proposed as a key mechanism in contributing to PARPi cytotoxicity.

Notably, the PARP-trapping ability of different PARPi correlates with their toxicity in cell lines: Talazoparib > Niraparib > Olaparib = Rucaparib > Veliparib. Moreover, there is clinical evidence supporting the correlation between PARP trapping and therapeutic response to these drugs, with veliparib (a weakly/non-trapping PARPi) showing a 20% response rate in clinical trials, compared to olaparib for example where the response rate is ~40% [34][35]. However, there is currently a lack of understanding in the mechanisms linking PARP activity, trapping, and clinical toxicity.

The vast range of work done until now, and the controversy surrounding PARP trapping highlights the ongoing debate over what PARP trapping actually is, how it is modulated, and what its effects on cytotoxicity really are.

1.2.5 Cell cycle dependency

The clinical activity of PARPi is heavily reliant upon the failure to repair complex DNA damage using HR. This repair can occur during the late S/G2 phase of the cell cycle exclusively, when a sister chromatid is available to act as a repair template. PARPi are thought to induce DNA DSBs progressively over multiple cell cycles in *BRCA1/2*-deficient cells, which fail to slow down cell cycle progression to repair damage [36]. This may explain why mitotic progression promotes PARP-inhibitor-induced cell death in these cells, and supports the rationale behind combination therapies such as Wee1 inhibitors [37,38]. Thus, it is important to determine how basal cell cycle state and cell cycle progression following treatment impacts the response to PARP inhibition. This is even more paramount in HGSC, where the universal loss of p53 function produces profound effects on cell cycle control [39].

In summary, various mechanisms of action for PARPi have been suggested. However, conflicting evidence exists for each of these mechanisms, and no single explanation fully accounts for the effects of PARPi. Whether it involves a combination of additive effects or is dominated by specific mechanisms remains an area for further investigation. Whilst it is widely accepted that PARPi inhibits DNA damage response through homologous recombination (HR) and causes replication fork collapse via PARP trapping, other mechanisms likely contribute to its toxicity. Understanding the role of each mechanism is important for the future development and optimization of PARPi therapies. Additionally, insights into these processes may shed light on potential mechanisms of resistance to PARPi, some of which are discussed below.

1.2.6 Suggested mechanisms of resistance to PARPi

The mechanisms driving resistance are currently poorly understood. Several mechanisms have been proposed, including loss of PARP1, restoration of HR repair, mitigation of replicative stress, increased drug efflux, and the mechanism this study will focus on mainly here, decreased PARP trapping (**Figure 5**) [26,40,41]. For a more complete review of potential mechanisms of PARPi resistance see [25,42].

1.2.6.1 Restoration of HR activity

If HR activity is restored in previously-HRD cells, DSBs can be repaired much more readily, decreasing overall genomic instability and hence, toxicity. This restoration can occur via two main mechanisms, somatic mutation reversion or loss of epigenetic silencing (**Figure 5A**).

1.2.6.2 Reversion mutations

The reactivation of *BRCA1* or *BRCA2* through reversion mutations can restore HR activity in *BRCA1/2* mutated cancers. This is most often a secondary mutation in *BRCA1/2* that either restores the open

reading frame or splices out the mutated exon, creating a truncated and partially functional protein. Such reversions have been found both in patients with somatic or germ-line mutations in ovarian cancer and breast cancer, reducing PFS under rucaparib treatment from 9 to 1.8 months. This offers clinical evidence that these mutations somewhat contribute towards PARPi resistance [43]. However, these secondary mutations are rare, occurring in ~25% of *BRCA1/2* mutated tumours, which only represent 20% of total HGSC cases. Moreover, this mechanism does not contribute towards explaining the rise of PARPi resistance patients lacking *BRCA1/2* mutations.

1.2.6.3 *Suppression of NHEJ*

The toxic effects of HRD experienced in *BRCA1/2* mutated cells can be somewhat overcome by the suppression of error-prone repair pathways such as NHEJ. This can occur through the disruption of genes such as *53BP1* that regulates NHEJ, leading to the rescue of HR in *BRCA1* deficient patients, thus reducing the effects of PARPi therapy [44].

1.2.6.4 *Increased drug efflux*

Like many chemotherapy drugs, PARPi seem to be subject to the challenge of an increased rate of efflux pump activity upon treatment (**Figure 5B**) [45]. Specifically, *ABCB1a/b* are thought to be involved in the case of olaparib, whereby gene expression is increased up to 85-fold in olaparib resistant cell lines. Similarly, *ABCB1a/b* expression has been found to correlate with rucaparib resistance, a phenotype that was reversed upon treatment with *ABCB1a/b* inhibitors such as verapamil or elacridar [46]. However, contrary to this, simply overexpressing *ABCB1a/b* does not cause resistance to all PARPi, suggesting it is not the sole driver of PARPi resistance. Moreover, in the context of a patient tumour the complex and heterogeneous nature of the tumour micro-environment is likely to modulate drug influx/efflux, and thus resistance, in a case-by-case manner.

1.2.6.5 *Stabilization of stalled replication forks*

Preventing the collapse of replication forks is crucial to avoid double-strand breaks (DSBs). In cell line experiments, it has been observed that stabilizing replication forks may lead to resistance against PARPi (**Figure 5C**). This resistance mechanism is associated with the PAX transcription activation domain interacting protein (PTIP), which prevents fork stalling in *BRCA*-deficient cells treated with PARPi. Notably, the loss of PTIP1 alone is sufficient to confer PARPi resistance in *BRCA1/2* deficient cells [47].

1.2.6.6 *Downregulation of PARG protein expression*

PARYlation is a reversible post-translational modification, and Poly (ADP-ribose) glycohydrolase (PARG) serves as the primary enzyme responsible for degrading PAR. In terms of modulating PARP1 activity, PARG operates in the same direction as PARP inhibitors (PARPi), aiming to reduce PARYlation rates and halt DNA damage repair. Specifically, PARG hydrolyses the ribose-ribose bond to produce

ADP-ribose. Loss of PARG in PARPi treated cells results in an accumulation of PAR, reducing PARP trapping [48]. Notably, PARG depletion is commonly observed in PARPi-resistant models. Recent research has revealed more intricate roles for PARG inhibitors (PARGi), suggesting that they are not merely the opposite of PARPi. Instead, sensitivity to these two types of drugs appears to be mutually exclusive [49]. Consequently, PARGi may hold therapeutic potential as a complementary treatment alongside PARPi for ovarian cancer.

1.2.6.7 *Reduction in PARP trapping/retention*

A genome-wide CRISPR-Cas9 screen identified one mutation of PARP1 (R591C) that has since been found in patients with *de novo* PARPi resistance and has been found to reduce the PARP trapping ability of the PARPi talazoparib [50]. This suggests that a reduction in PARP trapping could contribute to clinical PARPi resistance (**Figure 5D**). A subsequent study using hydrogen/deuterium exchange-mass spectrometry (HXMS) discovered the specific structural interdomain interaction between PARP1 Zn1-WGR-HD domains that is disrupted by this specific R591C mutation [51]. This provides a mechanistic rationale for the only currently known clinical mutation that reduces PARP trapping, highlighting how PARP inhibitors engage the PARP1 allosteric pathway to induce trapping.

As outlined here, PARPi resistance involves multiple potential mechanisms and exists as a multi-faceted challenge. Considering this, the therapeutic strategy utilised in the clinic should be shaped accordingly. PARP inhibition exerts selective pressure on highly mutagenic tumour cells, leading to the emergence of resistant clones that contribute to relapse. However, a theoretical approach involves combining inhibitors targeting different pathways simultaneously. Doing so would ensure constant stress is applied to cancer cells while mitigating the selective pressure from PARPi alone. Furthermore, understanding each resistance mechanism more completely, and identifying genetic biomarkers allows us to tailor secondary inhibitors to individual patients, advancing personalised medicine. This work addresses a clinical area of huge unmet need, as there are currently no treatment options for patients who have progressed to the stage of PARPi resistance. Furthermore, emerging evidence suggests that these patients have reduced subsequent response to platinum therapy, negating some of the positive effects of PARPi therapy [52].

1.3 A closer look at PARP trapping

PARP trapping describes the physical interaction of DNA-PARP1-PARPi complex that persists at DNA damage sites upon PARPi binding. An important factor to consider in this context is that different PARPi drugs have been repeatedly shown to have differing PARP trapping potencies, through a range of assays such as chromatin immunoprecipitation and mass spectrometry. For example, talazoparib exhibits the strongest PARP trapping potency of the currently approved PARP estimated at 100-1000 fold stronger than olaparib and rucaparib, which are considered moderate trappers. Veliparib is a further 10-fold weaker in terms of trapping ability [41].

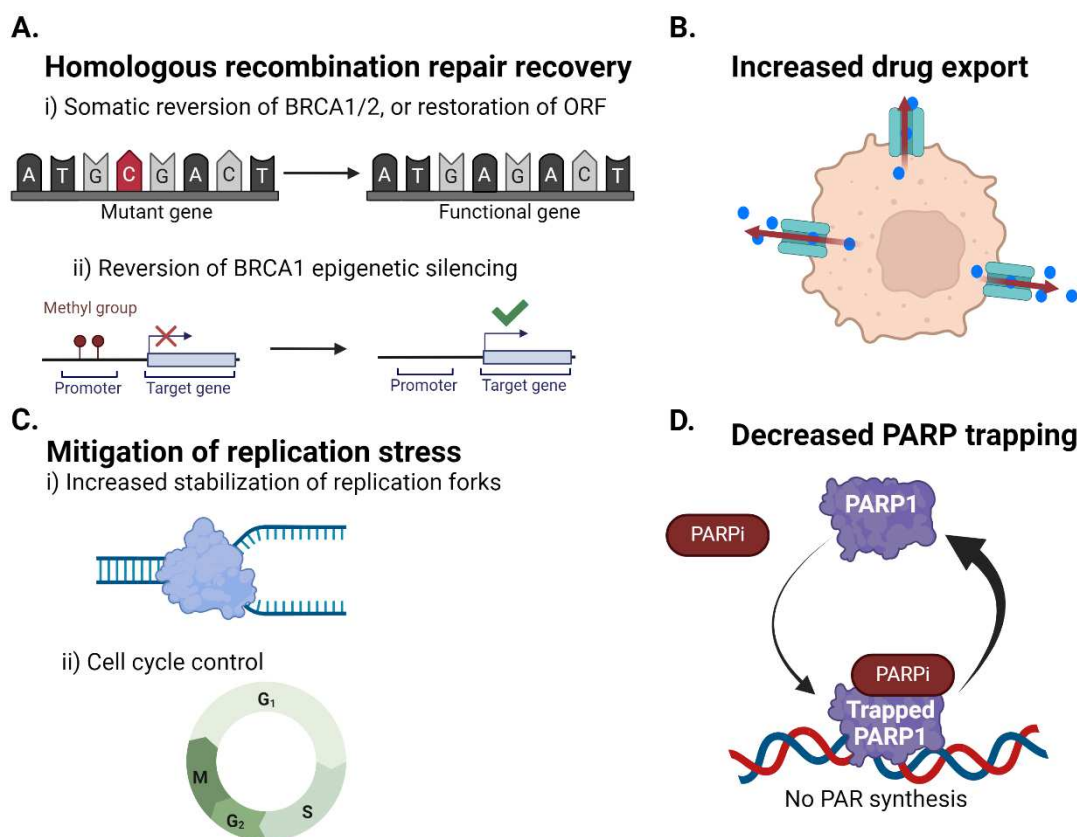


Figure 5: Some of the proposed mechanisms of resistance to PARPi. **A:** The restoration of HR through either reversion mutations or epigenetic modulation of BRCA1/2 leading to expression of the functional gene. **B:** Increased drug efflux may reduce the cytotoxicity of PARPi. **C:** Replication stress may be overcome through increased stabilization of replication forks, or cell cycle control. **D:** A decrease in the chromatin-bound fraction of PARP1, termed PARP trapping, may reduce the downstream cytotoxic effects of PARPi. Figure created in BioRender.

Two distinct mechanisms have been suggested to describe the increased association of PARP1 to DNA damage sites. The first of these mechanisms suggests PARPi allosterically control the DNA binding ability of PARP1. Work by Zandrasashbili *et al.* classified PARPi into three groups based on the nature of these allosteric effects, suggesting some PARPi elicit mild pro-trapping activity, whilst others have no ability to trap PARP1 [51]. For some PARPi, they even suggest that they stimulate the release of

PARP1 from damage sites. This is contrary to the beliefs of many in the field, who currently support a mechanism by which PARP1 catalytic inhibition (blocking NAD⁺ substrate binding) results in the suppression of auto-PARylation and thus drives the prolonged association of PARP1-DNA complexes, with no allosteric modulation involved at all.

Although the mechanisms that explain the increased association of PARP1 to DNA damage sites are unclear, two have been proposed. Firstly, via catalytic inhibition by binding directly to the NAD⁺ binding pocket of PARP1 (the common binding site of all currently approved PARPi) results in the suppression of auto-PARylation and thus drives the prolonged association of PARP1-DNA complexes, with no allosteric modulation involved at all. The second form of inhibition comes from the impact of PARPi molecules occupying the NAD binding site on the helical domain (HD), a vital allosteric regulatory domain of PARP1. PARPi have differing impacts on the HD, with some causing a destabilization of specific regions, and others having no impact. Those that destabilise the HD were found to increase PARP1 affinity for DNA, inducing further PARP trapping. In contrast, PARPi that stabilise the HD decreased this affinity for DNA.

One potential reason that the mechanism of PARP trapping remains elusive is the lack of methodologies for assaying PARP trapping. Traditionally, to assess PARP trapping, cells would be treated with PARPi and then fixed and pooled for chromatin immunoprecipitation (ChIP) assays. The protein is then extracted and chromatin-bound proteins separated through a series of treatments with detergents. The chromatin-bound fraction of protein is then separated on a gel, probing with an anti-PARP1 antibody. This method provides a fixed time-point, pooled overview of PARP trapping in a population and thus offers a very low throughput. Proteomics has been used more recently as well as fluorescence anisotropy using proteins in solution [26,53]. Some microscopy assays have also been performed, expressing fluorescently labelled PARP1 such as PARP1-GFP constructs, and then inducing micro-irradiation to cause DNA damage at a spatially controlled location. The persistence of PARP1-GFP signal is then assessed, as a readout of PARP trapping. However, these methods often fail to offer live-cell read-outs with high throughput and single-cell resolution. A more recent study performed fluorescence recovery after photobleaching (FRAP) and single-molecule imaging for single particle tracking (SPT). Although these methods offer live cell imaging with single molecule resolution, this comes at the trade-off of very low throughput, often measuring ~30 single molecules. However, the approach does have the benefit of using CRISPR-Cas9 knock-in for endogenous labelling of PARP1, but the HaloTag labelled fraction of PARP1 assessed in their assay is much lower than the endogenous expression of wild-type PARP1 [54].

Building upon this work, *Kanev et al.* employed FRAP with mathematical modelling to define two parameters of PARPi induced PARP1-chromatin retention [55]. Similarly, they performed micro irradiation experiments assessing FRAP of PARP1-GFP. Firstly, they defined PARP1 retention as “the prolonged dissolution of PARP1-EGFP damage foci in cells subjected to PARPi”, or more simply the half-life of PARP1-chromatin complex. They described PARP trapping as a decrease in the exchange rate or turnover of PARP1 at damage sites, like an increased dwell time. Using these two definitions and defining some parameters to compare untreated to treated conditions, they performed complex mathematical modelling to assess the kinetics of PARP1 at damage sites in live cells. They found that overall PARP1 retention at damage sites is controlled by catalytic inhibition and allosteric trapping, in a completely independent manner.

It is important to note, however, that the throughput of this method is also very low. Furthermore, this method only offers information about damage sites created in this very controlled and specific manner for irradiation. Hence, it is a leap to suggest that such kinetics would certainly be present at other DNA damage break types, through endogenously created lesions. Although they mention this approach may help to study sensitive vs resistant populations, they do not do so in their study. Nevertheless, this study takes a step forward in terms of studying PARP1 trapping and retention in live-cell models.

One study applied fluorescence polarization imaging in live-cells and suggested that talazoparib and niraparib do not reduce PARP1 turnover at damage sites, but instead postulated that the prolonged association of PARP1-DNA complexes is due to rapidly repeating binding and dissociation cycles of PARP1 molecules that are catalytically inactive [56]. More recently, another study has built upon this work, using single-molecule FRET microscopy in purified proteins [57]. This work supported the paradigms proposed in the polarization studies, proposing a two-step mechanism where catalytic inhibition of PARP1 occurs first, followed by allosteric modulation, in order to retain PARP1 at DNA damage sites.

Despite the interest in the field, and the clear advancements in recent years in terms of modelling of PARP1 allosteric/catalytic inhibition in purified protein and live cell experiments, a unified mechanism explaining PARPi induced changes in PARP1 dynamics is currently lacking. Hence, explaining the mechanisms driving cytotoxicity remains difficult, blocking progression to developing improved PARPi and overcoming potential PARPi resistance.

In light of this, there is currently an unmet need in this area for an assay that can fulfil these criteria, and further complement the existing tools in this field. Hence, my project aims to develop such an

assay, utilising Förster resonance energy transfer (FRET), a microscopy method that allows the study of protein-protein interactions in live cells.

Inspiration was drawn from a previous study that developed a FRET-based fluorescent sensor to assess structural dynamics and allosteric regulation of PARP1 in response to DNA damage [58]. This study used purified PARP1 proteins in solution, genetically labelled with GFP and mCherryFP at the C and N-terminus respectively, to form a FRET pair (**Figure 6A**). The sensor operates on the rationale that PARP1 exists in a more linear, relaxed state when not bound to DNA damage sites. Upon binding to DNA damage sites, the protein undergoes a structural compaction, and this conformational change is enough to bring the N and C terminus of the protein within sufficient proximity for FRET to occur, and/or for sufficient change in orientation of the donor and acceptor to modulate the FRET signal. In the dual-labelled PARP1, this is sufficient to induce a FRET signal, which can be modulated by the introduction of DNA with double strand breaks into the solution (**Figure 6B**). This model provides a method for real-time monitoring of PARP1 protein structure, offering a temporal resolution that many other assays cannot achieve. The authors then used this sensor to assess changes in PARP1-DNA binding in the context of multiple perturbations, such as DNA binding mutations, allosteric mutations, and treatment with PARPi olaparib. However, they did not introduce their FRET biosensor into live-cells, likely due to the complexity of performing protein-labelling in live cells, especially to label the endogenous PARP1 protein whilst maintaining functionality. Therefore, the opportunity to develop this FRET biosensor in live cells seemed logical if appropriate labelling methodologies were available, which is the main aim of this project.

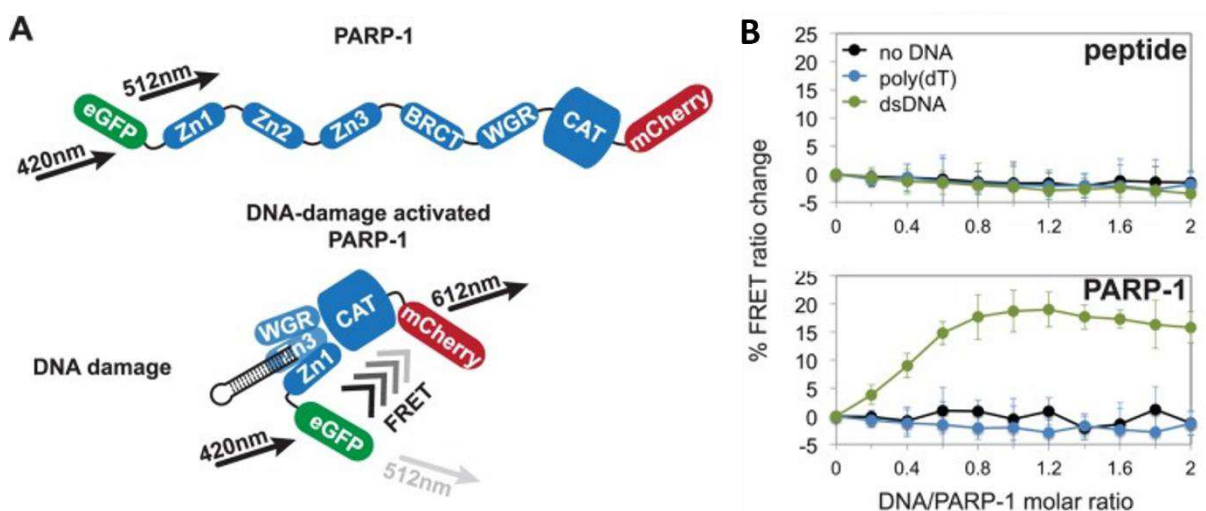


Figure 6: A FRET-based sensor to detect PARP1 global compaction in purified protein. **A:** Schematic model of dual-tagged PARP1 with donor and acceptor (EGFP and mCherryFP) fluorophores. The proximity of the N- and C-termini produces a FRET signal that allows assessment of the global conformational change of PARP1. **B:** Specificity of PARP1 biosensor compared to a peptide consisting of just GFP and mCherryFP without the functional domains of PARP1. dsDNA, poly(dT) or no DNA was titrated at 0.2 μ M steps with 1 μ M of PARP1 sensor. The emission ratio was normalised to zero in the absence of DNA and the percentage FRET ratio is plotted, based on a spectral ratiometric FRET readout. Taken from [58].

1.4 Microscopy

The optical microscope has become a fundamental instrument for biological and medical research following centuries of innovation. This began with the invention of the first compound microscope in the late 16th century, created by Hans and Zacharias Janssen. This early instrument consisted of a convex objective lens and a concave eyepiece lens mounted in a tube, allowing for the magnification of small objects. In the early 17th century, Robert Hooke and Antione van Leeuwenhoek developed improved microscopes, enabling the first visualization of cells and microorganisms [59,60]. These early discoveries laid the foundation for cell biology and shaped the way microscopy is utilised in the field today.

The development of achromatic lenses in the 18th century by Joseph Jackson Lister led to significant improvements in image clarity and resolution, enabling researchers to discern finer details within biological specimens. The introduction of transmitted light microscopy approaches such as phase contrast microscopy in the early 20th century by physicist Frits Zernike further enhanced the ability to visualise transparent structures. This allowed the imaging of living cells, without the need for staining or fixation, allowing for label-free imaging. However, the advancements in fluorescence microscopy have been most valuable for studying cell physiology.

1.4.1 Photophysics of fluorescence microscopy

Luminescence describes the emission of light from a substance due to electronically excited states. Fluorescence is a form of luminescence, defined as the emission of light when an optically excited molecular electron returns from an electronically excited state to the ground state, releasing energy as a photon. While some biological tissues and molecules naturally exhibit autofluorescence, most cellular molecules require labelling with fluorochromes or fluorophores for visualization.

Jablonski diagrams provide a graphical representation of the events occurring from light absorption to the emission of a photon, as shown in **Figure 7**. The singlet ground, first and second electronic states are shown by S_0 , S_1 , and S_2 respectively. The electrons within a fluorophore can exist at several vibrational energies in each of these states, depicted by 0,1,2 etc. When a fluorochrome absorbs photons, its electrons are excited to the first excited singlet state (S_1) where it is paired with a second electron of opposite spin in the ground state orbital. Therefore, the return of this electron to the ground-state (S_0) orbital through spontaneous emission is 'spin-allowed' and occurs rapidly. This results in the emission of a photon of lower energy and longer wavelength than that of the excitation photons, occurring mainly due to the loss of energy via vibrational relaxation but can also be due to

solvent rearrangement. This phenomenon was first observed by British physicist George Stokes and hence is named the Stokes shift [61].

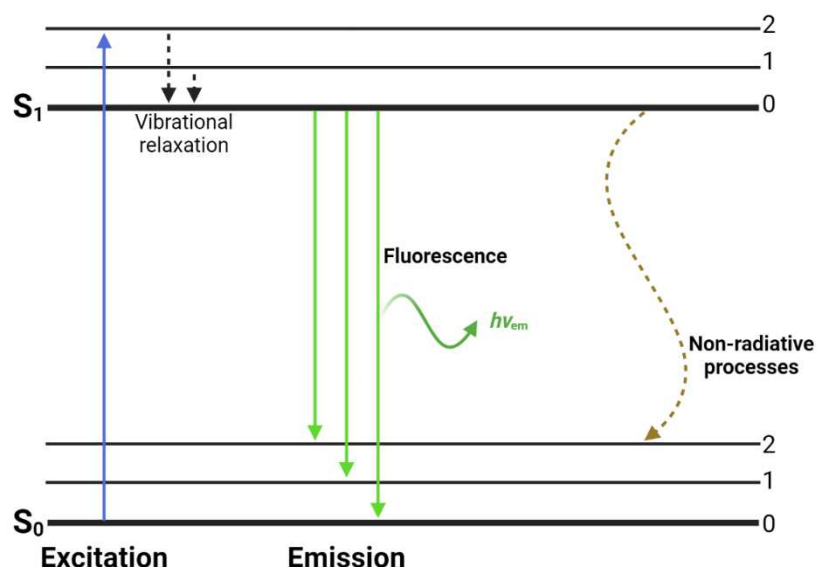


Figure 7: A simple Jablonski diagram depicting the general concept of fluorescence. Upon excitation of a fluorophore by the absorption of light, the electrons are excited from the singlet ground state (S_0) to the excited state (S_1). Some energy is lost through vibrational relaxation, before the excited electron returns to the more stable ground state, emitting a photon in a process termed fluorescence. Note, non-radiative processes can also occur. Figure created in BioRender.

Two key characteristics of a fluorophore are its quantum yield and fluorescence lifetime. Quantum yield measures the number of photons emitted relative to those absorbed, with higher yields indicating brighter emission. Fluorescence lifetime refers to the average time a fluorophore remains in its excited state before returning to the ground state. In the absence of non-radiative processes this is known as the intrinsic, or natural lifetime. The study of fluorophores relies on the development of instrumentation, which has been ongoing for ~100 years.

The first recorded fluorescence microscope, developed by August Köhler in 1904, used ultraviolet illumination with a cadmium arc lamp as the light source. Subsequently, in 1911 Oskar Heimstädt created the first fluorescence microscope used to image autofluorescence in organic and inorganic compounds [62]. These early fluorescence microscopes encountered various limitations including inadequate light source power to excite fluorochromes at desirable rates and the challenge of effectively separating the fluorescence signal from the excitation light. A major advancement occurred in 1929 with the invention of the epifluorescence microscope by Philipp Ellinger and August Hirt (**Figure 8**) [63]. In this configuration, both the illumination and detection occur from a single side of the sample, enhancing the signal-to-noise ratio by allowing only reflected excitatory and emitted light

to reach the objective lens. The later invention of dichroic mirrors in 1967 would see this configuration become the standard for fluorescence microscopes [64].

During the 1930s, Max Haitinger introduced 'secondary fluorescence,' using fluorescent dyes to stain samples and enhance the visibility of weakly autofluorescent biological specimens [65]. Haitinger later coined the term 'fluorochrome' for such fluorescent stains. Subsequently, in the early 1940s, Albert Coons pioneered the labelling of antibodies with fluorescent dyes, enabling the specific labelling of proteins and subcellular structures with unprecedented contrast and resolution [66]. Since then, this technique has become a standard practice in cell biology, biomedical research, and clinical diagnostics for fixed samples, including tissues and individual cells. At a similar time, the advent of lasers in the 1960s offered a solution to the persisting problem of limited excitation power. Innovations by Townes, Schawlow and Maiman led to lasers offering high spatial and temporal coherence, facilitating focused, diffraction-limited monochromatic and coherent beams with high local irradiance [67,68]. This also improved signal separation using specialised interference filters such as dichroic mirrors, which selectively transmit light in specific wavelength ranges while reflecting others. However, due to the lack of labelling methodologies, the application of fluorescence microscopy in biological studies remained rare during this time.

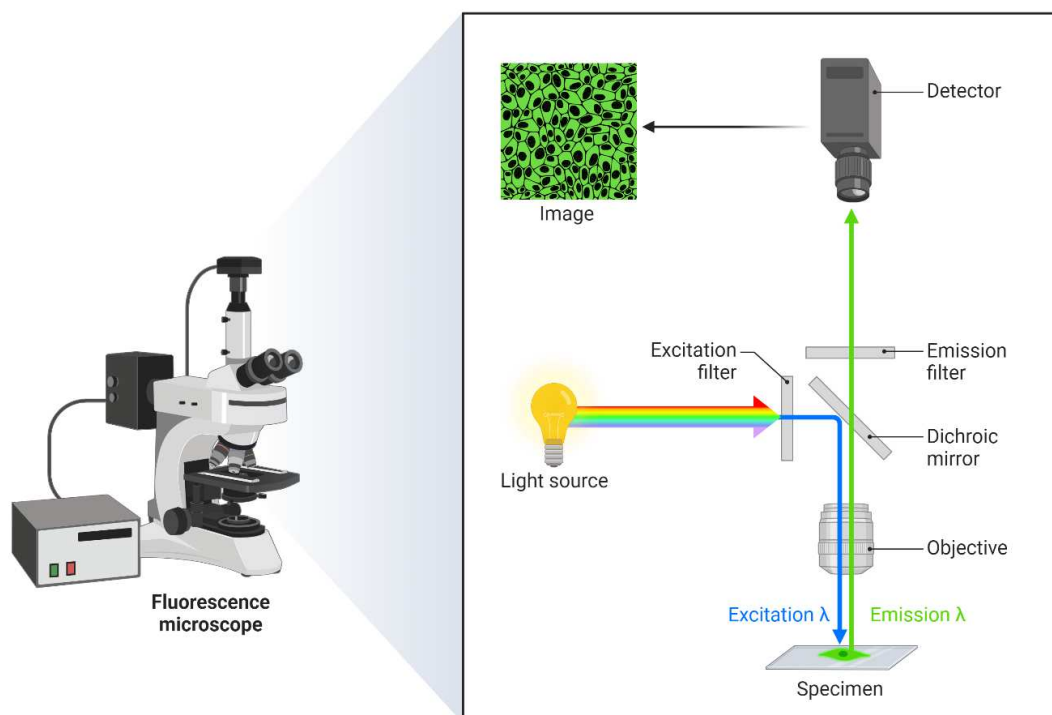


Figure 8: Fluorescence microscopy using an inverted epi-illumination microscope configuration. An excitation filter is placed between the lamp and the sample, narrowing the wavelength range of light reaching the sample. This allows flexibility to excite specific fluorochromes through filter selectivity, whilst minimizing unwanted excitation. The emitted light has a longer wavelength than the excitation filter. Hence, placing an emission filter between the sample and the detector effectively blocks the excitation light, preventing interference with the final image. Figure created in BioRender.

1.4.2 Fluorescent proteins, structural and spectral properties

The 1960s marked a pivotal moment in the field of bioimaging, transforming the applicability of fluorescence microscopy to cell biology with the discovery, isolation, and subsequent sequencing of green fluorescent protein (GFP). Originally found in the jellyfish *Aequorea Victoria*, GFP has become one of the most widely studied and utilised proteins in cell biology. GFP was later used as a fluorescent marker in the 1990s, leading to Chalfie, Shimomura and Tsien being awarded the Nobel Prize for its discovery and development [69–72]. The 26.9 kDa fluorescent protein consists of a β -barrel structure containing a triplet of amino acids Ser65, Tyr66 and Gly67 that form the chromophore moiety of the fluorescent protein (**Figure 9**).

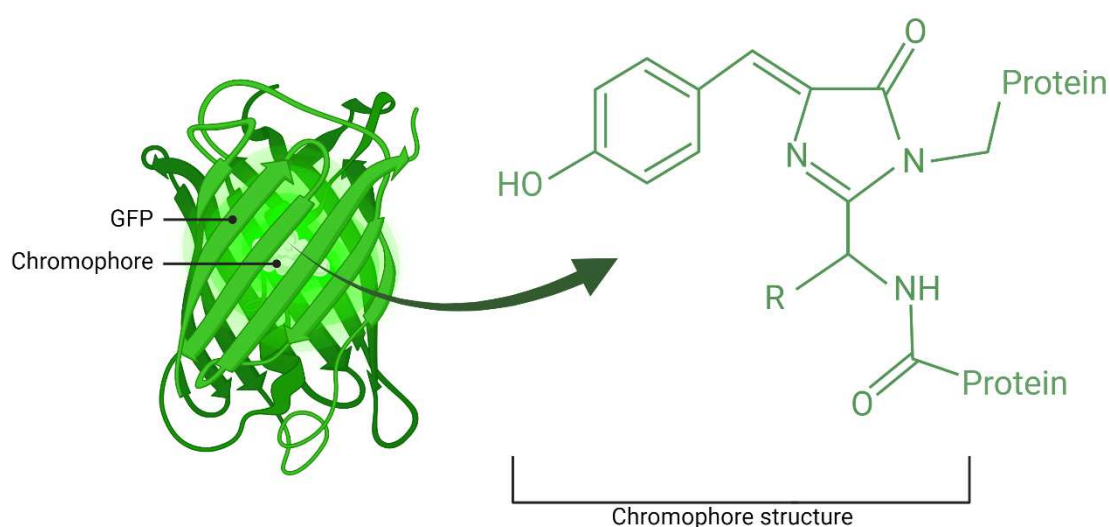


Figure 9: Green fluorescent protein (GFP) β -barrel protein structure. The chromophore structure consists of an α -helix structure containing three amino acids (Ser-Tyr-Gly), positioned in the centre of the β -barrel protein. This forms an imidazolidine ring with conjugated bonds and a planar structure. Figure created in BioRender.

Since then, hundreds of novel fluorescent proteins (FPs) have been derived from wild-type GFP, utilising directed and random mutagenesis techniques, primarily targeting the imidazolidine ring structure. These efforts aimed to develop FPs with specific desirable traits, such as increased quantum yield and specific emission wavelengths. Subsequently, a plethora of FPs is now available for applications in biological imaging, including multiplexed labelling, protein localization, and real-time visualization of dynamic cellular processes. These advancements in fluorescent protein engineering facilitate the application of many advanced fluorescence microscopy techniques.

1.4.3 Förster Resonance Energy Transfer (FRET)

One such advanced technique, Förster Resonance Energy Transfer (FRET), capitalises on the unique photophysical properties of fluorescent proteins, where a resonance energy transfer can occur from a donor fluorochrome to an acceptor. Resonance energy transfer describes a process whereby the

energy from a donor fluorochrome is transferred to a ground-state acceptor fluorochrome through a non-radiative mechanism (no photon is emitted) (**Figure 10A**). The theoretical process of this phenomenon was first described by Theodor Förster in the 1940s, providing the foundations for FRET microscopy [73,74]. For FRET to occur, the donor and acceptor molecules must be within roughly 10 nm of each other, and their electronic energy levels must be well matched (**Figure 10B**). Consequently, FRET is highly distance dependent and can be used to assess molecular interactions or proximity far beyond the resolution limits of traditional light microscopy approaches. Additional requirements must also be fulfilled, including sufficient spectral overlap between their emission and excitation spectra respectively (**Figure 10C**), as well as weak long-range dipole-dipole coupling between the donor and acceptor (**Figure 10D**). Notably, the acceptor does not need to be fluorescent.

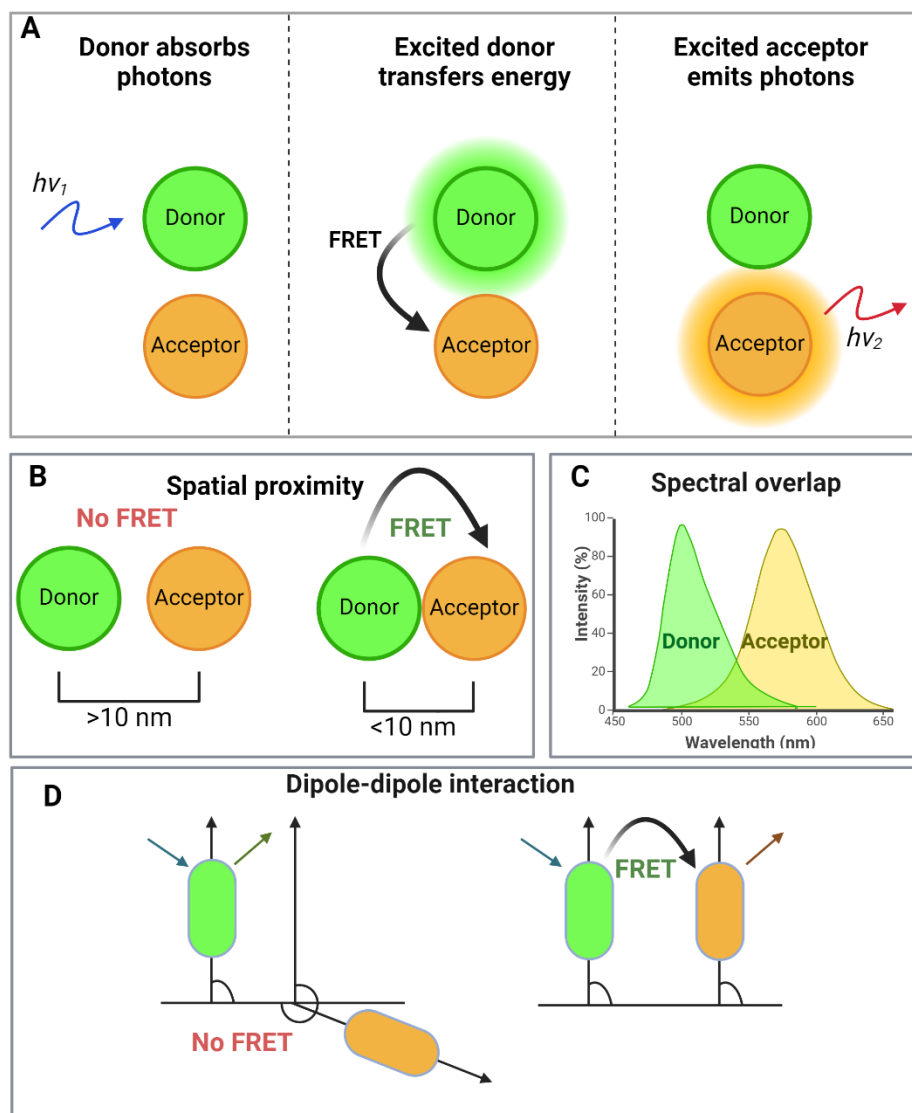


Figure 10: The process of FRET and criteria required for its occurrence. **A:** Sequential steps of FRET. **B:** Spatial proximity requirement for FRET, donor and acceptor must be within 1-10nm of each other. **C:** Donor and acceptor molecules must have sufficient spectral overlap between the emission spectrum of the donor and excitation spectrum of the acceptor. **D:** Dipole-dipole alignment of electrons transferring energy is required. Figure created in BioRender.

If only the donor is excited and the acceptor is too far (>10 nm) from it, the donor will be exclusively detected. However, when the acceptor molecule is closer to the excited donor, FRET can occur and hence both the intensity and fluorescence lifetime of the donor will be decreased and the acceptor emission can be detected. Therefore, one method to quantify FRET involves collecting and calculating the ratio of donor: acceptor fluorescence intensity, termed spectral ratiometric FRET.

The Förster theory of FRET states that the efficiency of the energy transfer (E_{FRET}) is dependent on the physical distance between the donor and acceptor molecules, the spectral overlap of the donor emission and acceptor absorption spectrum, and the relative orientation of the donor and acceptor dipole moments. However, E_{FRET} scales to the inverse 6th power of the donor-to-acceptor distance (r) as described by *equation 1*:

$$E_{FRET} = \frac{1}{1 + (\frac{r}{R_0})^6}$$

Which can be rearranged to *equation 2*:

$$r = R_0 \left(\frac{1}{E_{FRET}} - 1 \right)^{1/6}$$

The Förster radius (R_0) describes the distance at which 50% FRET efficiency occurs and can be calculated theoretically from the spectroscopic and mutual dipole orientational parameters of the specific donor and acceptor molecules in question (**Figure 11**). Hence, R_0 defines the length scale of the interactions assessable by FRET in each specific donor-acceptor pair. Generally, the effective range of R_0 is 3-8 nm, corresponding to the 5%-95% range of E_{FRET} where changes can still be detected. However, experimentally this range may be smaller due to noise. Generally, for fluorescent proteins, R_0 is around 5 nm. At such small distances, the occurrence of FRET offers strong experimental evidence that the donor and acceptor associate closely. However, this sensitivity also limits the approach, making it difficult to study multiple or very large proteins.

Furthermore, it is worth noting that due to the requirement of spectral overlap between the donor and acceptor molecules, the FRET signal is dramatically affected by donor emission spilling into the acceptor channel and by any excitation of the acceptor molecules caused by the donor excitation wavelength (spectral bleed-through). Hence, careful consideration is required when selecting a donor-acceptor FRET pair. Moreover, the FRET signal in the acceptor channel should be corrected for both biological and instrument-based noise and/or autofluorescence that will otherwise affect the measured FRET signals.

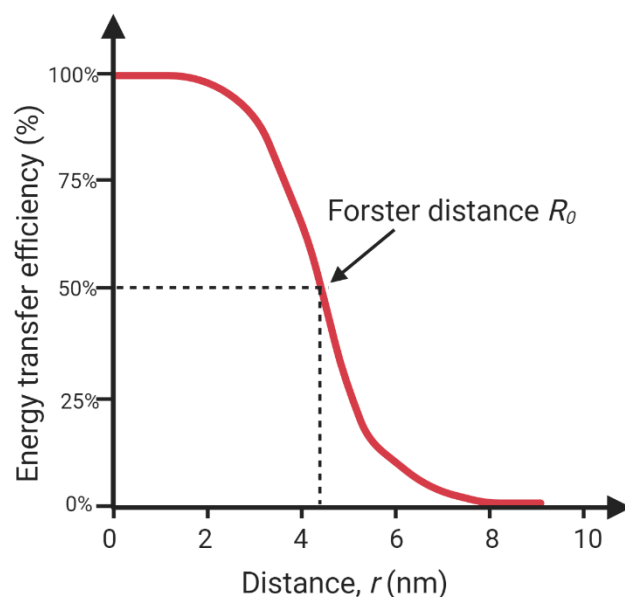


Figure 11: Förster radius (R_0) describing distance and energy transfer efficiency. R_0 represents the distance between the donor and acceptor molecules at which the FRET efficiency is 50%. This value varies for each specific donor-acceptor pair. Figure created in BioRender.

1.4.4 Applying FRET to cell biology

Due to the photophysical processes underlying FRET, the technique is often described as a “spectroscopic ruler,” making it particularly valuable in cell biology for assessing protein-protein interactions across various biological contexts, and addresses questions that are otherwise challenging to explore. FRET proves to be a highly effective technique when used in conjunction with *in vitro* expression of proteins of interest (POI) labelled with fluorescent proteins (FPs) or other fluorophores, introduced through multiple approaches such as plasmid overexpression or CRISPR-Cas9 knock-in [75].

FRET has been used to study protein-protein interactions in diverse cellular contexts such as signal transduction, conformational changes in proteins (e.g., enzyme activation, Ca^{2+} signalling), nucleic acid studies and gene expression characterization. For a comprehensive review of FRET applications in cell biology, see [76]. These diverse applications of FRET are heavily dependent on the availability and proper selection of suitable fluorescent proteins and dyes.

Various fluorescent proteins and dyes have been used for FRET, with common pairs and their properties summarised in **Table 2**. While FRET has traditionally employed FPs, organic dyes and quantum dots are also viable candidates for donor and acceptor molecules, although FPs tend to have lower emission intensity compared to these alternatives. Generally, these are used as single labels of

individual proteins (inter-molecular FRET) or by dual labelling the same protein to study structural changes (intramolecular FRET).

Donor	Acceptor	Donor excitation λ (nm)	Acceptor Emission λ (nm)	Donor QY	Acceptor ϵ	Förster radius (nm)
CFP	YFP	433	526	0.4	77,000	~5
EGFP	mCherryFP	507	610	0.6	72,000	5.1
VenusFP	mPlumFP	528	649	0.57	41,000	5.2

Table 2: Exemplar fluorescent protein FRET pairs spanning the visible light spectrum. Highlighting the differences in quantum yield (QY), efficiency (ϵ), and Förster radius.

The numerous studies that have applied FRET to biological research can be broadly categorised into two classes. The first study the induction of FRET upon protein interaction. In this approach, two distinct proteins are labelled with a donor-acceptor pair, and upon interaction, a FRET signal is generated. When the separation of the two proteins (and thus the bound fluorophores) surpasses the Förster radius, the FRET interaction ceases (**Figure 12A**). This offers an almost binary read-out of protein binding, due to the relationship of FRET efficiency to distance described in *Equation 2*. In such approaches, a flexible linker is often introduced between the POI and the fluorophore, reducing any steric hindrance that may negatively impact the basal functionality of the protein and its binding.

The second approach involves labelling a single protein at two spatially separated sites, allowing for the study of conformational changes in the POI. Upon a biological stimulus, the POI undergoes a conformational change, bringing the donor-acceptor pair within 10 nm of each other, inducing a FRET signal (**Figure 12B**). As the donor and acceptor molecules come closer together, the FRET efficiency will increase. Hence, this makes FRET an extremely powerful tool for detecting and quantifying conformational changes in proteins or other biomolecules, in real-time.

These two examples illustrate the distinction between intramolecular and intermolecular FRET. In the first case, the donor and acceptor fluorochromes are bound to the same molecule, typically a protein. In contrast, intermolecular FRET describes the labelling of two distinct molecules with a donor and acceptor pair. Upon the interaction of these two proteins, the donor and acceptor pair come within the Förster radius and FRET is possible. This subtle difference is worth noting, expanding the applications in which FRET can be applied to biological studies.

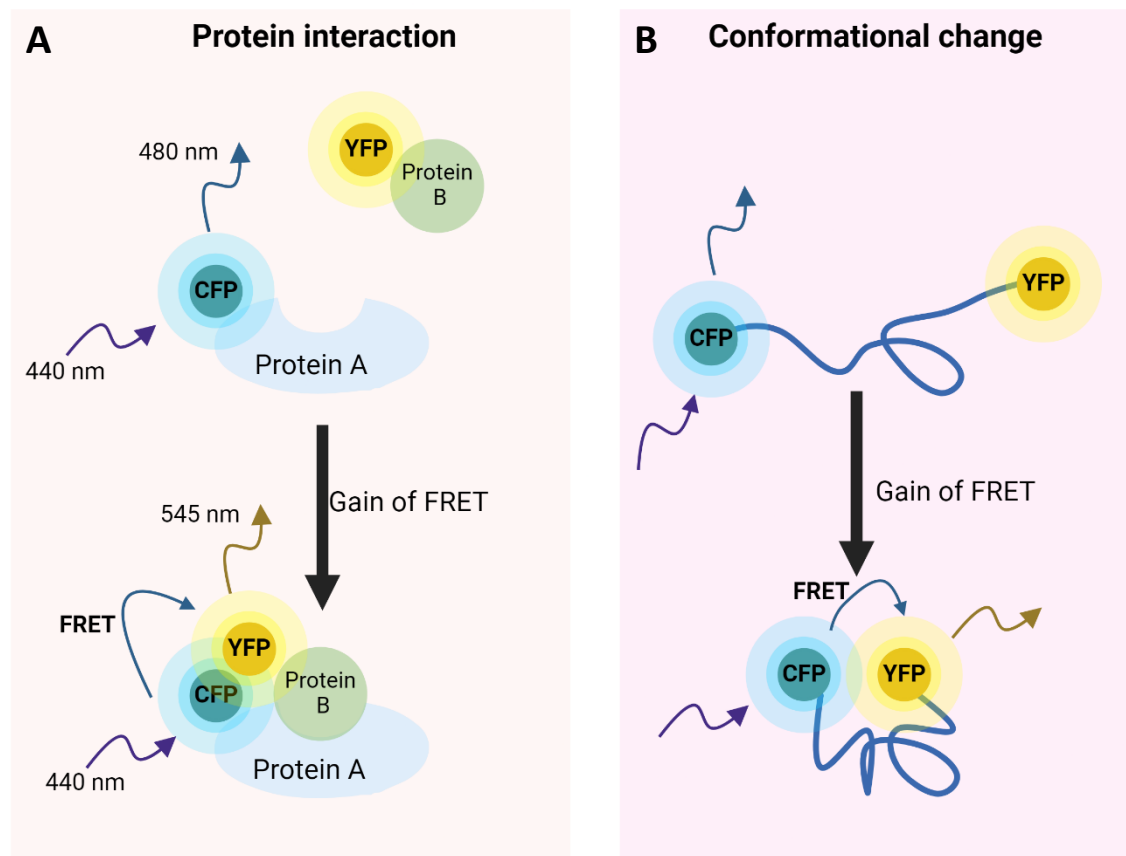


Figure 12: Schematic of FRET induction. **A:** Protein interaction of two proteins, labelled with donor and acceptor fluorophores respectively induces FRET upon interaction as the FRET pair comes within the Förster radius. **B:** Conformational change of a dual-labelled protein induces FRET, bringing the donor and acceptor together from their spatially separated positions in the proteins basal state, upon biological stimulation. Figure created in BioRender.

1.4.5 Fluorescence lifetime imaging microscopy

Fluorescence lifetime imaging microscopy (FLIM) is a powerful technique for studying biological processes by measuring the fluorescence lifetime of probes. This lifetime (average time a fluorophore remains in its excited state) is an intrinsic property of the fluorophore and is largely independent of its concentration. As a result, FLIM provides reliable quantitative measurements, overcoming the limitations of traditional intensity-based fluorescence microscopy, where probe concentrations can vary significantly due to factors such as diffusion, photobleaching, and efflux.

The fluorescence lifetime can change in response to environmental conditions (e.g., pH, temperature) and molecular interactions, including FRET. Hence, FLIM can indirectly measure and quantify FRET when these other variables are controlled for. However, the experimental design and hardware

required to measure fluorescence lifetime are complex and thus there must be sufficient value in this technique to warrant its use.

FLIM offers numerous benefits over ratiometric spectral FRET measurements. Firstly, FLIM is independent of concentration changes, or in the cellular context, the variable expression levels of a protein. This results in a methodology that is less sensitive to excitation and detection efficiencies, and the impact of scattering and sample absorption that are often troublesome in biological samples. Furthermore, the need to only measure donor fluorescence means the approach is less compromised by spectral crosstalk than ratiometric spectral methods. This also means that non-fluorescent acceptor molecules can be used in a FRET pair.

Conversely, FLIM produces data that necessitate more complex analysis than ratiometric FRET, and acquisition times may be longer depending on the specific FLIM methodology used. Further advantages and disadvantages of using FLIM for assessing FRET compared to ratiometric FRET are summarised in **Table 3**.

Advantages	Disadvantages
Fluorescence lifetime is a molecular property generally independent of variations in fluorophore concentration, illumination intensity, or photobleaching	Photophysical and time-resolved fluorescence spectroscopy expertise required for data interpretation (e.g. fluorescent proteins usually have complex fluorescent decays)
FLIM can be performed in normal cell culture conditions, accounting for changes between conditions such as refractive index, pH etc.	Interpretation of specific changes in fluorescence lifetime in terms of the underlying cell biochemistry may not be straightforward
Allows comparative experimental results, across different repeats, days and even instruments.	Requires expensive equipment and complex to operate

Table 3: Some of the advantages and disadvantages of FLIM-FRET vs spectral ratiometric FRET.

1.4.6 Instrumentation for FLIM:

Fluorescence lifetime can be measured either in the frequency or time domains and is often implemented in either wide-field or laser scanning microscopes. Frequency domain techniques are less intuitive, as fluorescence decay processes are generally conceptualised in terms of time. In this approach, a fluorophore is excited with light that is continuous, but intensity modulated with a given frequency. If the fluorophore emits photons immediately after excitation, the lifetime would be zero and the waveform of the emitted photons will be identical to the shape of the excitation waveform, with identical frequencies. However, with a finite excited-state lifetime, the emitted waveform exhibits a phase shift that can be quantified; greater wavelengths correlate with larger phase shifts. Traditionally this has been implemented using heterodyne or homodyne detection, whereby the excitation light source and detector can be modulated at specific frequencies.

Time-domain techniques utilise pulsed excitation, resulting in a convolution of that pulse with the excited state decay of the fluorophore, which is usually an exponentially decaying signal. One prominent method is time-correlated single photon counting (TCSPC), which detects single photons and measures their arrival times relative to reference pulses, making it suitable for laser scanning confocal or multiphoton microscopy.

Another method, wide-field time-gated FLIM, employs ultrashort optical pulses from a laser to excite the sample. The resulting fluorescence decay is recorded across the entire 2D fluorescence intensity distribution at various delays (t_1 , t_2 , t_3 , ...) after excitation (**Figure 13B**) using a gated optical intensifier (GOI) with an ultrashort gate width (around ~ 100 ps). This effectively acts as an ultrafast shutter. For each pixel in the field of view, the fluorescence decay constant (τ) is calculated, producing a fluorescence lifetime map, or a fluorescence lifetime image (FLIM). This modality is built upon an optical instrument, which can be a wide-field epifluorescence microscope.

In general, the fact that TCSPC acquires pixels sequentially renders it slower than wide-field FLIM approaches. The precision by which fluorescence lifetimes can be measured is a function of the number of detected photons [77]. Hence, the ability of wide-field time-gated FLIM to acquire signals from multiple pixels in parallel makes it advantageous. In short, TCSPC is more photon efficient, but time-gated wide-field FLIM is more time efficient.

Wide-field time-domain FLIM can be further improved by introducing optical sectioning using a Nipkow spinning disc scanner, enabling quasi-confocal FLIM (**Figure 13A**). This implementation is particularly valuable for quantitative live cell FLIM as its high degree of parallelization (compared to laser scanning microscopy) significantly reduces the instantaneous light exposure to the sample, thereby minimizing photobleaching and phototoxicity.

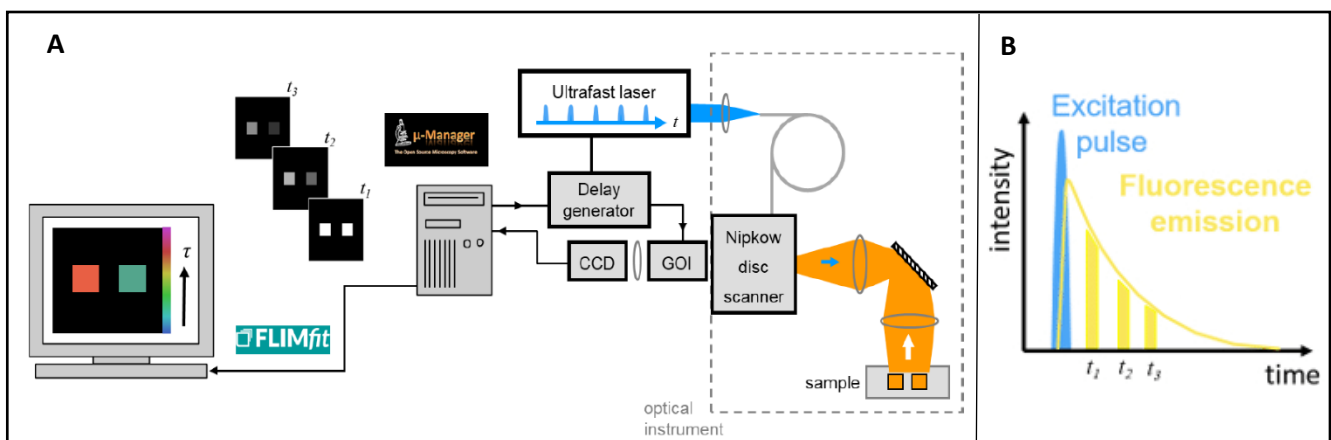


Figure 13: Imaging approach of wide-field time-gated FLIM. **A:** The system is comprised of four main elements. Firstly, an ultrafast excitation source. Secondly, a gated optical intensifier (GOI) coupled with a computer-controlled delay generator. Thirdly, a standard optical instrument frame and finally, a computer for data acquisition and analysis. **B:** The premise of time-gated FLIM, sampling the fluorescence decay at differing time gates, denoted t_1 , t_2 , t_3 .

1.4.7 Examples of FRET used to study PARP1

FRET has been applied to several studies in the context of PARP1, including both its protein-DNA interaction and its enzymatic role in PARylation. For instance, *Serebrovskaya et al.* designed a genetically encoded bipartite sensor for PARylation [78]. This sensor combines the fluorescent proteins Turquoise2 and Venus, both bound to the WWE domain of the RNF146 E3 ubiquitin ligase protein. The WWE domain serves as a PAR-targeting molecule, allowing the assessment of PARylation rates. Using a confocal spinning disc microscope with ratiometric FRET, the sensor successfully monitored PARylation rates in live mammalian cells under various conditions, including UV irradiation, hydrogen peroxide treatment, and hyperthermia.

Sallin et al. adopted a novel approach by creating semisynthetic fluorescent biosensors to quantify free nicotinamide adenine dinucleotide (NAD⁺) molecules and assess the ratios of reduced to oxidised forms (NADPH/NADP⁺) in live cells [79]. Given that NAD⁺ is consumed during PARP1-mediated PARylation, this approach indirectly reflects PARP1 enzymatic activity. The biosensor design involved an NADP-binding protein attached to a tethered ligand containing Halo and SNAP tags. NADPH and NADP⁺ competed for the binding domain, leading to a conformational change that brought the SNAP-tag donor near the Halo-tag acceptor, resulting in FRET. The authors quantified FRET using both ratiometric and fluorescence lifetime imaging microscopy (FLIM) techniques with a TCSPC system.

Another study applied single-molecule FRET (smFRET) to study PARP1 N-terminal zinc domain functionality in converting DNA single-strand breaks from an unperturbed conformation to a highly kinked state [80]. To do this, the authors used a single-stranded DNA molecule designed with two fluorophores positioned on either side of an inducible nick. The organic dyes ATTO 550 and Alexa Fluor 647 were used as the FRET pair, and the DNA conformations were monitored via changes in FRET efficiency. PARP1 was then introduced to the assay and changes in the conformation of the DNA break structure were observed to understand at a structural level how PARP1 modulates and signals for DNA repair to occur.

1.4.8 HCA FLIM-FRET: Instrumentation and applicability to studying heterogeneity in biological populations

In the context of cancer biology, drug resistance remains a significant hurdle which has generally gone unsolved. Resistance frequently arises due to the diverse behaviours of cells within a tumour population, or simply, the vast amount of heterogeneity in the disease. A subset of cells often have some adaptive advantage that renders them capable of evading treatment and thus expanding into a

resistant population, leading to cancer recurrence. Until now, the vast majority of anti-cancer drug discovery assays have overlooked this behaviour, typically measuring the average response of cell populations to drug candidates. Although this is powerful, it fails to account for the importance of outlier cells in a wider population. To address this limitation, there is growing interest in utilising high-throughput experiments that are capable of sampling many cells in varying conditions, whilst maintaining single-cell resolution in their readouts.

This ongoing trend towards automated high-content analysis assays has spurred the development of fluorescence microscopy systems with features such as motorised sample stages, auto-focusing, and multi-well plate compatibility. A number of such systems are now commercially available, but these typically only allow fluorescence intensity imaging. Therefore, there is a need to employ more advanced imaging techniques e.g. using FRET-based readouts, in HCA systems.

Until recently, FLIM had not been widely applied to HCA, mostly due to a lack of instrumentation. FLIM is often implemented using TCSPC, but the sequential pixel acquisition in this approach results in typical acquisition times of 10s-100s seconds per FOV. Hence, to allow rapid FLIM-FRET for HCA automated wide-field time-gated methods have been used. This approach is capable of acquiring an FOV in less than 10 s and hence, can image a whole 96-well plate in around 16 min, making it applicable to a vast range of biological FRET-based assays [81–84]. The discrimination of differences in fluorescence lifetime relies on both the temporal instrument response function (IRF) and the signal-to-noise ratio (SNR). Notably, wide-field time-gated FLIM has demonstrated the capacity to detect lifetime changes as short as 10 ps [85]. This combination of high sensitivity and throughput renders FLIM-FRET based HCA approaches extremely powerful for studying heterogeneity in response to cancer therapies.

1.4.9 Labelling methods used in FRET

In order to perform fluorescence microscopy studies on live HGSC cells, they must first be labelled with appropriate fluorescent markers. In the case of FRET experiments, a suitable donor and acceptor pair. Thankfully, the aforementioned fluorescent proteins such as EGFP are genetically encoded and thus can be synthesised by the cell itself. Genome editing must be applied to introduce the DNA encoding the gene of choice, the methods to achieve this have been evolving rapidly over recent years, as outlined below.

1.5 Genome editing for labelling live cells with fluorescent markers

1.5.1 Methods for genome editing

Following the discovery of DNA by Franklin, Watson and Crick and the evolving understanding of genetics thereafter, the possibility to modify DNA sequences has held significant promise spanning the fields of molecular biology and medicine. These efforts are commonly termed “genome editing” and have been a longstanding objective within these scientific communities to allow the modelling of disease.

The late 1970s saw a breakthrough with the discovery of restriction enzymes, which kickstarted the era of recombinant DNA technology, allowing the manipulation of DNA in the lab. However, precise DNA editing in living eukaryotic cells emerged later. By the mid to late 1980s, researchers like Capecchi and Smithies had made significant progress in demonstrating that the homologous recombination pathway is able to integrate exogenous DNA into mammalian cells [86]. This technique revolutionised gene studies but faced challenges such as low integration rates, efficiency variations across cells, and the risk of random integration.

To address these limitations, researchers began utilising targeted nucleases for genome editing and discovered that creating a double-strand break (DSB) at a target site greatly enhanced targeted gene integration. Initially, they utilised rare cutting endonucleases like I-SceI to achieve this [87]. However, these meganucleases encountered two key challenges: unique recognition sequences and error-prone repair mechanisms, resulting in possible random DNA changes at break sites (mutations).

The discovery and utilization of eukaryotic zinc finger proteins marked a new era in genome targeting and editing, as these small motifs regulated by zinc ions bind to DNA in a sequence-specific manner, with each recognizing a 3-bp DNA sequence. Researchers quickly began creating programmable nuclease proteins by fusing zinc finger proteins with the DNA cleavage domain of the Fok I endonuclease, allowing for enhanced DNA binding specificity. These zinc finger nucleases (ZFNs) significantly increased targeted homologous recombination, offering improved efficiency in genome editing and paving the way for therapeutic applications, while the subsequent discovery of transcription activator-like programable effector nucleases (TALEN) proteins inspired further excitement about their potential as genome-engineering tools [75].

However, the early 2000s brought about a true paradigm shift in the field, with the discovery of repetitive DNA sequences called CRISPR (clustered regularly interspaced short palindromic repeats) by Dr Nakata's group [88–90]. These sequences were first observed in bacteria, with “spacer” DNA sequences in between repeats that exactly matched viral DNA sequences. This led to the discovery that bacteria transcribe these repetitive elements upon viral infection as a defence mechanism. This

RNA functions to guide a nuclease named “Cas” (CRISPR-associated) to viral DNA in order to cut it and defend itself against the virus. The human genome project's computational analysis unveiled CRISPR's prevalence in bacteria and archaea genomes, adjacent to conserved CRISPR-associated (Cas) genes, with spacer sequences sourced from viruses and mobile genetic elements. Experimental evidence, notably from Horvath and colleagues, demonstrated the bacterial immune system's functionality, where *Streptococcus thermophilus* bacteria integrate viral spacers into their genome for defence, guided by CRISPR RNA (crRNA). Further discoveries, including protospacer-adjacent motifs (PAMs), Cas9's DNA catalytic activity, and the biogenesis of crRNA, laid the foundation for CRISPR's transformation into a versatile genome-editing tool. Groundbreaking studies, beginning in late 2012, showcased CRISPR's adaptability for *in vivo* genome editing in eukaryotic cells, leading to widespread adoption across scientific disciplines for its high efficiency and ease of use, reflected in over 9000 research articles since its inception [90].

In comparison to the previously available tools outlined above, CRISPR has demonstrated comparable, if not superior, editing efficiency. Moreover, its simplicity and flexibility make it notably more user-friendly. The CRISPR gene-editing technology comprises an endonuclease protein, the specificity and cutting activity of which can be precisely programmed using a short guide RNA.

While various CRISPR/Cas systems have been repurposed for genome targeting, the type II CRISPR-Cas9 system sourced from *Streptococcus pyogenes* is the most widely used. This consists of the nuclease Cas9, the crRNA array that encodes the guide RNAs and a required auxiliary trans-activating crRNA (tracrRNA) that facilitates the processing of the crRNA array into discrete units. Each crRNA unit contains a 20-nt guide sequence that directs Cas9 to a 20-bp DNA target via Watson-Crick base pairing. In the CRISPR-Cas system, the target DNA must immediately precede a 5'-NGG PAM [91]. This RNA-guided functionality of Cas9 can be utilised in mammalian cells through the expression of both a human codon-optimised Cas9 and the required RNA components. Moreover, the crRNA and tracrRNA can be fused to form a chimeric, single-guide RNA (sgRNA). Efficient delivery of both the Cas9 and the sgRNA components to the target cell is required to successfully perform the desired genome editing. Hence, Cas9 can be re-directed to almost any genomic locus of interest in the vicinity of the PAM sequence by altering the 20-nt guide sequence within the sgRNA. This renders the approach highly versatile across diverse applications. Therefore, the advent of CRISPR-Cas9 technology has allowed increased complexity and accessibility for all aspects of genome editing, from knock-out, knock-in and gene expression modulation.

Upon Cas9 cleavage, a DSB forms which subsequently undergoes one of the two major DDR pathways: error-prone- NHEJ, or the much higher-fidelity HDR pathway (**Figure 14**). However, both pathways can

be exploited to achieve a desired gene editing outcome. Firstly, NHEJ will re-ligate DSBs in the absence of a repair template, leaving errors in the form of insertion/deletion (indel) mutations. Additionally, NHEJ can be utilised for gene knockouts if the resulting indels are in coding exons of a protein, it can lead to a frameshift and thus form a premature stop codon [92]. Alternatively, the less frequently used but much more favourable HDR pathway can be used to enable precise modifications at a target locus when an exogenously introduced repair template is available. This template is typically a double-stranded DNA construct including homology arms flanking the insertion sequence. However, single-stranded oligonucleotides have also been used as repair templates, offering an effective method for making small genomic edits such as the introduction of single-nucleotide mutations [93]. By providing a donor repair template, HR can be exploited to introduce a gene of interest at a specific genomic locus, in a process termed “knock-in”. In contrast to NHEJ, HDR is generally only active in dividing cells and hence its efficiency varies significantly depending on factors such as cell type, state, genomic location, and repair template.

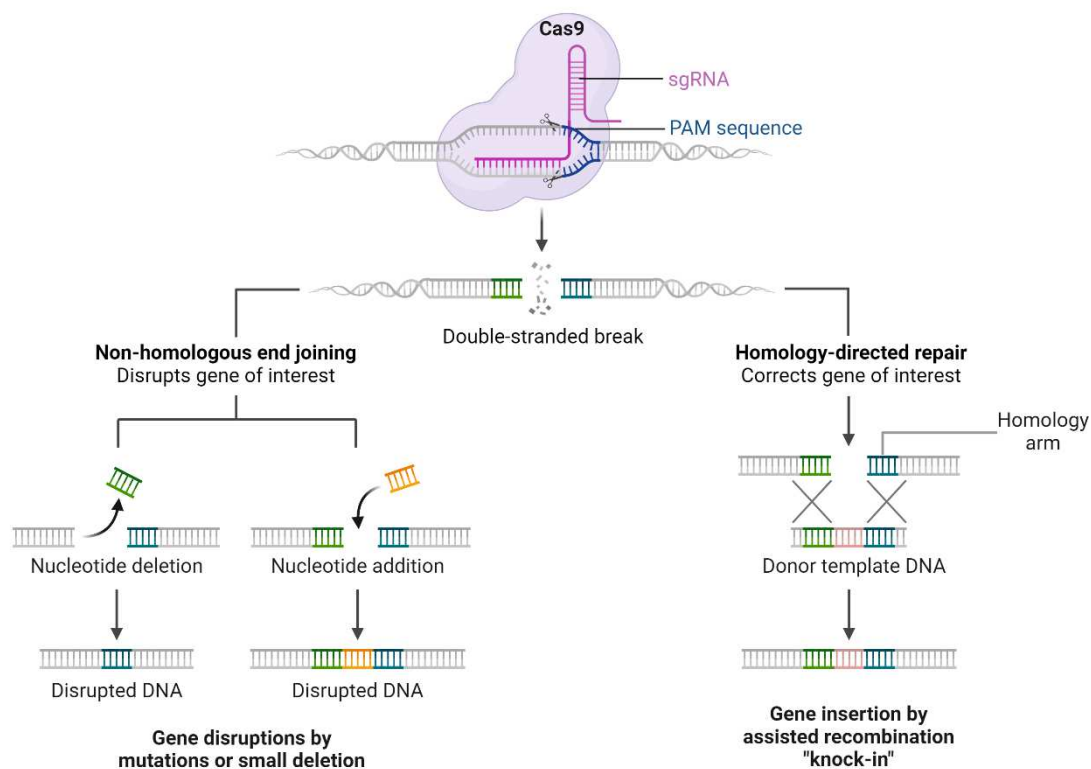


Figure 14: Schematic of CRISPR-Cas9 for gene knock-in. Cas9 enzyme induces double-stranded breaks at specific genomic loci, directed by the sgRNA, inducing the cut close to the PAM sequence. This dsDNA break can then be repaired by either NHEJ or HR. NHEJ results in the disruption of the targeted gene, as nucleotide deletion results in a deletion mutation and nucleotide addition results in insertion mutations. These may result in frameshifts and thus knock out as the protein is non-functional. Conversely, HR corrects the gene of interest. If a donor-template DNA is presented, this process can be hijacked to introduce a gene of interest if it is flanked by appropriate homology arms. This results in a gene knock-in. Figure created in BioRender.

Subsequent modifications to the Cas9 nuclease have led to increased functionality. For example, the introduction of an aspartate-to-alanine (D10A) mutant allows the Cas9 mutant to introduce nicks

rather than DSBs, leading to preferential repair through HDR, thus decreasing the frequency of unwanted indel mutations from off-target DSB formation [94].

For the context of this study, it is important to reiterate the fact that ovarian cancer is characterised by HRD state, presenting a significant challenge for relying on HR-dependent repair for CRISPR knock-in.

1.5.2 Benefits of CRISPR for endogenous labelling

The rapid advancements in the field of CRISPR-Cas9 technology in recent years have revolutionised the ability to label proteins of interest with fluorescent tags in living cells. Compared to traditional methods like transient overexpression of a transgene, performing knock-in of a fluorophore by CRISPR-Cas9 offers several advantages. Namely, the ability to label endogenous genes that are therefore expressed close to the level of the native protein and by its same regulatory machinery. Moreover, CRISPR allows a more targeted approach to genome editing than methodologies like transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs), with superior efficiency.

The CRISPR/Cas9 system works by inducing a DNA double-strand break (DSB) using the Cas9 nuclease. This process is precisely targetable to any genomic site through a 20-bp guide RNA (gRNA) sequence that complements the target site. This method allows for the introduction of fluorescent proteins or any gene of interest with spatial control in the genome via homologous recombination. Hence, the approach has an element of programmability, whereby you can selectively introduce exogenous genes into the endogenous gene of a target protein.

While endogenous gene tagging using fluorescent proteins is powerful, it often requires a significant time investment. For instance, GFP (green fluorescent protein) is 27 kDa, necessitating careful structural design considerations when using HDR for its knock-in, and computational planning is crucial for optimal results. Additionally, it is important to be cautious when using this approach to tag endogenous proteins, as the introduction of large, sterically bulky, fusion proteins can impact folding, stability, and cleavage patterns. Introducing a short flexible linker, such as a glycine-serine (GS) rich linker, can mitigate these effects [95].

1.5.3 Limitations of CRISPR-Cas9 approach for tagging POI for microscopy

The presence of a PAM sequence is the only selecting factor for directing Cas9 to a specific genomic locus. Although this somewhat limits the targeting range, the 5'-NGG PAM sequence of spCas9 can be found on average every 8-12 bp throughout the human genome [96]. Thus, significant pre-planning and design are required to perform a specific edit in a desired site. In terms of gene knockouts this may be less important, but for the introduction of a gene using a knock-in approach, this is vital.

The major concern point for CRISPR-Cas9 approaches is off-target effects, which have been observed at a frequency of >50% [97]. These off-target effects arise due to accuracy issues. The severity of the off-target effects will govern the impact that this may have, but it could lead to deletions or frame shifts that can result in non-viable cells. Hence, this should be considered when performing CRISPR gene editing. As a safety check and as part of all characterization, sequencing should be performed to assess potential off-target effects induced.

The delivery of the CRISPR-Cas9 machinery can also be challenging, especially in cancer cells that are notoriously difficult to transfect. This creates a practical hurdle that may require significant time and optimization to overcome but should be possible as there are now a growing number of approaches to improve delivery efficiency.

Most targeted genes can be tagged homozygously with fluorescent proteins whilst retaining physiological levels of functional expression. However, it has been reported that some proteins cannot be homozygously tagged, often due to the steric hindrance of the large FP perturbing the function of the fusion protein. Hence, for these genes, it may only be possible to tag them heterozygously. Another important factor to consider is the absolute number of molecules of the POI expressed, as this will ultimately define the fluorescence intensity and thus the range of applications you can use the tagged protein for, without being limited by the detection limits of a particular technique. In light of these limitations, it is vital to perform adequate characterization of protein labelling and functionality before utilising these cellular models.

1.5.4 Characterization methods for genotyping CRISPR-Cas9 knock-in

Genotyping is an essential step during the characterization and validation of a CRISPR-Cas9 knock-in. This step is vital for several reasons, primarily to verify the desired genome edit has been performed at the desired genomic locus. Moreover, this process allows the detection of off-target effects as CRISPR is known to occasionally induce unintended modifications at other genomic locations. Some genotyping methods also allow allelic discrimination, distinguishing between different alleles of a gene and assessing whether they are edited or wild type (i.e., with/without the desired modification).

Hence, genotyping allows quantification of the editing efficiency, by evaluating the edited to unedited allele ratio and identifying homozygous or heterozygous knock-ins.

Spanning a number of years since their invention and first application, there are several available approaches for genotyping, each offering slightly different ways to assess the success of CRISPR knock-in. Fluorescence In Situ Hybridization (FISH) is a cytogenetic technique that uses fluorescently labelled DNA probes to detect specific DNA sequences within cells. In the context of CRISPR genotyping, FISH can be used to visualise the integration of the edited sequence into the genome, by directly detecting the target DNA. This method provides spatial information on which alleles of the gene are edited. However, compared to the methods outlined below (such as nanopore sequencing), FISH is less quantitative and does not provide information on the exact sequence changes present. Furthermore, specific probes are required for each target, which can be time-consuming to design and validate.

A second possible approach is using junction PCR, which involves amplifying the genomic regions that span the junction between the edited and wild-type sequences. Through this approach, the exact knock-in sequence can be confirmed. Despite the simplicity of this approach, it may not distinguish between heterozygous and homozygous knock-in, which can be disadvantageous, especially in cancer cell lines with high ploidy.

Another PCR approach is digital droplet PCR (ddPCR), this quantitative method partitions individual PCR reactions into droplets, allowing the amplification of target DNA in separate compartments. This allows the quantification of alleles with a knock-in and offers high enough sensitivity to detect rare variants, which in this context may detect off-target effects. However, in the context of this study where a single protein was dual-labelled at both the C and N-termini, this could not be quantified in a single ddPCR reaction as short primers at each end are assayed. Currently, the method is limited to fragments on the order of 1000 bp each.

One method that aims to overcome the fragment size limitation is long-read sequencing. Several platforms such as PacBio and Oxford Nanopore aim to generate extended DNA sequences. For the application of genotyping CRISPR knock-in, these long reads can span the entire edited region, including junctions and flanking sequences, hence providing accurate information on structural variants. However, these long-read sequencing methods are limited by errors that increase with length. The DNA sequence of PARP1 is ~47 Kb in length, with an additional ~1kb added by the EGFP and mCherryFP knock-in. Hence, this would be near the practical upper limit of fragment length for these methodologies, but it may be a feasible approach. However, if long-read RNA sequencing were performed, this fragment would have been well within the read-length possible by such techniques as the respective RNA transcripts are only ~5 kb.

Oxford Nanopore technology (ONT) sequencing utilises small devices containing flow cells, containing an array of nanopores that directly read DNA sequences as they pass through each pore. Nanopores are embedded in an electro-resistant membrane, with each nanopore corresponding to an individual electrode connected to a channel and sensor chip. The sensor measures the electric current that flows through the nanopore which is disrupted when a DNA or RNA molecule passes through the pore, generating a characteristic signal which allows detection.

ONT sequencing offers long-read sequencing and thus, is traditionally used for whole-genome sequencing. However, in the context of this study, sequence information was only needed for the genome-edited gene of interest, *PARP1*. To perform targeted long-read sequencing there are two possible approaches. Firstly, transcript enrichment is a method which involves an elaborate sample-prep protocol to go from bulk RNA to a pool of transcripts enriched only with the target transcripts [98].

The second approach is adaptive sampling [99]. This involves the selective sequencing of DNA by analysing an initial segment and deciding whether to continue or discard the DNA strand based on its sequence. Traditionally, the selection process is based on fixed regions of interest identified before the sequencing starts. The pore can be programmed to reject or accept a DNA strand based on its sequence, enriching the sequencing of target strands, resulting in high coverage sequencing without the need for complex sample preparation that is required for transcript enrichment.

2 Project hypothesis and aims:

2.1 Hypothesis

This work explores the hypothesis that a reduction in PARP trapping is a possible mechanism driving resistance to PARP inhibitors in ovarian high-grade serous carcinoma. The aim of this body of work was to generate a novel FRET biosensor to assess PARP trapping and allosteric regulation in live cells. Previously, this model had been applied in purified proteins and was shown to produce FRET changes upon PARP1 binding to DNA damage sites, as demonstrated by *Steffen et al.* [58].

To achieve this in live cells, a range of techniques that have been rapidly advancing in recent years such as high content analysis microscopy assays and genome editing through CRISPR-Cas9 were leveraged. Initial developments focused on the development and expression of this biosensor in live cells, through a CRISPR-Cas9 genome editing, incorporating the fluorescent proteins into the endogenous PARP1 gene. This was followed by extensive characterization of the biosensor at the molecular and functional level. Additionally, validation that the biosensor produces reproducible FRET signals upon treatment of DNA damaging agents and PARPi was performed. Results were then cross correlated with prior findings of differences in PARP trapping potency of approved PARPi in order to validate the approach. The assay was then intended to be applied to novel models of acquired PARPi resistance to assess any differences in PARP trapping in sensitive and resistant populations. It was hypothesised that this FRET-based biosensor would not only allow the investigation of PARP trapping and allosteric regulation but also offer single-cell resolution to assess heterogeneity in response. This approach was anticipated to provide a better understanding of the mechanisms driving PARPi resistance. Information gained from these assays will ultimately inform ongoing clinical developments to improve the efficacy of this widely used class of chemotherapy drug. The overarching hypothesis addressed in this work will be investigated with the following aims:

2.2 Aims

Aim 1: Label HGSC cell models with fluorescent proteins using CRISPR-Cas9 genome editing approaches to form a FRET biosensor of PARP1 dynamics, allosteric regulation and trapping.

Aim 2: Use FLIM-FRET high-content analysis assays to assess PARP trapping and allosteric regulation in live cells, in the context of various PARPi and chemotherapy treatments.

Aim 3: Explore the ability of this biosensor to assess any changes in cells made resistant to PARPi *in vitro*.

Aim 4: Test if this biosensor can be employed in more complex, biologically relevant models, such as 3D spheroids, organoids, and *in vivo* models.

3 Material and Methods:

Ethics statements

All *in vivo* experiments performed in mice were approved by the Animal Welfare & Ethics Review Body (AWERB) at Imperial College London. Experiments were performed under the project license numbers PA780D61A and PP1321516 at Imperial College London. All experiments conformed to UK Home Office regulations under the Animals (Scientific Procedures) Act 1986, including Amendment Regulations 2012. Ascites from patients with ovarian cancer were collected and utilized under the ethical approval of the Imperial College Healthcare Tissue Bank (HTA license 12275, Research Ethics Committee number 17/WA/0161, Project ID R18060).

3.1 Cell Culture

Cell lines were routinely cultured in T75 flasks at 37 °C , 95% humidity and 5% CO₂. All immortalized cell lines grown as 2D monolayers were grown in Dulbecco's Modified Eagle medium (DMEM, Gibco, 11965092) supplemented with 5 µg/mL L-Glutamine (Thermo Fisher Scientific, 25030-081), 100 ug/mL penicillin/streptomycin (P/S) (Thermo Fisher Scientific, 15070-063) and 10% heat inactivated foetal bovine serum (FBS, Gibco, 10270-106). This medium composition is referred to throughout as "complete medium", assuming all supplements are added unless otherwise stated (e.g., serum-free culture medium). Cells were passaged by aspirating complete medium, washing once with phosphate buffered saline (PBS) and 1x Trypsin EDTA (Gibco, 25200-072) in PBS was added for approximately 5 min at 37 °C. Cells were dislodged by gentle tapping of the plate and resuspended in culture medium. The OVCAR4 cell line was kindly gifted by Dr Michelle Lockley's group (Queen Mary University of London).

Most cells grown in 3D as spheroids were grown in complete medium supplemented with 2% B-27 plus supplement (Gibco, A35828-01), insulin (1.72 µM), transferrin (0.07 µM), selenium (0.08 µM) (ITS, Gibco, 41400-045), 20 ng/mL epidermal growth factor (EGF, Thermo Fisher Scientific, PHG0311), 20 ng/mL Fibroblast Growth Factor (FGF, Thermo Fisher Scientific, 100-18B). Referred to as 3D medium throughout.

Before commencing this work, all human cell lines were authenticated by 16 locus short-tandem repeat analysis (Eurofins) and intermittently throughout the duration of the project. Cells were tested for mycoplasma infection on a regular basis, using MycoAlert® Mycoplasma Detection Kit (Lonza, LT07-218).

After sterile collection, human ascites samples were passed through a 40 µm membrane. Captured spheroids were subsequently cultured in T75 ultra-low attachment flasks (Corning, 3814) in advanced DMEM/F12 medium (Life Technologies, 12634010). Medium was supplemented with 10% autologous ascites fluid (from the same patient), 10 mmol/L HEPES, 1x N2 supplement (Thermo Fisher Scientific, 17502048), 1x B27 supplement (Thermo Fisher Scientific, 17504044), 100 U/mL penicillin with 100 µg/mL streptomycin (Thermo Fisher Scientific, 15140-122) and 2 mmol/L L-glutamine (Thermo Fisher Scientific, 25030-081).

All cells were stored as frozen stocks in liquid nitrogen in “freezing medium” consisting of FBS with 10% Dimethyl Sulfoxide (DMSO) (VWR, AAJ66650-K2). Cells were centrifuged at 200 x g for 5 min to form a pellet and resuspended in 1 mL freezing medium and allowed to gradually freeze in a Mr. Frosty. Cells were defrosted by rapid thawing in a 37 °C water bath and then resuspended in 10 mL of complete medium. Cells were then centrifuged at 200 x g for 5 min to form a pellet, the supernatant containing DMSO aspirated, and cells resuspended again in complete medium before plating into flasks.

3.2 3D cell culture

3.2.1 Casting 3D Petri Dish and subsequent culture

To prepare the 3D Petri Dishes® using micro-molds, the following procedures were conducted under sterile conditions. Initially, the micro-molds were rinsed with deionized water sterilized by autoclaving. Separately, 1 g of agarose powder (A9539-250G, Sigma-Aldrich) was mixed with 50 mL of sterile water. The mixture was heated in a microwave oven, with intermittent swirling every 10 sec, until the agarose powder was completely dissolved, ensuring no undissolved agarose remained.

Once the agarose was fully melted, it was allowed to cool to approximately 60-70 °C. Using aseptic techniques within the biosafety cabinet, ~200 µL of the molten agarose was pipetted into each well of the micro-mold, avoiding the formation of bubbles. Any bubbles trapped in the micro-mold features were gently removed by pipetting before the agarose set. After gelling, the 3D Petri Dishes® were carefully extracted from the micro-molds by flexing the molds, taking care to avoid cracking. The 3D Petri Dishes® were then transferred to appropriate wells of a 6-well tissue culture plate.

To equilibrate the 3D Petri Dishes®, cell culture medium was added (4 mL per well for a 6-well plate) and incubated for at least 15 min. This medium was then removed and replaced with fresh medium to complete the equilibration process.

Next, cells were trypsinized, counted, and resuspended to achieve the desired seeding density suitable for the 3D Petri Dish® (generally 2×10^5 cells per well). The culture medium surrounding the exterior of the 3D Petri Dish® was carefully removed, and the medium within the cell seeding chamber of the 3D Petri Dish® was also extracted by gently tilting the tissue culture plate.

The cell suspension was then carefully seeded dropwise into the cell seeding chamber of the 3D Petri Dish®. After allowing approximately 30 min for the cells to settle into the dish's features, additional 5 mL culture medium was added to the exterior wells. The tissue culture plate was subsequently placed in a cell culture incubator, and the medium surrounding the 3D Petri Dishes® was exchanged as necessary to maintain optimal culture conditions.

3.2.2 PolyPico

The Pico Spotter (Polypico, Cork, Ireland) was used to dispense small volumes of live cells suspended in BME. The machine was operated in a cold room at 4 °C to ensure the BME remained liquid. Cells were trypsinized and diluted to the desired working concentration and resuspended in BME. This BME/cell suspension was loaded into the disposable PolyPico tips and loaded into the cartridge head. The PolyPico software was used to control the droplet volume and position before automated dispensing of a 200 nL droplet to each well of a 96-well plate. The plate was then placed in a 37 °C incubator for 20 min to polymerise the BME droplets. Subsequently, 400 µL of 3D medium was gently added to each well to cover droplets. Cell medium was changed every three days.

3.2.3 Matribot

To dispense small volumes of cells embedded in hydrogels in an automated manner, the Matribot (Corning) was used. First, the temperature of the plate bed was equilibrated to 37 °C, while the nozzle was set to 4 °C to ensure optimal conditions for printing. Concurrently, basement membrane extract (BME) was defrosted on ice for ~30 min. Cells were trypsinized from a flask of monolayer cultures to achieve a single-cell suspension. The cells were then pelleted by centrifugation at 200 x g for 5 min. The pellet was initially resuspended in 100 µL of PBS and subsequently in 500 µL of BME (to a final 1:6 dilution), ensuring the mixture was free of air bubbles. This BME/cell suspension was then transferred into a syringe, which was carefully inverted and adjusted to remove any air pockets.

Following this, the syringe containing the BME/cell mixture was mounted onto the Matibot printer, and a 27-gauge nozzle (approximately 210 μm inner diameter) was attached. The plate was manually calibrated by selecting the centre of the bottom-left well. To ensure accurate dispensing, three to four test wells were printed prior to commencing the actual pattern. Each well received 3 μL of the BME/cell mixture, equating to a density of 6,000 cells per droplet. After dispensing, the plate was placed in a 37 °C incubator for 20 min to polymerise the BME droplets. Subsequently, 400 μL of 3D medium was gently added to each well to cover the solidified BME droplets. To change the culture medium, the plate was slightly tilted, and the medium was carefully removed from one side, minimizing disturbance to the printed structures.

3.3 Drug treatment

In experiments where cells were treated with a drug, the appropriate dilutions were made from stock solutions into complete medium. Drugs at final concentration were then added either 24 hr or 48 hr post cell seeding, as stated for each experiment. For continuous treatment of cells with rucaparib or olaparib for acquired resistance generation *in vitro*, cells were treated daily during medium changing at a constant dosage of 20 μM . Drugs used throughout are depicted in **Table 4**, including the maximum working concentration of the drug.

Compound name	Maximum working concentration	Provider	Catalogue number
Olaparib	50 μM	Selleckchem	S1060
Rucaparib	50 μM	APExBIO	A4156
Veliparib	100 μM	Selleckchem	S1004
Talazoparib	3 μM	Selleckchem	S7048
Carboplatin	100 μM	Consilient health	PL 15413/0068
Cisplatin	100 μM	Accord healthcare	PL 20075/0123

Table 4: Drug treatments and maximum working concentrations.

3.4 DNA transfection

2×10^5 cells were seeded per well of a 6-well plate in complete medium and allowed to adhere for 24 h. The following day, transfection reaction mixtures were made up in separate 1.5 mL Eppendorf tubes as follows. In one tube (per well) 1 μg per DNA construct was added, then 3 μL Viafect (Promega, E4981) per μg of DNA. Next, 100 μL of serum and antibiotic free medium was added. The mixtures were gently mixed by pipetting with a P20 and left at room temperature for 20-30 min to allow formation of liposome-DNA complexes. Medium was then removed from the cells, and the DNA-

liposome mixture was added to 1.4 mL of complete medium in each well for a final reaction volume of 1.5 mL. Cells were then left for 24-48 hr and transfection efficiency was assessed via fluorescence microscopy as all of the transfections performed were of fluorescently labelled constructs. Note, the presence of antibiotics can inhibit the initial formation of liposome-DNA complexes and hence, antibiotic & serum free medium was used for the 20-30 min incubation.

3.5 Cell viability assays

3.5.1 MTT cytotoxicity assay

Cells were grown in 96-well plates and treated with differing drug dilutions for 72 hr in a total volume of 200 μ L medium per well. At endpoint, 22 μ L Thiazolyl Blue Tetrazolium Bromide (MTT, 5 mg/mL) was added to each well, to a final concentration of 0.5 mg/mL and incubated for 4 hr at 37 °C. Supernatant was then removed and plates were left to air dry overnight at room temperature. 100 μ L DMSO was then added as a solubilization solution to dissolve formazan crystals. Plates were shaken for 10 min to ensure complete solubilization and then absorbance was measured at 560 nm on a TECAN plate reader.

3.5.2 SRB assay

Cells were grown in 96-well plates and treated with differing drug dilutions for 72 hr in a total volume of 200 μ L medium per well. At end point, cells were fixed by adding 25 μ L of 50% w/v trichloroacetic acid (TCA) to the 200 μ L supernatant in each well and incubated at 4 °C for 1 hr. Plates were then gently washed 5 times with tap water, ensuring that the cellular layer is not disrupted. Plates were then left to dry overnight at room temperature. Plates were then stained with 50 μ L sulforhodamine B (SRB, 0.04% w/v in 1% v/v acetic acid) for 1 hr at room temperature. Excess dye was removed with 5 washes with 1% v/v acetic acid. Plates were again allowed to dry overnight. 100 μ L of 10 mM Tris base solution (pH 10.5) was then added per well as a solubilization solution and left on an orbital shaker for 10 min to dissolve protein bound dye. The absorbance was then measured at 510 nm using a TECAN platereader to determine the optical density. Acquired data were then stored and viability curves were then plotted using GraphPad Prism, fitting a non-linear regression with log(inhibitor) vs response (three parameters) model. IC₅₀ values were calculated on a per-condition basis.

3.6 Cloning reactions

3.6.1 Polymerase chain reaction (PCR) for amplification of DNA fragments

PCR reactions were used for the generation of DNA constructs for expression plasmid formation and for the diagnostic analysis of cloning experiments. Reactions were prepared on ice, and most PCR reactions were performed with 10 ng DNA if plasmid DNA, or 50 ng if using linear DNA source. Typical reactions contained 10 pmol of both forward and reverse primers, 5 μ L 10X Pfu buffer to a final 1X concentration, 0.4 μ L dNTP, adding water to make up to a final reaction volume of 50 μ L. Reaction mixtures were gently mixed and then run on a thermocycler machine (³Prime, Techne). Cycling conditions of 5 min at 94 °C for initial denaturing, followed by 30 cycles of [1 min at 95 °C for denaturing, 1 min at 60 °C for annealing, 1 min at 72 °C for extension] and 5 min at 72 °C final extension. Note, where optimization was required for a specific reaction and conditions deviate from this, they are stated.

3.6.2 Restriction enzyme digestion

Restriction enzyme digestions were performed to cleave DNA to generate fragments for plasmid formation, or for diagnostic purposes. A typical 20 μ L reaction consisted of 1 μ g DNA, 0.5 μ L per restriction enzyme, 2 μ L 10X reaction buffer. Recommended buffers for single digestions were used, and for dual digestions Thermo Fisher guidance was used: <https://tinyurl.com/r4hnjam4>. MilliQ water was added to a final reaction volume of 20 μ L. The reaction was mixed well by pipetting and left in a humidified environment overnight at 37 °C. The next day, 5 μ L of reaction mixture was run on a diagnostic 0.8% agarose gel to visualize and confirm the expected digestion products were present. If the desired bands were present, the remainder of the reaction mixture was run on another 0.8% agarose gel for DNA extraction. Enzymes used are summarized in **Table 5**.

Enzyme	Manufacturer	Buffer
Bpil (BbsI)	Thermo Fisher Scientific, ER1011	Thermo Buffer G
EcoRI	Thermo Fisher Scientific, ER0271	Unique Buffer EcoRI
HindIII	Thermo Fisher Scientific, ER0502	Thermo Buffer R
XbaI	Promega, R618A	Promega Buffer D
XhoI	Promega, R616A	Promega Buffer D

Table 5: Restriction enzymes used for restriction digestion of DNA.

3.6.3 Phosphorylation of primers and ligation reaction

Ligation reactions were performed to insert the desired gRNA sequence into an expression plasmid. First, primers had to be phosphorylated as follows. A 10 µL reaction mixture was made consisting of 1 µL (10 pmol) each of the forward and reverse primers, 1 µL of T4 polynucleotide kinase (Thermo Fisher Scientific, EK0031), 1 µL 10X PNK buffer, 1 µL ATP (10 mM), 5 µL MilliQ water. The T4 PNK phosphorylates the 5' end of the oligos which can then react with the 3' OH group for ligation of the primers to occur. The reaction was placed in a thermocycler at 37 °C for 30 min, then 95 °C for 5 min for ligation to occur and then gradually cooled at 5 °C per min down to a final temperature of 25 °C, and subsequently stored at 4 °C before future processing.

Phosphorylated oligo reaction was diluted 1:200 in MilliQ water before being using in the following reaction whereby the plasmid backbone was restriction enzyme digested and the gRNA sequence was ligated into it, in a single tube reaction.

A 20 µL reaction mixture was prepared with 1 µL (100 ng) uncut pX330-U6-chimeric-BB-Cbh-hSpCas9 plasmid (Addgene #42230), 2 µL diluted phosphorylated oligos, 2 µL Tango buffer 10X, 1 µL DTT (10 mM) , 1 µL ATP (10 mM) 0.5 µL T4 ligase, 1 µL BbsI restriction enzyme, 11.5 µL MilliQ water. The reaction was placed in a thermocycler and heated at 37 °C for 5 min then 21 °C for 5 min, for a total of 6 cycles.

3.6.4 Gel electrophoresis

DNA products amplified by PCR reactions were run on agarose gels for separation and visualization, for either diagnostic confirmation, or subsequent DNA extraction. For 1% gels, 1 g agarose was dissolved in 100 mL of TAE buffer by short cycles of heating in a microwave until fully dissolved. Solution was allowed to cool at room temperature for 10 min before adding 2.5 μ L SYBR Safe DNA gel stain (Invitrogen, S33102, (1:40,000 dilution)) to allow DNA visualisation. Molten agarose solution was poured into a gel tray and allowed to solidify. Gels were then submerged in 1X TAE buffer (Jena Bioscience, BU-199-50). 5 μ L HyperLadder 1Kb DNA ladder (Meridian bioscience, BIO-33053) was loaded into the first lane for reference. DNA samples were loaded with a loading buffer (Bioline, 2035-013) and MilliQ water, typically to a total volume 20 μ L per lane. Gels were run in a Plusi gel electrophoresis unit (Scie Plas, 15845541) at 80V for 60 min (Cleaver Scientific, CS-300V) and imaged using a gel imaging system (Syngene Pxi). Agarose gels for DNA extraction were made at 0.8% and run for a longer period if required for sufficient band separation.

3.6.5 Gel extraction and DNA purification

DNA bands were visualized on a UV transilluminator, and desired bands were cut from the gel using a sterile scalpel into a pre-weighed Eppendorf tube. Extraction was performed using QIAquick gel extraction kit (Qiagen, 28706). Gel weights were calculated, referred to as gel weight. QG buffer was added at 3X w/v of gel weight and heated at 50 °C until the gel had fully melted. One volume isopropanol was added and mixed well. This mixture was then added to a spin column (750 μ L max at a time) and centrifuged at 16,100 x g for 1 min, capturing DNA onto the membrane. Supernatant was then discarded, and process was repeated until total volume had been spun. Columns were then washed with 750 μ L buffer PE before being spun at 16,100 x g for 10 s and liquid discarded. Columns were then dry spun for 1 min, discarding any remaining liquid. DNA was eluted to a clean Eppendorf using 10 μ L nuclease free water.

3.6.6 Determination of DNA concentration

DNA concentrations were measured using a Nanodrop Lite spectrophotometer (Thermo Fisher Scientific, 2634). DNA samples were carried forward for use if the 260/280 wavelength ratios were between 1.8-2.0.

3.6.7 Transformation of competent bacteria

Complete circularised DNA plasmids were amplified by culturing in transformed competent bacteria, with varying strains used including JM109 (Promega, P9751) and high competency *Stb13* (NEB,

B90205). 20 µL aliquots of bacteria were thawed on ice. DNA was diluted to 10 ng/µL and 1 µL was added per tube and mixed gently using a P20 pipette tip, to avoid shearing cells. Tubes were left on ice for 30 min to defrost. Heat-shock was performed, placing tubes at 42 °C for 45 s then back on to ice for at least 2 min to cool. Bacteria were then added to 1 mL LB broth and placed into a 50 mL falcon tube on a shaker at 37 °C, 200 rpm for 1 hr, leaving the lid loose to allow for oxygen uptake and bacteria outgrowth. 10-100 µL of this was then seeded on agar plates and left upside down at 37 °C overnight for colony formation. Plates contained ampicillin or kanamycin, depending on the antibiotic resistance gene encoded in the transformed plasmid.

3.6.8 Mini/midi prep of DNA from bacterial culture

Transformed bacterial colonies were expanded by inoculation into tubes containing 5 mL LB broth (Sigma-Aldrich, L3397) supplemented with appropriate antibiotic. Inoculated tubes were cultured overnight in a shaking incubator around 220 rpm, at 37 °C. Cells were then centrifuged into a pellet for miniprep or used to inoculate 100 mL of LB for midiprep. For minipreps, cells were pelleted by spinning at 4000 rpm for 30 min and the supernatant was discarded. QIAprep Spin Miniprep Kit (Qiagen, 27104) was used. Pellet was resuspended in 250 µL Buffer P1 and transferred to an Eppendorf tube. To lyse the cells, 250 µL Buffer P2 was added, inverted to mix, and left for 3 min. Next 350 µL Buffer N3 was added to neutralize the lysis buffer, inverted to mix, and left 2 min. The mixture was then centrifuged at 16,100 x g for 1 min and the clear supernatant was transferred to a spin column. This was then spun for 1 min, discarding flowthrough before washing with 500 µL PB, then 750 µL PE. Column was then dry spun before eluting DNA from the membrane into a new Eppendorf using nuclease free water.

3.6.9 Gibson assembly

Gibson assembly reactions are a single-tube, multi-step isothermal reaction that combine several linear DNA fragments into a newly circularised DNA plasmid. This was performed using the NEBuilder HiFi DNA assembly cloning kit (New England Biolabs, E5520S). DNA fragments with overlapping sequences are combined at equal molar ratios with the digested backbone sequence, to a PCR tube. The Gibson master mix is then added to a final 1X concentration, and the reaction is made up to a final volume of 20 µL with nuclease free water and mixed well. The reaction is then performed at 50 °C in the thermocycler, for 1 hr. The reaction steps are as follows. Exonuclease activity chews back nucleotides from the ends of each DNA fragment, creating a ssDNA 3' overhang that allows annealing of fragments with complementarity. Polymerase activity then fills in any gaps within each annealed fragment. Finally, the ligase seals nicks in the assembled DNA.

The assembled plasmid can then be stored at 4 °C, or be used for transformation of bacteria following standard protocol above. From the resulting colonies, six candidates were picked and minipreps were performed to assess the reaction product was as desired by sequencing.

3.7 CRISPR-Cas9 genome editing

3.7.1 Guide RNA design

Guide RNA sequences were taken from previous studies performing CRISPR knock-in at the same genomic locus and then cross-check with online guide design tool <https://chopchop.cbu.uib.no/CHOPCHOP>.

3.7.2 Plasmid design: targeting vector

The python package pyDNA was used for simulating DNA assembly steps for plasmid formation. This allowed simulations of restriction digestion, ligation, PCR, Gibson assembly, primer design and gel electrophoresis reactions. All cloning experiments were run as *in silico* simulations via pyDNA before ordering primers into the lab to ensure the reactions were theoretically feasible.

3.7.3 Screening for positive clones

Following transfection cells were allowed to recover and expand in complete medium. 24-72h later cells were assessed for expression of fluorescent protein (EGFP or mCherryFP depending on transfection vector) via fluorescence microscopy using the Incucyte S3 live-cell imaging system. Both phase contrast and fluorescence images were acquired (500 ms acquisition time) and fluorophore expression was identified.

3.7.4 FACS for isolation of transfected clones

Experiments were carried out at the FLOW facility at MRC, Hammersmith Hospital. Polyclonal populations were expanded into T75 flasks and at each passage, a subpopulation (~10%) of cells were plated into separate plates for assessment of fluorophore expression via fluorescence microscopy to ensure a percentage of positive cells remained in the population. Cells were then trypsinized at roughly 70-80% confluency and centrifuged at 200 x g for 5 min, removing the supernatant. Cells were then resuspended gently in 1 mL FACS buffer (PBS, 0.5% v/v FBS and 2 mM EDTA). Cells were then counted using a Countess 3 automated cell counter (Thermo Fisher Scientific) and diluted to a final concentration of around 8×10^6 cells/mL. One FACS tube (Corning, 352235) per sample was then pre-equilibrated with 100 μ L FACS buffer passed through the 35 μ m cell-strainer cap. 1 mL of each sample

was then transferred to a FACS tube gently passing the cells through the strainer cap to remove cell aggregates.

Samples were then sorted using BD FACSAria III or BD FACSAria Fusion Sorter with a 130 μm nozzle, first setting a gating strategy based on the fluorescence intensity of the sample compared to both negative and positive control samples (i.e. WT cells and GFP positive cells). Gates were based on forward scatter (FSC) and side scatter (SSC) to exclude debris and doublets. Due to the low percentage transfection efficiencies achieved, bulk sorts were performed, sorting positive single cells into one well of a 6-well plate containing complete medium. This avoided the need to centrifuge the cells again, maintaining higher cell viability.

3.7.5 Screening for gene edited clones

Polyclonal populations were expanded after FACS and then harvested for collection of DNA and protein for screening for success of CRISPR knock-in at both the genomic and protein level.

3.8 Sequencing

3.9 Protein expression levels

3.9.1 Isolating whole cell protein lysates

CRISPR edited cells were collected from routine culture in T75 flasks. Protein from treated cells were collected from $\sim 1 \times 10^6$ cells cultured and treated in 6-well plates. Cells were harvested at 70-80% confluency, or upon finishing treatments. Cells were trypsinized and pelleted via centrifugation. Supernatant was removed and the pellet was washed once with PBS before resuspending in 50 μL Phosphosafe extraction reagent (Millipore, 71296) with added Roche cOmplete™ Protease Inhibitor Cocktail (Roche, 11873580001, added at 1 tablet per 2 mL phosphatase buffer). Next, samples were centrifuged at 17,000 $\times g$ for 15 min at 4 °C to pellet non-protein cellular debris and the supernatant was transferred to a clean pre-chilled 1.5 mL Eppendorf tube. The sample was then snap-frozen in a mixture of dry ice and methanol before storing at -80 °C until further analysis was performed.

3.9.2 Determination of protein concentration

BCA assays were performed to determine protein concentrations of samples using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, 23227). Firstly, each sample was diluted 1:10 in PBS (5 μL sample in 45 μL PBS). A series of concentrations of protein standards were then prepared by diluting a 2 mg/mL stock solution of bovine serum albumin (BSA) in extraction buffer, generating concentrations from 0 to 1 mg/mL. Subsequently, 5 μL of either the prepared standards or the diluted

samples were dispensed into the wells of a 96-well plate, followed by the addition of 100 μ L of working solution (prepared by 1:50 dilution of BCA reagent A to BCA reagent B) onto each well. Plates were then covered with aluminium foil and placed on a plate-shaker for 30 s, before incubating at 37 °C for 30 min. Next, absorbance readings were recorded at 595 nm using a TECAN microplate reader. Standard curves were then plotted using the absorbance values of the known protein concentration standards, and the protein concentration of samples was subsequently extrapolated from the standard curve.

3.9.3 Preparation of polyacrylamide gels

Polyacrylamide gels were prepared at 7.5%, using the Bio-Rad Mini-PROTEAN Tetra Handcast System. Gel recipes are summarised in **Table 6**. Note, the two crosslinking reagents ammonium persulphate (APS, Sigma-Aldrich, A3678) and N,N,N',N'-Tetramethylethylenediamine (TEMED, Sigma-Aldrich, T9281) were added last, as the gel quickly polymerises upon their addition and gentle mixing.

Reagent	Resolving gel	Stacking gel
30% acrylamide (National diagnostics, EC-890)	2.5 mL	1.3 mL
dH ₂ O	2.5 mL	3.7 mL
2M Tris-HCL pH 8.8	5 mL	-
1M Tris-HCL pH 6.8	-	5 mL
10% APS	100 μ L	100 μ L
TEMED	10 μ L	10 μ L

Table 6: 7.5% acrylamide gel components for western blots used throughout.

To cast the gels, ~5 mL of resolving gel solution was dispensed into the cassette sandwich and 1 mL of 100% ethanol was added along the top of the cassette to remove air bubbles. This was allowed to set for ~20 min whilst the stacking gel was made. Stacking gel solution was then added atop of the resolving gel until the volume overflowed, to stop air bubble introduction. Immediately, a 10-well comb was gently inserted into the stacking gel and allowed to set.

3.9.4 Preparing protein samples

All samples were prepared on ice. A loading buffer was prepared with β -mercaptoethanol with 4x Laemmli Sample Buffer (Bio-Rad, 1610747) in a 1:10 dilution. Protein samples were then diluted in this loading buffer to a final concentration of 20 μ g protein and vortexed before heating at 95 °C for

5 min to denature the proteins. Combs were then removed from polyacrylamide gels and samples loaded. 5 μ L protein ladder (Thermo Fisher Scientific, 26619) was loaded into the first well to estimate molecular weights of separated proteins and 20 μ L of sample was loaded per lane.

3.9.5 SDS-PAGE and Western blotting, Wet transfer

SDS running buffer (20 mM Tris HCL, 190 mM Glycine, and 0.1% SDS in dH₂O) was used to fill the tank, ensuring there were no leaks between the two gels. Gels were then run at 80 V for 30 min and then 140 V until the loading dye had visibly reached the bottom of the gel.

A wet transfer was then performed to transfer separated proteins to a nitrocellulose membrane. Bio-rad Mini-PROTEAN Tetra Transfer System was used, soaking sponges, filter papers and nitrocellulose membranes in transfer buffer solution (20 mM Tris HCL, 190 mM Glycine, 20% methanol in dH₂O). A sandwich was assembled in the following order: one sponge, one filter paper, gel, membrane, filter paper and sponge. A roller was used throughout to remove air bubbles from the sandwich. This was then placed into the transfer cassette in the tank with 1L of transfer buffer. Ice packs were placed in the tank and the transfer was run at 200 mA for 90 min. Ponceau Red (Sigma-Aldrich, P7170) was used to visualize transferred proteins on the membrane and subsequently removed by 3 washes in TBS-T.

3.9.6 Antibody staining

Following membrane transfer, membranes were blocked in 5% (w/v) BSA (Sigma-Aldrich, A3294) in TBS-T for 1 hr at room temperature. Antibody dilutions are summarised in **Table 7**. Primary antibodies were made up at their appropriate dilutions in BSA and membranes were incubated in this solution overnight at 4 °C on a tube roller. Unbound primary antibodies were then removed by 3 x 5 min washes with TBS-T. Next, membranes were incubated in secondary antibodies diluted in 5% (w/v) powdered milk (Millipore, 70166) for 1 hr at room temperature on a tube roller. Excess secondary antibody was removed by 3 x 5 min washes with TBS-T. Membranes were developed using enhanced chemiluminescence (Cytivia life sciences, RPN2106) and imaged on the GE ImageQuant LAS 4000 Biomolecular Imager system.

Antibody (species)	Dilution	Supplier, Catalogue number
Anti- γ H2Ax (mouse)	1:1000	Sigma-Aldrich, 05-636
Anti-PARP1 (mouse)	1:1000	Cell Signalling, 9542S
Anti-GFP (mouse)	1:1000	Merck, 11814460001
Anti-mCherryFP (rabbit)	1:1000	Abcam, ab167543
Anti- β Actin (mouse)	1:5000	Sigma-Aldrich, A1978
Anti-Laminin A/C (mouse)	1:2000	Cell Signalling, 2032
Secondary anti-mouse	1:2000	DAKO, P0448
Secondary anti-rabbit	1:2000	DAKO, P0446

Table 7: Antibodies used for western blot experiments. The working dilution used and supplier information are also stated.

3.10 RNA extraction

RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen 74104). Cells were cultured until 80-90% confluency in 6-well plates and then washed twice with PBS before trypsinization. Cells were spun at 200 x g for 5 min to form a pellet, the supernatant was fully removed, before adding 350 μ L of lysis buffer RLT and vortexing to homogenize the lysate. Next 350 μ L 70% ethanol (in nuclease-free water) was added and mixed well by pipetting. The sample was transferred to a RNeasy spin column in a collection tube and centrifuged at 12,000 x g for 15 s and flowthrough was discarded. The sample was then washed once with RW1 buffer and twice with RPE buffer. The empty column was then spun at 12,000 x g for 2 min to dry the membrane and increase RNA purity. The column was placed in a clean 1.5 mL RNase-free Eppendorf tube. 30 μ L of RNase-free water was added to the column and spun at 12,000 x g for 1 min to elute the RNA. The RNA quantity was then assessed using the Qubit™ 2.0 fluorometer (Thermo Fisher Scientific), with the Qubit™ RNA Broad Range assay kit (Thermo Fisher Scientific, Q32852). Assay reagents were left at RT for 30 min to defrost. Qubit™ RNA BR Reagent was diluted 1:200 in Qubit™ RNA BR buffer and vortexed to make up the Qubit™ working solution to a final volume of 200 μ L per sample. To prepare the standards, 10 μ L of each Qubit™ standard (#1 and #2) were mixed with 190 μ L working solution. Sample RNA was then diluted 1:100 in working solution to a total volume of 200 μ L. All tubes were vortexed and incubated at RT for 3 min. The standards were read first, then the samples, calculating the RNA concentration. Quality checks were performed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA was aliquoted and stored at -70 °C until downstream use.

3.11 Immunofluorescence assays

Cells were cultured in fibronectin coated 8-well chamber slides (#1.0 8-well glass chamber slides with a working volume of 400 μ L (Thermo Fisher Scientific, Nunc LabTek, 155411) or glass 96-well plates (Greiner, 655892), working volume 200 μ L. At the end of treatment, the sample was placed on ice and

the medium was removed from each well and ice-cold 100% methanol was added to each well for 10 min on ice for fixation. The methanol was gently removed, and 3 x PBS washes were performed. Cells were then permeabilized in permeabilization solution (PS) (0.5% Triton X-100 (Amresco, 0694) in PBS) for 1 hr at RT. Blocking solution (BS) (PS + 10% FBS) was then added for 1 hr at RT. Primary antibodies were diluted to their working concentration in BS and incubated for the appropriate time, at 4 °C. The wells were then washed three times with PBS to remove unbound antibodies and then imaged.

3.12 Preparing HCA imaging plates

Poly-L-lysine (Sigma-Aldrich, P8920) was used to coat glass imaging plates, diluting 1:100 in PBS to a final concentration of 0.001% (w/v), 200 µL was added per well of a 96-well plate for 5 min at room temperature followed by 3 PBS washes to remove residual PLL. Fibronectin (Sigma-Aldrich, F0895) was also used, diluting in PBS to a final concentration of 3 µg/cm², left at room temperature for 1 hr, followed by 1 PBS wash and then allowed to air dry.

3.13 Sample preparation for FLIM-FRET HCA assay

7.5x10⁴ cells were cultured in fibronectin coated 96-well glass plates for 48h in complete medium. Medium was then removed and replaced with CO₂ dependent imaging medium, containing the appropriate drug treatments. CO₂ dependent imaging medium was freshly made for each experiment with the components found in **Table 8**.

Reagent	Quantity	Supplier	Catalogue Number
DMEM powder	0.415 g	Sigma	D5030-10L
Sodium bicarbonate 7.5% solution (893 mM)	1231 µL	Gibco	25080
Sodium pyruvate (100 mM) solution	500 µL	Sigma	58636
Glucose 1M solution	1250 µL	Sigma	G7528
L-glutamine (200 mM) solution	500 µL	ThermoFisher	25030081
MilliQ Water	46.19 mL	N/A	N/A

Table 8: CO₂ dependent imaging medium recipe. Component quantity is noted, with the supplier and catalogue number for each. Fresh medium was made up before each experiment and filter sterilized through a 0.2 µm filter (FischerScientific, 15206869).

In each experiment, three control cell lines were included. OVCAR4 wildtype cells as an autofluorescence control, OVCAR4 PARP1-GFP (N-terminal) as a FRET donor only control and OVCAR4 PARP1-mCherryFP as an acceptor only control. The drug treated cells were immediately transferred to the microscope enclosure (pre-heated to 37 °C, 5% CO₂) and left for 1 hr to equilibrate to a steady-state temperature. In this time, gate delays, FOV positions and acquisition times were selected. Automated FLIM acquisition commands were set using a custom-written MicroManager (v2.0.0) plugin for HCA.

3.14 *In vivo* experiments

All animal experiments were carried out by Katie Tyson. Female CD1-nude mice aged between 6-7 weeks old were ordered from Charles River (developed from the transfer of the nude gene from Crl:NU-Foxn1nu to a CD-1® mouse through a series of crosses and backcrosses beginning in 1979 at Charles River Laboratories, Wilmington, MA. These animals lack a thymus, are unable to produce T-cells, and are therefore immunodeficient, meaning that they are less likely to reject the injection of human cancer cell lines, unlike an immune competent animal, hence its use here with human the OVCAR4 cell line. Mice were left one week to acclimatise to the animal house before any procedures.

OVCAR4 GFP-PARP1-mCherryFP cells were injected intraperitoneally with 5×10^6 in 200 μ L PBS. The mice were subsequently monitored daily and weighed twice weekly to assess the formation of tumours. Drug treatments begun 14 days later. Olaparib was given daily (5 days on, 2 days off) for two weeks, at a dosage of 25 mg/kg. Olaparib was dissolved in 18% SBE- β -CD (MedChemExpress, HY-17031), 10% DMSO in saline.

Cisplatin was given once weekly, at a dosage of 5 mg/kg. Mice were then maintained until the first mouse in each cage reached endpoint, classified as any of the following: weight loss $\Rightarrow$ 15%, sudden weight gain of 2 g in 24 h, isolation, facial grimace, reluctance to move or swelling. Once this occurred, all cage mates were also culled, and tumours harvested straight into PBS on ice. Tumours were then homogenised, and cells were extracted for culturing. Cells were selected from distinct tumour sites to try to capture heterogeneity of disease on an intra- and inter-mouse basis. A peritoneal lavage (5 mL of 2 mM EDTA in PBS) was performed to isolate ascites spheroids which were centrifuged at 200 x g for 5 min and resuspended in TrypLE Express Enzyme (Gibco, 12604013) for 10 min, 37°C. Disassociated spheroids were subsequently resuspended in medium and cultured under standard conditions.

3.15 Plasmids used throughout

Plasmid name	Source
pX330-U6-Chimeric_BB-CBh-hSpCas9	Addgene, #42230
pST1374-N-NLS-flag-linker-Cas9-D10A	Addgene, #51130
AW69_pDonor ^{PARP1.TS}	Kindly gifted by M Gonçalves, described in [100]
AM70_pgRNA ^{PARP1}	Kindly gifted by M Gonçalves, described in [100]
Rhodopsin targeting vector (EGFP)	Kindly gifted by I Oinuma, described in [101]

Table 9: Plasmids used throughout this work. Those available from addgene are listed.

3.16 Primers used for PCR reactions

Primer Name	Sequence (5' -> 3')
topA (gRNA)	CACC-GTCAATTTTAAGACCTCCCTG
topB (gRNA)	CAGTTAAAATTCTGGAGGGAC-CAAA
gfp_f	ATGGTGAGCAAGGGCGAGGAGC
gfp_r2	CGGCTACCTCTCCCAAttaccacagTTACTTGTACAGCTCGTCCATGCC
mCherry_f	ATGGTGAGCAAGGGCGAGGAGG
Arm5_F	GTGCCACCTGGGCCGCCATTTAAATggcAGACAAGGATTAGAGGCTG
Arm5_r2	GCTCCTCGCCCTTGCTCACcatGGAGGTCTTAAAATTGAATTCAGTTTCAGCAG
Arm5_r_mc	CCTCCTCGCCCTTGCTCACcatGGAGGTCTTAAAATTGAATTCAGTTTCAGCAG
arm3_f2	ctgtggtaaTTGGGAGAGGTAGCCG
arm3_r3	GCCGATTCATTAATGCAGCGGCGCcgcTTTCCAAAATCAAACTAAAGACGAACACTTAG

Table 10: Primer sequences used for PCR reactions throughout for fragment synthesis.

3.17 Microscopy

3.17.1 Incucyte live cell imaging

Incucyte S3 Live Cell Analysis System with Incucyte S3 software (Sartorius) was used for live cell imaging. Multi well plates were placed inside the environmental chambre and kept at 37 °C, 5% CO₂ throughout imaging. Images were acquired with either a 4X, 10X or 20X objective with image resolution of 2.82, 1.24 and 0.62 µm/pixel respectively. Fluorescence images were acquired with green channel excitation at 441-481 nm and emission collected at 503-544 nm and red channel with excitation at 567-607 nm and emission collected at 622-704 nm. Images were exported as calibrated 32-bit TIFF file images without compression and later visualized in ImageJ.

3.17.2 Epifluorescence imaging

Imaging was performed on an Olympus IX73 body with a CoolLED pE300 illumination diode. An Olympus TH4-200 lamp was used with a Hamamatsu camera controller. Emission filters used: CFP-2432C-000 BrightLine Single, mCherryFP-40LP-A-000, GFP-3035C-000.

3.17.3 Quantification of γH2AX foci from immunofluorescence images

Cells were fixed, permeabilised and blocked as described above. Antibody labelling with γH2AX-AF488 conjugated antibody (Cell Signalling Technology, 20304) at a 1:10,000 dilution overnight was then performed. In order to label DNA SYTO Deep Red (Thermo Fisher, S34900) staining was performed,

adding 200 μ L (1:1000 in PBS) for 1 hr at room temperature. Three PBS washes were performed, and samples were subsequently imaged using a 100x, 1.4 NA objective Olympus UplanSApo objective lens with HILO illumination. AF488 was imaged with 462 nm laser excitation and SYTO Deep Red was imaged with 635nm laser excitation.

Images were stored as TIFF files for each imaging channel and subsequently analysed using ImageJ (NIH, US). A custom macro script was written to facilitate automated analysis and foci counting per nucleus. In brief, this involved automated intensity thresholding of the SYTO Deep Red image using Otsu thresholding. This was then converted to a mask for watershed segmentation. Analyse Particles function was used to count individual nuclei and define them as ROIs. These defined ROIs were overlaid on the γ H2AX images, and the Find Maxima function was used to count individual foci, storing the value per nucleus in a csv file for further statistical analysis.

3.17.4 Acquiring training data for label free cell cycle classification

CRISPR-Cas9 endogenously labelled A375 RELA-EGFP, PCNA-mScarletFP cells were kindly gifted by the Bakal Lab, Institute of Cancer Research, London. On the day of imaging, cells were trypsinized to a single-cell suspension and resuspended in phenol red free DMEM (Gibco, A1443001 with 10% FBS, 1% L-Glutamine, 1% Penicillin-Streptomycin) for imaging. Cells were counted and subsequently seeded on ibi-treat glass 24 well μ -Plates (Ibidi, 82426) at increasing concentrations per row, ranging from 2000-12,500 cells per row, in 1.2 mL medium per well.

Cells were immediately placed in the stage-top incubator on the M³M system at 37 °C, 5% CO₂ whilst imaging parameters were set. Image acquisition began 2 hr after seeding, allowing time for the cells to adhere to the coverslip, although some cells were still attaching in the first few hours of data acquisition. Images were acquired every 5 min in both the *pDPC* and fluorescence (mScarletFP) channels, with 2 FOV per well for a total of 48 FOV per timepoint. This time series was repeated over 96 hr of continuous imaging. For the biological repeat dataset, the same culture conditions, imaging parameters and machine learning model were used.

HeLa PCNA-EGFP, H2B-RFP and U2OS RPA-EGFP, PCNA-RFP cells were trypsinized to a single-cell suspension, counted, and then 35,000 cells were seeded per well in a total volume of 1.2 mL medium. The cells were placed in the M³M incubator and given ~4h to begin to adhere to the glass plate. This allowed for more accurate setting of the autofocus Z position before beginning the timelapse imaging. 2 FOV were selected per well, ensuring cells were present in the FOV at timepoint 0. Imaging for this experiment acquired 3 images per FOV, the *pDPC*, EGFP and RFP channels, all captured on separate cameras.

The raw data were saved in multiple files per FOV, separated by *pDPC* and fluorescence images. For data processing, a background subtraction (time-varying background) was performed to increase the SNR and standardize the images over time. Next, the fluorescence and *pDPC* images were co-registered. The fluorescence data were then segmented using CellPose [4]. The resulting segmentation masks were then used as the input for cell tracking, performed by TrackMate [5]. Tracking parameters were first optimized on a small sub-set of the data, before batch-processing all FOVs by running TrackMate through python scripts. Resulting cell tracks contained information of fluorescence intensity over time which was then used for cell-cycle stage classification, adapting methods used in *Zerjatke et al* [102].

3.17.5 Multi-beam, multi-photon, multi-well plate (M³M) imaging system

The TCSPC FLIM experiments were performed on a multi-beam, multi-photon, multi-well plate imaging system that is under development in the group (**Figure 15**). The system is built on the open-source and modular *openFrame* microscope (Cairn Research, UK).

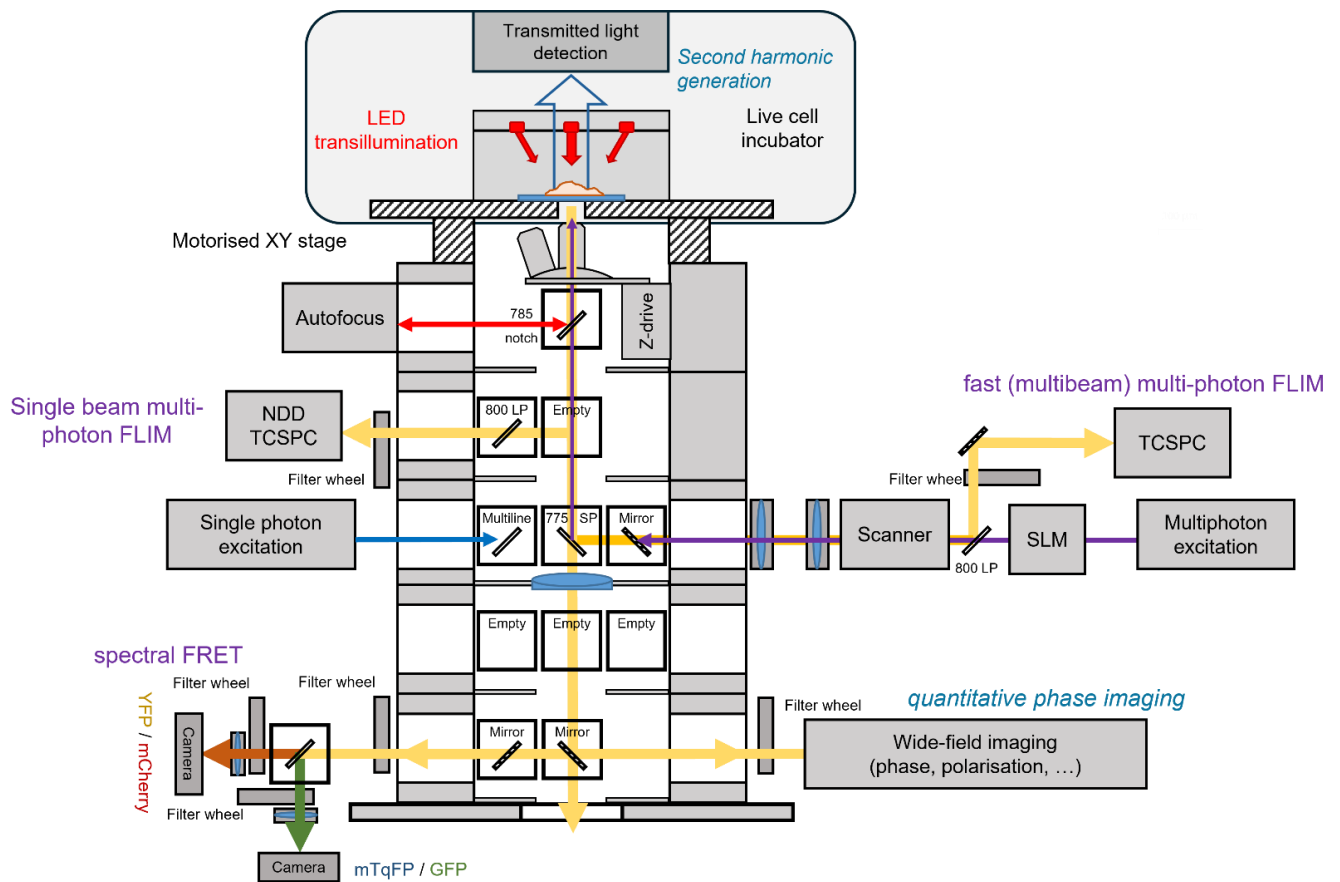


Figure 15: System diagram of the M³M imaging system. Built on the modular open-source *openFrame*. Different imaging modalities are implemented at each layer, with single beam multi-photon FLIM achieved by non-descanned detection (NDD) TCSPC. A spatial light modulator (SLM) is used for fast multibeam multi-photon FLIM. Widefield and quantitative phase imaging is implemented. A motorised XY stage and autofocus module allow for HCA imaging.

3.17.6 Imaging rucaparib intrinsic fluorescence

Two-photon fluorescence lifetime imaging microscopy (FLIM) was employed using the M³M microscope equipped with time-correlated single photon counting (TCSPC) capabilities. The imaging was performed with a 20X 0.95NA water immersion objective (Nikon, MRD77200). Cells were cultured in glass-bottom imaging plates suitable for high-resolution microscopy. Prior to imaging, cells were treated with 10 μ M rucaparib which was excited at approximately 820 nm. This excitation wavelength was chosen to optimise overlap with the two-photon excitation spectrum of rucaparib while minimizing potential photodamage to the cells.

To exclude the fluorescence from GFP, a series of optical emission filters were employed, specifically designed to block GFP emission wavelengths while allowing the detection of rucaparib's intrinsic fluorescence. The emitted fluorescence was collected and directed to the TCSPC detector, where the lifetime measurements were recorded. The data acquisition and subsequent analysis was performed using SymPhoTime 64 (PicoQuant), which allowed for precise determination of fluorescence lifetimes across different cellular regions.

3.17.7 Automated wide-field time-gated FLIM microscope and FLIM data acquisition

Here, the excitation source used was a picosecond supercontinuum laser source (Fianium UK Ltd, model SC-400-6), or a tuneable femtosecond Ti:Sapphire laser (Mai Tai HP, Spectra-Physics), providing the ultrashort pulse excitation required for time-gated FLIM. The instrument used is depicted in **Figure 16** and was implemented on the frame of a motorized inverted epifluorescence microscope (IX-81, Olympus, Tokyo, Japan) featuring a ZDC autofocus that was used for all experiments (This instrument is described in detail in *Görlitz et al.*[103]). OVCAR4 cells expressing the FRET biosensor were cultured in 96-well glass bottomed plates (SensyPlate, Greiner Bio-One GmbH, 655892). Following drug treatment, plates were mounted on a motorized X-Y stage (Märzhäuser Wetzlar GmbH) inside a stage-top incubator at 37 °C and 5% CO₂. Optically sectioned (quasi-confocal) FLIM was implemented using a spinning Nipkow disc unit (CSU-X1, Yokogawa Electric Corporation). A 20X air objective lens (UPlanApo 20X, Olympus) with an NA of 0.7 and working distance of 0.65 mm was used, unless otherwise stated. For excitation of green fluorescent proteins (e.g. EGFP) at 488 nm, the output beam from a tuneable femtosecond Ti:Sapphire laser (Mai Tai HP, Spectra-Physics) was directed to a second harmonic generation unit (Spectra-Physics, 3980) to provide pulsed excitation radiation at a 80 MHz repetition rate.

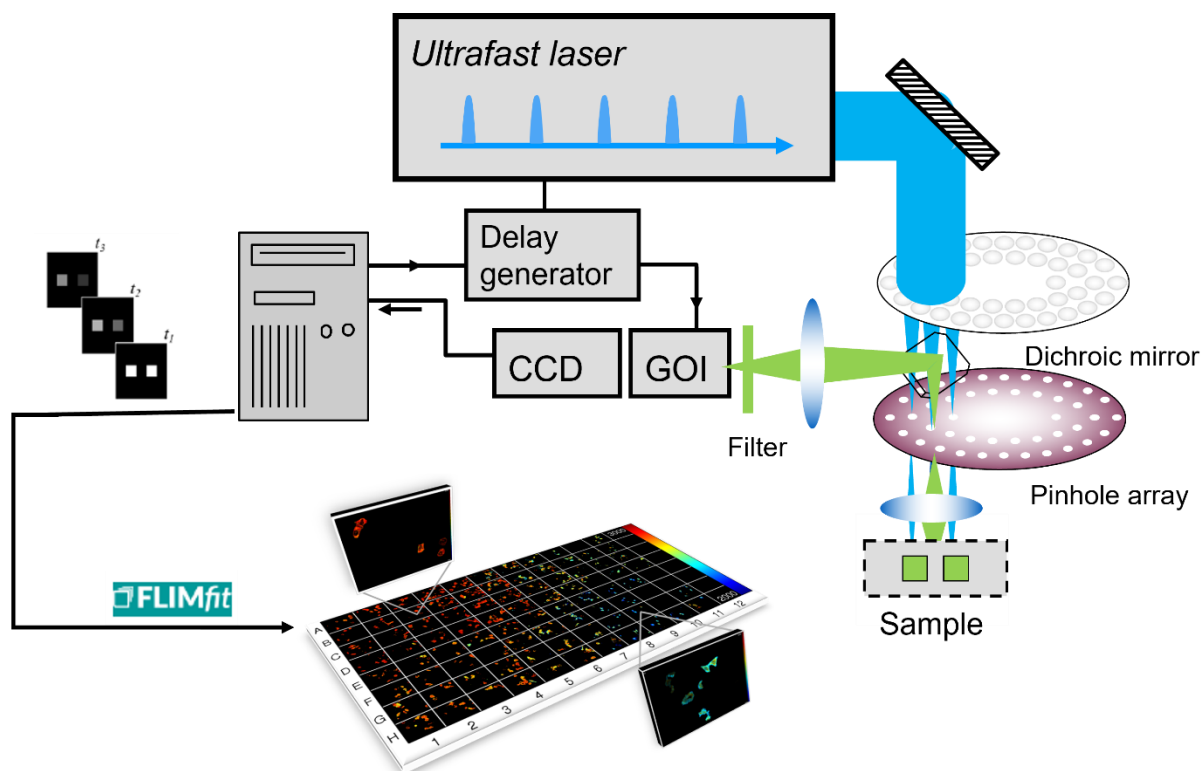


Figure 16: Optical setup for HCA FLIM-FRET. Optical sectioned FLIM with wide-field time-gated detection using a spinning Nipkow disc scanner. An ultrafast laser creates pulsed illumination which passes a pinhole array before illuminating the sample. Emission light passes back through the pinhole array and is reflected by the dichroic mirror into the gated optical intensifier (GOI), before forming an image on the cooled CCD camera. Fluorescence decays are fitted in FLIMfit, using images of differing delays (t_1 , t_2 , t_3) to produce fluorescence lifetime images.

The femtosecond excitation pulses were passed through a 5 cm pathlength in a Brewster-angle cut quartz rod to increase the pulse width (and thereby reduce the peak intensity and nonlinear photobleaching) before being directed via a polarization-preserving single-mode optical fibre to the spinning Nipkow disc unit. A 488/532 dichroic mirror (Semrock, Di01-T488/532-13x15x0.5) was used and the resulting excitation laser power was adjusted to ensure the power at the objective lens back focal plane was $\sim 200 \mu\text{W}$, as measured by an optical power meter console (Thorlabs, PM100D). The fluorescence emission was imaged onto a gated optical intensifier (GOI; HRI-HL, Kentech Instruments Ltd., Wallingford, UK) via a 520/35 nm emission filter (Semrock, FF01-520/35-25), and the resulting time-gated images at the phosphorus screen were imaged onto a cooled CCD camera (Retiga R1, Photometrics) with a relay of $0.7\times$ demagnification. The GOI gating voltage signal was set to 4 ns width and had been synchronized and delayed with respect to the laser excitation pulses. The delay between the excitation pulses and the gate was controlled using the $\mu\text{Manager}$ plugin *openFLIM-HCA*. Time-gated images of EGFP fluorescence were typically acquired at seven different delays with respect to the excitation pulses for FLIM experiments.

The FLIM data acquisition is controlled using *μManager* software, and the FLIM data were analysed using an in-house developed open-source MATLAB software, *FLIMfit* [103,104]. For these quantitative single-photon excited FLIM experiments, the sample was excited with a maximum power of 200 μW, and up to nine time-gated images were acquired, with a total integration time for FLIM acquisition of around ~10 s per FOV.

A previously reported *μManager* plugin for *openFLIM-HCA* was used to control the automated FLIM HCA data acquisition, imaging 4 FOVs within each well and acquiring FLIM data from all wells across the array without manual intervention [105]. On average, it required ~30 s to image each FOV, therefore an experiment imaging 4 FOV/well across 60 wells had required ~2 hr for the entire FLIM HCA acquisition. The FLIM data was saved as a series of OME-TIFF files (i.e., one TIFF file per FOV) in this study. More information and a detailed description of the *μManager* software had been available on the *openFLIM-HCA* wiki (<https://github.com/imperial-photonics/openFLIM-HCA>).

3.17.8 Instrument response function (IRF) measurements

To support correct analysis of the acquired FLIM data, for each experiment the fluorescence decay of a reference dye (75 μM Coumarin 6 in ethanol) was measured to provide an IRF. The decay was finely sampled at 25 ps intervals across the whole 12.5 ns pulse period. Additionally, the fluorescence decay profile of quenched fluorescein was measured, which exhibited a fluorescence lifetime around 100 ps. Briefly, 2.5 μL of 250 μM fluorescein (in water) was added to 100 μL 2M potassium iodide in water. The IRF was calculated from these two measurements via reference deconvolution and applied to subsequent FLIM data analysis [106]. This quantified any instrument drift over time to ensure that FLIM data would be reproducible. Note, IRF measurements were routinely taken before and after each imaging session to ensure no instrument drift had occurred over the course of imaging experiments.

3.17.9 FLIMfit data analysis

Fitting of fluorescence decays was performed in *FLIMfit v4.1.2*. Instrument response functions were measured and applied for each experiment. A time varying background was also measured, by measuring the decay of a fibronectin coated well containing the same imaging medium, but no cells. Global fitting of a double exponential lifetime was performed per FOV. Weighted mean lifetime values were extracted on a per-condition, per-cell basis and reported throughout.

3.17.10 Data analysis and statistical analysis

All images including immunofluorescence, FLIM images, widefield images and Incucyte images were processed and analysed using ImageJ (NIH, US). All numerical data were analysed and plotted on Prism v10.0.2 (GraphPad).

For mean comparisons between two groups, a *t*-test was used for populations with normal distribution and a Mann–Whitney test for nonparametric distribution and presented as mean $\pm$ standard deviation (SD). Analysis of variance (ANOVA) with Bonferroni correction and Tuckey post-hoc analysis was used for comparison of more than two groups. Matched-pair *t* test was used to compare mean values. Non-parametric regression was used to plot viability curves. Frequency distribution analysis was performed on single-cell FLIM data, fitting a gaussian curve to the dataset. The statistical test conducted in each experiment is stated in the legend of the corresponding figure. Significance was reported as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as standard.

4 Generation of FRET biosensor using CRISPR-Cas9 gene editing

4.1 Background

As outlined throughout the introduction, there are numerous approaches available to achieve genome editing. However, few, if any, can match the precision that CRISPR-Cas9 approaches offer. Hence, this method was employed with the goal of addressing *Aim 1* of this project, generating a dual-labelled PARP1 biosensor that can be expressed in live cells.

It was rationalised that two sequential rounds of CRISPR knock-in could be performed, first labelling the N-terminus of PARP1 with the donor (EGFP), followed by a second round of genome editing in the same cells, to introduce the acceptor (mCherryFP) at the C-terminus of the protein. Although this could theoretically be achieved in a single labelling step, the editing efficiencies would be likely to be low, due to factors including difficulty in transfecting cancer cells, high ploidy number and non-optimised gRNA sequence. Instead, it was decided to invest the time required for two rounds of genome editing, along with the subsequent quality control checks and characterization, in order to reduce the chances of introducing errors at each stage.

The introduction of fluorophores to label a gene of interest via CRISPR-Cas9 knock-in has recently become a commonly used approach, and there are many examples throughout the literature targeting various genomic loci. However, this task is not trivial, and dual labelling of a single gene is much less common throughout the literature. In addition, to form dual-labelled PARP1 at the protein level requires both genes (EGFP and mCherryFP) to be introduced on the same allele of *PARP1* to ensure that they are transcribed as a single EGFP-PARP1-mCherryFP transcript, that would be translated into a dual-tagged protein for use in live-cell FLIM-FRET assays downstream. To our knowledge, this had not been done before in the context of PARP1. Below, the required components to achieve this aim are outlined.

4.2 Aim and objectives:

This chapter reports the development of a genome editing approach aiming to introduce donor and acceptor fluorophores (EGFP and mCherryFP) to label endogenous PARP1 protein to create an endogenously expressed FRET biosensor for use in downstream FRET assays. The CRISPR system was first designed *in silico* before carrying out experimental steps to synthesise the required DNA constructs and plasmids to perform CRISPR knock-ins.

Firstly, computational tools were utilised for the *in silico* design and development of the molecular biology pipeline, with the hope of enhancing efficiency of the following experimental steps. This involved employing multiple molecular biology cloning techniques in order to synthesise novel linear DNA fragments that would be combined into dsDNA plasmids that could easily be delivered to cells

via transfection. These fragments were designed with complementary overlapping sequences to subsequently be combined into a plasmid using Gibson assembly reactions. This approach allowed control of the genetic design at each stage through careful design of DNA oligos for PCR amplification reactions. The generated plasmids were then used to deliver the components of a CRISPR-Cas9 system to induce genetic modifications (gene knock-in) at specific genomic loci. A thorough characterization of the generated CRISPR knock-in cell lines was then conducted at both the DNA and protein levels. This provided insight into the outcomes and efficiency of the gene editing approach used. The results are presented in three parts, first addressing the C-terminal labelling of PARP1, then the N-terminal labelling, and finally, dual labelling.

4.3 Results

4.3.1 CRISPR-Cas9 knock-in approach exploiting homologous recombination

The CRISPR knock-in strategy for targeting the PARP1 locus was developed by integrating key elements from previous research. Firstly, *Matsuda et al.*, demonstrated that they had synthesised a functional targeting vector (TV) for EGFP utilising homology arms for the rhodopsin gene rather than *PARP1* [107]. In this work, these homology arms were replaced with sequences complementary to the PARP1 region surrounding the intended cut site. This customised targeting vector was designed to serve as the repair template for inserting EGFP into the *PARP1* C-terminal locus.

Additional insights were gained from the work of *Perez-Leal et al.* who successfully performed CRISPR knock-ins at the C-terminus of *PARP1* [108]. Their research demonstrated the efficacy of a specific gRNA sequence for knock in of EGFP in HEK293T cells and therefore, the same gRNA sequence was chosen for use here. Of course, the gRNA performance can vary based on the cell line deployed in, but this offered a logical starting point.

From this, the desired CRISPR-Cas9 knock-in toolkit consisted of two separate plasmids (**Figure 17**), allowing delivery of the required components via lipofection or electroporation. The first component, the targeting vector, comprises the plasmid backbone, a 3' homology arm for *PARP1*, the EGFP sequence and a 5' homology arm for *PARP1*. Note, the homology arm sequences were also taken from the previously mentioned *Perez-Leal* study [108]. The second plasmid would consist of the gRNA sequence and the Cas9 enzyme. Therefore, the CRISPR knock-in would rely on exploiting homologous recombination repair upon the formation of a double strand break induced by the Cas9 enzyme. The targeting vector would then act as a donor repair template, resulting in the knock-in of GFP at this locus. Hence, combining elements of each of these studies was deemed an appropriate route to achieve the goal of EGFP knock-in at the PARP1 C-terminal locus.

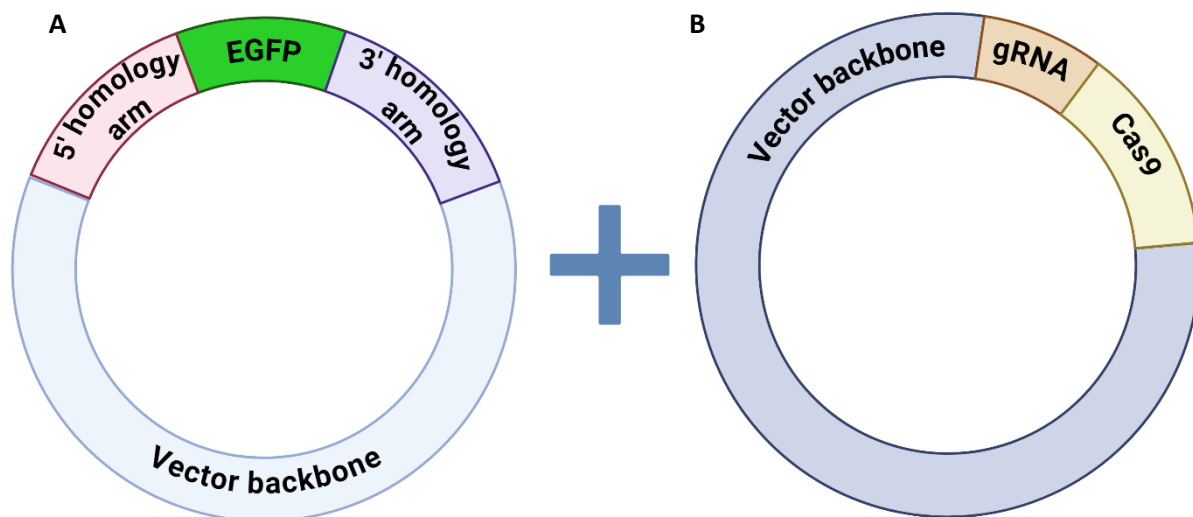


Figure 17: Two plasmid system for CRISPR KI to introduce EGFP at the C-terminus of PARP1. **A:** Targeting vector consisting of: vector backbone, 3' and 5' homology arms, EGFP repair template. **B:** Plasmid encoding the specific gRNA sequence and the Cas9 enzyme for targeting the C-terminus of PARP1.

Once this approach was finalised the *in silico* design work began, prior to the wet lab molecular biology experiments. Many *in silico* simulation experiments were performed, in the hope of reducing the chances of running into practical hurdles in the lab. As this strategy required the synthesis of two plasmids, the challenge was broken into smaller steps, addressing each individually.

The first plasmid synthesised was the PARP1 targeting vector, consisting of four individual fragments: a vector backbone, 5' and 3' homology arms, and the EGFP sequence. Each of these four fragments were designed in pyDNA to include 20-30 base pair overlaps between each consecutive fragment, with the intent of performing a Gibson assembly reaction to ligate them into a single functional dsDNA plasmid. Note, all PCR reactions in this work were performed with a Pfu DNA polymerase enzyme. This high-fidelity polymerase has a low error rate thus minimizing the introduction of mutations that could hinder the Gibson assembly approach. Moreover, the possible introduction of unwanted point mutations in the homology arms would reduce the efficiency of knock-in, as the CRISPR knock-in would be reliant on homologous recombination which is dependent on complementary homology arm sequences. In light of this, sequencing of all synthesised plasmids was performed as part of the quality control (QC) procedure.

4.3.1.1 Fragment 1: Vector backbone

The digested vector backbone fragment was synthesised first. ApE software (v3.1.4) was used to simulate restriction digestion products of the starting plasmid (Rhodopsin targeting vector (EGFP), kindly gifted by *Matsuda et al.*) with the aim of isolating the vector backbone (**Figure 18A,B**) [90,107]. This sequence would subsequently be used to design 20-30 bp overlaps with the 5' and 3' homology arm fragments that would later be annealed to this fragment in a Gibson assembly reaction.

A dual digestion was performed, using 0.5 μ L of both *Swa*I and *Asc*I restriction enzymes and 4 μ g of starting plasmid DNA. Reaction mixtures were prepared and mixed well, then left at 37 °C overnight in a humidified environment. The reaction product was then run on a diagnostic gel (0.8% agarose), loading 5 μ L of reaction per lane and compared to the molecular weight ladder to confirm the desired product was present. Upon confirmation of the presence of the desired product, a second gel was run, loading the remaining reaction mixture. The gel showed the presence of the desired DNA fragment, 2004 bp in length (**Figure 18C**). This band was then eluted and stored at -20 °C until required, securing the first of four fragments required.

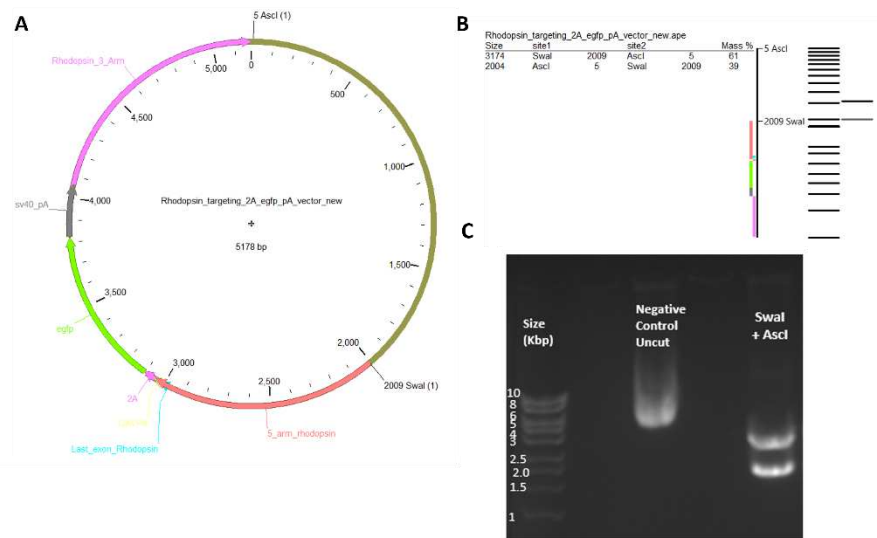


Figure 18: Targeting vector plasmid backbone fragment generation. **A:** Plasmid map from ApE software of the starting product, the rhodopsin-EGFP targeting vector from Matusda et al. **B:** ApE simulation of dual enzyme digestion product with *Swa*I and *Asc*I. **C:** Experimental diagnostic gel of dual digested (*Swa*I and *Asc*I, right) targeting vector backbone. The molecular weight ladder was run in the left lane. A negative control (no restriction enzyme) was run in the right lane.

4.3.1.2 Fragment 2: EGFP donor template

Next, the EGFP fragment was synthesised with 20-30 bp overlaps at each end, designed to ligate with the 3' and 5' homology arm fragments in the Gibson assembly reaction. Plasmid DNA (TV-Rhodopsin-EGFP) served as the template for a PCR reaction to amplify the desired fragment. The PCR used specific primers to introduce the overlaps, with an optimised extension step of 2 min at 72 °C per cycle, ensuring successful amplification.

The PCR product was confirmed on a diagnostic gel, revealing a band at approximately 800 bp, and no band in the negative control sample that was run under the same conditions with no source DNA (**Figure 19**). Subsequently, the desired band was purified from the gel, and the DNA concentration was quantified using a spectrophotometer for use in subsequent Gibson assembly reactions.

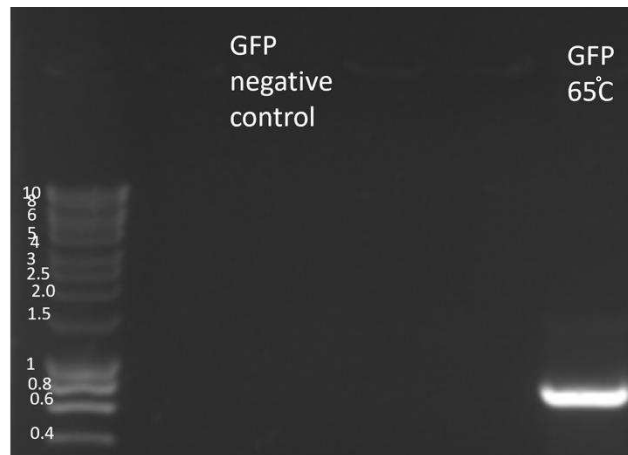


Figure 19: Diagnostic agarose gel of the PCR product for the EGFP fragment. DNA ladder was run on the left. The negative control condition had primers but no DNA. The PCR product (right) showed a band around the expected length of ~800 bp with an annealing temperature of 65°C.

4.3.1.3 Fragments 3 & 4: 5' and 3' homology arms complementary human PARP1 gene

The Cas9 cut site was designed based on a PAM sequence located immediately after the start codon of PARP1 exon 1, as previously described in the Perez-Leal paper (**Figure 20**). This would be dictated by the designed gRNA sequence (GTCAATTTTAAGACCTCCCTG). The homology arms therefore needed to be designed to flank the cut site. Generally, a length of 300-500 bp on either side of the DNA break site is sufficient for homologous recombination-directed repair to insert the protein of interest. Primers were designed for each homology arm, again introducing the ~20-30 bp overlap required for future Gibson assembly. 25 ng of human DNA from an HGSC ascites sample (ASC20-21) was used for the 3' homology arms, as a source of human PARP1 DNA.



Figure 20: gRNA and PAM sequence for C-terminal KI of PARP1. The DNA sequence of PARP1 (- strand), including the position of gRNA (TCAATTTTAAGACCTCCCTG) and PAM sequence (TGG) for C-terminal CRISPR knock-in. The PARP1 stop codon (TAA) is also shown. The flanking regions are also shown for context.

Initial attempts at amplifying the 5' homology arm fragment were unsuccessful. Therefore, to optimise this reaction a gradient PCR was conducted with annealing temperatures ranging from 45-59 °C. Subsequent gel electrophoresis of the reaction mixture showed faint bands at the expected length of 465 bp (**Figure 21**). Despite the low DNA yield observed, the bands were purified, and DNA quantity and purity were assessed using a nanodrop. The purified DNA was then stored at -20 °C until further use.

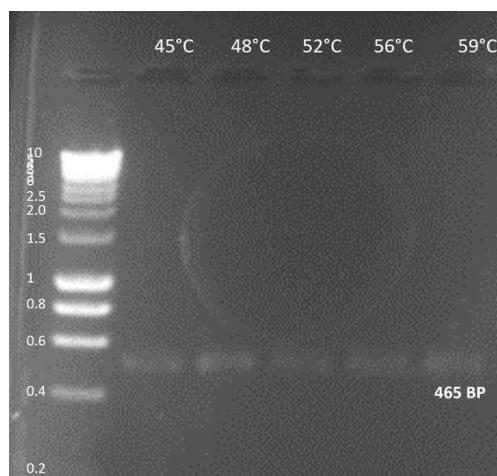


Figure 21: Diagnostic gel of PCR products to amplify 5' homology arm fragment. The temperature of annealing of PCR is annotated per sample. A molecular weight ladder is run in lane one (left) and weak bands are present in the PCR samples at the expected 465 bp.

The 3' homology arm fragment was synthesised via PCR amplification, using 60 ng human DNA extracted from a patient ascites sample as the DNA source with a Pfu polymerase supplied by Agilent. The PCR reaction mixture was analysed on a 0.8% agarose gel and the desired band was eluted. Three different conditions were used in optimization to find the optimal condition for PCR amplification of the fragment. Firstly, 1 min at 55°C annealing (**Figure 22A**), 2 min at 55°C annealing (**Figure 22B**) and finally 2 min at 55°C annealing, with 2 µL Pfu polymerase rather than the standard 1 µL (**Figure 22C**). All of these conditions produced a band at the expected 444 bp for the 3' homology arm fragment, but condition C produced the greatest quantity of DNA. Thus, this was the eluted band carried forward for Gibson assembly reactions. The successful synthesis and purification of the final fragment required for the TV marked the end of the synthesis stage. The subsequent step would involve ligating these four linear fragments into a single dsDNA plasmid through a Gibson assembly reaction.

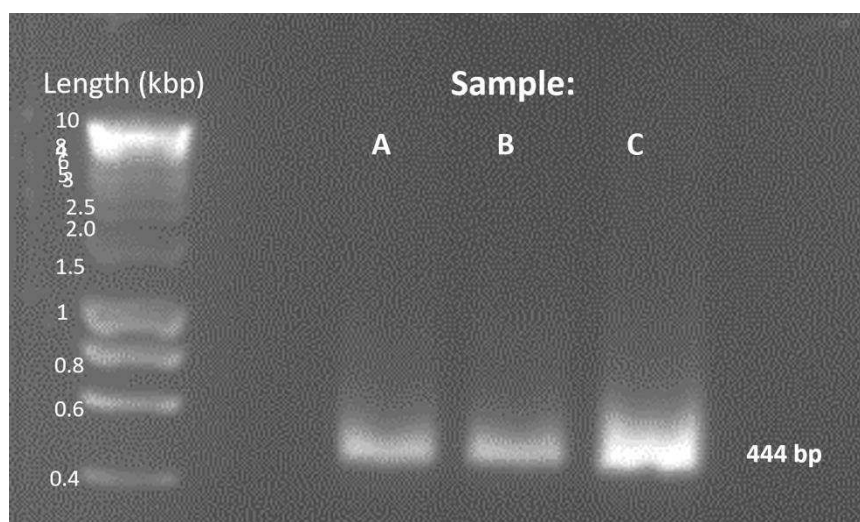


Figure 22: Diagnostic gel comparing PCR products from 3 differing optimised conditions. A molecular weight ladder was run in lane one (left). PCR samples are on the right with varying conditions. **A:** 55 °C annealing for 1 min, **B:** 55 °C annealing for 2 min, **C:** 55 °C annealing for 2 min, with 2 μ L Pfu polymerase enzyme.

4.3.1.4 Gibson Assembly reaction: combining the fragments and backbone

In order to have a functional plasmid, the three synthesised fragments had to be combined with the digested plasmid backbone (**Figure 23**). This necessitated a complex reaction with four reactants, all needing to align as desired. The efficiency of Gibson assembly reactions has been documented to decrease in efficiency as the number of fragments in the reaction increases [109].

Due to these challenges, optimizing the Gibson assembly reaction proved difficult, with several unsuccessful attempts resulting in no bacterial colonies upon transformation. The only readout of success was the presence of colonies and hence there was no way to determine if the process was failing at the Gibson assembly step, or the bacterial transformation. Each step of the reaction was therefore systematically optimised, with a single condition being altered at a time until bacterial colonies were produced under the following reaction conditions:

100 ng of each DNA fragment (EGFP, 3' and 5' homology arms) and 200 ng of digested plasmid backbone were added to a single PCR tube and mixed. This mixture of fragments was then left at 35 °C for 2 hr in the thermocycler to evaporate the water and concentrate the DNA products. This was then resuspended in 2 μ L nuclease free water and mixed well. 2 μ L of HiFi Master Mix (New England Biolabs, E5520S) was then added to a final 1:1 ratio with DNA and mixed gently. This was then heated at 50 °C for 1 hr in the thermocycler, at which time the isothermal, single tube Gibson assembly reaction was carried out.

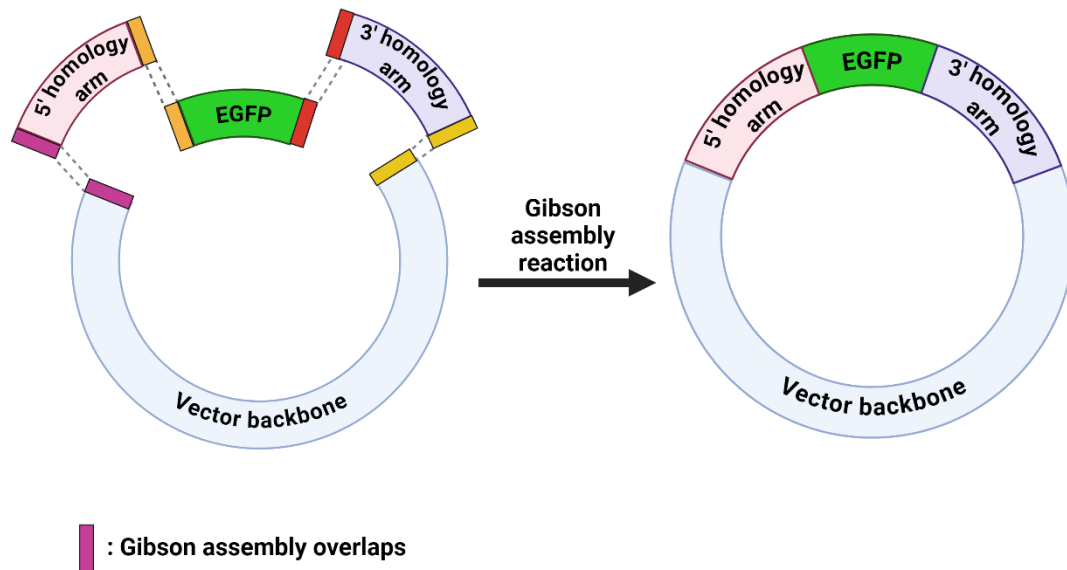


Figure 23: Schematic of Gibson assembly approach to form EGFP targeting vector. Four linear fragments (left) are combined into a single circular dsDNA plasmid (right) through a Gibson assembly reaction. Overlaps of ~20 nucleotides were added to each fragment during PCR amplification. The combined plasmid forms the PARP1-EGFP targeting vector.

The resulting Gibson assembly product was then transformed into the high copy number (1×10^8 efficiency) cloning grade bacteria JM109 (Promega L200A, 0000464492, 2022-02-23). For the transformation, 1 μ L of the Gibson assembly reaction was added to 50 μ L of JM109 bacteria. The following day, bacteria colonies had formed. Colonies were then picked, and plasmid DNA was isolated by miniprep and midiprep. The resulting purified DNA was then run on a diagnostic restriction digestion gel and sent for sequencing to confirm the completed targeting vector plasmid had the desired sequence.

These experiments led to the successful synthesis of the PARP1-EGFP targeting vector, in a stepwise fashion. This would make up one component of the CRISPR-Cas9 system to perform knock-in of EGFP at the C-terminus of PARP1.

4.3.1.5 Plasmid 2: Cas9-gRNA

The second plasmid to synthesise contained both the sequence for the Cas9 enzyme and the specific gRNA sequence to target the defined cut-site on PARP1. The commercially available pX330-U6-chimeric-BB-Cbh-hSpCas9 plasmid (Addgene #42230) that encodes for the Cas9 enzyme was purchased. Next, primers for the specific gRNA sequence required were designed using pyDNA. Note, during the design of the gRNA sequence, a guanine residue was added to the start of the sequence to ensure there is a functional start site for RNA polymerase to allow the synthesis of this fragment. These primers were then annealed and ligated into the plasmid in a single reaction. This process underwent multiple rounds of optimization, with the critical discovery that phosphorylating the

primers before this single-tube reaction was essential for success. Phosphorylation of the primers was achieved using T4 polynucleotide kinase (Thermo Fisher Scientific EK0031), which phosphorylates the 5' end of the oligos. This phosphorylated end then reacted with the 3' OH group of the digested plasmid, facilitating ligation in the subsequent reaction.

The phosphorylation reaction was conducted in a thermocycler at 37 °C for 30 min, followed by a ligation step at 95 °C for 5 min. The temperature was then gradually decreased at a rate of 5 °C per min until reaching a final temperature of 25 °C. Subsequently, the reaction mixture was stored at 4 °C for future use. The resulting phosphorylated oligos were diluted 1:200 in MilliQ water before being utilised in a reaction where the primers were ligated by T4 ligase, and the plasmid backbone was digested by BbsI restriction enzyme in the same reaction (see **Table 11** for reaction components).

Component	Volume
Uncut Cas9 Plasmid	1 µL (100ng)
Diluted phosphorylated oligos:	2 µL
Tango Buffer 10X	2 µL
DTT (10 mM)	1 µL
ATP (10 mM)	1 µL
MilliQ	11.5 µL
T4 Ligase	0.5 µL
BbsI restriction enzyme	1 µL

Table 11: Single-tube reaction components for plasmid DNA digestion and oligo ligation. The phosphorylated oligos are ligated into the restriction enzyme digested plasmid backbone.

The reaction was then heated at 37 °C for 5 min then 21 °C for 5 min for 6 cycles. Subsequently, the resulting reaction mixture was then transformed into the Stbl3 bacteria strain (Life Technologies, C737303). For transformation, 50 µL Stbl3 bacteria was mixed with 5 µL of the resulting ligation/digestion reaction. The bacterial mixture was then spread onto agar plates. The following day, six separate colonies were selected for mini preps and downstream sequencing to confirm the presence of the expected gRNA sequence in the plasmid. A diagnostic restriction digestion was performed to quickly assess whether the desired plasmid sequence had been successfully produced (**Figure 24**). The diagnostic gel suggested the presence of the desired plasmid, with the gel showing most of the expected bands from the simulation run in ApE software (v3.1.4). However, this method was only used as a quick quality control and is not definitive. Hence, the synthesised plasmid was later sent for sequencing.

4.3.2 Transfection of CRISPR components

Once the two plasmids had been successfully synthesised and quality-checked by Sanger sequencing, testing of the CRISPR system's functionality was performed by transfecting cells. The targeting vector was designed not to contain promoter activity, so that EGFP could not be expressed by itself. However, once the targeting vector was correctly inserted into the target locus with the aid of CRISPR/Cas9, a fusion protein between PARP1 and EGFP would be produced from the endogenous *PARP1* locus.

As the exogenous gene encoded a fluorescent protein, knock-in success could be screened by assessing the expression of EGFP 24-48 hr later using simple wide-field epifluorescence microscopy. However, this should only be regarded as a qualitative readout of success, as it is possible that the exogenous gene could be integrated at an undesired location in the genome. Therefore, further sequencing was also required.

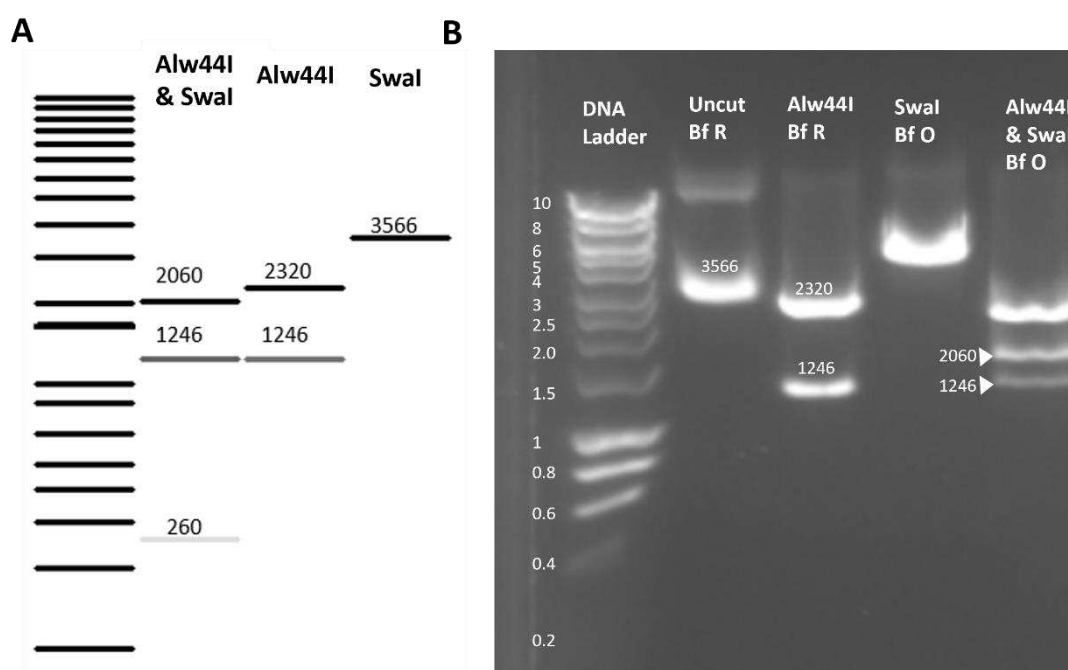


Figure 24: Restriction enzyme digestion of the synthesised PARP1-EGFP targeting vector plasmid. **A:** ApeX software simulation of the restriction digestion from the desired plasmid sequence. **B:** Experimental results from the diagnostic gel. The DNA ladder was run in the first lane, uncut control in the second lane, single enzyme digests in lanes three and four, and dual enzyme digest in lane five. Bands that could be identified are annotated on the gel.

Initially, to assess whether the CRISPR system was functional, both plasmids were transfected into the HEK293 cell line via lipofection using Viafect reagent (Promega, E4981), due to their high transfection efficiency compared to other cell lines. For this, 2×10^5 cells were seeded per well of a 6-well plate in complete medium. The next day a transfection mixture was made up as below (**Table 12**), mixed well, and then left for 30 min at room temperature, mixing every 10 min to allow time for the DNA-liposome complexes to form. The medium was then aspirated from the cells and 1.4 mL of complete

medium was added. The transfection mixture was then added to this, in a final volume of ~1.5mL. The cells were then cultured for 24-48h in this mixture and then transfection success was assessed via fluorescence microscopy, assessing for expression of EGFP in the nucleus, where PARP1 is found in its non-cleaved state under basal conditions.

Component	Amount
PARP1-EGFP targeting vector	1 µg
Cas9-gRNA plasmid	1 µg
Serum and antibiotic-free DMEM	100 µL
Viafect	3 µL per 1 µg of DNA

Table 12: Transfection conditions for CRISPR-Cas9 knock-in of PARP1-EGFP. Lipofection was used to insert the two plasmids PARP1-EGFP targeting vector and Cas9-gRNA plasmids for knock-in of EGFP at PARP1 C-terminus.

Around 5% of the population showed nuclear expression of EGFP 48h after transfection when assessed via epifluorescence **Figure 25**. The cells were then fixed and stained with SYTO Deep Red to co-stain the nucleus. Based on the overlap of these two labels, all the EGFP positive cells showed nuclear expression, providing further supporting evidence that PARP1 may be labelled with EGFP.

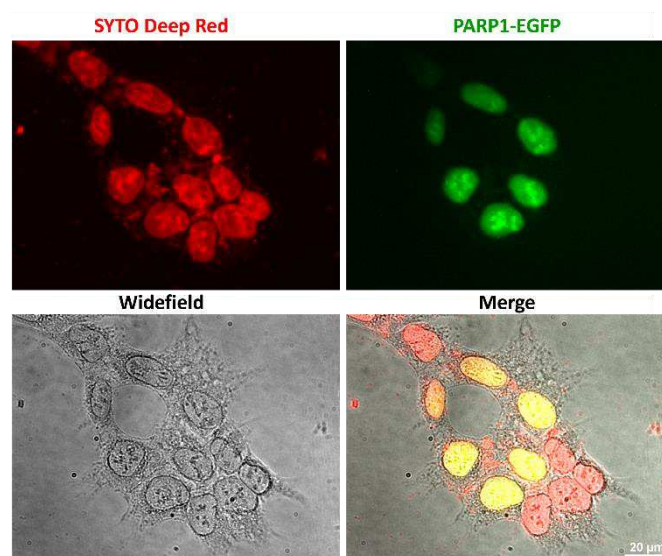


Figure 25: Transfection of HEK293 cells with PARP1-EGFP targeting vector. SYTO Deep Red (top left, red), EGFP (top right, green), widefield (bottom left) and merged images (bottom right) are shown. Epifluorescence images were acquired with a 60X objective.

Following the successful transfection of HEK293 cells, transfections were performed in OVCAR4 cells, the desired target of the CRISPR genome editing, using the same conditions outlined in **Table 12**. When assessed by epifluorescence ~48 hr later, approximately 1% of the cell population were EGFP positive. Cells were subsequently expanded, and 7×10^6 cells were used for fluorescence-activated cell sorting (FACS) to isolate the EGFP-positive cells. The cells were collected as a pooled population rather than

single-cells, to ensure the viability of the population. This homogenous population (all EGFP+) were then expanded for further characterization.

4.3.2.1 Assessment of PARP1-EGFP expression at the protein level

To determine if the genome editing successfully tagged the endogenous *PARP1* gene, a western blot was performed to assess EGFP tethering at the protein level. OVCAR4 wild-type cells were used as a negative control, showing a single strong band at PARP1 molecular weight of PARP1 (116 kDa) (**Figure 26**). In contrast, CRISPR-edited cells (OVCAR4 PARP1-EGFP) exhibited an additional band at approximately 143 kDa, indicative of EGFP (27 kDa) binding to PARP1. The presence of both bands in these cells suggests a heterozygous knock-in in at least a portion of the population. Subsequently, the nitrocellulose membrane was stripped and re-probed with an anti-EGFP antibody, selectively labelling the higher molecular weight band found exclusively in the PARP1-EGFP cells, thereby providing supporting evidence of EGFP being bound to PARP1. The unlabelled lower molecular weight band likely represents the wild-type PARP1 protein fragment at 116 kDa. As this was performed on a pooled population following FACS sorting and prior to any single-cell cloning, it indicates a mixed population comprising both heterozygous and potentially homozygous modified cells. However, a semi-quantitative assessment suggests that the labelled alleles constitute approximately 50% of the total PARP1 protein pool.

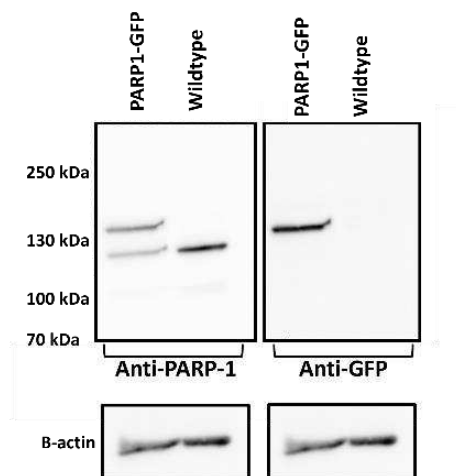


Figure 26: Western blot confirming C-terminal labelling of PARP1 with EGFP. Protein samples from the OVCAR4 wild-type cells and the CRISPR-edited OVCAR4 PARP1-EGFP populations. Nitrocellulose membranes were probed with an anti-PARP1 antibody (left) then stripped and re-probed with an anti-EGFP antibody (right), anti- β -actin was used as a protein loading control. The EGFP labelled cells show two molecular weight bands when probed with PARP1, suggesting a heterozygous knock-in as the wild-type bands ~116 kDa is still present. The higher molecular weight band ~143 kDa is labelled by the PARP1 antibody and selectively by the anti-EGFP antibody, suggesting this is PARP1-EGFP.

Next, an experiment was performed to assess if the labelled PARP1 was still capable of undergoing cleavage, an integral process for maintaining genomic stability and ensuring proper cellular responses to DNA damage. To induce DNA damage and PARP1 cleavage, PARP1-EGFP labelled cells were treated

with 40 μ M carboplatin for 48 h. Subsequently, a comparison between untreated and treated cells was made using western blot analysis, probing with an anti-EGFP antibody (**Figure 27**). A single band was observed for the untreated cells, whereas two bands were observed for the carboplatin-treated cells. This suggests that the EGFP-labelled PARP1 protein remains capable of undergoing protein cleavage, preserving a critical aspect of native PARP1 functionality. Note that PARP cleavage in the OVCAR4 wild-type cells under the same carboplatin dosage was also assessed, revealing similar levels of cleavage compared to the PARP1-EGFP population when probed with an anti-PARP1 antibody. However, comparisons between different antibodies are subject to variations in binding affinity, etc., thus this can only be considered a qualitative assessment.

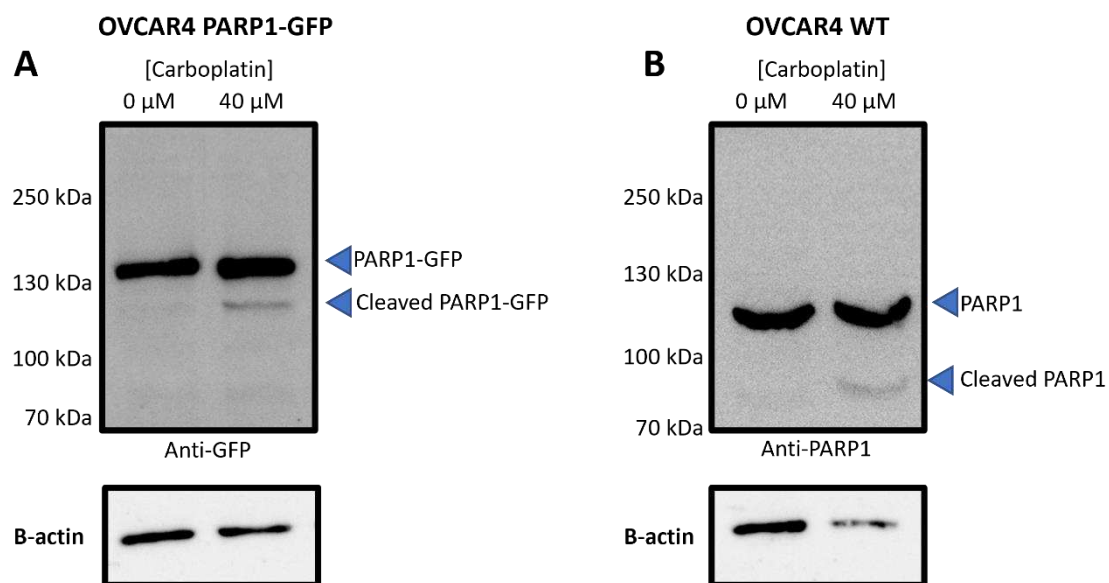


Figure 27: Western blot of PARP1 cleavage in CRISPR-edited cells. Fluorescent labelled PARP1-EGFP still undergoes PARP cleavage upon exposure to DNA damage/apoptosis. **A:** OVCAR4 PARP1-EGFP cells treated with 0 or 40 μ M carboplatin for 48 h, probed with anti-EGFP antibody. Treated cells show a lower molecular weight band ~116 kDa representing the cleaved PARP1 fragment (89 kDa) labelled with EGFP (27 kDa). **B:** OVCAR4 WT cells under the same treatment conditions, probed with an anti-PARP1 antibody show the full length PARP1 fragment at 116 kDa and cleaved PARP1 at 89 kDa upon treatment. β -actin was used as a protein loading control for both membranes.

Next, viability assays were performed to evaluate whether PARP1 functionality had been maintained in the EGFP labelled cells, compared to the wild-type OVCAR4 line. Firstly, MTT assays were used to investigate any changes in the DNA damage repair responses. Cells were treated with carboplatin to induce DNA damage, relying on PARP1 functionality for repair and subsequent survival. Viability curves comparing PARP1-EGFP and wild-type cells indicated no significant difference, suggesting no alterations in the ability to repair DNA damage, particularly in response to platinum chemotherapy, indicating PARP1's continued functionality function (**Figure 28**). However, this assessment is at the population level, and the assay's sensitivity is limited. Nonetheless, overall sensitivity to carboplatin remained unchanged, which would not occur if PARP1 were non-functional in the labelled cells.

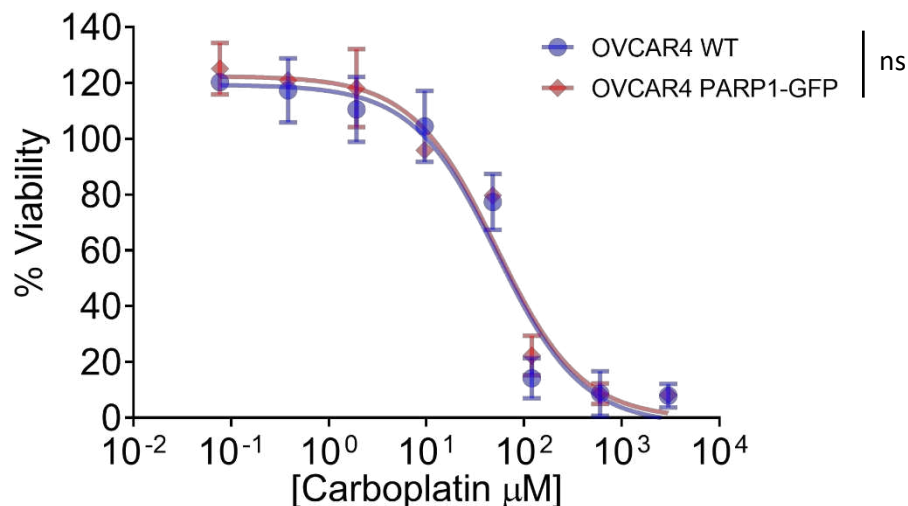


Figure 28: Endogenous labelling of PARP1 with EGFP does not alter the sensitivity to carboplatin. MTT assay assessing the viability of parental OVCAR4 WT cells compared to the CRISPR-labelled OVCAR4 PARP1-EGFP cells, no significant change in viability was observed. T-test was performed for statistical comparison of groups.

SRB assays were also performed to assess if there were any changes in the sensitivity to PARP inhibitors in these edited cells. When compared to the unlabelled OVCAR4 WT cells, the OVCAR4 PARP1-EGFP cells showed no significant difference in sensitivity to these two PARPi (**Figure 29**). This suggests that the PARPi are still able to induce cytotoxicity via PARP1 inhibition, despite the physical presence of the large EGFP molecule.

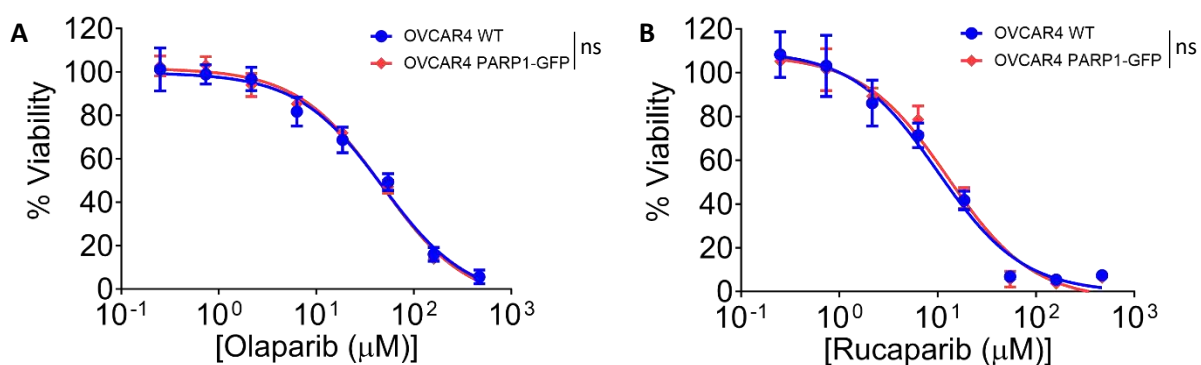


Figure 29: Endogenous labelling of PARP1 with EGFP does not alter the sensitivity to PARPi. SRB assays comparing unlabelled and labelled cells treated with PARPi **A:** olaparib and **B:** rucaparib for 72 h, $n=3$.

A potential consideration is that these cells are heterozygous for the PARP1-EGFP label, suggesting that wild-type PARP1 may compensate for any potential reduction in functionality from the EGFP-labelled PARP1 proteins. However, these assays demonstrate that there is no survival disadvantage resulting from the introduction of PARP1-EGFP into these cells. Furthermore, any off-target effects of

the Cas9 enzyme do not appear to confer a survival disadvantage, although it is possible that cells with such mutations were naturally selected against through clonal competition during passaging. Throughout this section, the ability to perform CRISPR-Cas9 genome editing, specifically to allow gene knock-in at a locus of choice, was demonstrated. This process is summarised in **Figure 30** below. However, despite the success achieved with knock-in at the C-terminal of PARP1, it was recognised that a slightly different approach would be needed to facilitate knock-in at the N-terminus of the gene.

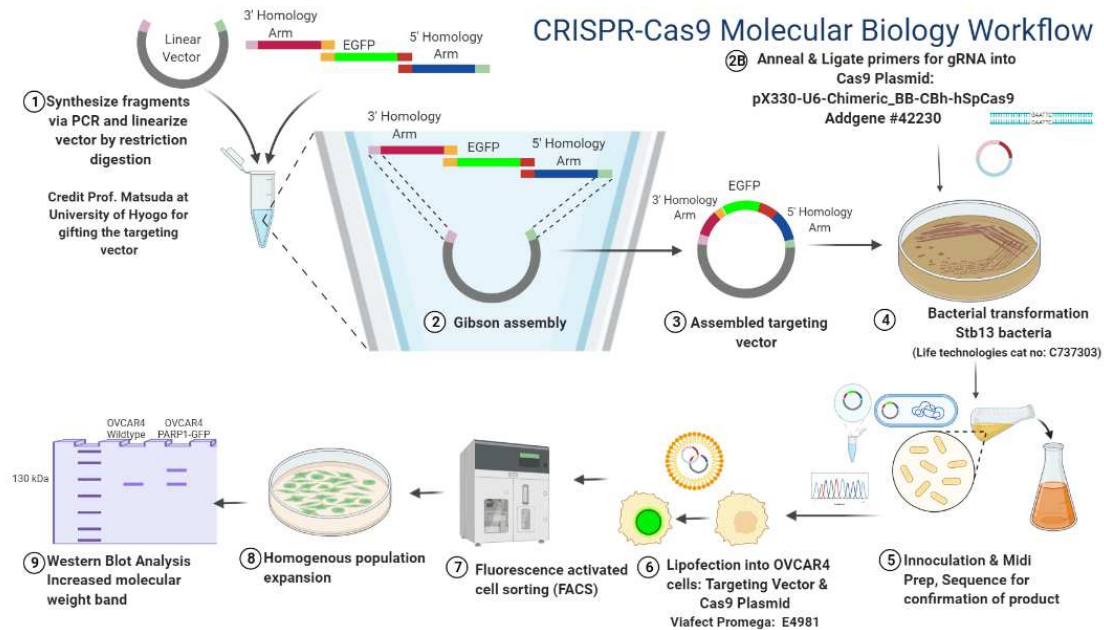


Figure 30: Schematic of CRISPR-Cas9 workflow for labelling PARP1. Individual fragments were synthesised (1) and combined by Gibson assembly (2). Primers for the gRNA sequence were ligated into the Cas9 containing plasmid backbone (2B). These plasmids were transformed and isolated from bacteria before lipofection into OVCAR4 cells (4-6). EGFP (C-terminal) positive cells were isolated by FACS, and western blots were performed for characterisation (7-9). Figure created in BioRender.

4.3.3 Part 2: Labelling PARP1-EGFP at the N-terminal

In order to dual-label PARP1, CRISPR approaches for knock-in were chosen for both the C and N termini. For N-terminal knock-in, a different approach was employed, utilizing simultaneous single-stranded DNA break formation at donor and acceptor DNA by CRISPR-Cas9 nickase (*in trans* paired nicking). This approach had previously been utilised successfully to label the N-terminus of PARP1 at the same genomic loci targeted by *Chen et al.* [100]. The rationale behind this approach is that single-stranded DNA breaks (SSBs) created by programmable and strand-specific nucleases (nickases) are inherently less disruptive than double-strand breaks (DSBs), as they do not serve as canonical NHEJ substrates. Therefore, CRISPR-Cas9 nickases, consisting of guide RNAs (gRNAs) and Cas9 proteins with disabled RuvC or HNH nuclease domains (e.g., Cas9^{D10A}), are potentially valuable programmable

nicking enzymes. Similarly to traditional cleaving CRISPR-Cas9 nucleases, nickases target DNA sequences containing a protospacer adjacent motif (PAM) and a region complementary to the 20 nucleotides at the 5'-terminal of the gRNA (spacer). Pairs of CRISPR-Cas9 nickases are often used to induce site-specific single-strand breaks (SSBs) through coordinated nicking on opposite target DNA strands. This dual-nicking approach enhances the specificity of double-strand break (DSB) formation, as SSBs at off-target sites are generally faithfully repaired. Hence, it is important to note that genome editing based on paired CRISPR–Cas9 nickases still carries a risk of mutagenesis and chromosomal rearrangements due to the eventual creation of DSBs.

Chen et al, utilised this approach to carry out directed gene-tagging of PARP1 at its N-terminal, achieving CRISPR knock-in of EGFP at the target locus [100]. Therefore, this approach was chosen to perform CRISPR editing at the same genomic locus. The gRNA and donor plasmids were kindly provided by Xiaoyu Chen, enabling the CRISPR knock-in of EGFP at the N-terminus following their approach. Note, the donor plasmid contained a XTEN linker that would be knocked in before PARP1. Previous studies have found that this flexible 16 amino acid linker is the optimal compromise between editing efficiency and specificity [110]. The gRNA (CGGCAGCTAGGGGAGGATGG) was designed to include the start codon of PARP1, immediately followed by the PAM sequence (CGG), ensuring that Cas9 induces a cut 3-4 bp upstream of the PAM sequence **Figure 31**. This positioning places the double-strand break precisely after the start codon of PARP1, facilitating the introduction of EGFP at this site through homology-directed repair.



Figure 31: gRNA and PAM sequence for knock-in at the N-terminal of PARP1. The DNA sequence of PARP1 (- strand), including the position of the gRNA (shown in bold) and PAM sequence (CGG) for N-terminal CRISPR knock-in. The PARP1 start codon (ATG) is also shown. The flanking regions are also shown for context.

The two plasmids supplied by *Chen et al.* (pgRNA^{PARP1}, pDonor^{PARP1}) were subsequently transfected into OVCAR4 wild-type cells along with the Cas9 plasmid (pST1374-N-NLS-flag-linker-Cas9-D10A, Addgene #51130), using 1 µg of each. These three plasmids constituted the CRISPR system for N-terminal targeting of PARP1. Transfection efficiency was then evaluated by epifluorescence microscopy, observing a low transfection efficiency with ~1% of cells showing nuclear EGFP expression (**Figure 32**).

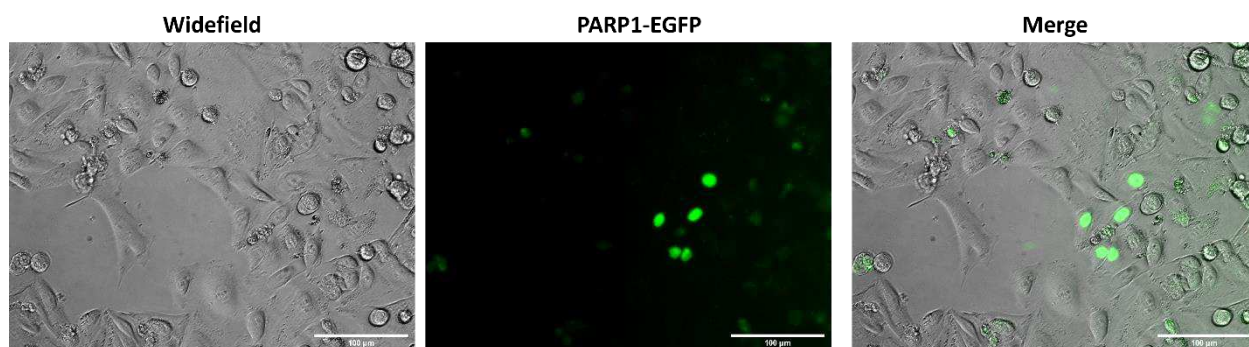


Figure 32: N-terminal PARP1-EGFP knock-in. Epifluorescence images of OVCAR4 cells 48 hr after transfection with the CRISPR system. Widefield (left), EGFP channel (middle) and merged images (right) are shown. Scale bar = 100 µm.

The transfected population was subsequently expanded and EGFP positive cells were isolated by FACS. The resulting homogenous EGFP positive population was then expanded and stored. To assess the labelling of PARP1 at the protein level, western blots were performed on protein extracted from these cells, compared to wild-type OVCAR4 cells. Protein samples were run across two gels and membranes were subsequently probed for either an anti-PARP1 or anti-EGFP antibody. The transfected cells showed two molecular weight bands when probed for PARP1, indicating a heterozygous knock-in. The higher molecular weight band of 143 kDa corresponds to PARP1-EGFP and the lower band at 116 kDa represents wild type PARP1, confirmed by the presence of this band exclusively in the wild-type OVCAR4 sample. The anti-EGFP antibody confirmed this, selectively labelling the higher molecular weight band in the OVCAR4 PARP1-EGFP cells only. Therefore, these results indicate successful N-terminal labelling of PARP1-EGFP. This marked a significant step towards synthesising the desired FRET biosensor *in vitro*, and this cell line would serve as the donor only control for subsequent FRET experiments. Moreover, once a suitable CRISPR system had been developed, this cell line would be transfected to form EGFP-PARP1-mCherryFP expressing cells.

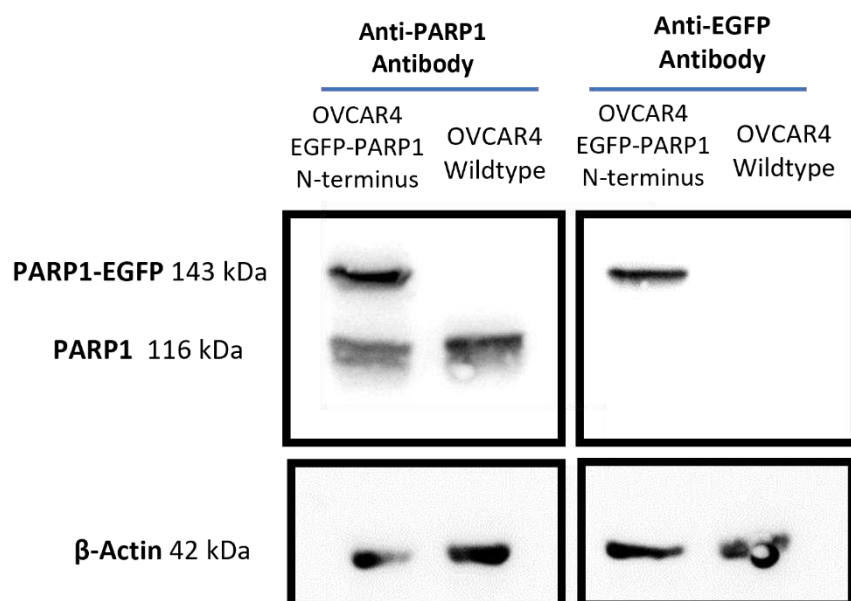


Figure 33: Western blot assessing N-terminal CRISPR knock-in. OVCAR4 wild-type and transfected counterparts (OVCAR4 PARP1-EGFP) were probed with either anti-PARP1 or anti-EGFP antibodies. B-actin was used as a protein loading control for all samples.

4.3.4 Part 3: Labelling PARP1 with mCherryFP at the C-terminal

The next objective was to endogenously label PARP1 at the C-terminal with mCherryFP, another fluorescent protein, which has been used widely as a FRET acceptor in combination with a EGFP donor. Since successful C-terminal labelling of PARP1 had been achieved already, the same gRNA used in the work by Perez-Leal et al., could be used for the introduction of mCherryFP at this locus [111]. The targeting vector synthesised for the previous EGFP C-terminal knock-in was utilised as a starting point, requiring replacement of EGFP with mCherryFP. A similar Gibson assembly approach was planned, involving the synthesis of four fragments with approximately 20 base pairs of homology overlaps at each end (**Figure 34**). *In silico* design and planning of the desired plasmid were conducted using pyDNA, identifying a 1 nucleotide variation between the starting sequences of EGFP and mCherryFP. Therefore, the forward primer and overlap sequence between the 5' homology arm fragment and the mCherryFP fragment had to be redesigned from those used in the C-terminal EGFP knock-in. Apart from this adjustment, the previously synthesised fragments could be utilised, as the N-terminal sequence of mCherryFP is identical to that of EGFP.

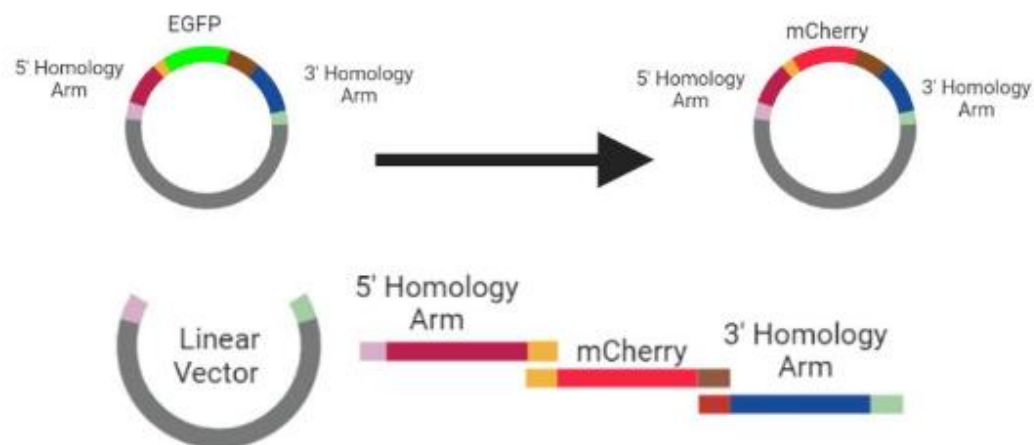


Figure 34: Schematic of PARP1-mCherryFP targeting vector. Top panel shows the starting product (PARP1-EGFP targeting vector) and the desired end product, the PARP1-mCherryFP targeting vector. Bottom panel shows the four individual fragments required for the synthesis of the PARP1-mCherryFP targeting vector with their respective complementary overlaps for ligation via a Gibson assembly reaction.

The desired plasmid sequence intended to be synthesised is depicted in **Figure 35**, illustrating the vector backbone along with the 5' arm, mCherryFP, and 3' arm fragments.

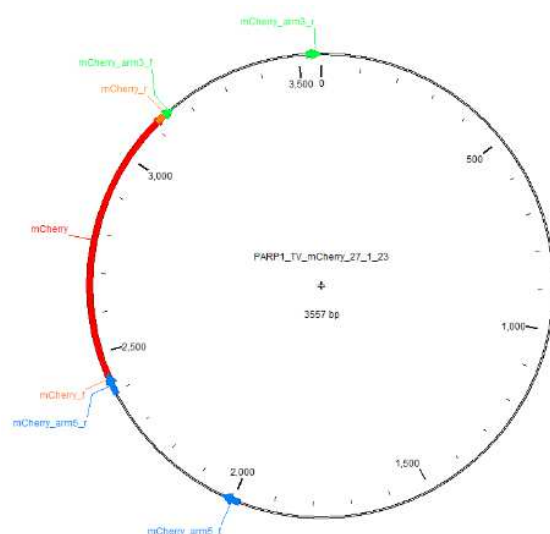


Figure 35: Desired plasmid sequence for PARP1-mCherryFP TV. 5' Homology arm is shown in blue, with the respective primer positions highlighted (mCherryFP_arm5_f, mCherryFP_arm5_r). 3' Homology arm is shown in green, with the primer positions labelled (mCherryFP_arm3_f, mCherryFP_arm3_r). The mCherryFP fragment is shown in red, with primers in orange (mCherryFP_f, mCherryFP_r). Total plasmid length is 3557 base pairs.

To synthesise the mCherryFP fragment, a PCR reaction was performed using 0.5 ng of an H2B-mCherryFP plasmid as the source DNA, and run across an annealing temperature gradient from 57-67 °C. The resulting PCR products were run on a 1% agarose gel and visualised against a molecular weight ladder, showing a single band of 465 bp, as expected (**Figure 36**). This band was then eluted and purified.

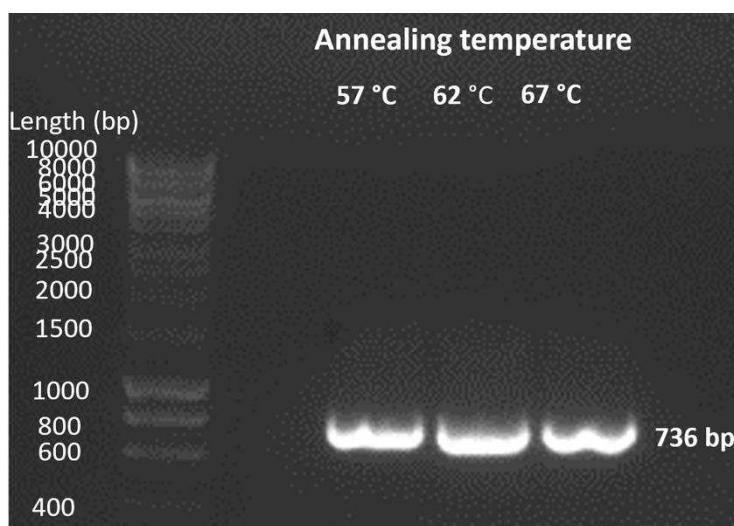


Figure 36: Conformation of mCherryFP fragment synthesis. mCherryFP fragment with flanking overlaps was synthesised via PCR amplification of H2B-mCherryFP plasmid. Products from a gradient PCR reaction (annealing temperature 57-67 °C were run on an agarose gel, with molecular weight ladder shown left. All conditions produced a band at the expected molecular weight of 736 bp which was subsequently eluted from the gel.

The 5' homology arm for mCherryFP was then amplified, using the previously synthesised 5' homology arm for EGFP as the DNA source, but using unique primers that account for the single nucleotide difference at the start of the mCherryFP and EGFP sequences, in order to have complimentary overlaps for future Gibson assembly reactions. The previously synthesised PARP1-EGFP (C-terminal) targeting vector was used as the DNA source for the PCR reaction. The resulting DNA was run on a 1% agarose gel and the desired band of 465 bp was extracted (**Figure 37**).

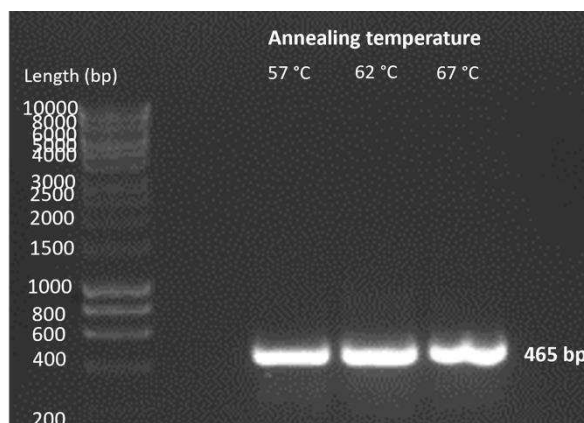


Figure 37: 5' homology arm fragment for the mCherryFP targeting vector. A gradient PCR was performed with annealing temperatures from 57 °C to 67 °C and band at 465 bp was eluted from the agarose gel. The molecular weight ladder is shown on the left.

The two newly synthesised fragments were then combined with the linearised backbone and the 3' homology arm, both of which were used in the previous C-terminal EGFP knock-in. A Gibson assembly reaction was performed to combine these four fragments. An equimolar (1:1:1:1) ratio of each fragment (mCherryFP, 5' arm, 3' arm, digested backbone) was mixed, 0.3 pmol of each (**Figure 38**). This DNA was then mixed 1:1 with HiFi master mix and placed at 50 °C for 1hr for the Gibson assembly

reaction to occur. Subsequently, 0.25 μL of the Gibson assembly reaction mixture was added to 50 μL of NEB bacteria (DH5 α) on ice and left for 30 min before heat shock was performed, placing the bacteria at 42 $^{\circ}\text{C}$ for 45 s and then back on ice for 2 min. This mixture was then transferred to 900 μL of NEB SOC medium to recover at 37 $^{\circ}\text{C}$ for 1 h, shaking at 200 rpm. 30 μL of this mixture was then seeded directly on ampicillin agar plates and left at 37 $^{\circ}\text{C}$ overnight. Colonies were present and 6 separate colonies were picked into separate tubes of 5 mL LB and grown out overnight.

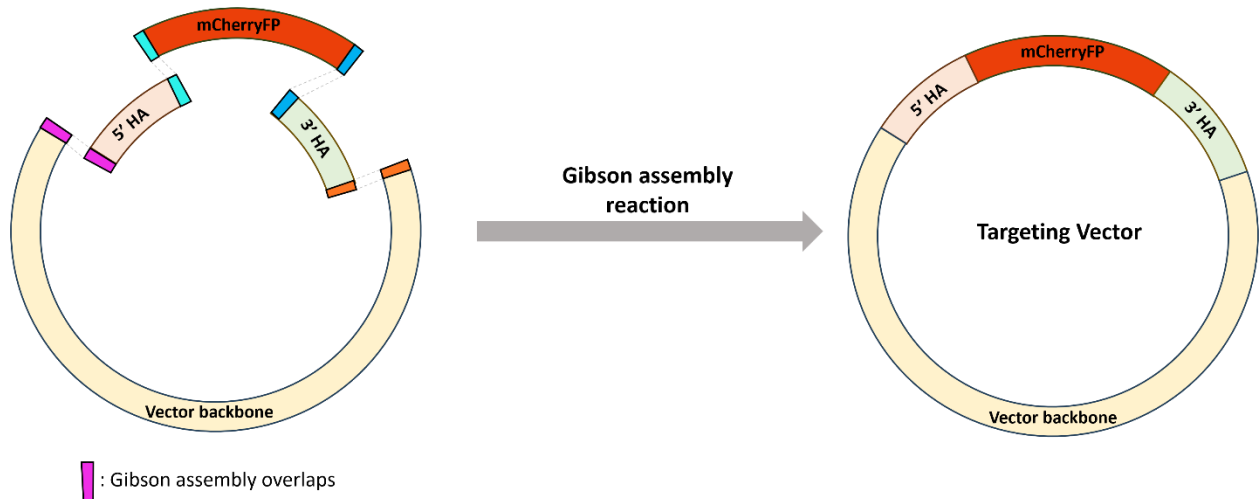


Figure 38: Schematic of Gibson assembly reaction forming the PARP1-mCherryFP targeting vector. Four linear fragments (left): vector backbone, 5' homology arm (HA), 3' HA, mCherryFP) with ~20 bp complementary overlaps are combined in an isothermal reaction to form the PARP1-mCherryFP targeting vector (right).

These six candidates were stored, and minipreps performed. Screening was then performed via transfection, evaluating the expression of mCherryFP through fluorescence microscopy to determine the functionality of the candidate plasmids.

To perform the CRISPR knock-in of mCherryFP, the newly synthesised targeting vector was utilised in conjunction with the Cas9-sgRNA plasmid that was generated for the EGFP knock-in. This approach targeted the same genomic locus as the one used for the C-terminal PARP1-EGFP knock-in (**Figure 39A**), employing the same PAM sequence (TGG) as the target of the gRNA sequence (GTCAATTTTAAGACCTCCCTG).

For screening purposes, HEK293 cells were transfected with 1 μg of each candidate plasmid, and 1 μg Cas9-sgRNA plasmid using 6 μL Viafect per reaction (3:1 ratio to DNA). Each well was monitored for nuclear mCherryFP expression at 24 and 48 hr post transfection. Out of the six transfected candidates, two showed mCherryFP expression in cells, with candidate #3 having the highest efficiency rate. Therefore, candidate #3 was selected for future work (**Figure 39B**). HEK293 cells were used for the screening of candidates due to their higher transfection efficiency compared to cancer cells.

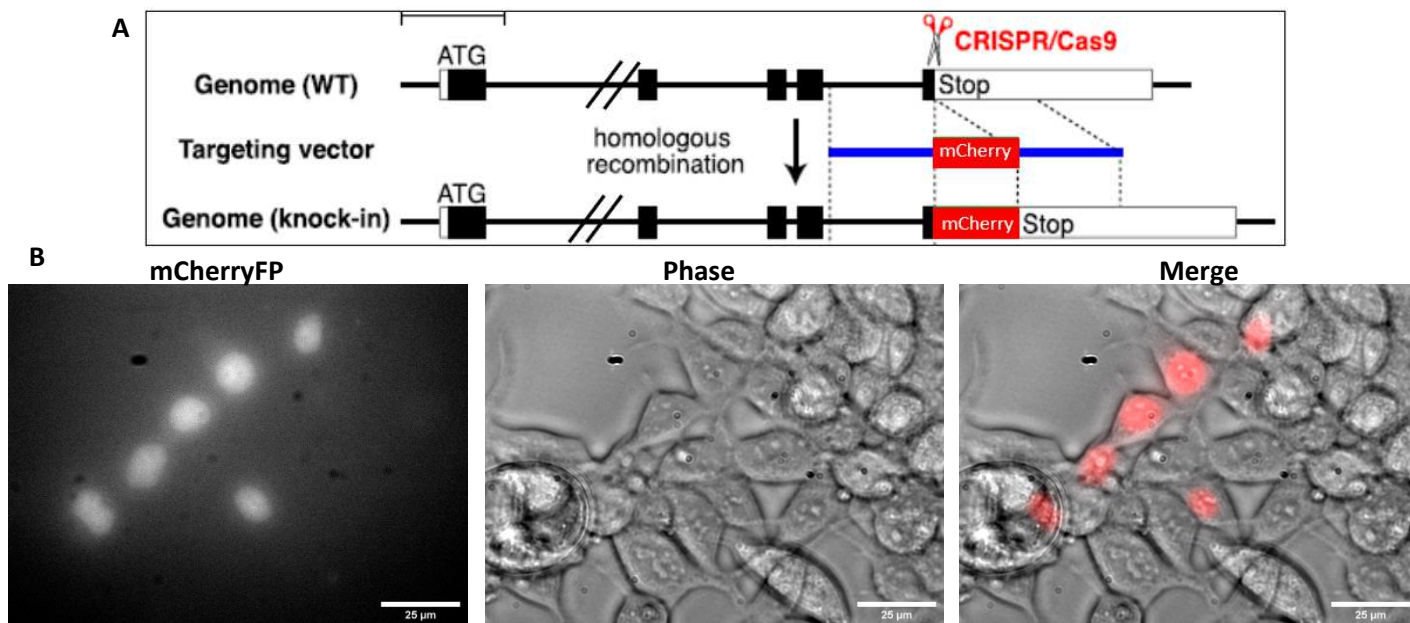


Figure 39: C-terminal PARP1-mCherryFP knock-in approach. **A:** Schematic of CRISPR-Cas9 editing regime for knock-in of mCherryFP at the C-terminus of PARP1. **B:** PARP1-mCherryFP targeting vector plasmid candidate #3 showing nuclear expression of mCherryFP in HEK293 cells upon transfection with Cas9-sgRNA plasmid. mCherryFP (left), phase contrast (middle) and merged image (right) are shown. Images acquired with 60X objective on an epifluorescence microscope. Scale bar = 25 μm .

Next, OVCAR4 WT cells were transfected to demonstrate that the CRISPR system was also functional in this cell type, and that the labelled cells were viable and able to divide. **Figure 40** shows colony formation of these cells mCherryFP positive cells in an unsorted population. These cells were then expanded and isolated using FACS to form a pooled population of mCherryFP positive cells. This secured the OVCAR4 PARP1-mCherryFP cell line which would be the acceptor only controls in subsequent FRET assays.

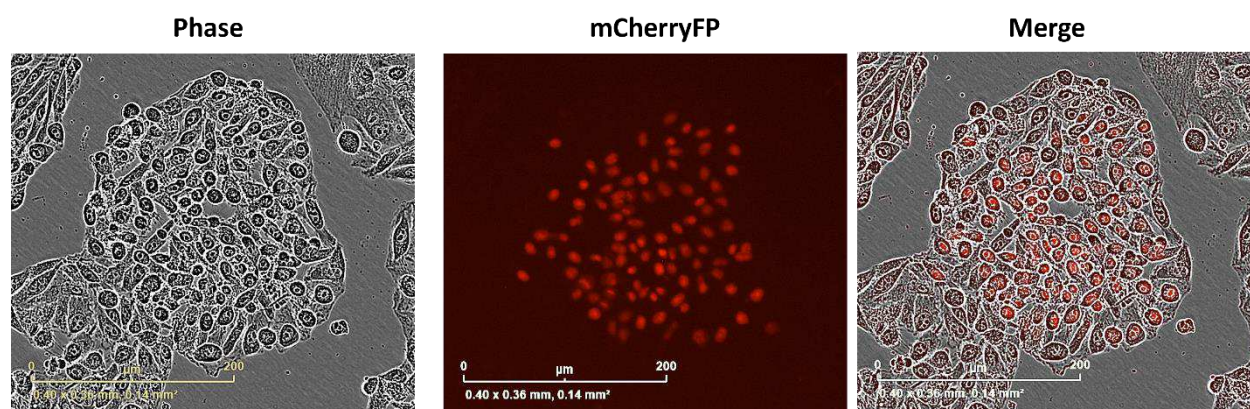


Figure 40: Transfection of OVCAR4 with PARP1-mCherryFP targeting vector. The synthesised targeting vector achieves nuclear mCherryFP expression in OVCAR4 cells upon transfection. Phase contrast (left), mCherryFP (middle) and merged (right) images are shown. Incucyte images taken with 10X objective, 600 ms acquisition time for mCherryFP channel. Scale bar = 200 μm .

4.3.5 Part 4: Dual labelling of PARP1 with EGFP (N-terminal) and mCherryFP (C-terminal)

Following this, work began to endogenously label PARP1 with EGFP and mCherryFP at the N and C termini of the protein, respectively. These fluorescent proteins would form the donor and acceptor pair as an intramolecular FRET biosensor. However, as this approach has not previously been reported, uncertainty existed regarding whether the introduction of two large fluorescent proteins bound to the PARP1 protein would interfere with its functionality, or if the cells would even be viable with the introduction of this second label. Moreover, a second round of CRISPR editing could further introduce off-target effects. Hence, thorough characterisation would be essential. The previously generated OVCAR4 PARP1-EGFP (N-terminus) cell line was used as the starting point. These cells were then transfected with the CRISPR-Cas9 plasmids for the knock-in of mCherryFP at the C-terminus of the protein that had demonstrated functionality in labelling PARP1 in OVCAR4 WT cells.

OVCAR4 PARP1-EGFP (N-terminal) cells were transfected via lipofection, using 1 µg of each of the mCherryFP-targeting vector and Cas9-gRNA plasmids. After 48 h, the cells were assessed for the nuclear mCherryFP expression. The Incucyte S3 imaging system was used to perform automated imaging, sampling many cells. Phase, EGFP and mCherryFP images were acquired for each FOV. A small percentage of cells in the population were mCherryFP positive, indicating initial viability of the cells despite dual-labelling PARP1 with EGFP and mCherryFP (**Figure 41A**). However, the possibility remained that mCherryFP integration occurred at an off-target site. Therefore, without further characterization at the protein and DNA levels, it could not be conclusively confirmed whether this approach successfully labelled PARP1 with mCherryFP at its C-terminus.

These transfected cells were then expanded, and 8×10^6 cells were sorted by FACS, directly into a 24-well plate (**Figure 41B**). From this sort, only ~2000 cells positive for both EGFP and mCherryFP expression were isolated, highlighting the low efficiency of this CRISPR knock-in. However, when the cells had been expanded, the population was no longer homogenous, with some cells expressing EGFP exclusively, and not mCherryFP. This was somewhat expected, as a non-stringent gating strategy had been employed for sorting as the percentage of mCherryFP positive cells was low. Hence, a second round of FACS was performed with a stricter gating strategy. This resulted in 468,000 dual-labelled cells which were then expanded and stored (**Figure 41C**).

Following the successful transfection and preliminary imaging, a western blot was performed on the polyclonal population to confirm the labelling of the PARP1 protein. This would allow the comparison of the newly generated dual-labelled OVCAR4 which express both PARP1-EGFP (N-terminus) and PARP1-mCherryFP (C-terminus), against single-labelled OVCAR4 cells that express either PARP1-EGFP

or PARP1-mCherryFP separately (**Figure 42**). This provided evidence supporting the presence of dual-labelled PARP1 in the polyclonal population, indicated by the increased molecular weight band at 171 kDa when probing for PARP1. Moreover, when probing for EGFP, the same higher molecular weight band is only present in the dual labelled sample, not the single labelled samples. Note, the polyclonal population also has PARP1-EGFP (143 kDa) and/or PARP1-mCherryFP (143 kDa) proteins, and the wild-type PARP1 (116 kDa).

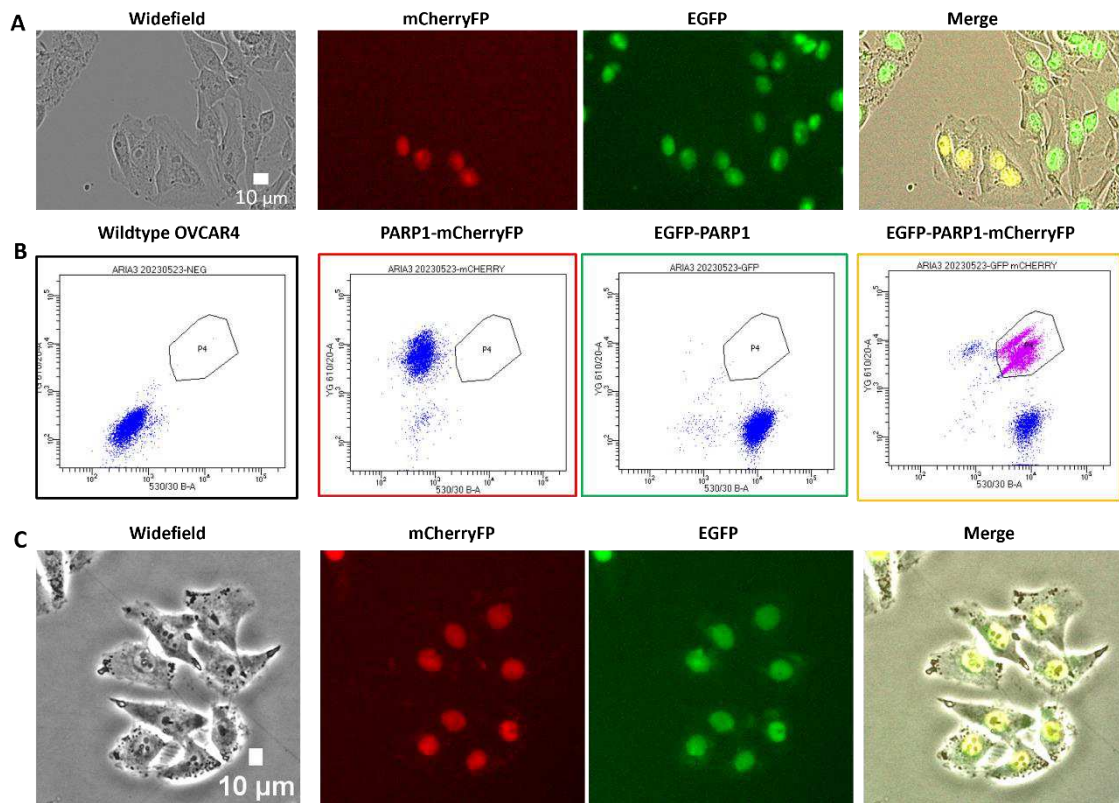


Figure 41: Unsorted population of dual labelled OVCAR4 EGFP-PARP1-mCherryFP cells. **A:** Individual phase, mCherryFP and EGFP channels are shown with the merged image. Images acquired on the Incucyte S3 with 20X objective, and 600 ms acquisition time for the green and red channels. Scale bar = 10 μ m. **B:** FACS gating strategy for selection of dual-labelled cells, using the unlabelled and single-labelled cell lines as negative controls to set the gates. **C:** Sorted population following FACS, dual labelled cells expressing both mCherryFP and EGFP with nuclear localization. Cells were imaged with 20X magnification, 600 ms acquisition time for the EGFP and mCherryFP channels respectively. Scale bar = 10 μ m.

In light of these findings, the next step was to attempt to isolate cells that were dual labelled on the same allele of PARP1 from this polyclonal population. This would involve carrying out single-cell cloning, initially isolating single cells, and then expanding them until they could be characterised for expression of dual-labelled PARP1.

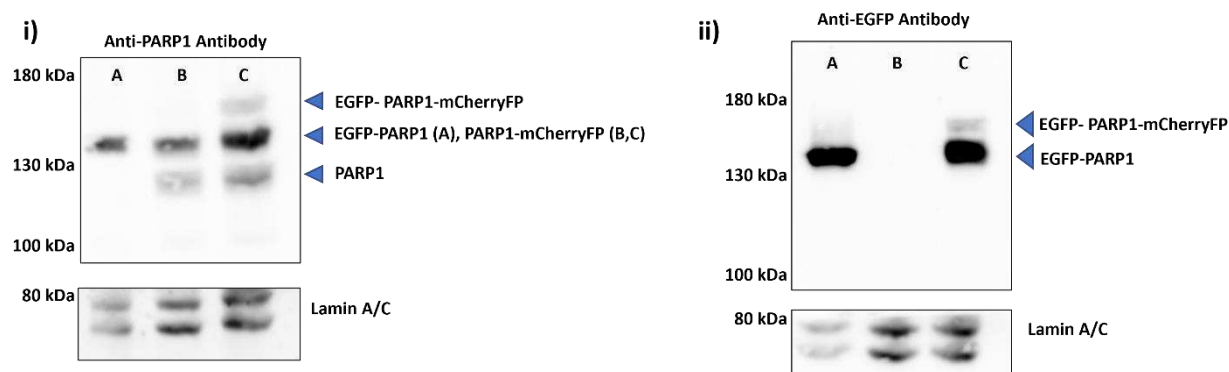


Figure 42: Confirming dual labelled PARP1 by western blot. EGFP-PARP1-mCherryFP OVCAR4 cells compared to single labelled EGFP-PARP1 and PARP1-mCherryFP cells. Membranes were probed with an anti-PARP1 antibody (i, left) then stripped and reprobed with an anti-EGFP antibody (ii, right). Three samples (A: OVCAR4 PARP1-EGFP (N-terminal), B: OVCAR4 PARP1-mCherryFP, C: OVCAR4 EGFP-PARP1-mCherryFP cells) were run per gel.

Cells were seeded at ~0.5 cells/well into 3 x 96 well plates, aiming to isolate single-cell clones from the polyclonal population. The plates were then grown and imaged *in situ* in the Incucyte to screen cell growth and fluorescent protein expression. Colonies had formed within 14 days, with most wells either containing more than one colony, or none (**Figure 43**). However, in 10 wells, a single colony derived from a single cell was observed (as documented by the Incucyte time-lapse images), and therefore these 10 clones were selected for further characterization to assess if any exhibited dual labelling with EGFP and Cherry on the same allele of *PARP1*. Clones were named after the well of the 96W plate they were grown from, e.g., B8.

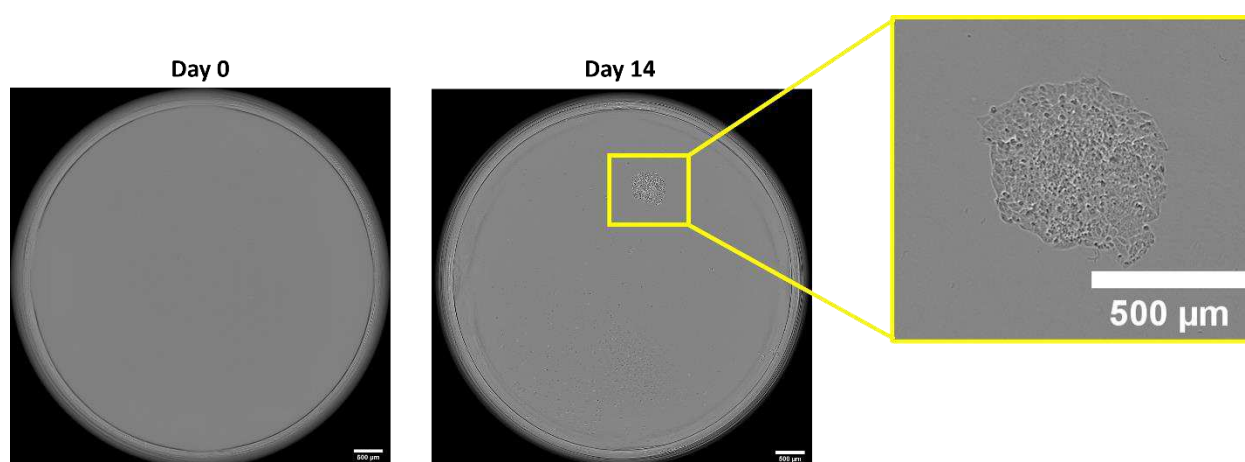


Figure 43: Tracking growth of single-cell clones of dual labelled cells. A representative well (B8) of a 96-well plate on day 0 (left) of cell seeding and day 14 (right), showing the presence of a single colony (inset). Images were acquired on the Incucyte S3 system using whole-well overview imaging setting. Scale bar = 500 µm.

The single-cell-derived colonies were then expanded to obtain sufficient cells for performing western blots to screen for dual-labelled PARP1 protein. Of the 10 isolated colonies, only 8 successfully grew out. Protein was subsequently extracted, and a western blot was performed sequentially probing for PARP1, EGFP, and mCherryFP. There were a number of possible outcomes of the second round of

CRISPR editing as the absolute copy number of OVCAR4 cells is at least $n=2$, according to data from the next-generation cancer cell line encyclopaedia (CCLE2) study, with OVCAR4 having an average ploidy of 2.5 [112]. Hence different alleles of *PARP1* may be labelled differently. For the intended application to FRET, it is necessary for PARP1 to be labelled with the donor and acceptor pair on the same allele. However, since the parental line of OVCAR4 PARP1-EGFP (N-terminal) was heterozygous, the second CRISPR knock-in could result in PARP1 alleles being labelled with mCherryFP alone, EGFP alone, or both EGFP and mCherryFP. From the western blot, it was determined that 3 out of the 8 clones had at least one allele of PARP1 dual-labelled with EGFP and mCherryFP (highlighted in green in **Figure 44**). This confirmed the successful dual labelling of PARP1 at the protein level, marking a significant milestone in this work.

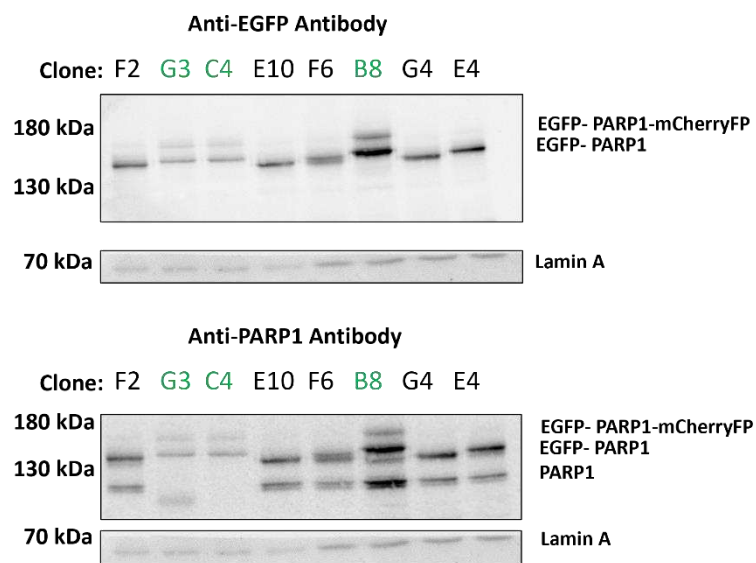


Figure 44: Screening for dual labelling of the same allele of PARP1 by western blot. Protein extracted from 10 single-cell clones. Membranes were probed and imaged with anti-EGFP (top) and then anti-PARP1 (bottom) antibodies sequentially after stripping and re-probing. The clones labelled in green show a higher molecular weight band indicating the presence of dual labelled EGFP-PARP1-mCherryFP in these samples. Lamin A was used as a protein loading control. Dual labelled clones labelled in green.

The dual labelled clones were then carried forward for further characterization, to ensure that PARP1 was still functional in these cells by performing viability assays against carboplatin and PARPi rucaparib to evaluate whether the cellular response to DNA damage and PARPi remained unchanged (**Figure 45A,B**). No significant difference in sensitivity to carboplatin or rucaparib was observed among the three clones. Due to the similarity in phenotypes among these three clones, it was decided to conduct additional screening based on the FLIM-FRET signals generated by each clone. Doubling time assays were also performed to compare the growth rate of the three clones, all showed similar doubling times of ~38 hr (**Figure 45C,D**).

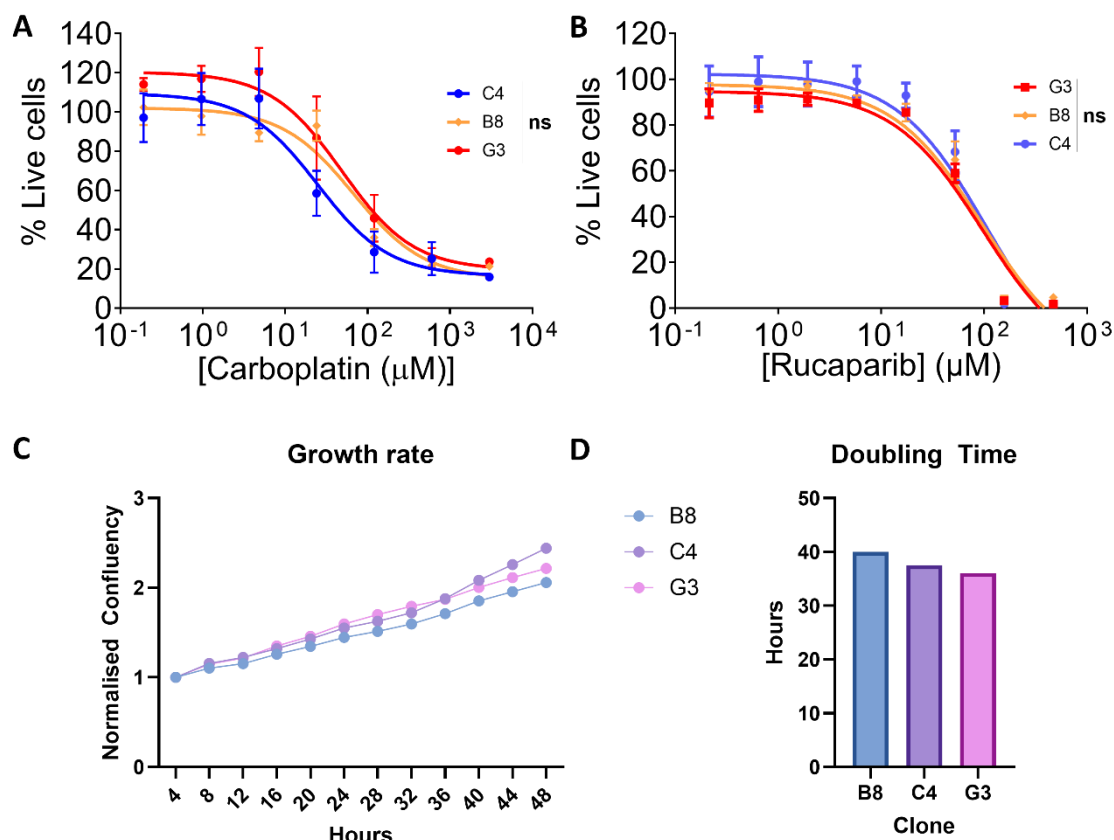


Figure 45: Characterisation of three dual labelled OVCAR4 EGFP-PARP1-mCherryFP clones. **A:** SRB assays of single-cell derived clonal populations (B8, G3, C4) treated with carboplatin **B:** or rucaparib. Percentage viability is plotted, normalised to vehicle treated controls. **C:** Growth rate is comparable between different dual-labelled clones. Images acquired on Incucyte S3 system at 10X magnification and segmentation and analysis performed in Incucyte software. Data were normalised to the confluency at t=0 for each subsequent time point. **D:** Doubling times were calculated by calculating the gradient of growth rate curves. Statistical analysis via Welch's ANOVA were performed. n=3 biological replicates.

Through sequential rounds of CRISPR-Cas9, both single and dual labelled cell lines were generated from the parental OVCAR4 cell line. Viability assays were conducted to assess if these genome editing processes had altered the cellular functioning of the labelled PARP1 protein, serving as a proxy for PARPi and carboplatin sensitivity, which could indicate perturbations in PARP1 functionality (**Figure 46**). However, no significant changes in viability were observed in all three generated cell lines compared to the parental OVCAR4 cell line.

As three dual-labelled single-cell clonal populations had been generated, these would all be carried forward for downstream FLIM assays, to screen which (if any) of the three clones provided the greatest signal to noise ratio in terms of FRET readout upon treatment with DNA damaging agents and PARPi.

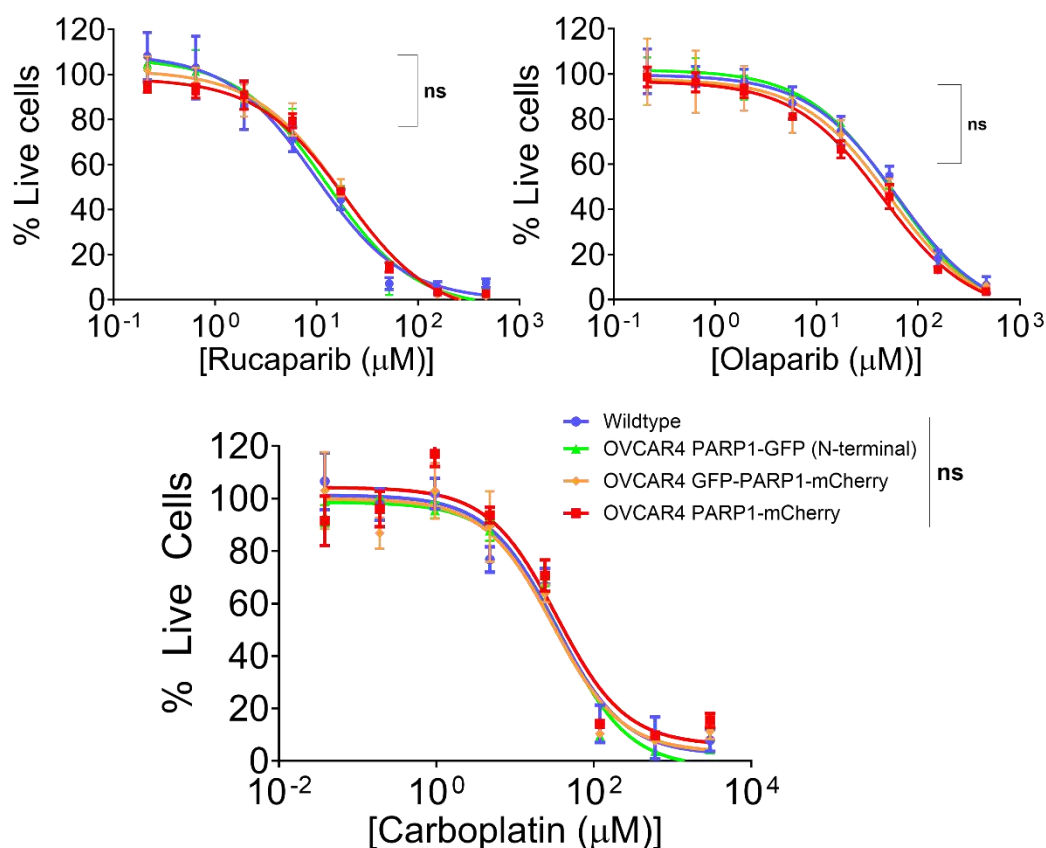


Figure 46: SRB assay comparing the viability of parental OVCAR4 and CRISPR-labelled cell lines. SRB assays for OVCAR4 WT compared to the 3 CRISPR generated cell lines treated with **A:** rucaparib, **B:** olaparib or **C:** carboplatin. OVCAR4 wild-type (blue), OVCAR4 EGFP-PARP1 (green), OVCAR4 PARP1-mCherryFP (red), OVCAR4 EGFP-PARP1-mCherryFP (orange). Absorbance values were normalised to untreated controls to calculate live cell values per treatment. Statistical analysis via Welch's ANOVA were performed. $n=3$ biological replicates.

The schematic in **Figure 47** demonstrates a working model of how the FRET biosensor works. When PARP1 is not bound to sites of DNA damage, it is in a relaxed near-linear conformation. In this state, the C- and N-termini are spatially separated and thus there is little to no FRET interaction occurring. However, upon the formation of DNA breaks, PARP1 rapidly binds these sites. In the process, the protein undergoes a conformational compaction, bringing the C- and N-termini into close proximity (<10 nm). As the donor is excited exclusively, there is a chance of FRET interactions occurring if the appropriate criteria are fulfilled. The FRET efficiency of this process increases in a distant dependent manner. It is postulated that PARP trapping will increase the proportion of biosensor molecules in the higher FRET efficiency state, thereby reducing the fluorescence lifetime of the donor.

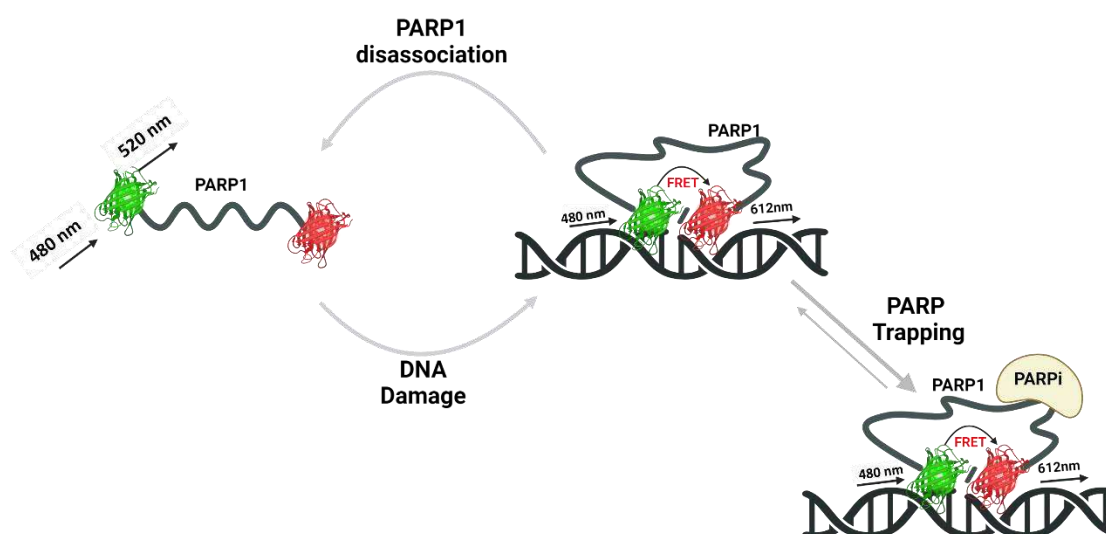


Figure 47: Schematic of FRET biosensor to assess DNA damage and PARP trapping in live cells. PARP1 is labelled with a FRET donor (EGFP) and acceptor (mCherryFP). When PARP1 binds to DNA damage sites it undergoes a conformational change, bringing the C- and N-termini of the protein in close proximity, resulting in FRET. PARP1 molecules continuously disassociate at damage sites, however, PARPi treatment prolongs the association of the PARP1-DNA complexes, increasing the proportion of biosensor molecules that are in their higher FRET efficiency state.

4.4 Summary

The work reported in this chapter involved the use of genome editing with CRISPR-Cas9 to endogenously label PARP1 with EGFP and mCherryFP to generate a FRET biosensor of PARP1 binding. This was addressed in stages. Initially, it was demonstrated that endogenous PARP1 could be labelled at the C-terminus. This was accomplished by modifying a previously used targeting vector, adapting the homology arms to target PARP1. Following this, mCherryFP was knocked in at the same locus to form the acceptor in the FRET pair. Extensive optimization of the molecular biology approach led to the successful achievement of the CRISPR knock-in. Subsequently, this approach was expanded to perform N-terminal tagging.

Targeting the N-terminal locus of PARP1 required a different approach, utilising *in trans* paired nicking to induce homology-directed repair without the formation of a DSB. This allowed the introduction of EGFP by delivering a repair template with homology arms flanking the cut site. The same methodology was subsequently applied to cells already labelled with C-terminal PARP1-mCherryFP, successfully achieving dual labelling. The generated cell line expressing the FRET biosensor would subsequently serve as the primary tool for FLIM-FRET HCA assays to evaluate PARP trapping in HGSC, as discussed in the following chapter.

To the best of our knowledge, this represents one of the earliest documented instances of dual labelling of a single endogenous protein using CRISPR-Cas9 knock-in, underscoring the ambitious nature of this endeavour. These efforts benefited significantly from the prior work of *Matsuda, Perez-Leal O, and Chen et al.*, who generously shared plasmids pivotal to this research. Despite this, extensive cloning techniques were required to construct the desired plasmid sequences tailored to this specific application, emphasizing the meticulousness and resource intensiveness of CRISPR-Cas9 methodologies. The technical difficulties presented by this approach are significant and hence, it is likely not a suitable approach for high-throughput labelling or labelling time-sensitive samples such as patient-derived samples.

Considering this, ongoing efforts are directed towards exploring alternative strategies to facilitate expression of the dual-labelled EGFP-PARP1-mCherryFP biosensor in patient-derived samples. One prospective approach involves generating a lentivirus encoding the biosensor based on the cDNA from cells expressing EGFP-PARP1-mCherryFP generated in this study, representing a promising avenue for future investigations. Improving the speed and throughput of the labelling methodology would further expand the applicability of this approach. This could potentially facilitate the assessment of PARP trapping status in patient derived samples, thereby guiding clinical treatment decisions (e.g., choice of specific PARPi), and potentially enhancing patient outcomes.

5 Establishing and validating a FLIM-FRET assay with endogenously expressed EGFP-PARP1-mCherryFP biosensor

5.1 Background

This chapter details the optimization of experimental design and treatment protocols for FLIM-FRET assays using OVCAR4 cells expressing the FRET biosensor developed in the previous chapter. Although prior studies had utilised this FRET biosensor in purified protein solutions, it has not been applied in the context of live cell studies. Here, live cell FLIM-FRET assays were performed with this biosensor to assess changes in PARP1 binding dynamics upon drug treatment. Wide-field time-gated FLIM was implemented to facilitate quantitative FRET measurements in high-content analysis, enabling the comparison of various drug treatments in automated multi-well plate experiments.

The work is divided into two sections. The first section discusses the technical optimization of sample preparation to ensure optimal cell growth in glass multi-well plates suitable for FLIM-FRET HCA experiments. The second section addresses initial FLIM-FRET experiments with the biosensor, involving the treatment of cells with various DNA damage-inducing small molecule drugs. Since this biosensor had not previously been used in live-cell *in vitro* experiments, these initial FLIM assays were conducted with simple untreated versus treated conditions. Assay conditions were established through optimization and cross-validation with traditional assays such as immunofluorescence and western blot. Subsequently, multiple drug treatments were compared in single multiwell experiments. Beyond assessing DNA damage, the biosensor was used to distinguish differences in PARP trapping potencies, cross-validated with results from traditional biochemical assays. This indicated the potential to develop a novel tool to assess PARP trapping in live cells with single-cell resolution.

5.2 Aims and objectives

The primary aim of the work reported in this chapter was to perform preliminary FLIM-FRET experiments to optimise treatment conditions for assaying PARP trapping using the FRET biosensor developed in chapter 4. Prior to commencing HCA experiments, cell culture conditions were optimised for the growth of cells on the glass well plates required for live cell imaging. Different glass coatings were assessed to ensure minimal cell stress under basal conditions.

Initial FLIM-FRET experiments were conducted with drug treatment conditions previously optimised for immunofluorescence assays, but these required further refinement for live cell experiments. This involved optimizing treatment timings and dosages, using western blot analysis alongside FLIM-FRET readouts to establish conditions that provided reproducible FLIM signals while inducing DNA damage, as assessed by western blot and immunofluorescence of γ H2AX.

Once these assay conditions were established, larger-scale multi-well plate FLIM-FRET HCA experiments were performed to quantify PARP trapping upon treatment with PARPi and DNA-damaging agents. Biological repeats demonstrated the reproducibility of these HCA measurements of cells treated with PARPi. Following this, screening experiments assessed the FLIM signal produced by three clonal populations of biosensor expressing OVCAR4 cells. Assay conditions were expanded to evaluate the PARP trapping potency of a range of clinically approved PARPi molecules, cross-referencing results with prior findings from established assays such as ChIP. This provided a method of validation for the FLIM-FRET assay and its ability to assess PARP trapping.

5.3 Results

5.3.1 Optimizing cell seeding for FLIM-FRET assays

Glass-bottomed wells are frequently employed in fluorescence microscopy due to their lower autofluorescence compared to standard plastic bottomed well plates, which allows for a higher signal-to-noise ratio. In the context of FRET experiments, reducing background fluorescence is crucial, as it can complicate the analysis. Glass bottomed wells also present lower optical aberrations and so enable higher quality imaging. Additionally, glass is less prone to warping upon temperature changes, which can cause focus drift and disrupt automated multi-well plate imaging. Therefore, it is necessary to optimise culturing cells on the glass surface in order to replicate conventional cell culture conditions in plastic.

Although the glass surface can be left uncoated, it is often coated with a functionalizing extracellular matrix polymer, such as Poly-L-lysine (PLL), to facilitate better cell adhesion. However, the application of this approach with the cell lines derived from OVCAR4 in this project revealed a negative impact on cell morphology and viability when PLL was used (**Figure 48**). Consequently, alternative coating polymers were trialled such as fibronectin. PLL is a positively charged molecule that modifies the surface charge of negatively charged substrates, promoting cell adhesion by strengthening the electrostatic interaction between the substrate and the cell membrane. This aims to create a charged and hydrophilic surface conducive to cell attachment through electrostatic interactions. Conversely, fibronectin, a glycoprotein present in the extracellular matrix, features an RGD sequence (Arg-Gly-Asp) that facilitates integrin-mediated cell adhesion to the substrate surface.

To compare these conditions, OVCAR4 PARP1-mCherryFP cells were seeded on a glass 8-well chamber slide that was either uncoated or functionalised with PLL or fibronectin. 2×10^4 cells were seeded for 24 h, after which live-cell imaging was conducted using epifluorescence to assess cell morphology and growth. Culturing the cells on fibronectin led to more uniform epithelial cell morphology, with no dead

or rounded cells observed 24 hr post-seeding, in contrast to the aberrant morphology observed in both the uncoated and PLL-coated conditions (**Figure 48**).

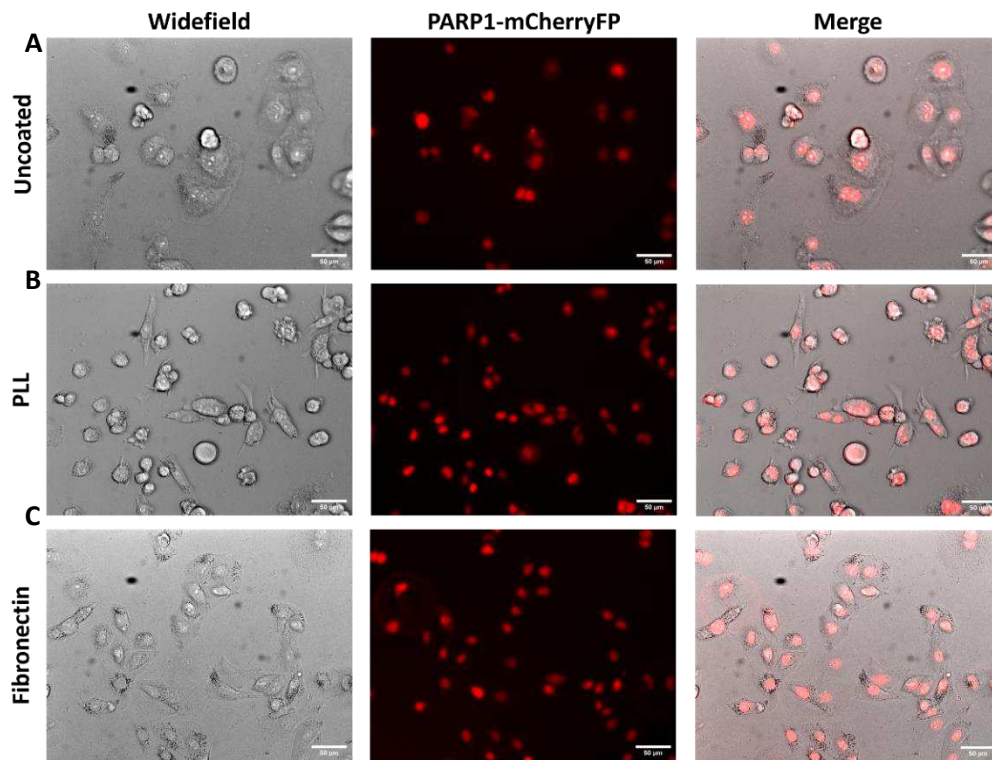


Figure 48: Comparing cell morphology on different glass coatings. OVCAR4 PARP1-mCherryFP cells imaged 24 hr after seeding on glass chamber slides. Wells were either **A:** uncoated, **B:** coated with PLL or **C:** fibronectin. Images acquired with 20X objective. Scale bar = 50 μ m.

5.3.2 Automated quantification of γ H2AX foci from immunofluorescence images

In order to obtain a quantified measurement of cellular stress in each of these conditions, the cells were subsequently fixed and immunostained for γ H2AX, a marker commonly used to indicate DNA double-strand breaks (DSBs). The nuclei were stained with SYTO Deep Red to allow nuclear segmentation, thereby defining regions of interest (ROIs) where γ H2AX foci could be quantified on a per-nucleus basis (**Figure 50A**). The average number of foci per nucleus was plotted for each condition. The image analysis pipeline used for segmentation and subsequent foci counting is shown in **Figure 49**.

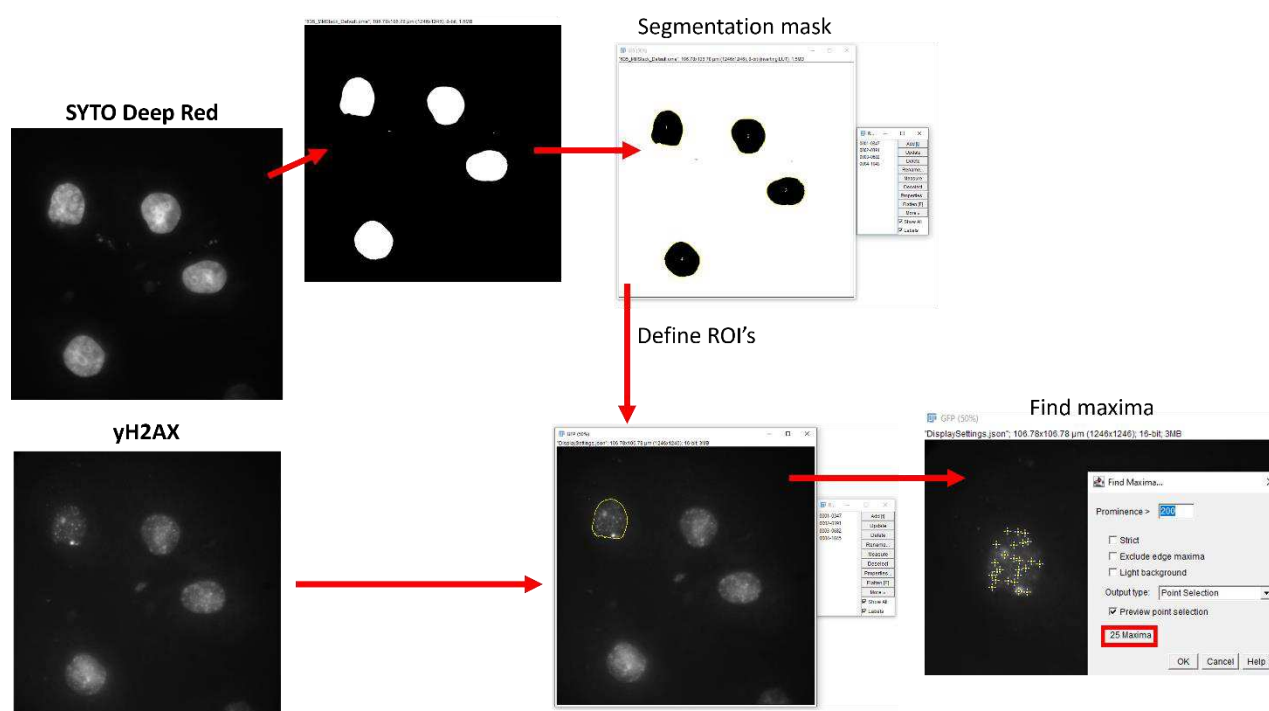


Figure 49: Image analysis pipeline used for automated γ H2AX foci quantification. The SYTO Deep Red image was intensity thresholded and watershed segmentation was performed, producing a segmentation mask with each nucleus defined as a ROI. These ROIs were overlaid on the γ H2AX image sequentially and local maxima were found with a prominence of 400 AU. Foci counts were stored in a csv file for further analysis.

The average number of γ H2AX foci per nucleus varied significantly between the different coating conditions. Uncoated glass surfaces resulted in the highest number of γ H2AX foci, indicating substantial DNA damage. PLL-coated glass reduced the number of foci, while fibronectin-coated glass showed the fewest foci, suggesting the least DNA damage (**Figure 50B**). These results corroborated the morphological differences observed under these conditions, with fibronectin coating inducing minimal stress, thereby providing conditions closest to optimal for standard cell culture. Moreover, given that this study focuses on inducing DNA damage with various small molecule compounds, it is crucial to start with the lowest baseline DNA damage to maximise the window for observing biological signals.

This series of results provided further evidence for selecting fibronectin as the coating substrate of choice for all subsequent FLIM assays. With optimal seeding conditions established, the next section focuses on performing preliminary FLIM-FRET experiments to fine-tune treatment conditions for assessing PARP trapping using the FRET biosensor.

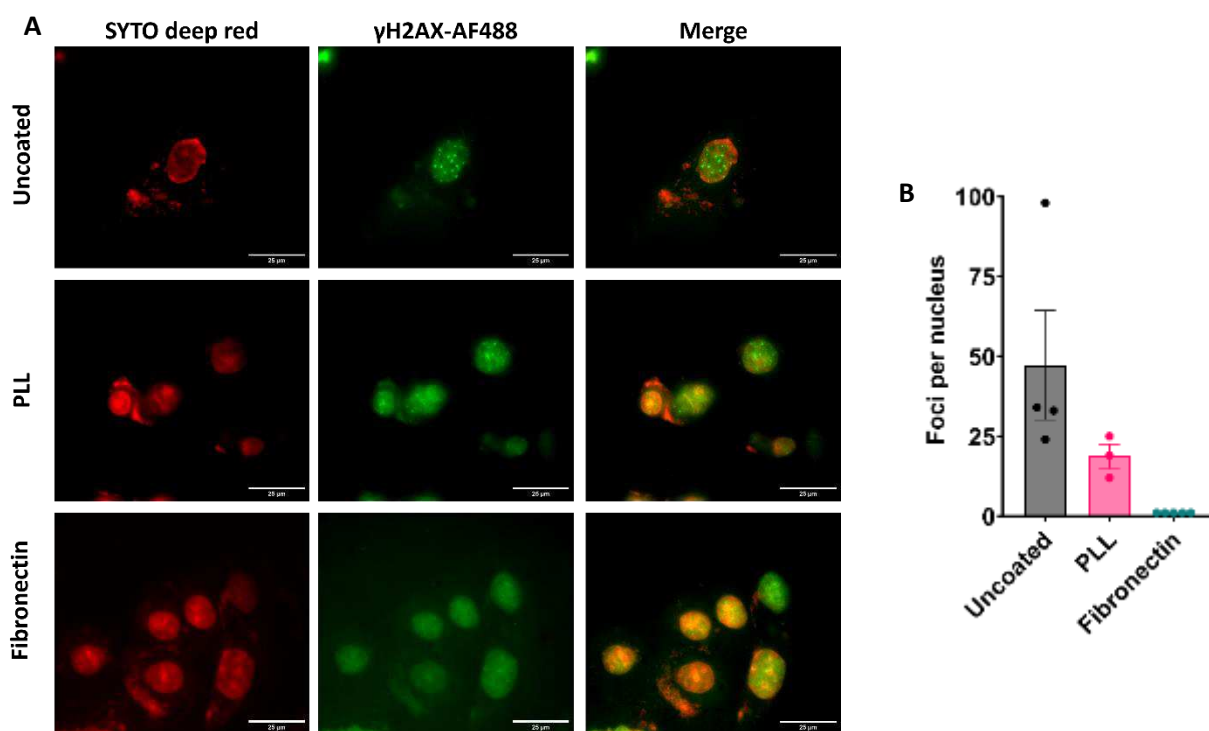


Figure 50: Comparing DNA damage induced by different glass coatings. **A:** Representative immunofluorescence images of OVCAR4 WT cells cultured on uncoated, PLL coated, or fibronectin coated glass multi-well plates. Images obtained at 100X magnification, oil objective, HILO fluorescence excitation configuration. SYTO deep red was excited with a 636 nm laser at 0.3V for 5 ms. AF488 was excited at 462 nm with 0.3 V for 100 ms. Scale bar = 25 μ m. **B:** Quantification of foci per nucleus, per condition. Individual points represent the mean value for each FOV with the error bars denoting the mean and SE, $n=4,3,5$ FOV. Statistical comparisons were not performed due to low sampling numbers.

5.3.3 Establishing environmental conditions for FLIM-FRET assays

Performing live-cell fluorescence microscopy experiments, especially FLIM-FRET, requires careful consideration of experimental conditions. Maintaining sample viability and health during preparation and imaging is crucial. Therefore, the sample is maintained in an environmental chamber atop the microscope stage at a temperature of 37 °C and 5% CO₂ in a humidified environment. Additionally, light exposure must be minimised in terms of frequency and irradiation power to reduce phototoxicity to the sample and photobleaching of fluorescent proteins (FPs). Consequently, all FLIM-FRET experiments were conducted using a 20X objective at 200 μ W power at the sample plane, as this power level has previously been demonstrated to preserve cell viability in this instrument [84]. Any autofluorescence in the same spectral window as the donor (EGFP) must be minimised or eliminated and so, all FLIM experiments were performed in a CO₂ dependent imaging medium (FBS and phenol red free) to reduce background fluorescence from the medium.

5.3.4 Performing high-content analysis FLIM-FRET assays to assess effect of DNA damage and PARP trapping

5.3.4.1 Instrument response function

To aid analysis of subsequently acquired FLIM data, information about the system instrument response function (IRF) was measured before and after each experiment. For this, the fluorescence decay profile of a reference dye, coumarin 6 (known lifetime of 2.5 ns), and quenched fluorescein (expected lifetime of ~100 ps) were measured with high precision (i.e., high temporal sampling) to provide information about the IRF. The data acquired from these two measurements was subsequently included in the decay analysis via reference reconvolution and applied to FLIM analysis of the data acquired from cells [113]. The fitted fluorescence decay of coumarin 6 is shown in **Figure 51A**, with a measured mean fluorescence lifetime of 2.48 ns. The decay from quenched fluorescein is overlaid in red. A frequency distribution histogram of the fitted fluorescence lifetimes per pixel presents a near normal distribution (**Figure 51B**). The fluorescence lifetime image of the coumarin 6 sample shows no significant spatial dependency on lifetime across the FOV (**Figure 51C**). To assess if there were any intensity dependent changes in lifetime in the measured decay, pixel intensity was plotted against calculated lifetime (τ_1) (**Figure 51D**). No strong intensity dependence was observed, supporting the application of this instrument for FLIM analysis of biological samples.

Moreover, the consistency in the measured lifetimes of the reference dyes before and after imaging the experimental samples indicates that the FLIM instrument was functioning correctly throughout each experiment. This internal control was important to confirm the reliability of the experimental results.

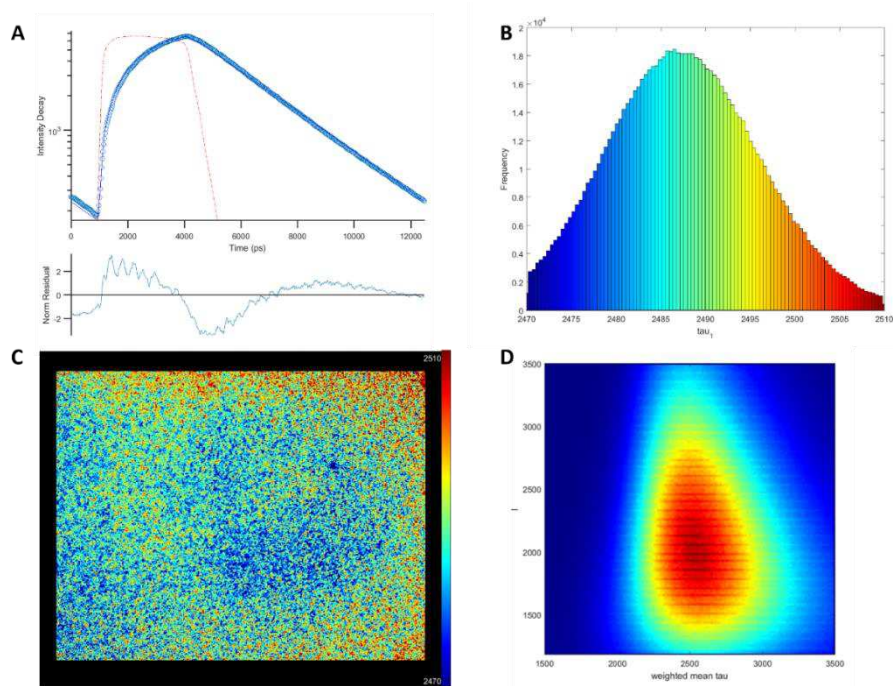


Figure 51: Instrument response function used as an experimental control. **A:** The measured fluorescence decay of Coumarin 6 (blue circles) sampled at 25 ps intervals, with the fitted lifetime overlaid (solid blue line). The quenched fluorescein decay is overlaid in red. In the bottom panel the calculated normalised residual values between the measured and fitted data across the 12500 ps decay is shown. **B:** Histogram of fluorescent lifetime (τ_{11}) from the fitted data. **C:** Fluorescence lifetime image of the coumarin 6 FOV. Image is pseudo-coloured by τ_{11} value, scalebar from 2470 to 2510 ps. **D:** Scatterplot of intensity values (I) against fluorescence lifetime (τ_{11}) for all fitted pixels.

5.3.4.2 Experimental FRET controls

As an internal control for each experiment, non-labelled parental OVCAR4 cells were imaged to assess autofluorescence from cellular components. Single-labelled OVCAR4 EGFP-PARP1 were imaged as a donor-only control in which FRET should not occur. OVCAR4 PARP1-mCherryFP cells were also imaged as an acceptor-only control, whereby no fluorescence signal should be detected in the FLIM channel. Representative intensity images are shown for each sample in **Figure 52**, along with the dual-labelled FRET biosensor expressing cells OVCAR4 EGFP-PARP1-mCherryFP.

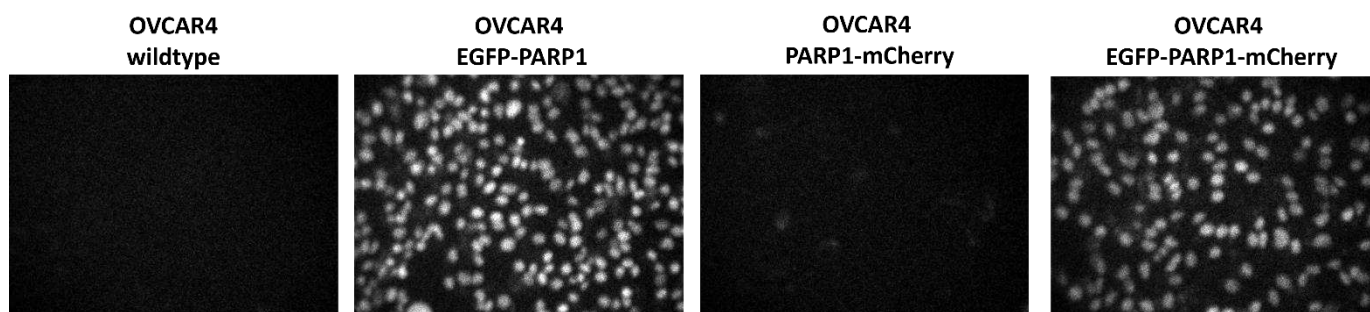


Figure 52: Unlabelled, donor-only and acceptor-only controls for FRET experiments. OVCAR4 wild-type, OVCAR4 EGFP-PARP1 (donor-only), OVCAR4 PARP1-mCherry (acceptor-only) cells were imaged in each FLIM-FRET experiment. Excitation at 488/20 nm with a 488/532 nm dichroic mirror and fluorescence emission was collected via a 520/35 nm, UPlanApo 20X (Olympus) objective lens, with an irradiation power of 200 μ W at the back focal plane of the objective. Image exposure times were 2000 ms for each well FOV.

5.3.4.3 Segmentation and fitting workflow

Prior to establishing and expanding assay treatment conditions, it was necessary to develop an optimised pipeline for analysing and fitting fluorescence lifetimes to the intensity images acquired from the donor-only channel (EGFP-PARP1). The output from wide-field time-gated imaging was a series of intensity images acquired at different delay time points, thereby sampling the fluorescence intensity throughout the period of fluorescence decay. To calculate fluorescence lifetimes, individual cells were segmented using the intensity images, which was particularly effective due to nuclear localization of PARP1 (and thus EGFP). This segmentation utilised the n^{th} algorithm in *FLIMfit* analysis software, as illustrated by an example field of view (FOV) showing the workflow from the intensity image (**Figure 53A**) to the segmentation mask (**Figure 53B**). Each segmented cell was designated as a region of interest (ROI), colour-coded, and indexed for further analysis. The segmentation mask was subsequently overlaid on the intensity image to create a binary image (**Figure 53C**). Global fitting of a double exponential decay was performed, producing fluorescence lifetime images, pseudo-coloured by mean weighted tau value (**Figure 53D**). Importantly, this process enabled extraction of single-cell level data from these ROIs, including weighted mean lifetimes, which was important for the analysis of PARP1 trapping dynamics in this study.

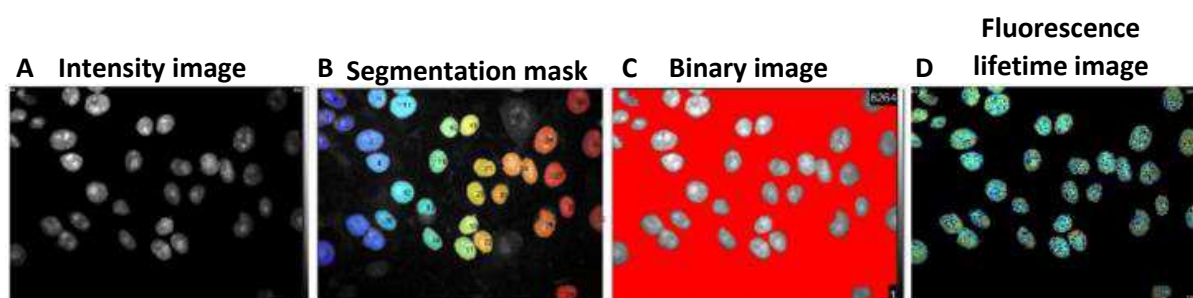


Figure 53: Segmentation and fitting workflow in *FLIMfit*. Representative wide-field time-gated data of OVCAR4 EGFP-PARP1-mCherryFP cells obtained on the *FLIM* platereader system with 40X objective. **A:** Widefield intensity image of a single FOV. **B:** Segmentation mask produced by the n^{th} segmentation algorithm in *FLIMfit*. **C:** Binary mask produced based on the defined ROIs from segmentation. **D:** The segmented image following global fitting of fluorescence lifetime, image is pseudo-coloured for fluorescence lifetime value.

5.3.4.4 Initial FLIM-FRET HCA assays

The next objective was to conduct the first fully automated FLIM-FRET HCA experiment using the endogenously labelled EGFP-PARP1-mCherryFP biosensor in live cells in order to investigate the feasibility of detecting FRET signals within the cellular context.

Although this FRET biosensor had previously been demonstrated to be effective with purified proteins in solution, its efficacy had not been evaluated in live cells. To address this, the FLIM assay was performed, treating cells with 10 μM carboplatin for 24 hr, which had previously been shown to induce increased levels of DNA damage compared to untreated controls using γH2AX

immunofluorescence (**Figure 54A**). Note that these comparisons are only qualitative, as imaging was performed with a 60X objective lens on a widefield fluorescence microscope, lacking HILO or TIRF illumination that would aid optical sectioning to resolve individual foci for quantification. The rationale for this approach was that inducing ssDNA damage would increase PARP1-DNA complex formation, thereby enhancing the FRET signal readout if the proportion of PARP1 molecules in their bound state increased. Moreover, the study from *Steffen et al.* observed an increase in FRET efficiency upon the introduction of DNA damage [58]. Hence, it was reasoned that using DNA damaging agents in live cells offered an approach to mimic these conditions *in vitro*.

This initial FLIM assay was imaged using the wide-field time-gated FLIM system, and representative intensity images and their respective fluorescence lifetime images of the untreated and carboplatin treated cells are shown in **Figure 54B**. The fluorescence lifetime images demonstrate considerable inter and intra-cellular variation in lifetime. However, between the two treatment conditions there is not an obvious observable difference in the fluorescence lifetime images.

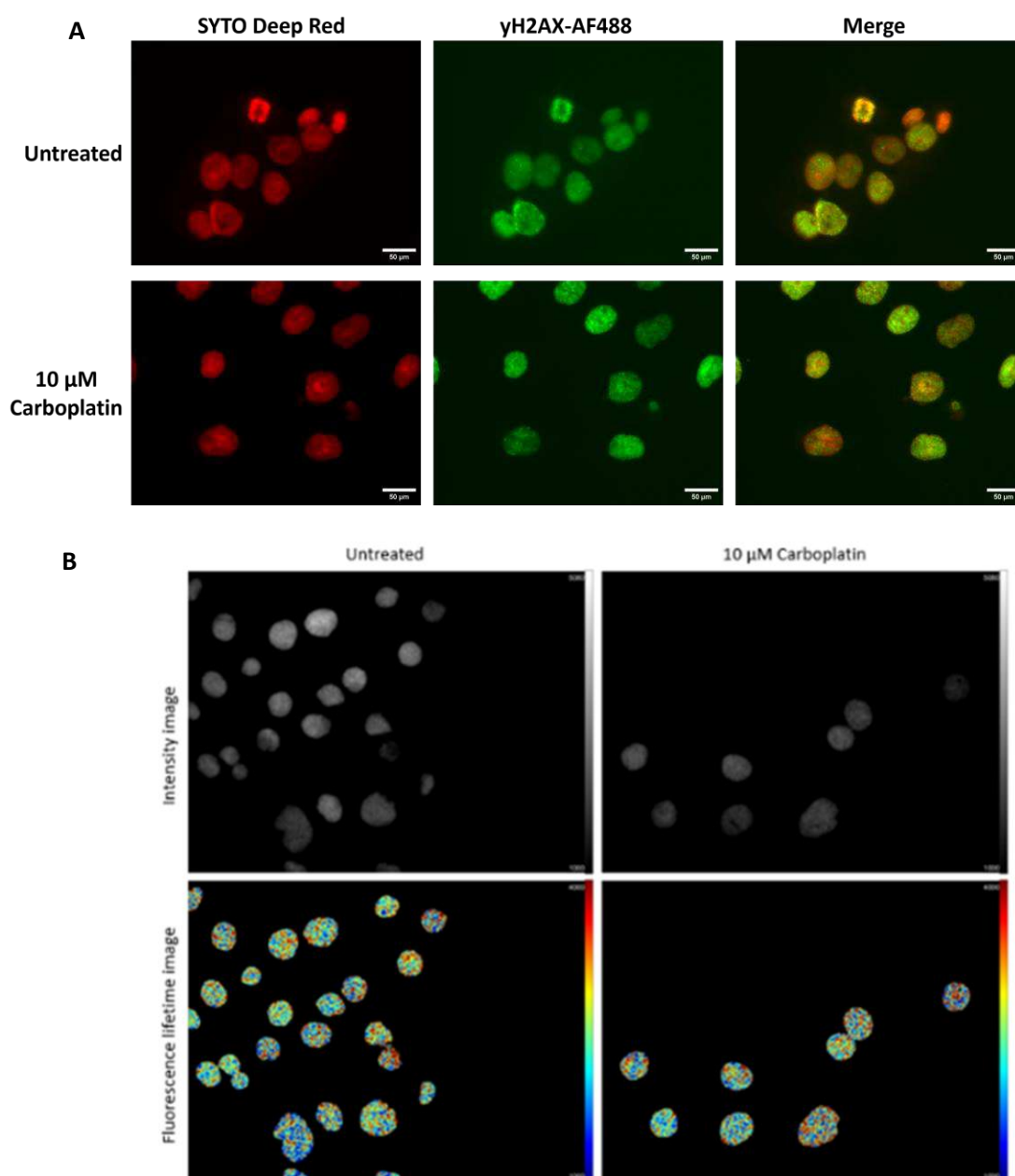


Figure 54: Immunofluorescence and fluorescence lifetime images of carboplatin treatment. **A:** Representative immunofluorescence images of SYTO Deep Red (left), γ H2AX (middle) and merge (right) in untreated (top) and carboplatin treated OVCAR4 wild-type cells (bottom). **B:** OVCAR4 EGFP-PARP1-mCherryFP cells imaged using wide-field time-gated FLIM imaging. Top panel shows the intensity images I_0 and the bottom panel shows the corresponding fluorescence lifetime images produced through global fitting of a double-exponential decay in FLIMfit. Left column shows untreated cells, and right column shows cells treated with 10 μ M carboplatin for 24h. Images are pseudo coloured for τ_1 value.

The produced fluorescence lifetime images are comprised of many individual nuclei, each of which was defined as an individual ROI. Therefore, single-cell data could be extracted for each treatment condition and subsequently quantified, averaging many cells together over a population, or maintaining single-cell resolution to assess heterogeneity in response. To quantify the effect of the drug at a population level, the average fluorescence lifetime per field of view (FOV) for each condition was plotted. This analysis revealed no significant change ($p = 0.4671$) in fluorescence lifetime upon

carboplatin treatment, with mean fluorescence lifetimes of 2641 and 2652 ps for the untreated and treated cells, respectively (**Figure 55**). Although this drug dosing was sufficient to induce significant changes in γ H2AX levels over 24 hr, no change in FLIM signal was observed suggesting that FRET was not occurring at a significant level across the population 24 hr after addition of carboplatin. Consequently, the focus shifted to optimizing treatment conditions specific to this live-cell FLIM-FRET assay, diverging from conditions previously optimised for fixed-cell immunofluorescence assays. Adjustments in drug dosage and assay readout timing were considered logical steps in this optimization process.

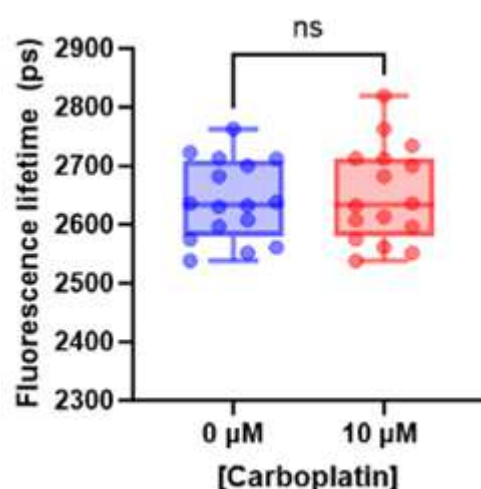


Figure 55: Comparing fluorescence lifetime of carboplatin treated cells. Weighted mean lifetime of untreated cells compared to cells treated with 10 μ M carboplatin for 24 hr (red) compared to untreated cells (blue). Individual values plotted represent average fluorescence lifetime per FOV. Statistical analysis performed at the FOV level, via unpaired t-test. $n=1$ biological replicate.

5.3.4.5 Optimising drug treatment dosage and timing for FLIM-FRET assays

The preliminary results from the FLIM assay suggested 10 μ M carboplatin treatment for 24 hr is not sufficient to induce changes in FLIM signal from the FRET biosensor. Therefore, the drug dosage was optimised by performing a western blot of γ H2AX expression at 4 and 24 hr post carboplatin treatment at two doses (**Figure 56**). This experiment indicated that the γ H2AX signal did not accumulate significantly until the 24 hr timepoint and required a dosage of 100 μ M carboplatin, much higher than the dosage used in the preliminary FLIM experiment. Therefore, another FLIM-FRET assay was conducted to assess if these optimised treatment conditions were sufficient of inducing a FLIM signal.

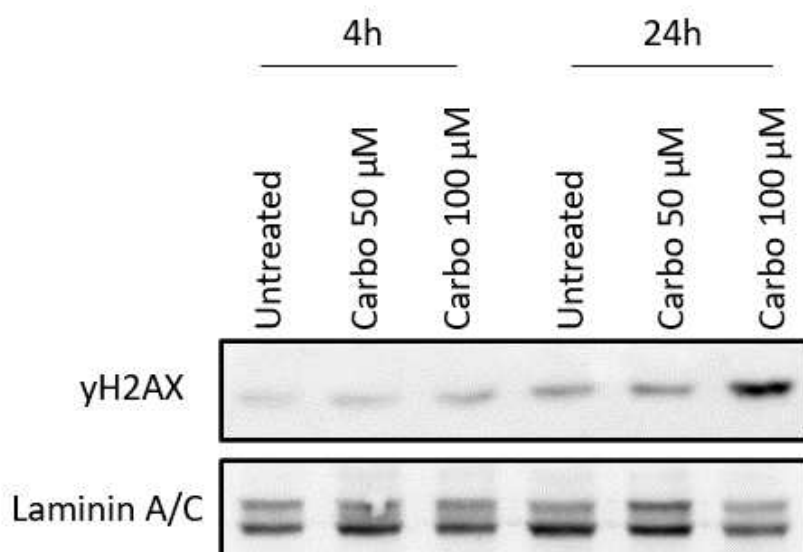


Figure 56: Western blot of γ H2AX at 4 and 24 hr carboplatin treatment. Cells were treated with 0, 50 or 100 μ M carboplatin for 4, or 24 hr. Laminin A/C was used as a protein loading control.

The biosensor-expressing cells were treated with 100 μ M carboplatin for 24 hr and compared to untreated wells. Multiple technical replicates were used across a 96-well plate. It was hypothesised that the accumulation of DNA damage sites would increase the chromatin-bound fraction of the biosensor pool, placing it in a structurally compact conformation where the FRET efficiency should be higher, which would be indicated by a reduction in fluorescence lifetime compared to the untreated cells.

The automated FLIM-FRET HCA acquisition was performed and the images were segmented before global fitting the cellular fluorescence to a double exponential decay (**Figure 57A**). Single-cell weighted mean lifetime values were subsequently extracted and plotted for each condition. The mean lifetime of untreated cells was 2661 ± 20 ps, while treatment with 100 μ M carboplatin for 24 hr resulted in a statistically significant reduction to 2530 ± 19 ps (**Figure 57B**). However, it should be noted that the sample size was relatively low, with only 164 and 94 cells sampled in the untreated and treated populations, respectively.

Interestingly, when looking at the intensity value of the image acquired at the first delay gate (I0), there is a statistically significant ($p < 0.0001$) difference between the treated and untreated cells with mean values of 2226 and 2862 AU respectively (**Figure 57C**). As the cells were treated with carboplatin for 24 h, this was likely long enough to induce significant DNA damage, potentially upregulating PARP1 expression. Consequently, more EGFP-PARP1-mCherryFP would be expressed as the biosensor is under control of the endogenous promoter, causing the increased intensity observed in this population. This supports the use of FLIM, an intensity-independent method, over spectral ratiometric

FRET. Moreover, extracting secondary information such as this illustrates the potential power of HCA for producing multi-parameter datasets that are rich in biological information.

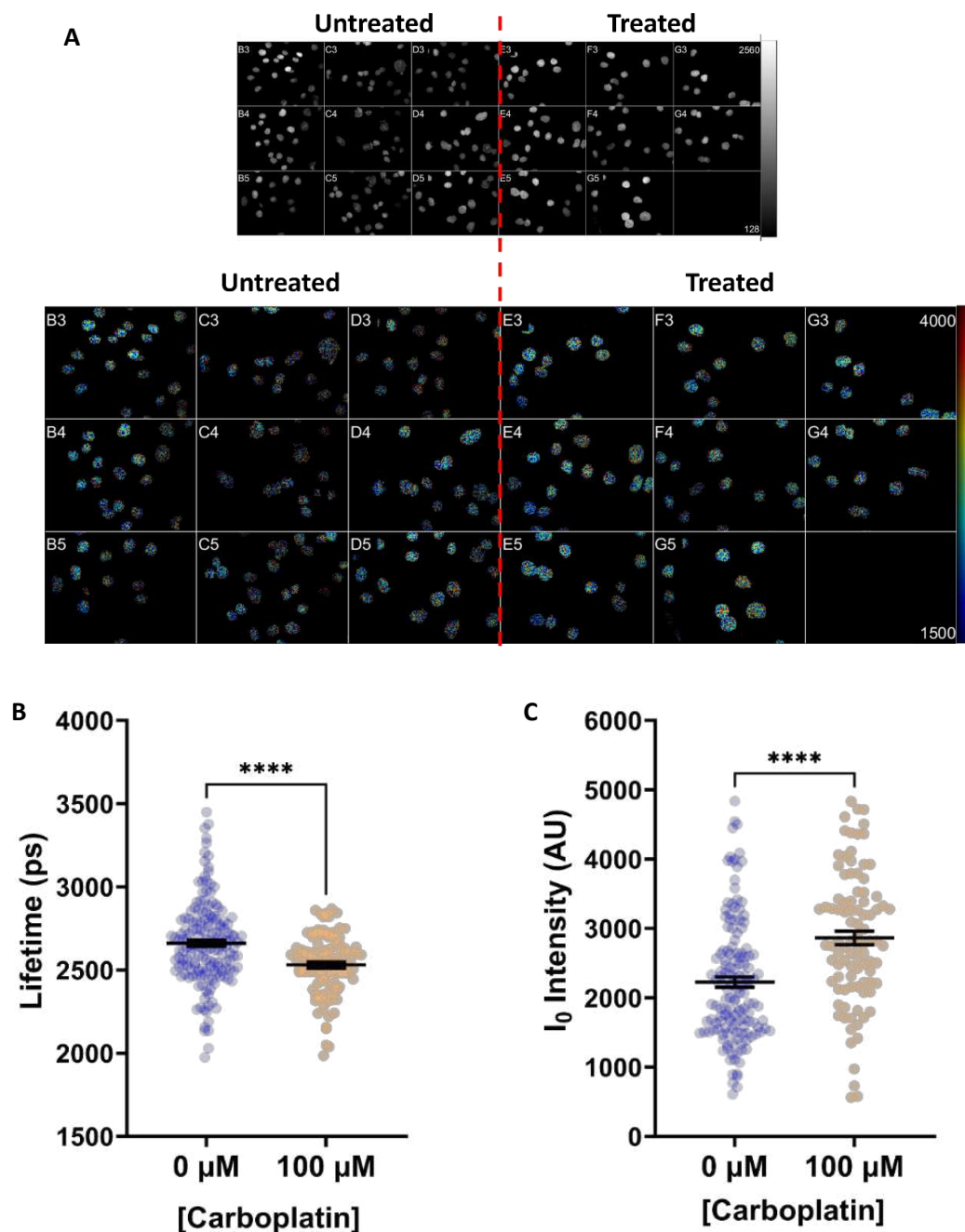


Figure 57: Comparing intensity and fluorescence lifetime images of carboplatin treated cells. Cells were treated for 24 hr with 100 μ M carboplatin. **A:** Intensity images (top panel) and respective fluorescence lifetime images (bottom panel) for untreated and treated cells across different wells of a mutliwell plate. Images acquired with 40X objective, 2000 ms acquisition time per frame. **B:** Weighted mean lifetime values per cell, with error bars denoting mean and SEM, $n = 164$, 94 cells per condition. **C:** Each point represents a single-cell mean intensity value at the first time gate (I_0), $n = 164$, 94. Statistical analysis performed at the single cell level, via unpaired t-test. ****: $p < 0.0001$.

In light of the discovery that rucaparib treatment alters fluorescence intensity in the donor channel, a crucial negative control experiment was conducted. Donor only (OVCAR4 EGFP-PARP1 N-terminal) cells were treated with a range of doses of rucaparib and underwent fluorescence lifetime imaging. In this population, no significant reduction in fluorescence lifetime was observed upon treatment, with mean values of 2568, 2567 and 2576 ps for 0, 10 and 50 μM rucaparib, respectively (**Figure 58**). The reduction in fluorescence lifetime of the biosensor expressing cells but not the donor-only (unable to FRET) negative control suggests that changes in lifetime observed are due to PARP trapping and not to the drug itself changing the properties of the fluorophores.

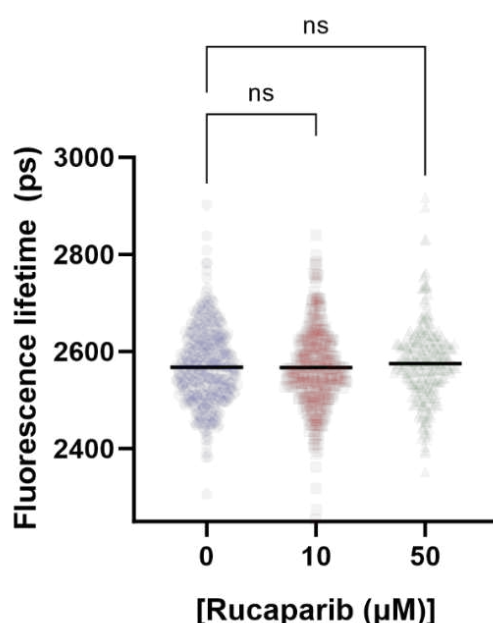


Figure 58: Donor only cells show no reduction in lifetime upon rucaparib treatment. OVCAR4 EGFP-PARP1 (N-terminal) cells were treated with 0 (blue), 10 (red) or 50 μM rucaparib (green). Single-cell weighted mean lifetimes are displayed and error bars show the mean and SEM values for each condition. $n=3$ biological repeats.

This marked a significant milestone towards achieving **Aim 2**, observing statistically significant differences across two treatment conditions in a live-cell FLIM-FRET assay for the first time. However, the high dosage used in this experiment (100 μM carboplatin) limited the ability to detect signal changes through treatment modulation. Consequently, efforts continued to optimise treatments to identify more physiologically relevant dosing regimens capable of eliciting biological signals in the FLIM-FRET assay.

Despite the initial western blot indicating that significant DNA damage repair did not occur until 24 hr post-treatment with 100 μM carboplatin, it remained unclear whether this signal would emerge within the 4-24 hr time window when no observation was made. To address this, another western blot was conducted with a comprehensive time course ranging from 2 to 72 hr (**Figure 59**). γH2AX

serves as a marker of DNA damage repair, providing information on events occurring downstream of those assessed with the FRET biosensor. PARP1 is known to bind to DNA damage sites within seconds of their formation; therefore, γ H2AX, which is commonly used as a proxy for DNA double-strand break formation, may not be a suitable reporter in this case due to the mismatch in temporal dynamics. While it can be considered a marker of damage accumulation; it reflects later events in the process of DNA damage onset and repair, rather than the earlier occurrence of PARP1 activity. However, it could also be the case that the western blot assay is simply not sensitive enough to detect these γ H2AX events earlier than 24 hr.

As illustrated in **Figure 59**, a clear increase in γ H2AX expression was not observed until 24 hr, suggesting that DNA damage events themselves were occurring well before this time point. However, pan-nuclear expression of γ H2AX is also a marker of apoptosis. This potentially explains why initial assays attempted at 24 hr post drug treatment failed to detect any significant reduction in fluorescence lifetime upon drug treatment, as most of the active DNA damage events had likely occurred much earlier. This hypothesis is further supported by extensive evidence in the literature characterizing DNA damage events occurring within minutes to hours post drug treatment. Additionally, the subsequent sensing and binding of PARP1 to damage sites is known to occur within seconds of break formation. Therefore, considering all the evidence it seemed reasonable that assessing PARP1-DNA binding dynamics at timepoints much earlier than 24 hr would be more informative in the context of the FRET biosensor used in this study.

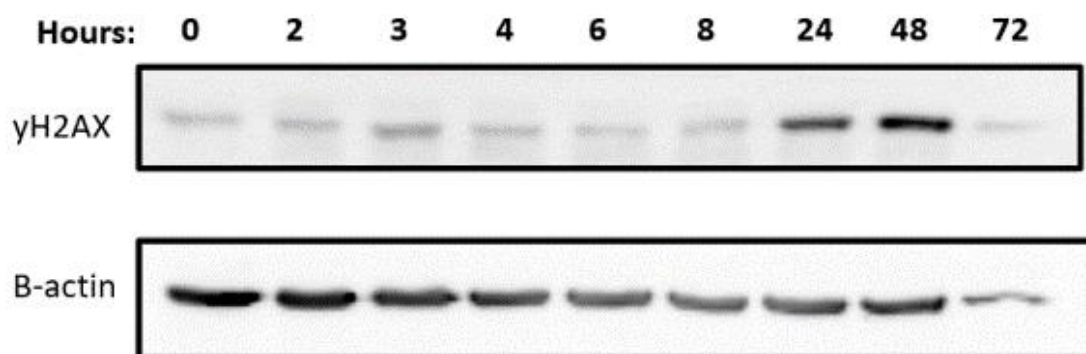


Figure 59: Time course western blot of γ H2AX signal upon carboplatin treatment. Protein was extracted from OVCAR4 cells after differing exposure times to 100 μ M carboplatin (0-72 h). β -actin was used as a protein loading control.

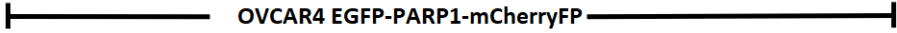
5.3.4.6 FLIM-FRET HCA assays performed 1 hr post treatment

To address this hypothesis, in the next FLIM assay measurements were taken 1 hr after drug addition. The rationale for this change was that PARPi bind extremely quickly to DNA damage sites (on the timescale of seconds). Moreover, the half-life of PARPi such as rucaparib and olaparib (as well as that of carboplatin) is in the range of 6-8 hr, meaning that by the 24 hr time point, although significant DNA damage is induced (as seen in γ H2AX immunofluorescence experiments at 10 μ M rucaparib), the assay

was observing a time window beyond which PARPi were primarily engaged in their functional role of DNA binding. Ideally, FLIM readings would be performed before, during, and after the addition of DNA damage inducing drugs. However, the temperature sensitivity of FLIM measurements makes this challenging since there are no automated microfluidics built into the imaging system, to allow the addition of drug treatments without opening the environmental chamber and thus altering the local temperature. In the current implementation of the wide-field time-gated FLIM system used here, these components are not available. However, this could be implemented in the future to facilitate the assessment of temporal dynamics of the FLIM signal upon treatment. For these reasons, it was deemed reasonable to commence the assay at the 1 hr time point, the earliest point at which both the enclosure and sample temperature had reached a steady state.

For this assay, cells were treated with rucaparib for the first time to compare the effects of PARPi and carboplatin treatment on the FLIM signal. Carboplatin, a cross-linking agent, induces direct DNA damage. Conversely, PARPi induces DNA damage indirectly by causing PARP trapping, which increases the complexity of stalled replication forks, leading to DNA damage. Therefore, the mechanistic differences between these two classes of drugs may result in differences in the FLIM-FRET signal. It was postulated that PARP trapping induced by PARPi treatment may increase the percentage of the FRET biosensor population in its FRETing state, thus resulting in larger decreases in fluorescence lifetime than when inducing DNA damage alone. This is attributed to the temporal dynamics and persistence of PARP1-DNA damage complexes under PARP trapping conditions. Hence, rucaparib treatment was assessed in the first FLIM-FRET assay performed at 1 hr post-treatment using the plate layout shown below (**Figure 60**); performing the assay in a 96-well format allowed assessment of several drug conditions and control wells with technical replicates, sampling multiple fields of view per well.

	1	2	3	4	5	6	7	8	9
B	OVCAR4 Wildtype	Untreated							
C									
D	OVCAR4 EGFP-PARP1	50 μ M Carboplatin	100 μ M Carboplatin	10 μ M Rucaparib	30 μ M Rucaparib	50 μ M Carboplatin	100 μ M Carboplatin	10 μ M Rucaparib	30 μ M Rucaparib
E									
F	OVCAR4 PARP1-mCherryFP	50 μ M Carboplatin	100 μ M Carboplatin	10 μ M Rucaparib	30 μ M Rucaparib	50 μ M Carboplatin	100 μ M Carboplatin	10 μ M Rucaparib	30 μ M Rucaparib
G									



OVCAR4 EGFP-PARP1-mCherryFP
Clone B8

Figure 60: Plate map for initial multi-well plate experiment to optimise FLIM-FRET HCA assay. OVCAR4 wild-type cells served as an autofluorescence control, and for overall cell viability before and after imaging. OVCAR4 PARP1-EGFP cells were the donor only control, OVCAR4 PARP1-mCherryFP were the acceptor only control. Biosensor expressing cells (clone B8) were either untreated, or treated with carboplatin (50, 100 μ M) or rucaparib (10, 30 μ M).

The first column consisted of controls for fluorescence lifetime measurements. Firstly, OVCAR4 wild-type cells (the parental cell line from which labelled cells were derived) were utilised to control for any autofluorescence present within the spectral window used for measuring fluorescence lifetime. Secondly, OVCAR4 PARP1-EGFP (N-terminal) cells were used as a donor-only control, that should not undergo FRET due to the absence of an acceptor fluorophore. Finally, OVCAR4 PARP1-mCherryFP cells were utilised as an acceptor-only control, from which no fluorescence signal was expected to be collected. This control can indicate any spectral bleed-through between the EGFP and mCherryFP channels that could contaminate fluorescence from the donor and impact the fitted donor lifetime. Additionally, two rows of untreated controls were included to ensure no plate effects were affecting the measured lifetime across the multi-well plate. The automated FLIM-FRET HCA acquisition was performed, and the FLIM data were global fitted to a double exponential decay model. Representative fluorescence lifetime images of each treatment condition are shown in **Figure 61A**.

The fitted lifetime values were plotted and visualised on a per FOV (**Figure 61B**) or single-cell (**Figure 61C**) basis, revealing similar overall trends. Carboplatin induced small, non-significant reductions in fluorescence lifetimes compared to the untreated conditions (2577 ps), with values of 2556 ps for 50 μ M and 2558 ps for 100 μ M carboplatin. In contrast, the PARPi rucaparib caused a statistically significant reduction in lifetime, with values of 2532 ps for 10 μ M and 2516 ps for 30 μ M rucaparib (**Figure 61B,C**). Note that these quoted fluorescence lifetimes are average values from the FOV analysis, although mean values of the single-cell analysis are in close agreement (average percentage variance between FOV and single-cell means was 0.07%). Hence, this suggests that pooled analysis averaging over each FOV is sufficient to capture differences between population averages.

From this initial experiment, the reduction in lifetime with rucaparib treatment appeared to be dose-dependent, with higher doses resulting in lower lifetimes. In contrast, increasing the carboplatin dose did not lead to a further decrease in fluorescence lifetime. Interestingly, treatment with 100 μ M carboplatin did not induce a reduction in fluorescence lifetime after 1 hr of treatment, unlike the previous experiment using 24 hr treatment at the same dosage (**Figure 57B**). This suggests that the accumulation of DNA damage upon carboplatin treatment occurs over longer time periods, highlighting an area for future research to assess the temporal dynamics of damage accumulation and subsequent PARP1 dynamics.

Interestingly, a near normal distribution of single-cell lifetime values was observed within each condition. Due to the relatively high sampling power, some heterogeneity within a population of strong, moderate and weak responding cells was expected. This heterogeneity could be attributed to the lack of control for cell cycle in these experiments, resulting in sampling across all stages of the cell

cycle. However, cell cycle distribution is not even for unsynchronised cells and may not produce the normal distribution observed here. Additionally, there is variation in the expression level of the biosensor between cells, due to the nature of the CRISPR knock-in resulting in the reporter being under endogenous expression. However, the stoichiometry of donor:acceptor molecules should be the same, despite differences in absolute number of reporter molecules between cells.

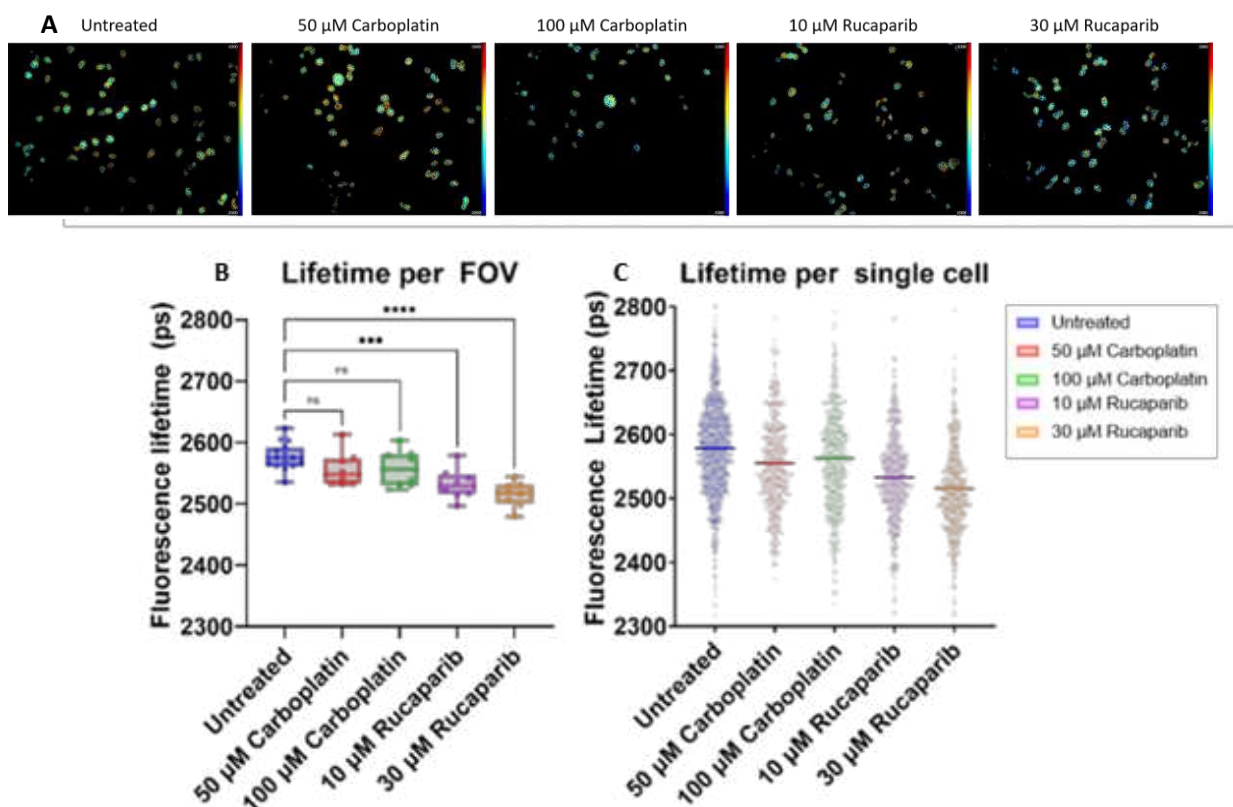


Figure 61: FLIM-FRET HCA experiment of cells treated with carboplatin and rucaparib for 1 hr. **A:** Representative fluorescence lifetime images of each condition, images are intensity-weighted, and colour coded by weighted mean lifetime scaled from 2000 to 3000 ps. **B:** Calculated fluorescence lifetime per FOV for treated and untreated conditions. Lifetimes were fitted in FLIMfit, using a double-exponential, global fit. $n=15,8,8,8$ FOVs. Carboplatin results in a non-significant reduction in mean weighted lifetime, whereas treatment with rucaparib at both 10 μ M and 30 μ M results in a statistically significant reduction in lifetime from a mean of 2577 to 2532 and 2516 ps respectively. **C:** Weighted mean lifetime values were extracted per single cell for each condition. Plotting the single-cell data follows the same trend as the FOV summarised dataset whilst giving the resolution to assess inter-cellular heterogeneity in the population. $n= 825,425,444,453,486$ cells per condition, from one biological replicate. Statistical analyses were performed at the FOV level, using a one-way ANOVA with multiple comparisons; ***: $p<0.001$; ****: $p<0.0001$.

As an additional control, single-cell values of untreated wells across the plate were plotted to assess potential spatial distribution effects. The weighted mean fluorescence lifetime of untreated cells per column was plotted, displaying single-cell values (**Figure 62**). Reassuringly, no significant change in the mean lifetime between columns was observed, suggesting the absence of unwanted plate effects. Practical measures were taken to minimise this, by using only the inner 60 wells of a 96-well plate and filling the outer wells with PBS to create a border, thereby reducing humidity gradients. This approach aimed to prevent evaporation effects that could alter working volumes in the wells at the edge of the plate, enhancing reproducibility and minimizing errors. Note that this also demonstrates a temporal

stability in the fluorescence lifetime of untreated cells, as the acquisition is performed in a column-wise manner, there is ~90 min separating the acquisition of data in columns 2 and 9.

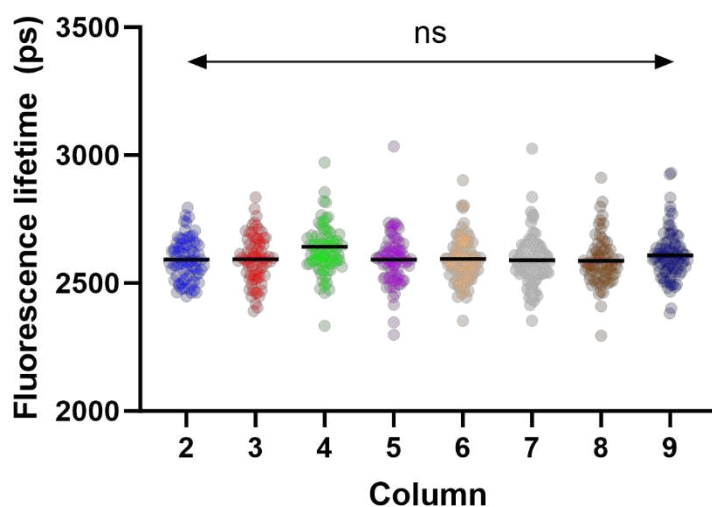


Figure 62: Untreated cells have stable fluorescent lifetimes across multi-well plates. EGFP-PARP1-mCherryFP expressing OVCAR4 cells in untreated conditions, spatially separated across columns of the multi-well plate. The weighted mean fluorescence lifetime per cell is plotted, separated by column. Mean and SEM of the population lifetime is displayed by the error bars. Statistical analyses were performed at the single cell level, using a one-way ANOVA with multiple comparisons.

Alternative data visualization methods were also explored, presenting the distribution of fluorescence lifetime across treatments as frequency distribution (histogram) plots at the single-cell level, using the python package JoyPlot (**Figure 63**). Single-cell lifetime values were used to visualise the distribution of mean lifetime throughout the population per condition. This provides an alternative method to visualise the reductions in lifetime across the population induced by rucaparib treatment, which were not observed with carboplatin treatment of 1 hr.

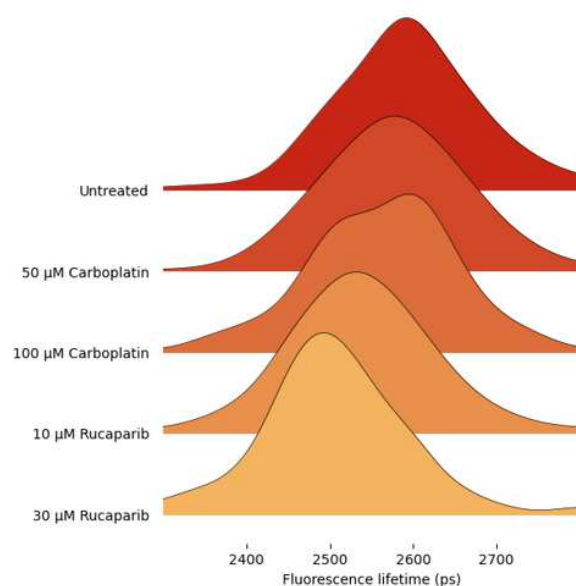


Figure 63: JoyPlot showing distribution of fluorescence lifetimes upon treatment. JoyPlot generated in Python, showing the frequency distribution histograms of mean fluorescence lifetime per single cell, pooled per treatment condition.

In summary, these experiments demonstrated that carboplatin did not induce a reduction in lifetime after 1 hr; however, a reduction was observed after treatment for 24 hr. This suggests that DNA damage sites do not accumulate significantly until the later timepoint. In contrast, 1 hr treatment with the PARPi rucaparib was sufficient to induce a reduction in fluorescence lifetime, suggesting FRET is occurring. This is perhaps unsurprising in light of previous evidence suggesting that the proportion of chromatin bound PARP1 molecules does not increase upon induction of DNA damage. Rather, the PARP1 pool exists in a range of slow-to-fast diffusing states and PARP1 molecules are continuously exchanged at DNA damage sites, rather than persisting at the damage sites [54]. However, in the presence of PARP inhibitors, trapping is induced thereby prolonging the interaction of the EGFP-PARP1-mCherryFP FRET biosensor at DNA damage sites. In theory, this would increase the proportion of the biosensor pool that is in its FRET-ing state (the condensed structural configuration where the C and N termini are in close proximity) and therefore decrease the mean donor fluorescence lifetime.

The observation of differences in fluorescence lifetime from these initial FLIM-FRET assays employing this novel biosensor in live cells for the first time was highly encouraging. The potential of this FRET biosensor to assess PARP trapping is explored in more depth throughout the remainder of this chapter, particularly addressing two new questions: Firstly, is this biological signal upon rucaparib treatment reproducible? Secondly, does this dose-dependent reduction in fluorescence lifetime extend to other doses and other PARPi? These questions, among others, were the focus of the subsequent work.

5.3.5 Comparison of FLIM signal from three clonal populations

This initial experiment was carried out using one (Clone B8) of the three dual-labelled clonal populations that had been derived. This clone demonstrated potential for producing FLIM-FRET signals. Consequently, the next step involved assessing whether similar signals were present in the two other clones that had been isolated. To evaluate this, another FLIM experiment was performed, during which the three separate OVCAR4 EGFP-PARP1-mCherryFP clones (B8, C4, G3) were treated with increasing concentrations of rucaparib (0, 10, 30 and 50 μ M). Imaging was conducted 1 hr after the addition of the drug. Multiple wells per condition were imaged, and 3 fields of view (FOVs) per well were examined to sample a sufficient number of cells, ensuring statistical robustness for comparisons between conditions. The plate map is shown in **Figure 64** and the outcomes are summarised in **Figure 65**, with single-cell values depicted in the top panel and averaged fluorescence lifetimes per FOV shown in the bottom panel.

	1	2	3	4	5	6	7	8	9	10									
B	WT	Untreated			Untreated			Untreated											
C																			
D	N-GFP	10 μ M	30 μ M	50 μ M	10 μ M	30 μ M	50 μ M	10 μ M	30 μ M	50 μ M									
E																			
F	mCherryFP																		
G																			
		Clone B8			Clone C4			Clone G3											

Figure 64: Plate map for comparing the FLIM response of 3 clonal populations. Aimed to screen the FLIM signal produced by each of the clones expressing the FRET biosensor, in response to increasing doses of rucaparib (0 to 50 μ M) for 1 hr. Plate map of experimental layout. Column 1 are FRET controls, WT cells for autofluorescence background. N-EGFP cells as donor only control and mCherryFP cells as acceptor only control. 3 clones are seeded in columns, with untreated cells and treated at differing concentrations of rucaparib.

Analysis of single-cell fluorescence lifetimes revealed a dose-dependent reduction in lifetime across all three clones (C4, G3, and B8) following rucaparib treatment (**Figure 65A**). As the concentration of rucaparib increased, a significant decrease in the mean fluorescence lifetime compared to untreated controls was observed. Notably, only clone B8 displayed a statistically significant reduction in lifetime, suggesting a stronger response to rucaparib in this clone, despite the sensitivity (as measured by the IC₅₀ values in the three populations) being very similar. The statistical analysis confirmed that the reduction in fluorescence lifetime was significant at higher rucaparib concentrations, indicating FRET and effective binding and trapping of PARP1 at DNA damage sites.

The FOV-averaged data further supported these findings, showing consistent trends of decreased fluorescence lifetimes with increasing rucaparib concentrations (**Figure 65B**), although this does not fully capture the heterogeneity of the single-cell data. The fluorescence lifetime for the untreated wells remained relatively high and stable across all FOVs, whereas a significant dose-dependent decrease was evident as the concentration of rucaparib was elevated. This trend was consistent across all three clonal populations.

Overall, these results again demonstrate that rucaparib induces a measurable decrease in fluorescence lifetime in a dose-dependent manner, suggesting that the EGFP-PARP1-mCherryFP biosensor effectively reports on PARP1 trapping at DNA damage sites. The observed reductions in lifetime align with the hypothesis that rucaparib enhances PARP1-DNA binding, thus increasing the proportion of the biosensor in its high FRET efficiency state, thereby validating the functionality of the biosensor in live-cell assays. The variance in response among the clones also highlights the potential for differential sensitivity to PARP inhibitors, which may be down to the ratio of dual-labelled *PARP1* alleles in each clone.

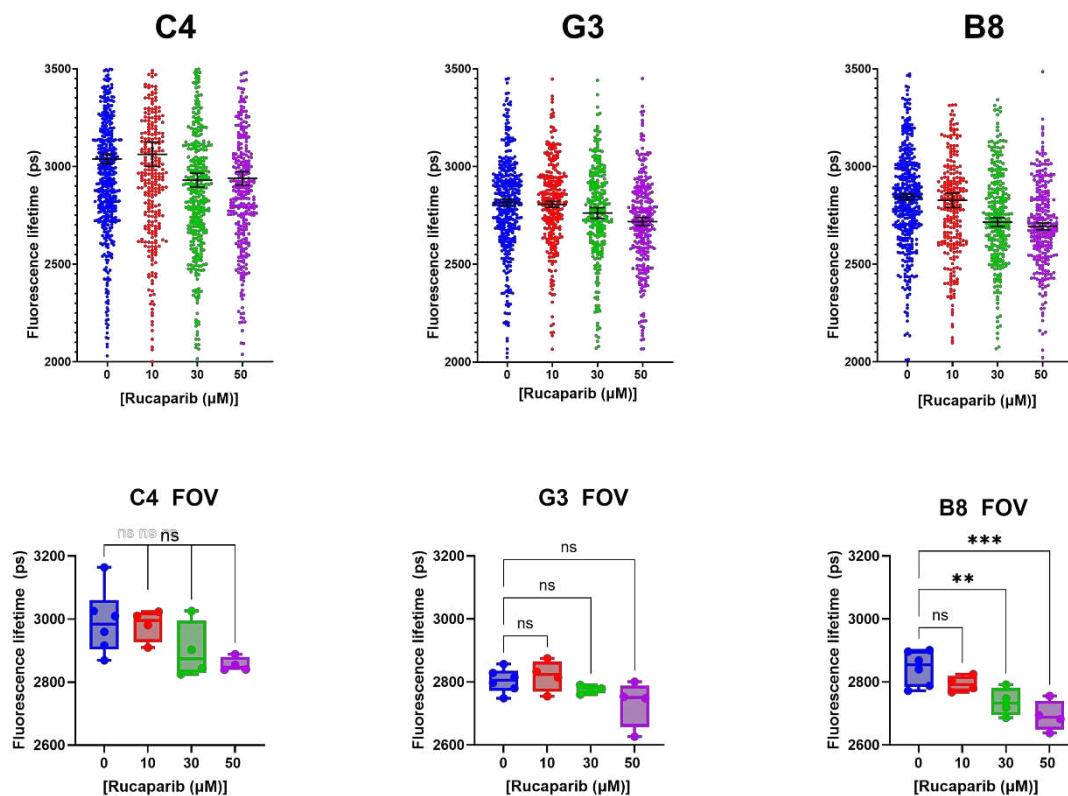


Figure 65: Quantification of fluorescence lifetimes of each clone from FLIM-FRET experiment. Single-cell and Field-of-view (FOV) analysis of fluorescence lifetime in different OVCAR4 EGFP-PARP1-mCherryFP clonal populations following rucaparib treatment. **A:** The graphs display the distribution of single-cell mean fluorescence lifetimes in three different clonal populations (C4, G3, B8) of OVCAR4 EGFP-PARP1-mCherryFP cells. Each dot represents a single cell's fluorescence lifetime, measured after treatment with increasing doses of rucaparib (0, 10, 30, and 50 μ M) for 1 hr. Each condition shows significant variability in single-cell fluorescence lifetime values, with the mean lifetime and SEM indicated by the error bars for each group. **B:** The box plots represent the average fluorescence lifetime per FOV for the same conditions as above, aggregating

*multiple FOVs per treatment group. The data illustrates that rucaparib treatment induces a dose-dependent reduction in fluorescence lifetime across all three clonal populations. Statistical analysis were performed at the FOV level via one-way ANOVA, $p \leq 0.01$ (**), $p \leq 0.001$ (***). $n=1$ biological replicates.*

This experiment demonstrated that all three generated clones underwent some level of reduction in fluorescence lifetime upon treatment with rucaparib. However, clone G3 exhibited a smaller reduction, requiring a much higher dosage to induce a relatively small change in fluorescence lifetime compared to the untreated control. In contrast, a dose-dependent reduction in lifetime, relative to the untreated control, was observed in both clones B8 and C4. Among these, clone B8 exhibited lower levels of variability, as indicated by the smaller standard deviation values per condition when compared to clone C4. Consequently, clone B8 was selected for future experiments due to its perceived higher signal-to-noise ratio.

One potential explanation for the differences in FRET signals observed between the clones could be the ratio of dual labelled, single labelled, and unlabelled *PARP1* alleles. It is known that the average ploidy of OVCAR4 wild-type cells is approximately three. However, without performing a copy number analysis specifically for *PARP1*, the exact number of *PARP1* alleles present remained unclear [112]. This presented a challenge in determining the ratio of single/dual labelled to non-labelled alleles in the CRISPR-generated cells, as sequencing methodologies do not offer sufficient resolution to assess this.

5.3.6 Assessing the reproducibility of FLIM-FRET signal upon rucaparib treatment

Given the preliminary evidence that had been acquired thus far, the next aim was to assess the reproducibility of the observed changes in lifetime. Therefore, three biological repeats of the assay were performed, comparing untreated cells with those treated with 30 μ M rucaparib for 1hr. In **Figure 66B** each biological repeat is represented by a distinct colour (blue for experiment 1, red for experiment 2, and green for experiment 3). The data points indicate the fluorescence lifetimes, with a notable difference observed between the untreated and rucaparib treated groups. The mean fluorescence lifetimes were 2592 ps for the untreated cells and 2506 ps for the rucaparib-treated cells, a statistically significant ($p=0.0060$) reduction in fluorescence lifetime at the plate-level, across three separate experiments, indicating an increase in FRET under these conditions. The reproducibility of these results across biological replicates demonstrates the FRET biosensor's effectiveness in assaying *PARP1* dynamics in live cells. The statistical significance at the plate level underscores the robustness of the HCA FLIM-FRET approach in consistently detecting *PARP* trapping changes.

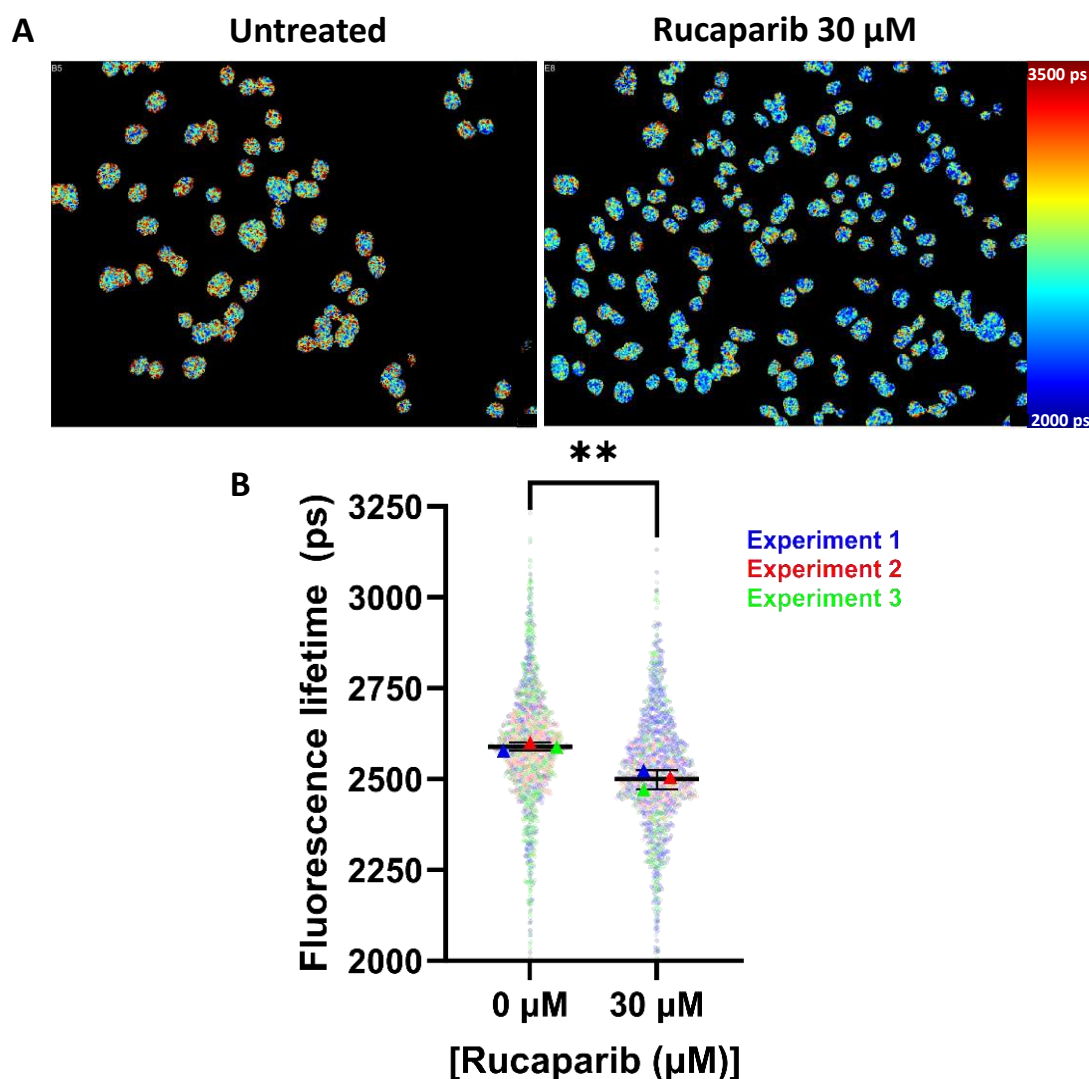


Figure 66: Rucaparib treatment results in a reproducible reduction in lifetime. **A:** Representative fluorescence lifetime images of untreated and cells treated with rucaparib for 1 hr. **B:** The violin plot presents the single-cell fluorescence lifetime measurements of a FRET biosensor following treatment with a drug, across three biological replicates (Experiments 1, 2, and 3). The untreated control group and the drug-treated group (30 μM) are shown on the left and right, respectively, with data points from each experiment distinguished by colour (blue for Experiment 1, red for Experiment 2, and green for Experiment 3). The error bars indicate the mean and SEM fluorescence lifetime values for each condition. Statistical analyses were performed at the experiment level, using an unpaired t-test **: $p < 0.01$.

As the transition from a 24 hr to a 1 hr drug treatment had been chosen, it was also necessary to ensure the presence of DNA damage after 1 hr of treatment with PARPi using traditional probes. To assess this, OVCAR4 cells were seeded on a fibronectin-coated glass 8-well chamber slide. After 24 hr, the cells were treated with 0, 10, or 50 μM rucaparib for 1 hr. Immediately after, the cells were fixed and stained for γH2AX . Cells were also stained with SYTO Deep Red in order to segment individual nuclei **Figure 67A**. An automated Fiji macro was written to perform thresholding-based segmentation on the SYTO Deep Red images, defining regions of interest to find maxima points of intensity in the γH2AX images, successfully identifying foci. Following this, mean single-cell fluorescence lifetimes per condition were plotted (**Figure 67B**). A dose dependent increase in γH2AX foci was observed upon

rucaparib treatment, with 50 μM rucaparib resulting in a statistically significant increase in γH2AX compared to untreated controls. The mean numbers of foci per nucleus were 1, 2 and 6 for 0, 10, 50 μM respectively. By performing analysis in this automated manner, human error and bias is removed from quantifying foci per nucleus. Furthermore, the fact that all images were analysed in an automated manner means that the treatments were effectively blinded. Although this pipeline may fail to identify some foci and thus not be 100% accurate, these inaccuracies are consistent over all treatments. This experiment confirmed that DNA damage is induced after 1 hr of treatment with PARPi. Whilst the analysis performed here is sufficient for this validation experiment, further refinement would be required if DNA damage quantification was using only the γH2AX assay. Also, these results suggest that the previously performed western blots were not sensitive enough to detect these early γH2AX events.

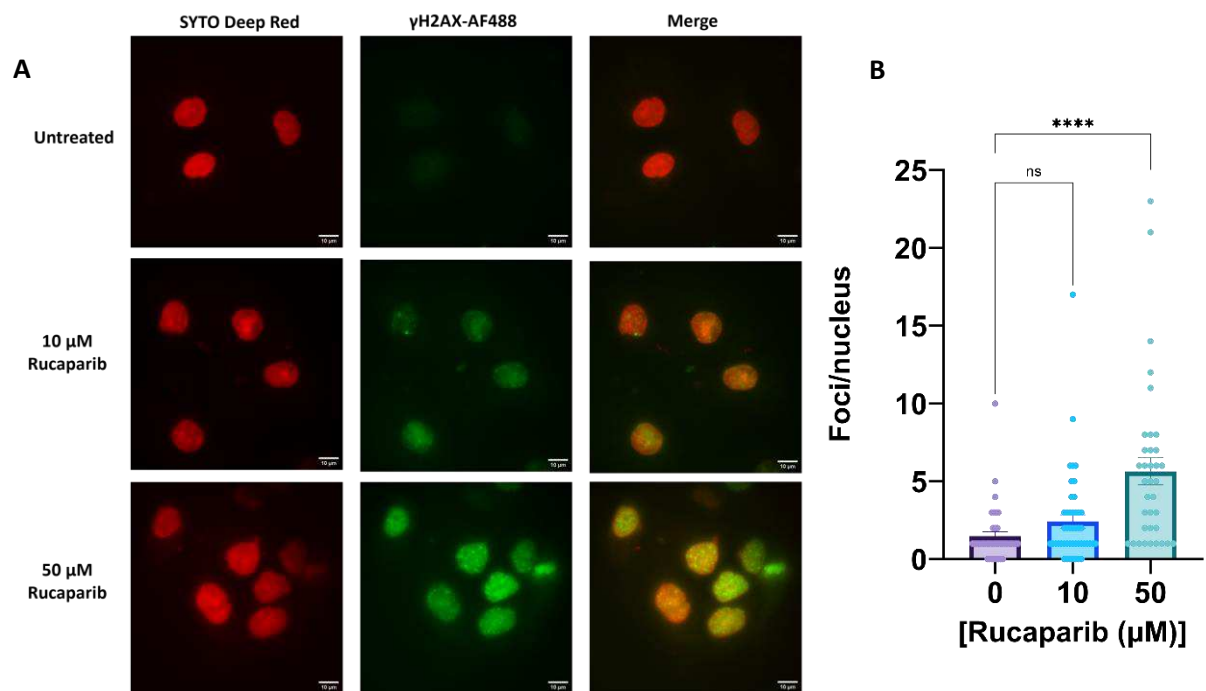


Figure 67: One hour treatment with rucaparib induces DNA damage. Analysis of DNA damage in cells treated with Rucaparib for 1 hr using γH2AX as a marker in OVCAR4 cells expressing the dual-tagged PARP1 FRET biosensor. **A:** Fluorescence microscopy images showing the cellular localization and intensity of γH2AX foci (green) in cells treated with Rucaparib. Cells were counterstained with SYTO Deep Red to visualise nuclei (red). Three conditions are presented: untreated cells (top row), cells treated with 10 μM Rucaparib (middle row), and cells treated with 50 μM Rucaparib (bottom row) for 1 hr. The merged images (right column) demonstrate the colocalization of γH2AX within the nuclei, indicating sites of DNA damage. **B:** Quantification of γH2AX foci per nucleus under different treatment conditions. The box plot illustrates the distribution of the number of γH2AX foci per nucleus for each condition, with individual data points representing single-cell measurements, automated quantification of γH2AX foci in at least 40 nuclei/condition. Each point represents a single nucleus. One-way ANOVA with Dunn's multiple comparisons test (One-way ANOVA with Dunn's multiple comparison's test. **, $p < 0.01$. ***, $p < 0.001$. ****, $p < 0.0001$.), $n = 40, 47, 36$ cells per condition.

Figure 68 depicts a schematic of the optimised treatment protocol employed throughout the remainder of the project. 7.5×10^4 cells were seeded in fibronectin coated multi-well plates and cultured for 48 hr in complete medium. The medium was subsequently removed, adding CO₂ dependent imaging medium containing the appropriate drug treatments. The plate was immediately placed into the incubator on the FLIM plate reader system and allowed to equilibrate up to 37 °C. Next, the FLIM acquisitions were performed, using eight delay gates to sample the fluorescence decay profiles. Acquisitions were carried out in an automated fashion, achieving HCA FLIM-FRET, imaging multiple (generally 4) FOV per well, across the 96-well plate. The acquired data were later analysed using *FLIMfit*, fitting global double exponential decays to the fluorescence decay of the donor EGFP to fit a fluorescence lifetime, represented by weighted mean lifetime values.

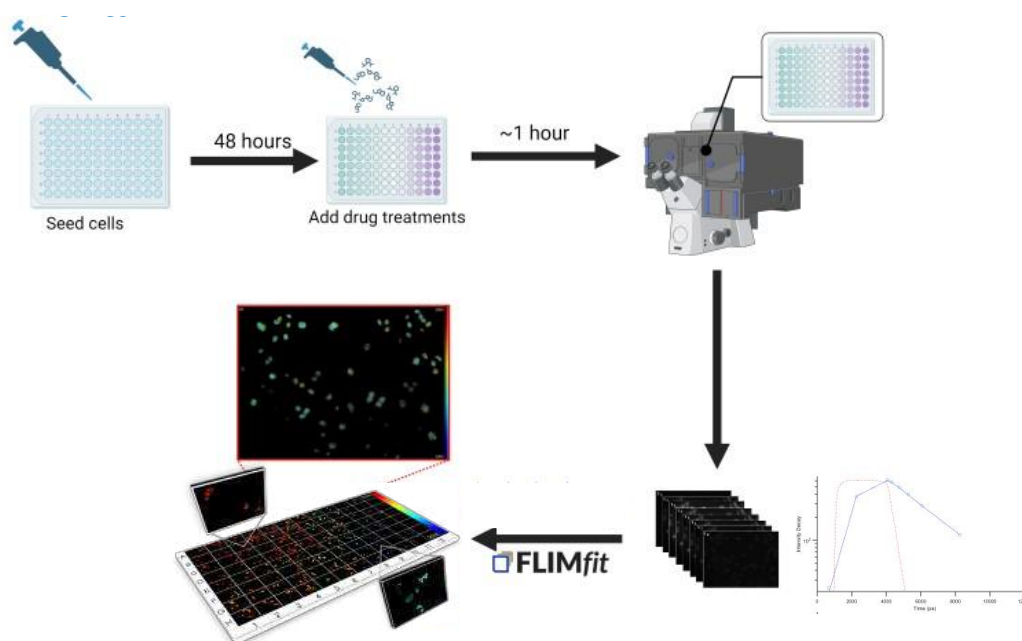


Figure 68: Schematic of experimental setup for HCA FLIM-FRET experiments. Cells are seeded into 96-well plates and cultured for 48h before the addition of drug treatments. Assay plates are placed in the microscope top environmental chamber and imaging is performed 1 hr later, acquiring intensity images at different time delays. FLIMfit software is used to perform fitting of fluorescence lifetimes.

5.3.7 Comparing PARP trapping potency of multiple PARPi

Next, assay conditions were expanded to assess whether a similar dose-dependent reduction in fluorescence lifetime observed upon rucaparib treatment was observed when treating with other PARPi. Using a range of biological assays, including ChIP, micro-irradiation assays, and fluorescence anisotropy, it has been well documented that different PARPi have vastly different PARP trapping potencies [53,54]. Hence, a further validation experiment was performed to test whether the FLIM assay could discriminate between PARP trapping potency of different PARPi. Biosensor expressing

cells were treated with a range of concentrations of four clinically approved PARPi: veliparib, olaparib, rucaparib and talazoparib. The prior literature suggests these are ordered from strongest to weakest PARP trapping potency: talazoparib > olaparib = rucaparib > veliparib [26]. However, there is not a linear relationship in trapping ability and talazoparib has been suggested to be 10,000-fold more potent PARP trapping ability than veliparib [41]. Hence, various doses of each drug were used, reflecting their respective potency. It is important to note that the cytotoxicity of these PARPi has been shown to correlate with their PARP trapping potency and thus, using much higher dosages of weak trapping-inducing inhibitors should not drastically effect viability.

Cells were seeded as above and treated with one of the mentioned PARPi at various concentrations. The FLIM acquisitions began 1 hr post-treatment. The mean weighted fluorescence lifetimes are plotted below, separated by drug (**Figure 69**). From this, veliparib showed non-significant changes in lifetime between the untreated cells even at the extremely high dose of 100 μ M, indicating that minimal PARP trapping is occurring, or at basal levels comparable to the untreated cells. Mean fluorescence lifetimes were 2854, 2838 and 2858 ps for 0, 10 and 100 μ M veliparib respectively. Treatment with olaparib resulted in a dose dependent decrease in mean weighted lifetime: 2842, 2850, 2782 and 2762 ps for 0, 10, 30, 50 μ M respectively. Treatment with 10 μ M olaparib did not induce a significant reduction in lifetime although higher doses of 30 μ M and above did. Conversely, rucaparib induced a significant reduction in fluorescence lifetime compared to the untreated cells at concentrations as low as 10 μ M (mean weighted lifetime of 2842, 2777, 2766 and 2765 ps respectively for 0, 10, 30 and 50 μ M). This may suggest that the PARP trapping potency of rucaparib is greater than that of olaparib, due to the lower dose required to induce changes in lifetime. However, these data derive from only one biological replicate and thus, should be confirmed by future biological repeats. Talazoparib induced significant decreases in lifetime at just 0.11 μ M and maintained this decrease at higher doses. Dosages two magnitudes less than those used for rucaparib and olaparib were sufficient to induce larger absolute decreases in fluorescence lifetime upon talazoparib treatment (2850, 2740, 2701, 2723 and 2713 ps for 0, 0.11, 0.33, 1 and 3 μ M respectively). Hence, this result suggests that talazoparib has by far the strongest PARP trapping potency. Although these data are from one biological replicate, it provides evidence that the assay can distinguish between different biological conditions, in this case differences in PARP trapping potency of PARPi. Furthermore, the general trends in trapping potency observed here are in agreement with the findings produced by traditional assays, providing supporting evidence for the application of this assay to study PARP trapping. In future, this assay could be expanded to bench mark the PARP trapping potency of novel PARPi in live cells, such as UKTT15, a structural variant of veliparib with enhanced trapping potency [51].

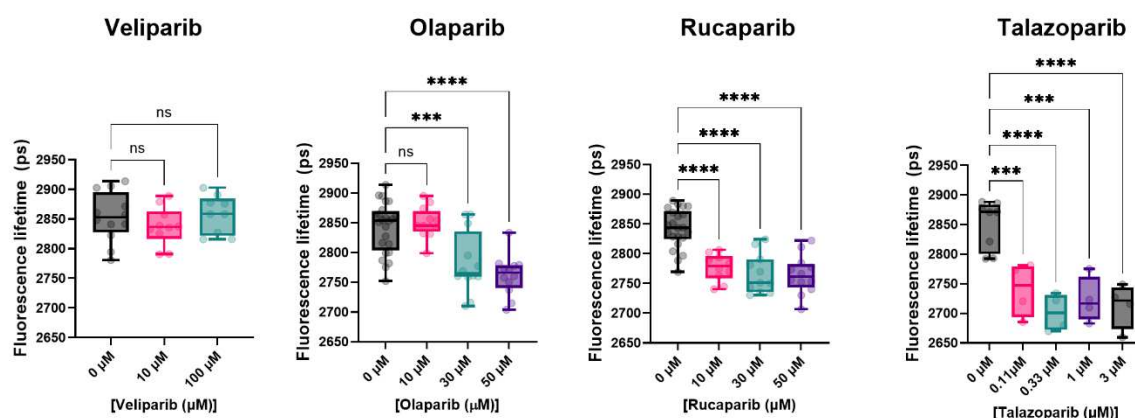


Figure 69: FLIM-FRET assay can distinguish between PARP trapping potencies of PARPi. OVCAR4 EGFP-PARP1-mCherryFP cells treated with a range of PARPi, global double exponential fitting is performed to calculate fluorescent lifetimes. Mean fluorescent lifetimes per FOV are plotted, with each FOV containing an average of 62 nuclei per image. Box plot shows the mean and range for each condition. Data were analysed by t-test with at least 4 technical repeats per condition, $n=1$ biological replicate. Statistical comparison performed at the FOV level using one-way ANOVA, with multiple comparisons.

Thus, this experiment provided a cross validation of prior knowledge of PARP trapping. Note however, that this assay can be performed in live-cells and can provide single-cell resolution if required, unlike methods such as ChIP. This is a major benefit of this approach, allowing the study of single-cell heterogeneity in response which is particularly important in the context of cancer biology and the generation of resistance to chemotherapy.

This series of experiments provided supporting evidence that this FRET biosensor could be used to assess PARP trapping and/or allosteric regulation in live cells. The fluorescence lifetime of the donor EGFP was measured to assess FRET, with a reduction in lifetime suggesting more FRET is occurring. The results acquired suggest that PARP trapping causes a persistence in the chromatin bound fraction of the biosensor population, thus increasing the proportion of biosensor molecules in the structural conformation where the FRET efficiency is its highest. This results in increased FRET, assessed by a reduction in fluorescence lifetime under these conditions.

5.4 Discussion

This chapter employed the endogenously expressed FRET biosensor in live cells for the first time, assessing changes in PARP1 trapping upon treatment with DNA damaging agents such as platinum therapy and PARPi. Carboplatin treatment was used to induce DNA damage in a clinically relevant manner, in contrast to the prior work of *Steffen et al.* where the biosensor was exposed in solution to strands of DNA damage [58]. However, here initial attempts using a dosage and timing previously optimised in fixed cell γH2AX immunofluorescence assays (10 μM for 24h) resulted in no change in FLIM signal. Therefore, treatment conditions were optimised using time-course western blot analysis,

showing that γ H2AX formation can be detected by western blot ~24h after treatment, requiring a significantly higher dosage of 100 μ M.

The purified protein version of the PARP1 biosensor used by *Steffen et al.* likely exhibited higher sensitivity to DNA damage, owing to its application in a less complex biological system [58]. When this biosensor was employed in live cells, the results reflected a higher level of biological noise due to the heterogeneity in cell states sampled across the population. Note also that noise of the optical measurement will contribute to this. Consequently, observing slightly different results in this live-cell context was not particularly surprising. Nevertheless, the general trends observed in both approaches were comparable, with the introduction of DNA damage leading to an increase in FRET.

Next, this carboplatin dosage was validated in live cell FLIM-FRET assays, producing statistically significant reductions in fluorescence lifetime, indicating an increase in FRET. This was the first demonstration of the FRET biosensor being used in live cells to distinguish between biological conditions and thus, marked a significant milestone. Note that this concentration of carboplatin is beyond that traditionally used, as 10 μ M for 24 hr had been demonstrated to be sufficient to induce significant DNA damage by γ H2AX immunofluorescence. However, peak plasma values of carboplatin in patients exceed 50 μ M, although the half-life is short (~1 hr), causing these levels to fall rapidly. Hence, the temporal dynamics of DNA damage formation and thus PARP1 binding were considered. This led to the FLIM-FRET assay being performed 1 hr post treatment, as PARP1 binds to DNA damage sites within seconds of their formation and subsequent immunofluorescence assays demonstrated the appearance of γ H2AX at this timepoint.

This was subsequently applied in the context of PARP trapping, a phenomenon that would traditionally be assessed through biochemical assays that involve analysis of a pooled population of fixed cells. Hence, these traditional approaches fail to capture single-cell heterogeneity in response and moreover, are subject to fixation artefacts. However, the FRET biosensor approach demonstrated here can provide single cell resolved readouts of fluorescence lifetime, inferring information about the dynamics of PARP1 conformation and allosteric regulation. This makes this approach an attractive alternative to the currently available biochemical assays. By coupling this novel biosensor with HCA FLIM-FRET automated multi-well plate experiments were achieved, allowing the sampling of thousands of cells per condition. This provided sufficient statistical power to make comparisons between treatment conditions. Moreover, the standardised and semi-automated approach to data analysis makes this approach less susceptible to human-bias and be used for assay-based screens. Here, cells were treated with known compounds and known concentrations, yet one could easily

employ a blinded-drug screen to assess changes in PARP1 trapping and allosteric regulation in response to various drug candidates.

In the context of this study, the fact that dose dependent reductions in fluorescence lifetime upon treatment with PARPi were induced reproducibly over several experiments suggests that it is biologically relevant. Furthermore, relating these results back to the original study that proposed this biosensor in solution, they demonstrated dose-dependent changes in FRET efficiency (all-be-it through a spectral FRET readout, not FLIM) in the presence of increasing amounts of DNA ds breaks. As shown in prior γ H2AX assays, PARP inhibition induces DSBs and thus the drug treatments reported here somewhat mimic the introduction of synthetic DSBs in the prior study by *Steffen et al* [58].

The findings from these validation experiments, specifically recapitulating trends in PARP trapping potency of differing PARP inhibitors, underscore the potential of this FRET biosensor to discern changes in PARP trapping/allosterics in other contexts, where the PARP trapping status is currently unknown. More specifically, this could be utilised to assess changes in PARP trapping in populations that are sensitive or resistant to PARPi therapy, given that a reduction in PARP trapping is one proposed mechanism of PARPi resistance. This relates to **Aim 3** of this project. If possible, this would greatly increase the clinical relevance of this biosensor, as it may aid the study of mechanisms of resistance to PARPi and thus inform therapy development to overcome this. Therefore, work towards this goal is discussed in the following chapter.

6 Assays of PARP trapping in PARPi sensitive and resistant ovarian cancer cell populations

6.1 Background:

In this chapter, the FLIM-FRET assay established in Chapter 2 was applied to evaluate novel models of PARPi resistance by studying differences in PARP trapping between populations that are sensitive or resistant to PARPi. A reduction in PARP trapping has previously been suggested as a potential mechanism driving resistance to PARPi [26,48,50]. However, experimental methods to directly measure these changes in live cells have been lacking. Consequently, the newly developed PARP binding FRET biosensor was applied to explore differences in PARP trapping. This assessment was conducted in a high-throughput manner using live-cell HCA assays with single-cell resolution, facilitating the examination of heterogeneity in responses. Although several studies have addressed generating models of PARPi resistance *in vitro*, and other distinct studies have investigated differences in PARP trapping, very few have combined these two aspects [114,115]. One such example is work by *Petit et al.*, where a genome wide CRISPR screen was performed to assess the effects of mutations leading to PARP trapping [50]. The work presented here aims to generate novel models of PARPi resistance through clinically relevant treatment protocols and subsequently assay changes in PARP trapping within these populations.

In numerous prior studies generating PARPi resistance models *in vitro* a range of treatment protocols such as constant dosing, increased dosage concentration, and pulsed treatments, have been utilised. However, the lack of a unified approach makes it difficult to compare results between populations generated via different resistance mechanisms.

Previous work by *Huang et al.* demonstrated that the specific treatment protocol used could alter the phenotypic pattern of resistance due to distinct selection pressures and short-term evolutionary paths [114]. In that work, two distinct treatment protocols resulted in distinct phenotypes of PARPi resistance. The first approach treated PEO1 cells with escalating dosages of olaparib from 0.5 μM to 40 μM over 15 weeks. The second scheme began treatment at a much lower dosage of 5 nM, escalating to 20 μM over 24 weeks, before selecting BRCA2 mutant clones from the population. The IC₅₀ to olaparib of the respective populations was 372.8 and 141.7 μM , compared to 2.4 μM in the parental PEO1 population.

This protocol-dependent variation complicates comparisons between studies that have generated PARPi resistance through different mechanisms. In this study, resistance to different PARPi was

generated in the same FRET biosensor-expressing cell line by mimicking clinical treatment protocols, where patients receive PARPi treatment daily.

Traditionally, drug resistance *in vitro* has been generated by exposing cells to incrementally increasing drug dosages until a stable resistant population arose. However, this approach is not clinically relevant for PARPi, where patients receive a constant daily dosage that is not increased, even if the therapeutic response declines. Therefore, to model clinical conditions more accurately, cells were exposed here to a continuous daily dose of the drug at a fixed concentration. This method aimed to replicate the selection pressures experienced in patients. However, this more subtle approach, using a constant low dose rather than continuously escalating to a high dose, may result in slower generation or less aggressively resistant populations.

6.2 Aims and objectives:

The work presented in this chapter aimed to develop novel models of PARPi resistance in the FRET biosensor-expressing cell line. The specific objectives were to characterise the sensitivity of these cells using viability assays and subsequently perform FLIM-FRET assays to assess any changes in PARP trapping between resistant and sensitive populations. FLIM-FRET HCA experiments were used to evaluate response heterogeneity with single-cell resolution. Additionally, changes in PARP trapping efficiency were assessed in cells resistance to olaparib or rucaparib, and cross-resistance to cisplatin was also explored. As an extension to this work, *in vivo* models of PARPi resistance were to be generated and similar FLIM-FRET assays were planned to assess changes in PARP trapping in this more physiologically relevant model.

6.3 Results

6.3.1 Generating novel PARPi resistance models *in vitro*

OVCAR4 GFP-PARP1-mCherryFP cells were continuously exposed to a daily dosage of 20 μ M olaparib or rucaparib, a concentration close to the measured IC50 value for this cell line for a 72 hr one-off treatment. Fresh drug-containing medium was administered daily, as shown in **Figure 70**, to recapitulate the constant daily dosage given to patients in the clinic. Cells underwent this treatment protocol for a total of 9 weeks, throughout which time cell viability and morphology were regularly assessed. Following this treatment regime, viability assays were employed to characterise any differences in sensitivity to PARPi between the continuously treated cells and the parental untreated populations.

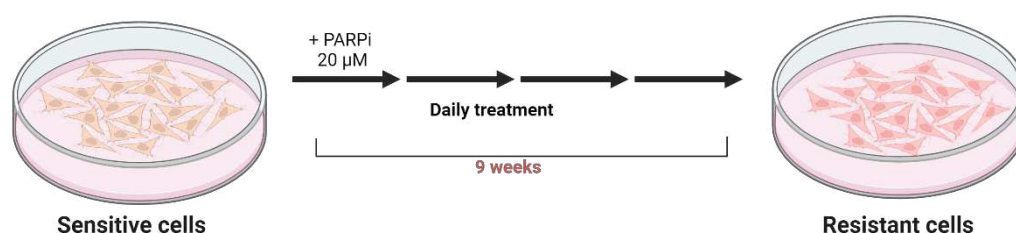


Figure 70: Treatment protocol for the generation of *in vitro* acquired resistance to PARPi. OVCAR4 EGFP-PARP1-mCherryFP cells were treated daily (5 days on, 2 days off) for 9 weeks at a constant 20 μ M concentration of either rucaparib or olaparib. Cells were passaged when they reached ~90% confluency and split 1:5. Figure created with BioRender.

6.3.2 Characterising novel models of *in vitro* PARPi resistance

A previous effort to model PARPi resistance *in vitro* utilised long-term exposure to incrementally increasing concentrations of olaparib. Treatment over 11 months resulted in a 9.8-fold increase in resistance relative to the parental UWB1.289 cell line [115]. However, this cell line harbours a germline *BRCA1* mutation, unlike OVCAR4, which is *BRCA1* wild-type. The time frame used in the present study was significantly shorter and this, together with the constant dosing protocol that more closely mimics the clinical treatment of patients with PARPi, leads to an expectation of a lower magnitude of resistance. In our study the majority of the population of cells died upon starting the PARPi treatment. This was not unexpected, as the dosage at around the IC₅₀ for a 72 hr one-off treatment is relatively high for continuous dosing. However, a small proportion of cells survived after ~1 week and over time these cells persisted in spite of the constant dosing and began to divide in the presence of drug containing medium. These cells may represent an intrinsically less responsive population, or alternatively the treatment may have induced acute changes that allow some cells to persist. The selection and expansion observed here somewhat mimic the selection process that occurs in patient tumours as chemotherapy resistance arises. Eventually, after 9-weeks of continuous treatment, the cells were growing and dividing readily and required passaging 2-3 time per week, similar to the untreated parental cell line from which they were derived. The morphology of these cells is shown in **Figure 71**, with the continuously treated cells showing a slight change in cell morphology compared to the untreated cells: the treated populations, particularly the olaparib treated cells, appear to have a more mesenchymal morphology, with an irregular, elongated, spindle-like shape compared to the untreated cells. This may suggest that continuous treatment induces an epithelial to mesenchymal transition (EMT) in these populations. A strong correlation between EMT and resistance to talazoparib has previously been demonstrated in *BRCA* wild-type tumours [116]. Furthermore, a separate study showed that PARPi resistance is characterised by EMT in rucaparib resistant PEO1 cells that also underwent similar changes in cell morphology as observed here [114]. These previous findings and

the observations reported here suggest that EMT may be a shared mechanism among resistance to various PARPi. However, this would require more complete gene expression profiling to confirm.

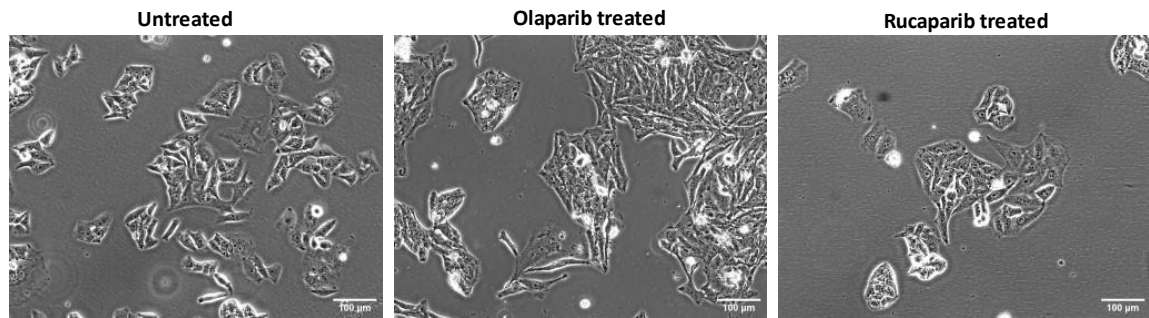


Figure 71: Cell morphology of cells continuously treated with PARPi. Phase contrast images are shown of the OVCAR4 GFP-PARP1-mCherryFP cells that have undergone no treatment (left), continuous treatment with olaparib (middle), or rucaparib (right) for 9 weeks. Images acquired with a 10X objective, scale bar = 100 µm.

The sensitivity of the continuously treated cells to the PARPi drugs olaparib and rucaparib, in comparison to the untreated parental OVCAR4 EGFP-PARP1-mCherryFP cell line was assessed using SRB assays (**Figure 72A, B**). In the rucaparib-treated cells, the IC₅₀ value increased by approximately 50%, from 22 µM to 36 µM. Similarly, in olaparib treated cells, the IC₅₀ value increased from 23 µM to 39 µM. This increase in IC₅₀ value suggests that the treatment protocol used here was sufficient to reduce the sensitivity to PARPi compared to the untreated populations, thereby generating increased resistance to both olaparib and rucaparib. Note that both of these increases in IC₅₀ were statistically significant and therefore, these EGFP-PARP1-mCherryFP expressing cells that had been continuously treated were referred to as “rucaparib resistant” or “olaparib resistant” for the remainder of this work.

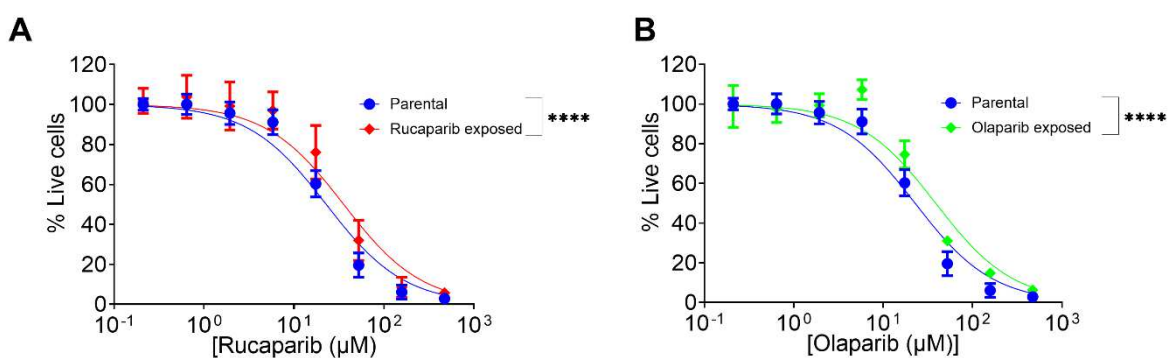


Figure 72: Continuous treatment with PARPi decreases sensitivity. SRB assays comparing viability in untreated parental OVCAR4 EGFP-PARP1-mCherryFP cells with continuously **A:** rucaparib treated or **B:** olaparib treated cells. Viability assays were performed after 9 weeks of continuous treatment.

It is possible that these decreases in sensitivity may be due to changes in the homeostatic response of these cells on re-exposure to drug, or possibly due to remodelling of the NAD metabolism state of

these cells, among many other possible reasons. Compared to the previously mentioned 11-month study that reported a 9.8-fold increase in IC50 values following escalating doses rucaparib [115], the magnitude of differences in sensitivity observed here with the more clinically relevant constant dosage regime is relatively small. However, recent studies have shown that differing treatment protocols can cause resistance to arise through different mechanisms due to the varying selective pressures, thereby providing further support for using a clinically relevant treatment protocol for modelling PARPi resistance.

6.3.3 Assessing cross-resistance to platinum therapy in PARPi resistant cells

Once decreased sensitivity to PARPi in the OVCAR4 EGFP-PARP1-mCherryFP cell line had been established and characterised, the subsequent work assessed whether this induced cross-resistance to platinum-based therapy by performing viability assays against cisplatin. Reduced sensitivity to cisplatin was observed in the cells that had decreased sensitivity to both rucaparib and olaparib, compared to the untreated parental cell line OVCAR4 GFP-PARP1-mCherryFP. This increase in IC50 to cisplatin suggests potential cross-resistance. This is consistent with a recently performed post-hoc analysis of the SOLO2 clinical trial dataset; the time to second progression following disease progression on maintenance olaparib therapy in BRCA mutant patients was assessed, showing a reduced efficacy of platinum-based therapy compared to patients who had not previously received PARPi [52].

Moreover, the percentage increase in IC50 to cisplatin was much greater than to rucaparib and olaparib respectively in the generated PARPi exposed lines, further supporting the hypothesis of cross-resistance (**Figure 73**). The rucaparib resistant cells had an IC50 to cisplatin of 17 μ M, olaparib resistant cells IC50 value was 13 μ M, compared to the untreated cells with an IC50 of 5 μ M. These data suggest that there may be shared mechanisms of resistance between these two drug classes, which has been reported in the literature [52,117]. Hence, it would be interesting to explore the mutual molecular mechanisms involved in response to these two distinct classes of therapy, as well as secondary effects that may be influencing the broader tolerance to cellular stress. However, whilst a correlation between resistance to PARPi and cisplatin was observed here, no causal relationship has yet been established.

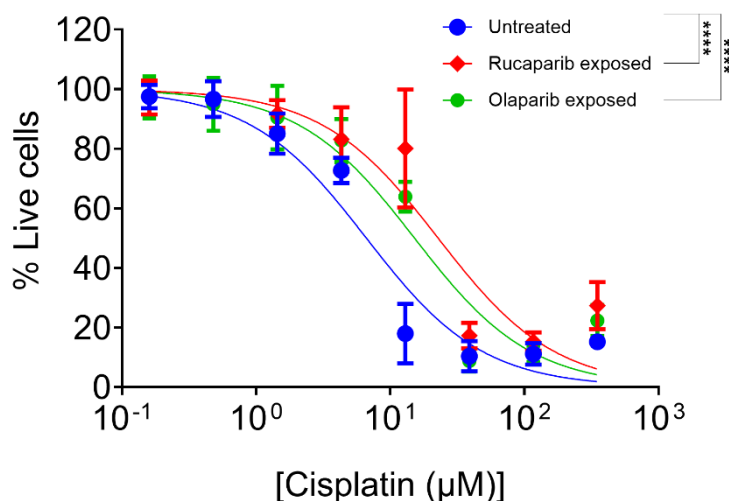


Figure 73: Generating PARPi resistance induces cross-resistance to cisplatin. Viability assay comparing untreated OVCAR4 GFP-PARP1-mCherryFP cells (blue) with their olaparib (green) and rucaparib (red) resistant counterparts treated with cisplatin for 72 hr. $n=2$ biological replicates, with 3 technical repeats at each concentration. Error bars show standard error across biological replicates.

Beyond the potential for cross-resistance to platinum therapy, another area for future research would be exploring cross-resistance between different PARPi. For instance, it would be valuable to assess whether resistance to rucaparib affects sensitivity to olaparib and vice versa. Such findings could indicate shared molecular mechanisms of resistance or a general increase in cellular stress resistance, as suggested by the observed increase in sensitivity to platinum treatment. While studies of this nature require careful distinction between association and causation, they offer valuable insights if the experimental design is robust. Although viability assays were not performed here to assess any effects of cross-resistance, the potential effects on PARP trapping in various PARPi are explored later in this work.

6.3.4 FLIM-FRET HCA assay of PARPi sensitive and resistance models

The PARPi resistant cell lines were subsequently assayed for any changes in PARP trapping, using the assay conditions established throughout chapter 5. Here, the sensitive parental OVCAR4 EGFP-PARP1-mCherryFP cells were compared to the cells that underwent continuous treatment with PARPi. For the FLIM assay, live cells were treated for 1 hr before commencing the FLIM acquisition. Cells were treated with a range of concentrations of rucaparib (0-50 μM). Fluorescence lifetimes were subsequently fitted to a double exponential decay model, from which representative images of weighted mean fluorescence lifetime are shown in (**Figure 74**). Despite the inter- and intra-cellular heterogeneity observed in fluorescence lifetime values, there is an observable trend towards lower lifetime as the drug concentration increases. This difference is less marked in the PARPi resistant populations. Single-

cell weighted mean lifetime values were extracted from all FOVs, and subsequently plotted in **Figure 75**, separated by treatment condition in the sensitive and resistant populations.

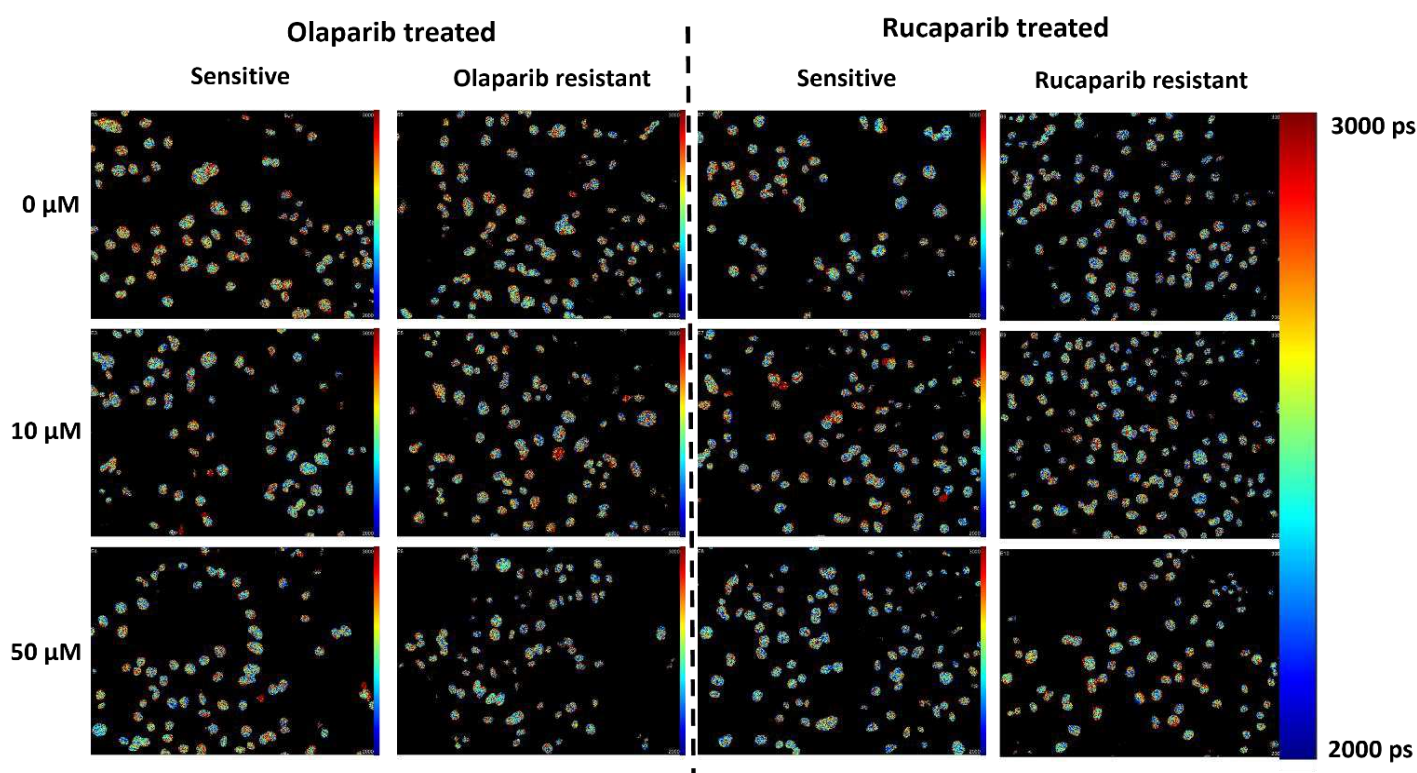


Figure 74: Fluorescence lifetime images comparing PARPi sensitive and resistant populations. Mean tau values from a double exponential fit on a per cell basis are shown, displaying representative images shown for each treatment condition. Olaparib resistant cells (left) were compared to the sensitive parental OVCAR4 EGFP-PARP1-mCherryFP cell line upon 1 hr treatment of 0-50 μ M olaparib. Rucaparib resistant cells (right) were compared to the sensitive parental OVCAR4 EGFP-PARP1-mCherryFP cell line upon 1 hr treatment of 0-50 μ M rucaparib. Images are pseudo-coloured by mean tau value per cell, with colour bar ranging from 2000-3000 ps.

Three biological replicates were performed for the FLIM-FRET HCA assay, and SuperPlots were produced to visualise heterogeneity between replicates [118]. This allowed the sampling of a large number of single cells in each population (an average of 2501 cells per condition were sampled across the three experiments). Single-cell mean fluorescence lifetime values from a double exponential fit (colour-coded by experiment) showed that in the sensitive population, a dose-dependent reduction in fluorescence lifetime was observed. Median lifetime values decreased from 2616 ps in untreated cells to 2580, 2578, and 2553 ps upon treatment with 10, 30, and 50 μ M rucaparib, respectively. Both 30 and 50 μ M treatments resulted in statistically significant reductions in lifetime. Conversely, in the resistant population, no statistically significant reduction in lifetime was observed with median lifetimes of 2594, 2586, 2609, and 2589 ps for 0, 10, 30, and 50 μ M treatments, respectively. This smaller decrease in fluorescence lifetime in resistant cells compared to sensitive cells suggests that

there is less PARP trapping occurring in the resistant cell population, and hence a smaller proportion of the PARP1 biosensor is in its high FRET efficiency state.

Overall, the distribution of single-cell values did not appear to cluster by experiment, providing a representation of the reproducibility of this assay across biological replicates. Moreover, the general trends in lifetime (i.e., a reduction in the sensitive population and not in the resistant population) are reproduced consistently between repeats. Therefore, although the observed changes in fluorescence lifetime absolute values are small, they are robustly reproducible and statistically significant over the population and support the hypothesis that PARP trapping may be reduced upon PARPi resistance.

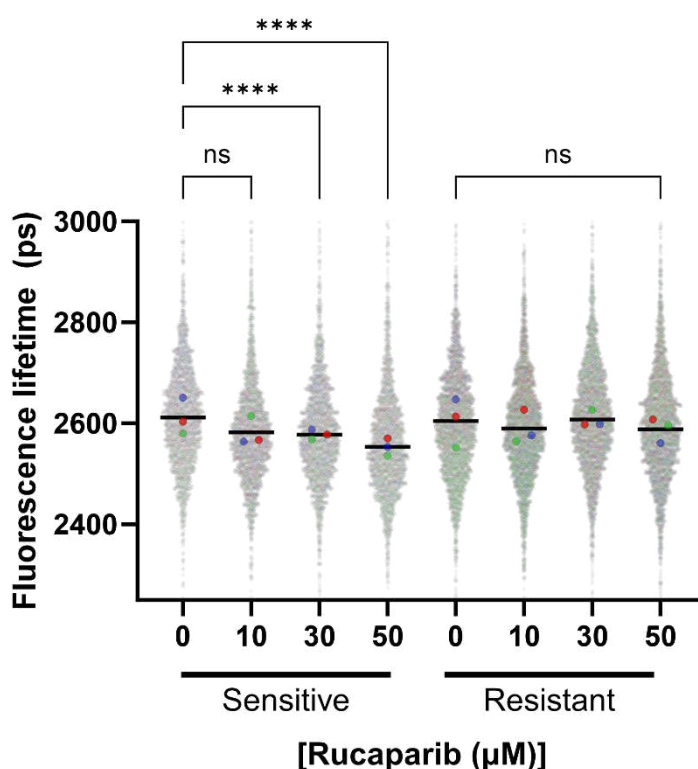


Figure 75: Rucaparib resistant cells undergo less PARP trapping than sensitive populations. Single-cell mean fluorescence lifetimes obtained from a FLIM-FRET experiment, assessing PARP trapping in PARPi-sensitive and -resistant cell populations. Cells were treated with doses of rucaparib ranging from 0 to 50 μM . The cell populations are categorised into rucaparib-sensitive (left) and rucaparib-resistant (right). The individual points correspond to single nuclei, with the colour coding indicating the specific experimental repeat each data point comes from (N1 =red, N2= blue, N3=green). The larger diameter points within each plot represent the mean fluorescence lifetime values for each experimental repeat under each condition and the error bars show the mean and SEM of these values. Statistical analysis was performed at the plate level by one-way ANOVA with Dunn's multiple comparison's test. ****, $p < 0.0001$, $n = 3$ biological repeats.

This series of HCA experiments also included the assessment of any changes in PARP trapping in the olaparib resistant population compared to the parental OVCAR4 EGFP-PARP1-mCherryFP cells. Again, the sensitive and resistant populations were treated with a range of concentrations of olaparib for

1 hr before FLIM acquisitions were performed. A similar general trend in the fluorescence lifetime values per condition was observed compared to the rucaparib resistant population (**Figure 76**). Namely, a statistically significant dose dependent reduction in fluorescence lifetime in the drug sensitive population was observed, compared to no significant reduction in lifetime in the olaparib resistant population upon drug treatment. Median lifetimes for the sensitive population were 2687, 2636 and 2625 ps for 0, 10 and 50 μM olaparib, respectively. For the resistant population median values were 2654, 2638 and 2635 ps for 0, 10 and 50 μM olaparib, respectively. The single-cell lifetime values demonstrated similar levels of heterogeneity and variability, with a near normal distribution around the mean value. Taken together, the results observed here are in agreement with those obtained with the rucaparib resistant populations, providing evidence towards supporting the hypothesis that PARP trapping may be reduced in resistant populations.

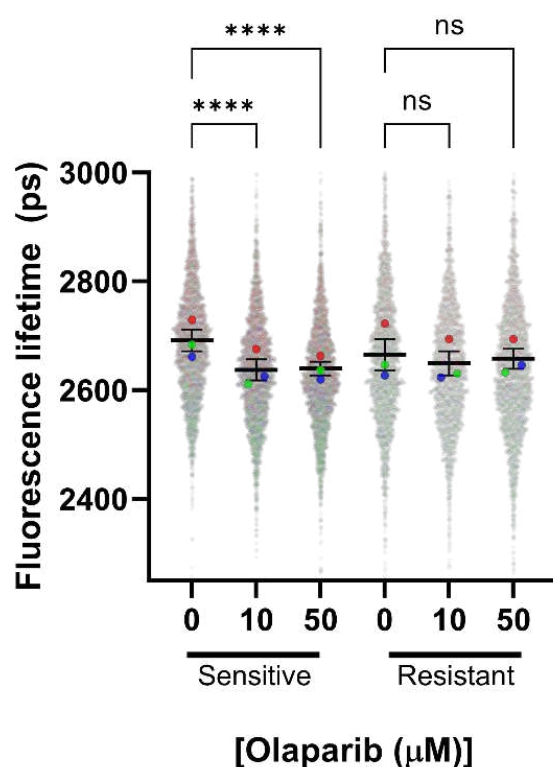


Figure 76: Olaparib resistant cells undergo less PARP trapping than sensitive populations. Single-cell mean fluorescence lifetimes obtained from a FLIM-FRET experiment, assessing PARP trapping in PARPi-sensitive and -resistant cell populations. Each violin plot represents a specific treatment condition, with doses of olaparib ranging from 0 to 50 μM . The cell populations are categorised into PARPi-sensitive (left set of plots) and PARPi-resistant (right set of plots). The individual points correspond to single nuclei, with the colour coding indicating the specific experimental repeat each data point comes from (N1 =red, N2=blue, N3=green). The darker spots within each plot represent the mean fluorescence lifetime values for each experimental repeat under each condition and the error bars show the mean and SEM of these values. Statistical analysis was performed at the plate level by one-way ANOVA with Dunn's multiple comparison's test. ****; $p < 0.0001$.

The SuperPlots above were used to visualise the distribution of the single-cell data values acquired from three biological replicates. This demonstrated the reproducibility of the observed effects. Therefore, the data could also be pooled per treatment condition and displayed as frequency distribution plots. This demonstrates a consistent trend in FLIM signal between the rucaparib and olaparib resistant cells, compared to the sensitive parental line (**Figure 77A,B**). Here, PARPi treatment induces a small reduction in fluorescence lifetime in the resistant populations in both cases. This infers that less FRET interaction is occurring, and, in the context of the biosensor, implies that PARP1 is less frequently in its compact, DNA bound state. This could be down to a reduction in PARP trapping, i.e., a reduction in the chromatin bound fraction of biosensor labelled PARP1 molecules. Alternatively, it could be due to a reduction in the allosteric modulation of PARP1 that usually occurs upon PARPi treatment. The biosensor does not currently offer any greater resolution to determine if this effect is exclusively down to one or the other of these two effects. However, in concordance with numerous studies in this space, it is most likely to be a combination of these two factors [55,56,58]. Additionally, there could be a lower intracellular concentration of drug in these cells, either via reduced uptake, or increased efflux. This in turn would also reduce the chromatin-bound fraction of PARP1, but through a distinct mechanism. Therefore, future studies should aim to correlate this FRET readout with quantifications of drug concentration at the single-cell level. Although fluorescently labelled paralogues of several PARPi exist, their uptake and efflux is unlikely to be identical to the unlabelled drug, thus making such experiments practically difficult. One solution to this is discussed further in chapter 7, focusing on the direct imaging the intrinsic fluorescence of rucaparib.

Although these data do not allow the direct measurement of a reduction in PARP trapping in resistant cells compared to sensitive cells, it presents a model in which have identified there are clear and reproducible differences between sensitive and resistant populations, in terms of the PARP trapping and allosteric regulation of PARP1. Moreover, this is achieved in live cells in an HCA fashion. To further untangle PARP trapping and allosteric regulation of PARP1, other assays are required to offer complementary information. For example, assessing PARP trapping using standard micro-irradiation assays may offer supporting or conflicting evidence to the approach used here.

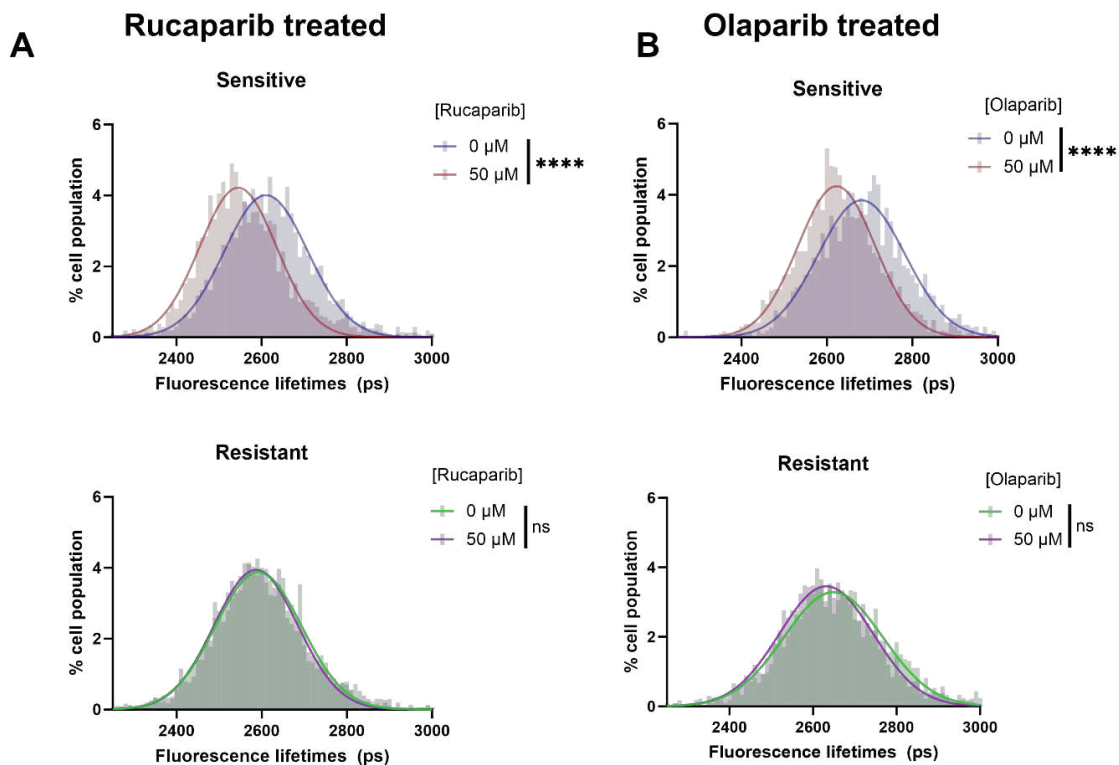


Figure 77: PARPi resistant cells show reduced PARP trapping. Frequency distribution of fluorescence lifetime values for rucaparib-sensitive and -resistant cell populations treated with rucaparib. Top panel (sensitive cells) shows the fluorescence lifetime distribution of untreated cells (blue) compared to cells treated with 50 μ M rucaparib (red). Bottom panel (resistant cells) shows the fluorescence lifetime distribution of untreated cells (green) compared to cells treated with **A:** 50 μ M rucaparib or **B:** 50 μ M olaparib (purple). A gaussian curve is fitted and overlaid on the frequency distribution plots. Statistical comparisons were performed using unpaired t-tests, ****, $p < 0.0001$, $n = 3$ biological repeats.

The frequency distribution plot of olaparib sensitive and resistant populations allowed a clear visualisation of the single-cell mean fluorescence lifetime distributions between conditions (**Figure 77B**). The degree of overlap or separation between the treated and untreated conditions in each cell population indicates the extent of change in PARP trapping efficiency, potentially shedding light on the differential response to olaparib in sensitive versus resistant cells.

Performing three biological repeats of these experiments allowed the assessment of the reproducibility of this signal, and an assessment of the stability of this effect. As the cells were passaged an additional time before each biological replicate was imaged, the FLIM-FRET HCA acquisitions spanned the space of three weeks. Thus, the signals produced seem reproducible over this time period, which may not be the case if loss of resistance or other adaptations occurred once the cells were routinely passaged in the absence of PARPi following PARPi resistance generation. Following this series of experiments, further characterisation of the resistant cell populations was performed, such as exploring the possible presence of cross-resistance to other clinically relevant therapies.

6.3.5 Assessing cross-resistance between PARP inhibitors

Cross-resistance between PARPi was also assessed, exposing olaparib resistant cells to rucaparib. Here, cells that had undergone continuous treatment with olaparib and had been characterised to have a reduced sensitivity to olaparib were exposed to rucaparib (another PARPi) before FLIM assays were performed. Assessing the PARP trapping response to a unique PARPi could imply that PARPi resistance, and any subsequent changes in PARP trapping, may be driven through shared molecular mechanisms. However, it was noted that these experiments were performed with only one combination of PARPi, and thus, any conclusions should not be generalised to all PARPi without further testing.

FLIM-FRET assays were performed, beginning acquisition 1 hr after the addition of 0, 10 and 30 μ M rucaparib. The acquired data were analysed in *FLIMfit*, fitting fluorescence lifetimes with global double exponential fitting. Single-cell mean weighted lifetime values were extracted and plotted per condition (**Figure 78**). The mean fluorescence lifetimes in the sensitive cells were 2690, 2676 and 2662 ps for the 0, 10 and 30 μ M treatments, respectively. This reduction in population mean values was statistically significant in both cases. Conversely, in the olaparib resistant populations, the fluorescence lifetimes upon rucaparib treatment were 2672, 2686 and 2668 ps, respectively. None of the changes in lifetime in the resistant population were statistically significant. Hence, this suggests that less FRET occurs in the resistant population, following a similar trend to when the olaparib resistant cells underwent olaparib treatment. Note however, statistical tests at the cell level are difficult to justify, as the assumption that each cell is independent of all other cells in the same well may not be true. Therefore, more biological repeats are required to verify these observations.

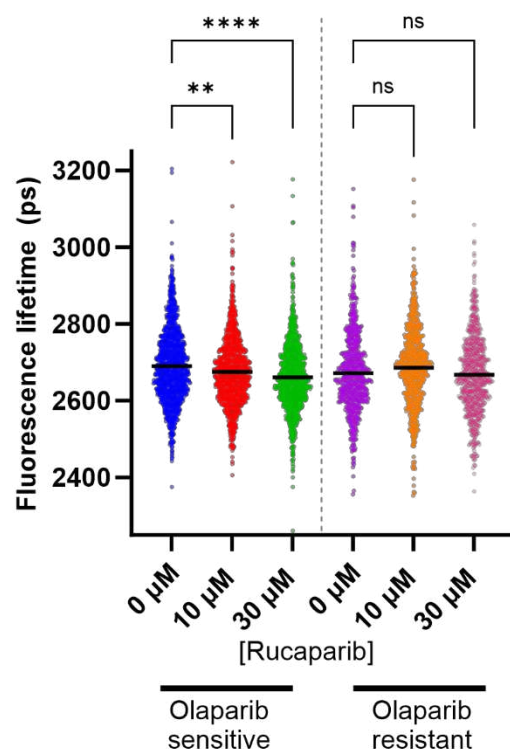


Figure 78: Assessing changes in PARP trapping in the context of cross-resistance. OVCAR4 EGFP-PARP1-mCherryFP cells (parental, sensitive) and continuously olaparib treated (resistant) cell populations were treated with a range of rucaparib for 1 hr and subsequently FLIM acquisitions were performed. Single-cell mean fluorescence lifetime values are plotted, per condition. Error bars show the mean and SEM values for each condition. Statistical analysis was performed by one-way ANOVA with Dunn's multiple comparison's test. **, $p < 0.01$. ****, $p < 0.0001$, $n = 1$ biological replicate.

To extend this further, any cross-resistance between other PARPi of varying PARP trapping ability should be explored, such as niraparib, veliparib and talazoparib. One could postulate that generating resistance to a PARPi with a stronger PARP trapping potency may present a larger selection pressure on overcoming this trapping, in order for cells to survive. However, this sort of correlation analysis is yet to be performed. With that in mind, one possible limitation of this study is that the resistance models generated were all to PARPi with moderate PARP trapping potency. Hence, as a direction of future study talazoparib resistant models should be generated to assess if acquired resistance to stronger trapping PARPi results in a larger decrease of PARP trapping in resistant cells. Similarly, this could be extended to a plethora of PARPi, including those that are yet to have their PARP trapping potency characterised. This may offer a route towards screening potential clinically potent PARP inhibitors that are in development, based on their *in vitro* trapping efficiency.

6.3.6 Generating resistance to PARPi *in vivo*

As previously mentioned, the method of resistance generation to PARPi may drive distinct mechanisms of resistance. In light of this, it is valuable to generate further models than the *in vitro* models developed here. Therefore, at the time of writing, work is ongoing to generate *in vivo* resistance in these same cell lines (**Figure 79**). OVCAR4 GFP-PARP1-mCherryFP cells have been injected into CD1 nude mice that have received daily treatment with olaparib (25 mg/kg), or weekly treatment with cisplatin (5 mg/kg). Tumour cells will be harvested from the ascites when the first mouse in each group reaches endpoint. The cisplatin treated arm of the study will allow preliminary studies investigating cross resistance between platinum therapy and PARPi, and any possible changes in PARP trapping. This will allow preliminary efforts to address a clinically relevant question, as generally all patients receive platinum-based therapy before PARPi. Hence, if resistance to platinum therapy is modulating PARP trapping affinity, this will be crucially relevant in the clinical landscape.

A further group of 3 mice will receive PBS treatment as a vehicle control. This is vitally important in such experiments, as prior experiments with OVCAR4 WT in the same mouse model have shown an estimated 90% of cells die following injection, leading to some clonal selection. In the context of this study, and the generation of acquired resistance, this is important to consider. Hence, cells will be harvested from each treatment group before characterising their drug sensitivity downstream, as well as the readout of the FLIM assays using the FRET biosensor they express. Generating these novel models will allow HCA FLIM experiments similar to those presented here, with resistance generated in a distinct model. If similar trends in PARP trapping are observed, i.e., a reduction in PARPi resistant populations, it will offer supporting evidence that could be indicative of clinical mechanisms of resistance.

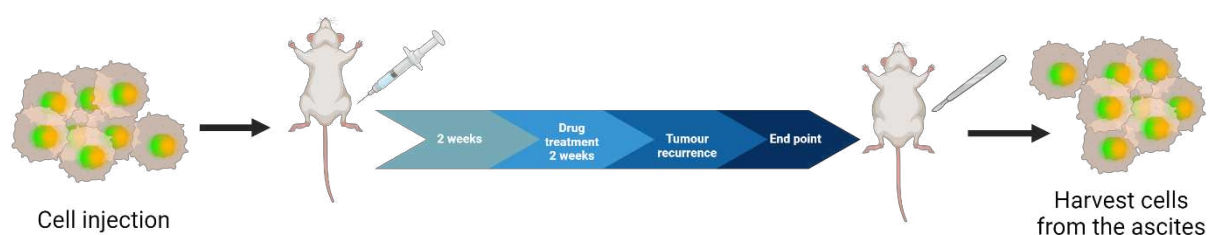


Figure 79: Schematic of *in vivo* resistance generation protocol. Intraperitoneal injection of 5×10^6 OVCAR4 EGFP-PARP1-mCherryFP cells was performed, 2 weeks later drug treatment began. The treatment groups were split into three; PBS, olaparib, or carboplatin treated. Olaparib was given daily at 25 mg/kg and cisplatin was given once a week at 5 mg/kg. Mice were maintained until the first mouse in each group reached endpoint. Tumours and ascites were subsequently harvested, isolating tumour cells via filtration. Figure created with BioRender.

6.4 Discussion:

The experiments conducted in this chapter utilised the FLIM-FRET HCA assay conditions, established in previous chapters, demonstrating the ability to investigate PARP trapping in novel models of PARPi resistance in HGSC. This approach successfully identified differences in PARP trapping between sensitive and resistant cell populations, revealing a reduction in PARP trapping in resistant cells. This finding offers evidence supporting the hypothesis that a reduction of PARP trapping is present in PARPi resistant populations and may be a mechanism driving resistance to PARPi.

Additionally, a correlation towards increased resistance to platinum upon PARPi resistance was demonstrated. Likewise, potential correlation between resistance to rucaparib and a reduction in PARP trapping upon olaparib exposure was also observed. These two examples demonstrate the ability of a HCA platform to assess multi-parameter effects of a cellular phenotype quickly and robustly. Thus, there are vast possibilities for future applications of this approach, whether that be exploring PARP trapping upon resistance to other PARPi or establishing a screening-like approach based on this assay.

Unlike this HCA approach, the current tools to study PARP trapping do not offer such high-throughput, or single-cell resolution, typically acquiring information from bulk population biochemical assays on heterogeneous populations. Thus, the ability of the approach demonstrated here to obtain single-cell information at scale offers a unique tool which complements and extends prior studies. Furthermore, the reproducibility of this approach was demonstrated by performing multiple biological replicates of HCA experiments, showing consistent trends upon drug treatment.

While these FLIM-FRET assays produced reproducible changes in FLIM signals, it is crucial to cross-validate these results with existing methodologies to strengthen the evidence. For instance, ChIP assays comparing sensitive and resistant populations, both treated and untreated, could provide a semi-quantitative assessment of the distribution of the chromatin bound PARP1 pool. Additionally, correlating these findings with traditional microscopy approaches such as micro-irradiation FRAP could offer further insights, albeit in the context of specific DNA lesion types, with limited throughput. This underscores the importance of HCA approaches, which allow sufficient sampling power to achieve statistical robustness to detect small changes in fluorescence lifetime that might be missed in low-throughput methodologies.

Building on these findings, it is pertinent to consider the clinical implications. Clinical studies have found that resistance to platinum-based chemotherapy is a strong predictor of resistance to PARPi treatment [119]. Therefore, it is possible that the mechanisms driving resistance to platinum and

PARPi therapies are similar. Hence, a logical next step would be to generate acquired resistance to platinum-based therapy and assess any potential changes in PARP trapping in these cells, even without extended exposure to PARPi. This would mimic the clinical treatment route of most patients, receiving platinum therapy before PARPi. At the time of writing, the generation of *in vivo* cisplatin-resistant cells from the OVCAR4 GFP-PARP1-mCherryFP cell line is underway. These cisplatin-resistant cells will be employed in subsequent FLIM-FRET assays to evaluate any alterations in PARP trapping associated with the acquisition of platinum resistance, independent of PARPi treatment. This research is expected to provide valuable insights into any changes in PARP trapping resulting from platinum-based chemotherapy, a topic that has not been extensively studied.

It is important to note in this study PARPi resistance was generated through a single mechanism, involving daily dosing at a constant dose. This was done to mimic the PARPi treatment regime used in clinical settings. However, studies have shown that the method chosen to generate PARPi resistance can drive diverse and heterogeneous resistance mechanisms [114]. This highlights a limitation in the current study, suggesting the need for exploring various resistance-inducing protocols in future research.

The models of acquired resistance developed in this study exhibited relatively modest changes in sensitivity compared to untreated cells, potentially limiting the observed differences in the FLIM assays between sensitive and resistant populations. To address this, future research should focus on generating a spectrum of resistance models with varying sensitivities to PARPi to assess whether changes in PARP trapping evolve over time as resistance progresses or occur at specific points during resistance development. Extending this study to other PARPi with varying trapping potencies would facilitate determining if a reduced PARP trapping is a common feature of resistance across different inhibitors. Additionally, the clinically documented PARP1 mutation R519C, known to reduce PARP trapping, could be induced into this FRET biosensor through site directed mutagenesis or CRISPR-Cas9. This would aid the understanding of molecular mechanisms modulating PARP trapping, potentially leading to better patient outcomes. Also, this study exclusively investigated models of acquired resistance due to practical considerations, as these models provided matched sensitive lines as positive controls. However, in the clinical context of HGSC, around 20% of *BRCA1/2* deficient patients exhibit *de novo* resistance to PARPi, failing to respond to therapy from the outset [120]. Therefore, it would be informative to assess the basal PARP trapping status in such patients.

In summary, FLIM-FRET HCA of OVCAR4 cell lines expressing the GFP-PARP1-mCherryFP FRET biosensor have been shown to be able to identify and quantify reductions in PARP trapping in PARPi resistant populations. Whether the observed reduction in PARP trapping is a causative factor of PARPi

resistance, or merely associated with it remains a crucial question. This study lays the groundwork for further exploration into the molecular dynamics of PARP trapping, potentially informing improvements to PARPi therapy, leading to better patient outcomes.

7 Ongoing work to expand HCA assay to multiparametric readouts

This chapter outlines ongoing work that aims to expand FLIM-FRET HCA assay conditions, implementing novel imaging modalities, and acquire higher-dimensional datasets for complementary cell biology readouts. Such information can be subsequently used to correlate drug responses to other conditions, such as DNA damage response and PARP trapping. The chapter is divided into three sections, presenting preliminary data for each. The first section addresses the assessment of cell cycle stage information by developing a novel workflow for label-free classification, utilising quantitative phase imaging and machine learning. The second section follows this theme of label-free imaging, providing a proof-of-concept for fluorescence lifetime imaging of the intrinsic fluorescence of the PARP inhibitor rucaparib, indicating drug uptake, localization, and drug-target binding. Finally, the third section addresses the development of 3D models of HGSC and cell culture methodologies that are amenable for automated imaging in HCA assays, decreasing manual handling whilst maximising reproducibility. These efforts converge to provide additional biological context and understanding of changes in PARP-DNA binding and trapping observed in previous chapters.

7.1 Label-free cycle stage classification using quantitative phase imaging and machine learning

7.1.1 Introduction

The clinical activity of PARPi relies on the induction of changes to the adaptive immune response and is also heavily upon the failure to repair complex DNA damage using homologous recombination. This process occurs exclusively in late S/G2 phase of the cell cycle when a sister chromatid is available as a repair template. Prior studies have suggested that PARPi progressively induce DNA DSBs over multiple cell cycles in *BRCA1/2*-deficient cells that fail to slow cell cycle progression to repair damage [36]. This possibly explains why mitotic progression promotes PARPi-induced cell death in these cells, supporting combination therapies with agents such as Wee1 inhibitors [37,38]. Given the cell cycle dependency of PARPi action, it is imperative to understand how basal cell cycle state and progression are modulated following treatment, as this impacts the response to PARPi. This is particularly important in HGSC, where the universal loss of p53 function profoundly affects cell cycle control [39].

Therefore, incorporating cell cycle stage information into the HCA assay developed in this study was deemed beneficial, providing contextual information on a single-cell basis to enable the correlation of responses to PARPi therapy and PARP trapping with cell cycle stage. Traditional cell cycle stage classification relies on fluorescence microscopy, utilising biosensors such as FUCCI for dynamic

assessment [121]. An alternative is the endogenously labelled proliferating cell nuclear antigen (PCNA) “all-in-one” reporter, as proposed by *Zerjatk et. al* [122]. This genetically encoded fluorescent reporter monitors cell cycle progression in live cells based on changes in localisation and intensity of PCNA, a protein critical for DNA replication and repair. This marker has the advantage of occupying only a single fluorescent channel, unlike the FUCCI marker, which requires two. Moreover, CRISPR-Cas9 technology has made the endogenous labelling of genes such as PCNA with a fluorescent marker accessible, offering a pathway to form stably expressing cell lines compatible with live cell time-lapse microscopy. However, phototoxicity and photobleaching limit the efficacy of fluorescence-based approaches during extended live cell time-lapse experiments.

To overcome these limitations, a novel label-free approach for cell cycle stage classification was developed using a new method of quantitative phase imaging (QPI), termed polarization differential phase contrast (*pDPC*) microscopy [123]. QPI has gained significant interest as it allows label-free imaging of cellular dynamics, facilitating segmentation and tracking. While numerous methods exist for QPI, many require specialised optical components, such as objective lenses with phase rings, Nomarski prisms, and wavefront sensors, which limit their applicability to standard fluorescence microscopes found in biology laboratories.

Here, a novel low-cost and open-source approach was implemented to achieve *pDPC* by modifying only the illumination path, eliminating the requirement for a specialised objective lens [123]. The optical setup orientation (**Figure 80A**) allowed for the sequential acquisition of both phase and fluorescence images of the same FOV. *pDPC* involves acquiring transmitted light images at different illumination angles to reconstruct semi-quantitative phase images. A semi-circular mask was placed in the back focal plane of the condenser lens, and transmitted light images were captured using four illumination directions onto a polarization-resolved camera sensor (**Figure 80B**). This method is applicable to almost any microscope system.

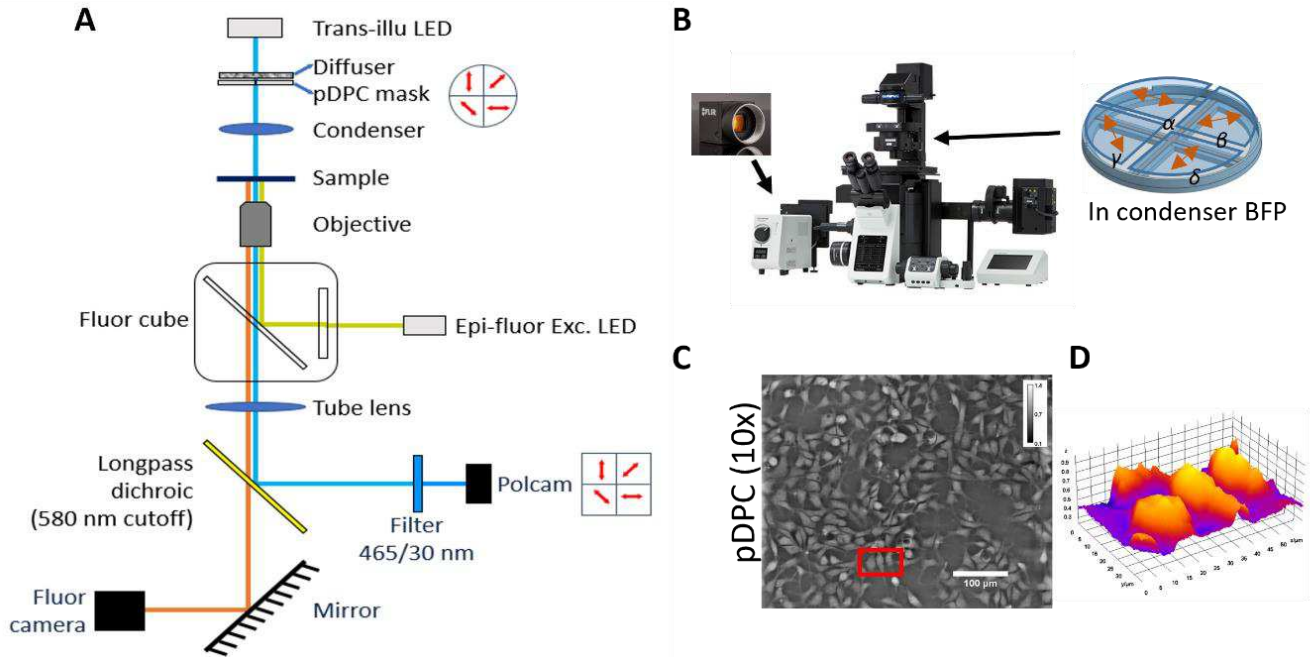


Figure 80: Implementation of pDPC in an existing fluorescence microscope. **A:** System diagram for the simultaneous acquisition of fluorescence and QPI images of the same FOV. **B:** pDPC implemented in existing fluorescence (Olympus IX-83) microscope using a condenser in the back focal plane (BFP) and a polarisation resolved camera (PolCam). **C:** Representative pDPC image of HEK293 cells imaged with a 10X objective. Scale bar = 100 μm . **D:** Surface plot of the red ROI in image, shown in C.

The primary benefit of utilizing QPI in this context is that it is minimally phototoxic and does not take up any fluorescent channels, unlike traditional fluorescence approaches. This allows the imaging of cells that can be labelled with almost any fluorescent probe (or not) over multiple days without detrimental effects on cell viability. This approach achieves high-contrast imaging of cell morphology, resulting in more efficient cell segmentation (**Figure 80C-D**). Additionally, QPI enables the measurement of the dry mass of cellular contents at a single-cell level, as the refractive index (and therefore the optical path length through the cell) is linearly proportional to the cell's density. This provides label-free biophysical information thereby complementing the multi-parametric readouts achieved in HCA FLIM-FRET assays. This study aimed to combine QPI technology with the evolving field of deep learning and convolutional neural networks (CNNs) for the extraction and classification of cell cycle stages in a label-free manner.

Over the last decade, the exponential advancement of machine learning and its application in bioimaging has become widespread. Advances in high-throughput microscopy have provided rapidly growing dataset sizes, offering a wealth of data for training and validating machine learning models. CNNs have been applied to numerous problems in fluorescence imaging, such as classification, object detection, segmentation, and feature extraction. CNNs are particularly well suited for image analysis

tasks due to their ability to automatically learn hierarchical features from raw image data. Additionally, CNNs have been used for cell cycle tracking of single cells from fluorescence images and to generate self-supervised methods of extracting cell cycle stage information without manual input [124,125]. However, these approaches share the inherent limitations of fluorescence imaging, including photobleaching and phototoxicity.

In this study, a novel proof-of-concept approach was tested, aiming to achieve label-free cell cycle stage readout without using fluorescence cell cycle reporters. Timelapse fluorescence microscopy data of PCNA-mScarletFP expressing cells were acquired, concurrently performing QPI imaging of the same FOV at each time point. The fluorescence data served as a ground truth for cell cycle stage, facilitating the training of a ResNet-50 architecture to identify cell-cycle specific morphological features solely from QPI images. This required co-registration of the two image sets, coupled with segmentation and tracking of individual cells over time. A custom analysis pipeline was developed for data annotation and subsequent training and validation of ML models. Importantly, the relatively simple implementation of *pDPC* used here can enable QPI on almost any fluorescence microscope, widely enabling studies of single-cell dynamics throughout the cell cycle, such as probing heterogeneity in image-based high-throughput drug screening. We note a similar approach was recently reported using a commercial QPI microscope with cells expressing the FUCCI cell cycle reporter [126]. However, due to the nature of the FUCCI marker, this approach cannot discriminate between all four stages of the cell cycle. Furthermore, that approach is only applicable on QPI specific microscopes whereas the approach presented here is applicable on any microscope.

7.1.2 Results:

7.1.2.1 *Live cell timelapse imaging*

The PCNA-mScarletFP biosensor provides distinct spatial and intensity distributions of PCNA at various cell cycle stages (**Figure 81A**). During the G1 phase, PCNA-mScarletFP fluorescence is expressed at low intensity levels and is diffusely distributed within the nucleus. As cells transition from G1 to S phase, the fluorescence intensity increases, leading to the formation of high-intensity foci within the nucleus, indicative of active DNA replication. The transition from S to G2 phase is marked by the dissolution of these foci, resulting in a uniform, elevated fluorescence distribution compared to G1. Finally, the transition from G2 to M phase is characterised by the redistribution of fluorescence from the nucleus to a homogenous expression throughout the entire cell. These distinct fluorescence patterns serve as the ground truth dataset for training a CNN to extract cell cycle stage information from QPI images.

The implementation of QPI with *pDPC* enabled the acquisition of phase images of CRISPR-Cas9 labelled A375 cells expressing PCNA-mScarletFP in a 96-well plate. Phase and fluorescence images of the same

FOV were acquired sequentially, which were later co-registered before image analysis. Timelapse images of 48 FOVs were acquired over 48 hr at 5 min intervals, capturing both phase and fluorescence channels. For creating a ground truth training dataset, the acquired QPI and fluorescence images were first co-registered. Segmentation was performed on both the fluorescence and QPI channels using CellPose, and cell tracking was done with TrackMate on the fluorescence images only (**Figure 82A**) [127,128]. The QPI and fluorescence images were subsequently coregistered before forming the training dataset. Representative images from each cell cycle stage are presented in **Figure 81B**, displaying both QPI and fluorescence images. While fluorescence images clearly depicted differences in fluorescence intensity and localization, QPI images exhibited little observable difference between cell cycle stages.

7.1.2.2 *Data annotation to form a training dataset*

In order to utilise the acquired data in a machine learning pipeline, an annotated training dataset had to first be established. Automated batch analysis was applied to the PCNA fluorescence images, analysing metrics defined in the work of *Zerjatke et al.* [102]. The segmentation masks were overlaid onto the fluorescence data and custom Python scripts were developed in-house to assess changes in mean fluorescence intensity and the distribution of intensity values across the timecourse of each segmented cell. A representative example of this analysis is shown in **Figure 81B**, displaying the mean pixel intensity and the 1-99th percentile range in intensity values per time point over 40 hr. This allowed for the automated annotation of each transition in the cell cycle (**Figure 81C**), thereby producing an annotated ground truth and training dataset that could subsequently be used as the input to the ResNet-50 CNN architecture.

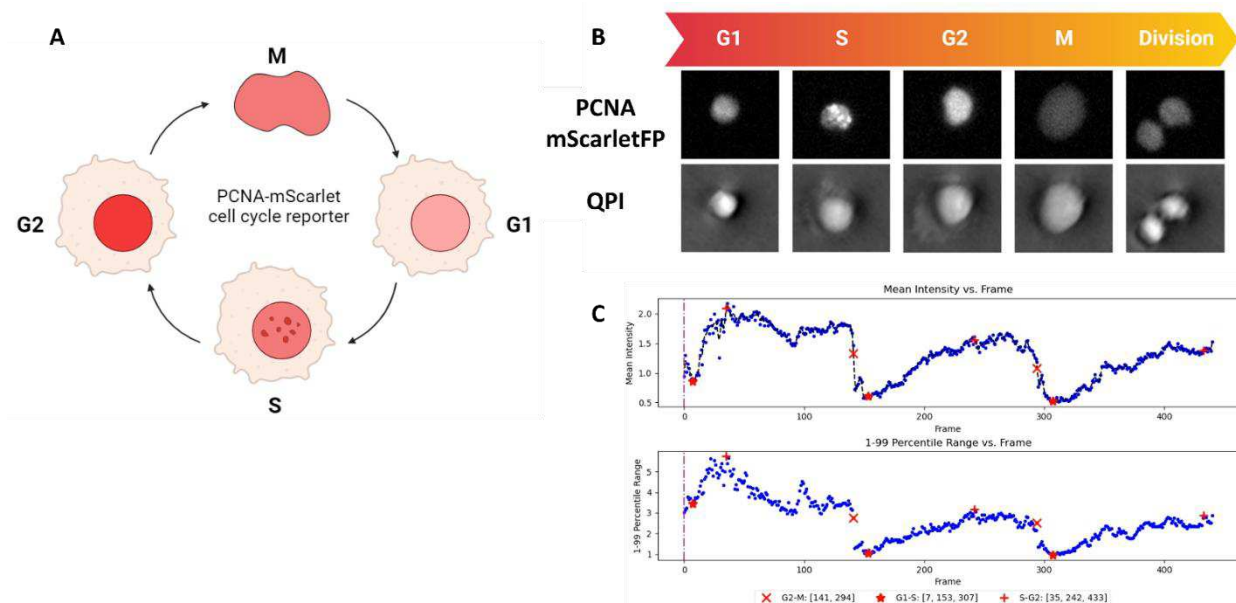


Figure 81: All-in-one cell cycle reporter provides ground truth dataset. **A:** graphical schematic of the all-in-one PCNA cell cycle reporter. **B:** Representative images of both the QPI and fluorescence images at each stage of the cell cycle. Note, this is the same cell from a single FOV, co-registered and segmented based on the phase image. **C:** Mean fluorescence intensity (AU, top panel) of the segmented image over time (480 frames, 5 min per frame). The 1-99th percentile range of fluorescence intensity (AU, bottom panel) over the same period. Graphs are annotated with estimated cell cycle stage transition points (X= G2-M, *=G1-S, +=S-G2).

7.1.2.3 Data analysis pipeline:

The full data analysis pipeline is outlined in **Figure 82A**, demonstrating how the two imaging channels (QPI and mScarletFP) are initially analysed before converging as the final training and ground truth datasets. Before passing the ground truth data through CNN analysis, it was first augmented by transformations such as image rotations and adding artificial noise, to enhance the model's generalisation capability (**Figure 82B**).

7.1.2.4 Training of deep learning architecture:

The initial accuracy results were poor when training on the raw QPI images. To address this the QPI images were cleaned, augmented, and resized to 64x64 pixels as the input and the PCNA-mScarletFP annotated cell-cycle stage was provided as ground truth. As this was mainly a feature extraction and image classification task, a deep neural network was trained to infer each cell's category as either G1, S, G2, or M. The ResNet-50 architecture was subsequently fine-tuned on QPI images (**Figure 83**).

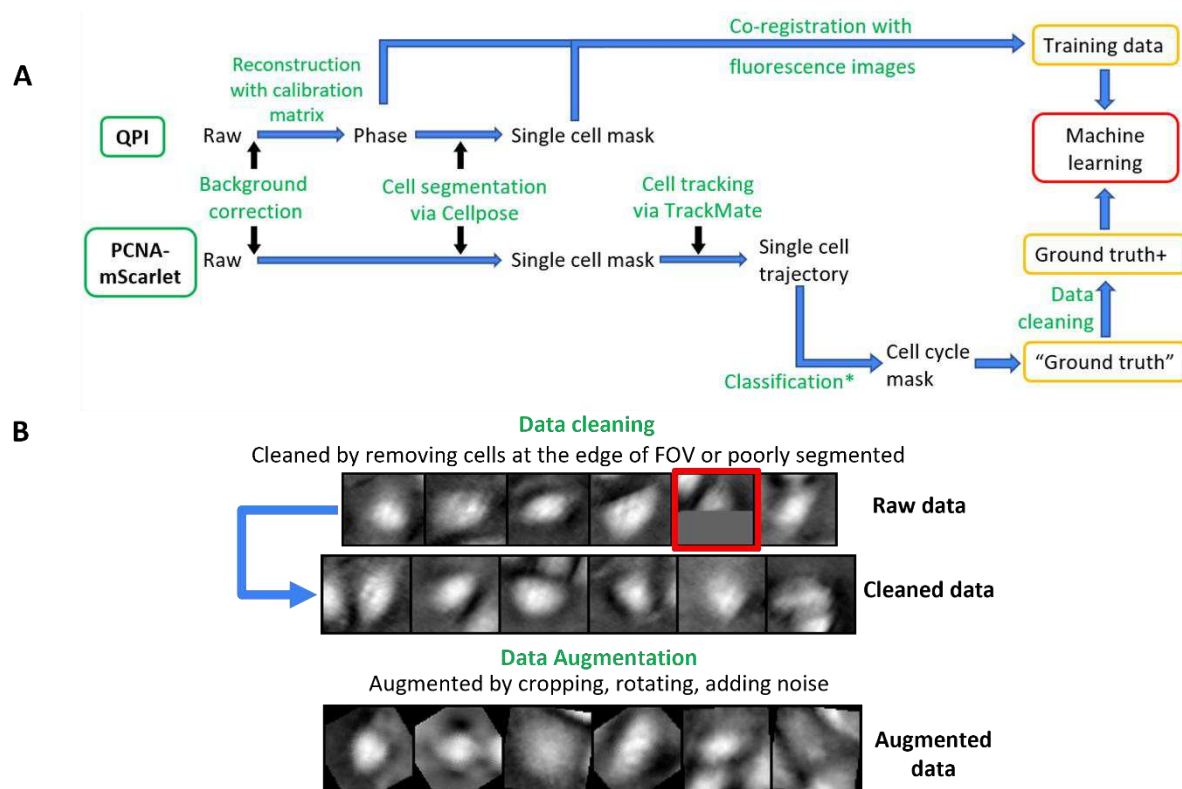


Figure 82: Workflow for label-free cell cycle classification. **A:** Data analysis pipeline is outlined, processing the acquired QPI and fluorescence images separately before co-registration and using the phase images in the training dataset, and the fluorescence images as a ground truth dataset. **B:** Representative images from the raw data and cleaned dataset produced by the cleaning process, removing cells that are at the edge of the FOV. Augmentation involves cropping, rotating, and adding noise to images to make a more varied training dataset.

7.1.2.5 ResNet-50 structure

The ResNet-50 architecture is a deep learning architecture widely used for image classification and other computer vision tasks. ResNet CNNs address the vanishing gradient problem commonly encountered in deep neural networks (**Figure 83**). This occurs when the gradients of the loss function with respect to the network parameters become extremely small as they are propagated back through the layers during training. This makes it difficult for the optimization algorithm (typically gradient descent) to update the weights of the early layers effectively, leading to very slow convergence or even halting the learning process altogether.

The fundamental compartment of the ResNet is the residual block, which allows the network to learn residual functions with reference to the layer inputs. Instead of directly learning the desired output, the network learns the difference (residual) between the input and desired output. The ResNet-50 is a deep architecture, consisting of 50 layers, including convolutional and batch normalisation layers which increase training speed, rectified linear unit (ReLU) activation functions, and fully connected layers. This deep architecture is more efficient in training due to the residual connections, which make it easier to train the deep network and tend to lead to improved accuracy. Additionally, the ResNet

can be scaled and is flexible. However, due to its large number of parameters, ResNet-50 can be susceptible to overfitting, especially if the training dataset is not sufficiently large or diverse.

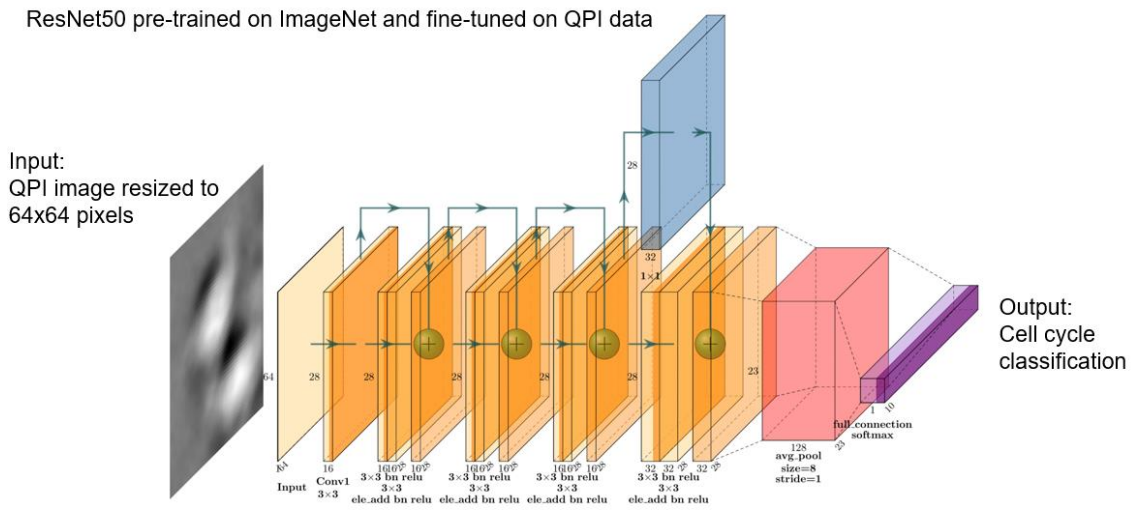


Figure 83: Schematic of the ResNet-50 architecture used for cell cycle classification. QPI images are resized to 64 x 64 pixels as the input, passing convolutions (conv), batch normalization (bn) and rectified linear unit (ReLU) layers. The output is a cell cycle classification into a data table, per cell, per time point.

The ResNet-50 was pre-trained on the large ImageNet dataset and benefited from advanced initial weights, enabling more efficient and accurate performance through transfer learning by leveraging prior knowledge of feature extraction. Performance metrics were quantified, with average scores across the four cell cycle stages of 86% for recall, 92% accuracy, 88.5% F1 score, and 91.5% precision (**Figure 84D-G**). The training dataset contained two million parameters, yet it was determined that manual annotation, combined with data cleaning and augmentation, allowed datasets in the tens of thousands to achieve prediction accuracy of approximately 90% (**Figure 84H**). Most imaging systems capable of performing single-cell resolved timelapse acquisitions can quickly accumulate enough training data for this approach. An example output FOV is displayed as a segmentation mask with each cell marked as an ROI with differing colours (**Figure 84I**) and the respective cell cycle classification mask (**Figure 84J**) produced by the label-free ML pipeline. As a next step, this analysis could be coupled with other fluorescence microscopy readouts, to contextualise signalling in terms of cell cycle state.

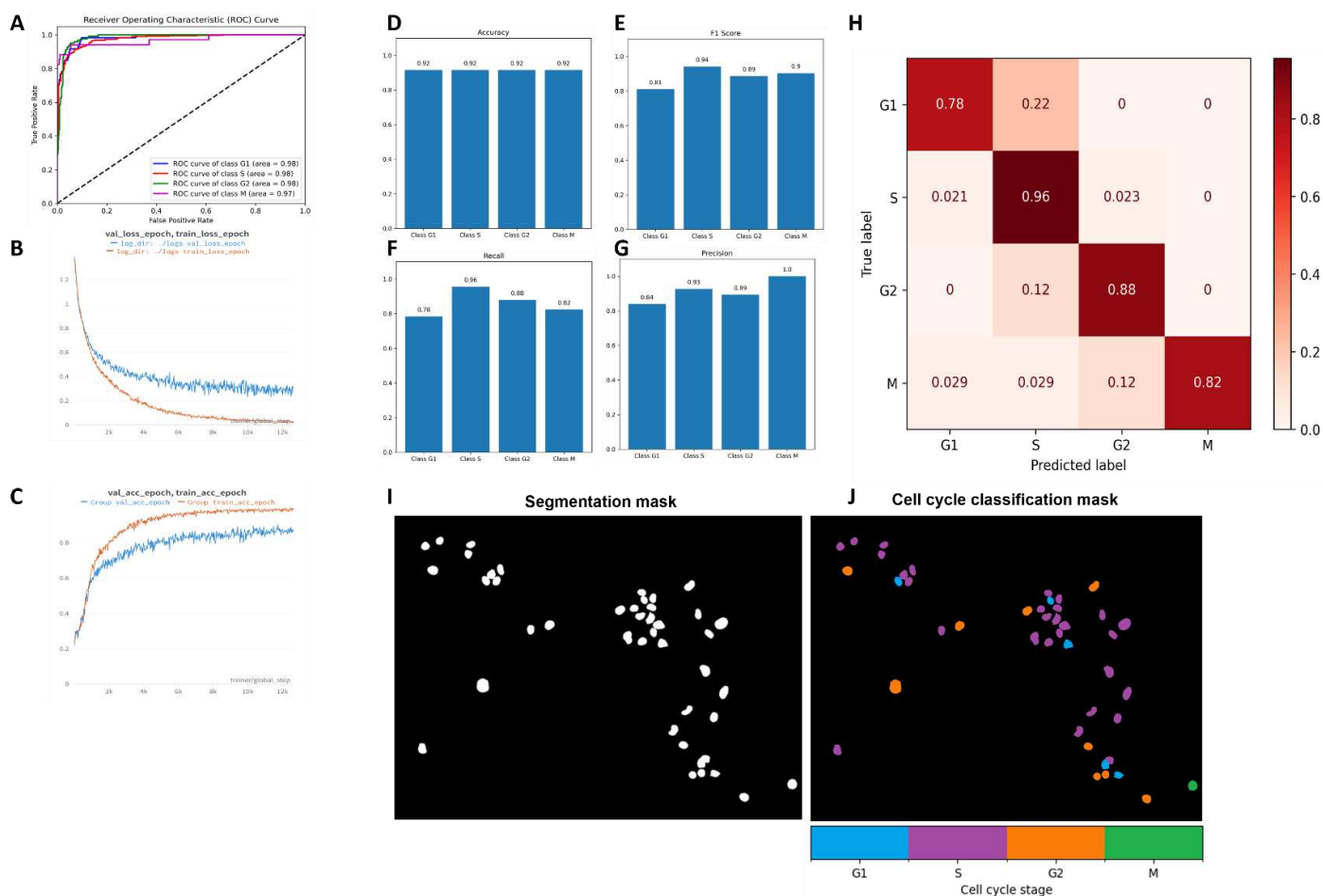


Figure 84: Results of machine learning for cell cycle classification. A: Receiver operating characteristic (ROC) curve for each stage of the cell cycle, showing low (~2%) false positive prediction rate. **B:** Loss function over ~12k epochs suggests the model is not overtrained to the training data. **C:** Model accuracy over the same period. **D-G:** Recall, accuracy, F1 score and precision scores for each stage of the cell cycle. **H:** Machine learning results, showing prediction vs ground truth per cell cycle stage, average prediction accuracy of ~90%. **I:** Segmentation mask from pDPC. **J:** Corresponding cell cycle classification mask, example of ground truth training data.

7.1.2.6 Extending to HGSC cell lines

To extend this approach to the FLIM-FRET HCA assays, OVCAR4 cells, the parental cell line used to express the FRET biosensor, were labelled with PCNA-mScarletFP. The same machine learning training would allow this label-free method to be integrated into FLIM-FRET HCA assays, categorizing single-cell responses to PARPi by cell cycle state and potentially explaining the heterogeneity observed at the population level. This would enable the acquisition of information-rich datasets, facilitating the assessment of novel biological questions.

OVCAR4 cells were transfected with 1 μ g of CRISPR-Cas9 targeting vector and Cas9-gRNA plasmids and assessed for mScarletFP expression ~48 hr later via Incucyte imaging (**Figure 85**). Nuclear PCNA expression, indicating successful CRISPR knock-in, was observed in <1% of the transfected cells. These cells were isolated via FACS for future pDPC experiments to generate a training dataset for inferring

cell cycle stages. Training could not be performed on dual-labelled OVCAR4 EGFP-PARP1-mCherryFP cells due to spectral overlap between mCherryFP and mScarletFP. Future work includes adapting the PCNA targeting vector to encode a spectrally distinct fluorophore, like CFP or a far-red fluorophore, to facilitate direct training on FRET biosensor-expressing cells, potentially increasing accuracy by reducing over-training errors due to morphological differences.

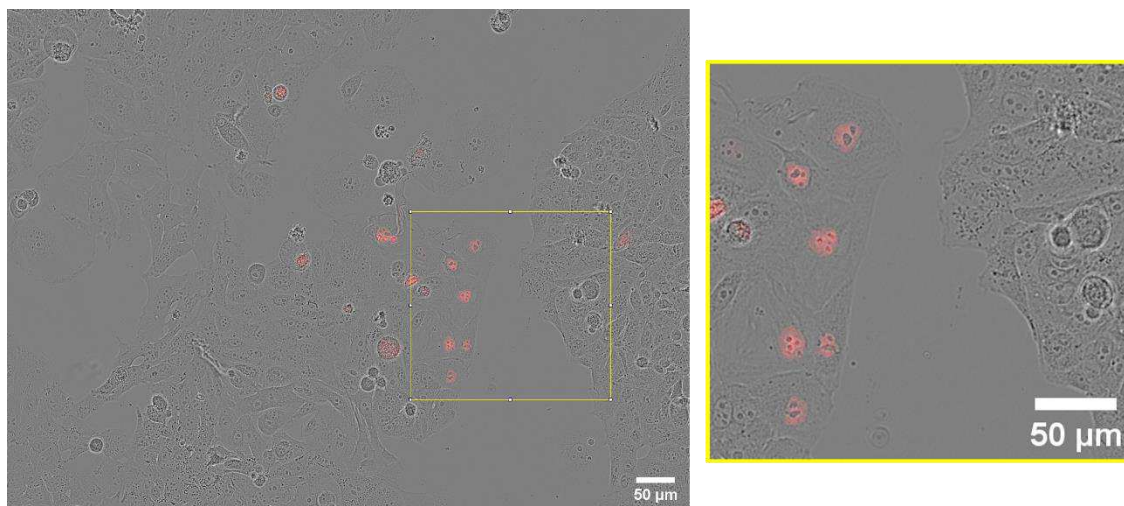


Figure 85: Labelling OVCAR4 with PCNA-mScarletFP. Cells were transfected with two plasmids encoding the CRISPR-Cas9 components for the labelling of endogenous PCNA. Fluorescence expression was assessed 48 hr later, with nuclear expression suggesting successful labelling. The merged phase contrast and epifluorescence image is shown, with the highlighted area enlarged (right) to show PCNA positive cells. Red fluorescence was collected with a 567-607 nm excitation filter and a 622-704 nm emission filter. 20X objective, scale bar = 50 μm .

This preliminary work offers a proof of concept that PCNA can be labelled in this HGSC cell line. However, the labelled cells must be isolated via FACS and gene editing accuracy needs to be assessed at the genomic and protein level. Following this, similar timelapse experiments as those conducted on A375 cells can be performed to create a ground truth and training dataset for subsequent ML pipeline training to assess cell cycle stage in a label-free manner in this specific cell line. This approach is expected to be applicable to the OVCAR4 FRET biosensor expressing cells used in FLIM-FRET assays, although training would be performed on the parental wildtype OVCAR4 cells to avoid spectral overlap between mCherryFP and mScarletFP. Ongoing development of the M³M system has also incorporated the *pDPC* modality, enabling simultaneous acquisition of FLIM and *pDPC* measurements, facilitating the correlation of cell cycle and FLIM-FRET readouts in future assays.

7.1.3 Discussion:

This work presents a novel workflow for label-free cell cycle stage classification based on phase contrast (*pDPC*) imaging and machine learning analysis, achieving ~90% prediction accuracy without occupying another fluorescence channel, avoiding increased phototoxicity and photobleaching. This

approach offers a significant improvement over conventional methods like FUCCI and PCNA fluorescence reporters, which are limited by these issues. Moreover, the *pDPC* method implemented here is both inexpensive and open-source, making it accessible for timelapse imaging on almost any fluorescence microscope system which further enhances the potential impact of this approach. Note, these results were achieved using the same microscope system and same cell line as used for the training dataset. Thus, using a microscope with a different magnification or a different cell line may require additional training data.

One challenge in generating ground truth training data is that the transitions from one cell cycle stage to the next are inherently continuous, making the definition of finite positions for stage boundaries somewhat subjective. To mitigate potential bias, three independent trainers performed the manual annotation, creating a more diverse and representative training dataset. Additionally, the ground truth data were augmented, further increasing the diversity of the dataset.

This method would offer multiparametric information to the PARP1 biosensor assay developed here, allowing the role of cell cycle stage in PARP trapping to be assessed. Such an approach has broader implications for a vast number of experiments, enabling the correlation of perturbations and cell cycle stage with single-cell resolution. Despite the high accuracy demonstrated in this preliminary work, its applicability to a wide range of cell types and morphologies remains to be established and represents an area for future research.

7.2 Imaging intrinsic fluorescence of rucaparib

7.2.1 Introduction

As discussed in the prior section, label-free imaging can offer complementary biological insight in a range of contexts. Following a similar rationale, here the direct imaging of the PARP inhibitor rucaparib was explored, as it had previously been demonstrated to exhibit intrinsic fluorescence due to the presence of an aromatic chromophore ring in its chemical structure [129].

Efforts have been made to synthesise fluorescently labelled PARPi to study their localization, penetrance, and interaction [130,131]. However, the discovery that rucaparib is intrinsically fluorescence facilitated the direct imaging of the drug itself, without structural modifications. The fluorescence absorption and emission profiles of rucaparib, along with its relatively long (compared to most biomolecules) fluorescence lifetime of ~7 ns were documented by *Kossatz et al.* [129]. The fluorescence lifetime was only measured in solution, not in cells. This was likely due to the short absorbance wavelengths of 275 and 255 nm for rucaparib, which necessitated UV excitation using

traditional single-photon approaches, which would be highly phototoxic to live cells. To overcome this, here two-photon imaging was employed here to excite rucaparib at approximately 850 nm, thus overcoming the phototoxic effects of UV excitation. This was done to perform fluorescence lifetime measurements of rucaparib *in vitro*, postulating that this may provide information on rucaparib localisation, uptake, and target engagement. In the context of the FLIM-FRET assay developed here, such an approach may offer complementary information, particularly in studying intracellular rucaparib concentration to correlate with readouts of PARP trapping.

7.2.2 Results

7.2.2.1 Wide-field imaging of rucaparib fluorescence

This work began serendipitously while imaging OVCAR4 EGFP-PARP1-mCherryFP cells following 24 hr of rucaparib treatment, when it was observed that the cells were emitting fluorescence in the UV channel (**Figure 86**). A literature search revealed the prior study by *Kossatz et al.*, demonstrating that rucaparib was intrinsically fluorescent. This suggests that fluorescence imaging could be used to assess rucaparib localisation and intracellular concentration.

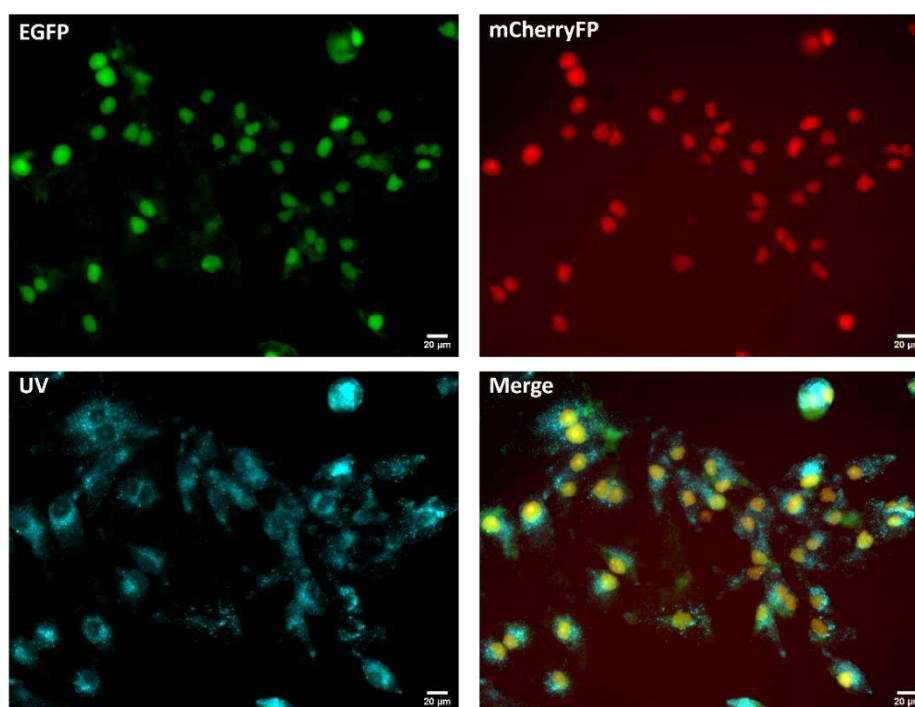


Figure 86: Widefield imaging of rucaparib intrinsic fluorescence. OVCAR4 EGFP-PARP1-mCherryFP cells were treated with rucaparib for 24 hr and epifluorescence images were acquired, with rucaparib showing a peri-nuclear localisation of fluorescence emission upon excitation in the UV channel (350/50 nm excitation filter and 460/50 nm emission filter). Images were acquired with a 20X objective lens. Scale bar = 20 μm .

7.2.2.2 Fluorescence lifetime of rucaparib *in vitro*

As the fluorescence lifetime of rucaparib was only previously measured in solution, here we aimed to measure its lifetime in the cellular context which to the best of our knowledge, had not yet been described in the literature. Multi-photon TCSPC imaging was used for this, aiming to achieve

fluorescence lifetime measurement without inducing excessive phototoxicity to the cells, as would occur with UV excitation. OVCAR4 wild-type cells and OVCAR4 PARP1-EGFP cells were therefore imaged at 850 nm excitation, with both untreated and rucaparib (10 μ M) treated conditions imaged (**Figure 87**). A 483/35 nm emission filter was used to remove unwanted autofluorescence from the sample. Despite this, a small amount of fluorescence from EGFP was collected as observed in the OVCAR4 PARP1-EGFP untreated cells. The mean fluorescence lifetime of rucaparib inside the cells was $\sim$ 6.5 ns. However, some heterogeneity in this measurement was observed at differing spatial localisations of the cell (**Figure 87**). Generally, the perinuclear regions exhibited a higher lifetime than cytosolic fluorescence.

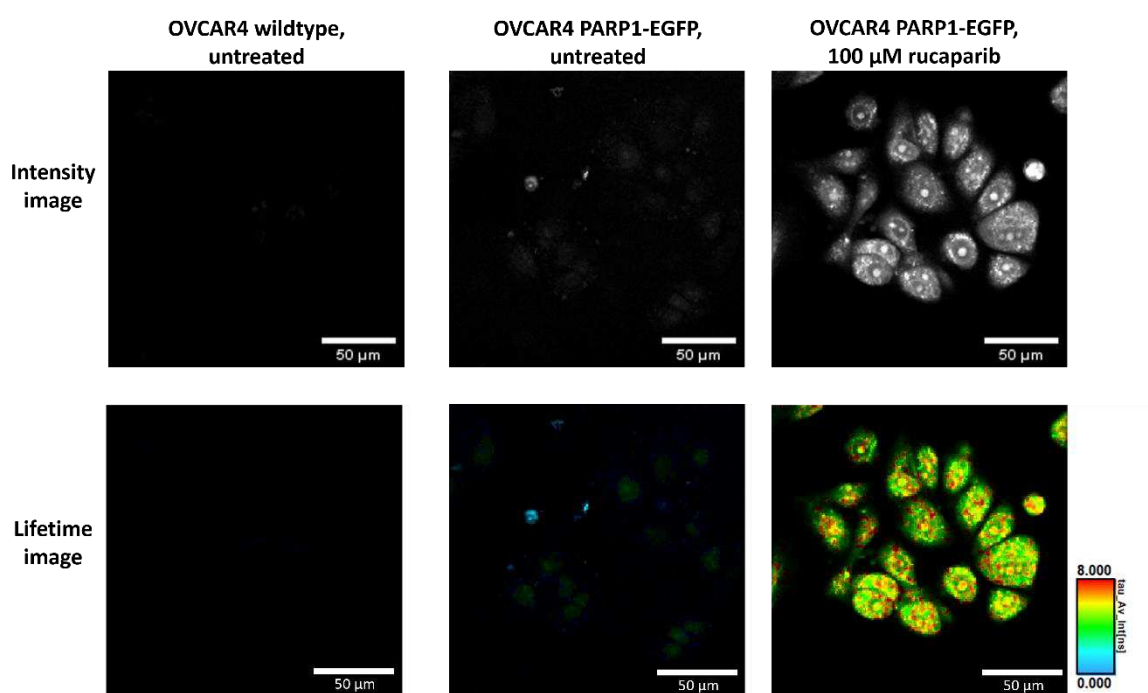


Figure 87: Measuring the fluorescence lifetime of intrinsic rucaparib fluorescence in live cells. The top row shows intensity images and bottom row shows fluorescence lifetime images. Untreated OVCAR4 wild-type cells (left), OVCAR4 PARP1-EGFP untreated (middle) and OVCAR4 PARP1-EGFP cells treated with 100 μ M rucaparib (right) are shown. Lifetime images are pseudo-coloured for τ_{1} values. Images acquired with a 20X objective, scale bar = 50 μ m.

7.2.2.3 Fluorescence lifetime of rucaparib in solution measured by TCSPC

The observed changes in fluorescence lifetime throughout the cell may be due to environmental factors such as viscosity, pH and refractive index. Within the intracellular environment, viscosity varies between different compartments and organelles. Thus, the fluorescence lifetime of rucaparib was measured in solutions with decreasing glycerol concentration to model viscosity changes. This demonstrated a clear viscosity dependency: the fluorescence lifetime was lower at higher viscosities (with higher glycerol percentages) decreasing from approximately 7 ns to 5 ns (**Figure 88**). However, the refractive index of this solution also changes in these solutions, which may affect lifetime

measurements. Moreover, this simple experiment only modelled one variable that can affect lifetime and therefore for a full characterisation parameters such as pH and viscosity must be considered.

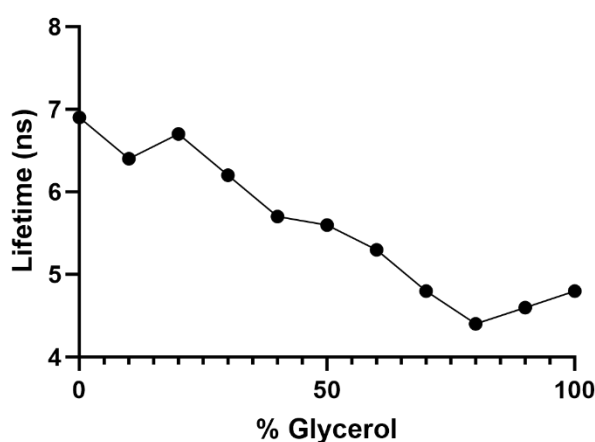


Figure 88: Modelling changes in the fluorescence lifetime of rucaparib with changing viscosity. Rucaparib was resuspended at 10 μ M in PBS and an increasing percentage of glycerol. Fluorescence lifetime was measured at 10% intervals of glycerol concentration.

7.2.2.4 Can rucaparib serve as a FRET donor with GFP?

In vitro fluorescence lifetime imaging of rucaparib could potentially facilitate FRET assays between PARP1-EGFP-labelled cells, with rucaparib as the donor and EGFP as the acceptor. The rationale is that as rucaparib and PARP1 interacted (<10 nm apart), an increase in FRET efficiency would occur, resulting in a reduction in the fluorescence lifetime of the donor, rucaparib. This may offer insights into drug-target engagement between rucaparib and PARP1.

To test this hypothesis, TCSPC FLIM measurements were acquired of OVCAR4 wild-type and OVCAR4 PARP1-EGFP cells treated with 10 μ M rucaparib (**Figure 89A**). The nucleus of each cell was segmented as ROIs as this is where PARP1-EGFP is localised. The mean fluorescence lifetime of rucaparib in the nucleus of OVCAR4 wild-type cells was measured at 6.4 ns, compared to 5.9 ns for the OVCAR4 PARP1-EGFP cells (**Figure 89B**). This reduction in fluorescence lifetime between the OVCAR4 wild-type cells (donor-only control) and the OVCAR4 PARP1-EGFP cells could indicate FRET. However, these preliminary data do not definitively confirm that these changes in lifetime are a result of FRET. To further characterise this, a competition assays could be performed in future, in which the rucaparib binding site of PARP1 would be saturated, preventing the drug and target from interacting. If the reduction in lifetime were no longer observed under these conditions, it would provide supporting evidence that the change might be due to a FRET interaction.

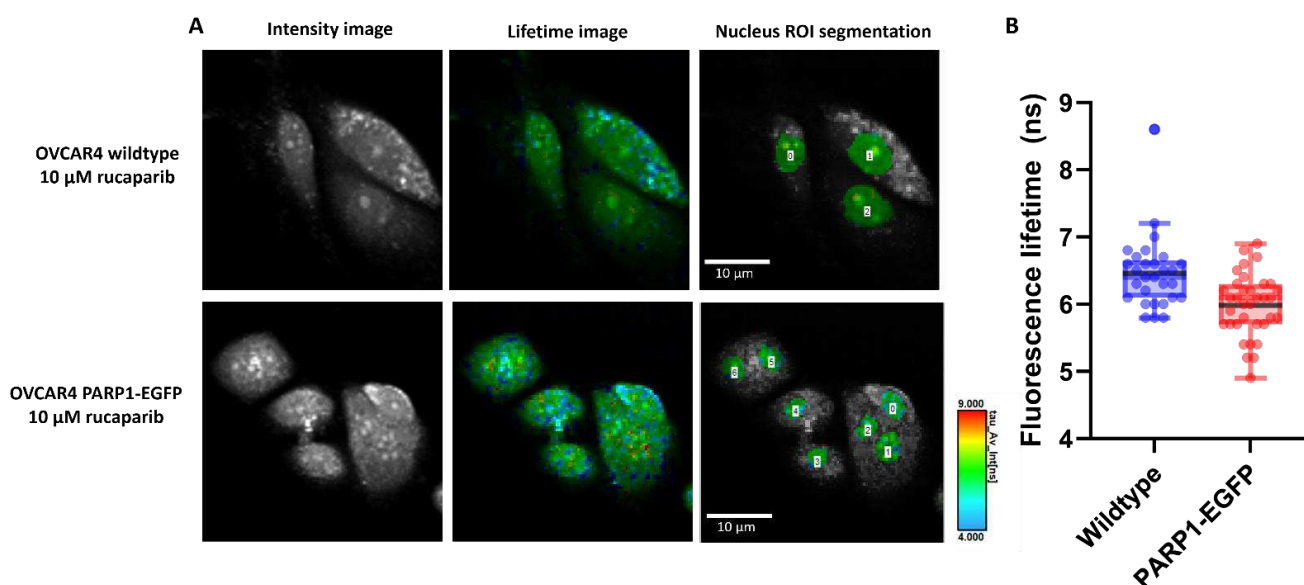


Figure 89: Assessing if rucaparib and EGFP can serve as a FRET pair. **A:** Representative intensity (left) and lifetime (middle) images of OVCAR4 wild-type (WT) and OVCAR4 PARP1-EGFP cells imaged using TCSPC upon treatment with 10 μ M rucaparib. The right column shows segmented nuclear ROIs. Lifetime images are pseudo-coloured by τ_1 values. 20X objective was used, scale bar = 10 μ m, $n=29$ and 35 cells for the wild-type and PARP1-EGFP cell lines respectively. **B:** Quantification of nuclear fluorescence lifetime upon treatment of 10 μ M rucaparib. Mean population values are shown by the black line.

7.2.3 Discussion

The work presented in this section demonstrated that imaging the intrinsic fluorescence of rucaparib could enable the investigation of drug localization and semi-quantitative readouts of concentration within cells. Understanding the localization of rucaparib may provide insights into its degradation pathways and potential interactions with cellular components. Furthermore, this could enable imaging studies to evaluate PARPi influx and efflux, as changes in these processes have been linked to resistance to PARPi [132].

TCSPC FLIM measurements revealed differences in fluorescence lifetime correlated with the spatial distribution of rucaparib throughout the cell, potentially indicating localization within low pH organelles, such as lysosomes. To confirm this hypothesis, future experiments should focus on the colocalization of lysosomal labelling with these areas of lower fluorescence lifetime. These findings would align with existing literature, where the autophagy-driven (lysosomal) degradation of PARPi is being actively investigated [133–135]. To the best of our knowledge, this marked the first reported measurements of the fluorescence lifetime of rucaparib inside live cells.

Preliminary evidence suggested that rucaparib could be used as a FRET donor when paired with EGFP as an acceptor. This finding suggests a potential FRET interaction upon treatment, as evidenced by the reduction in fluorescence lifetime of rucaparib in OVCAR4 PARP1-EGFP cells compared with donor-

only OVCAR4 wild-type cells. The ability to utilise rucaparib in this manner would represent a significant advancement in the study of drug-target engagement, simplifying experimental design by eliminating the need for donor labelling.

Overall, the label-free imaging of rucaparib offers information regarding drug localisation and interactions that could be incorporated into FLIM-FRET HCA assays to complement the PARP1 FRET biosensor. These multiparametric data would allow a better understanding of the mechanisms modulating PARP trapping. Such knowledge would inform treatment options to negate resistance to PARPi, thus improving patient outcomes.

7.3 Developing 3D cell culture models of HGSC that are amenable for HCA imaging

7.3.1 Introduction

Although the FLIM-FRET assays developed throughout this project were performed in 2D cell cultures, the microscopy methods used are also capable of 3D imaging. More specifically, the wide-field time-gated FLIM system uses quasi-confocal sectioning achieved by a spinning Nipkow disc to perform Z-stacks that can sample the Z-axis of 3D cell culture models. The M³M system, on the other hand, performs multibeam multiphoton imaging with scanned detection to achieve 3D imaging. Hence, this presents an opportunity to perform assays in more complex 3D biological models that may better recapitulate the physiological conditions of HGSC tumours than 2D cell monolayers. However, there is currently a lack of methodologies that allow the culture of 3D cell culture models such as spheroids and organoids in a format that is amenable to HCA imaging assays. A key issue is the limited working distance of high magnification objective lenses, which is often $< \sim 200 \mu\text{m}$. Therefore, multiple methods of generating 3D models were explored, aiming to establish a protocol amenable to growing spheroids near the coverslip in glass 96-well imaging plates for HCA assays.

Recent advancements in 3D imaging methodologies and 3D cell culture models present a converging area of opportunity offering novel data from models that more closely replicate disease physiology, complementing high spatial resolution with the capability to sample hundreds of fields of view per condition to assess heterogeneity. However, from a technical point this is a challenging task for multiple reasons. Firstly, there is not a unified approach for culturing cells in 3D due to the heterogeneous nature of tissues, especially patient derived samples that require specific culture conditions depending on the tissue from which they are sourced. Secondly, the relatively small ($< \sim 200 \mu\text{m}$) working distance of high magnification optics limit the formats in which these 3D tissue

models can be imaged. The working distance is influenced by several factors including numerical aperture, magnification, objective design, and immersion medium (**Figure 90**).

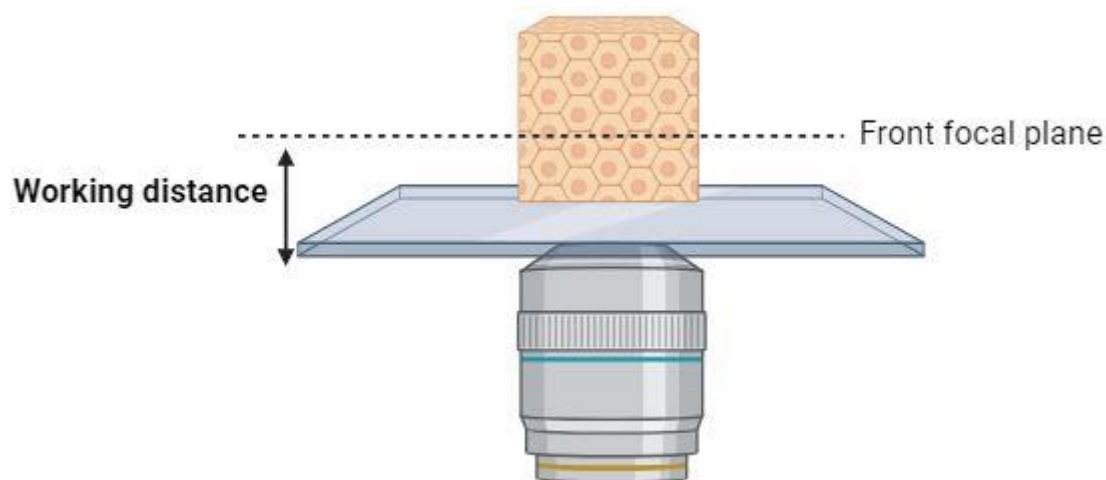


Figure 90: Working distance of the objective when imaging a thick biological sample section. The working distance is the distance from the top of the coverslip to the front focal plane (dashed line). Note that anything above the working distance of the objective in Z will not be possible to image with sharp focus.

7.3.2 Results:

Before optimising the culture method of choice, the effect of different culture media components on 3D cell growth was assessed. For this, the conventional 2D complete medium used for standard monolayer culture was compared to a specialised 3D culture medium previously reported by *Hoare et al.* [136]. Medium components are described in full in the Materials and Methods section. To assess the effect on growth, OVCAR4 EGFP-PARP1-mCherryFP cells were resuspended in a 50% BME solution and 20 μ L droplets were manually dispensed onto glass 96-well plates. This produced a small droplet of BME with cells embedded inside the droplet. After 96 hr, the spheroids grown in the 3D medium were much larger and had a more compact morphology (**Figure 91**). Therefore, all subsequent experiments used the 3D medium as standard whilst optimising culture methodologies. Additionally, this experiment demonstrated that the droplet method of 3D culture was effective for the growth of HGSC spheroids. However, manually dispensing BME droplets led to low reproducibility due to the difficulties of working with small volumes of a temperature-sensitive hydrogel. Thus, automated methods were subsequently explored.

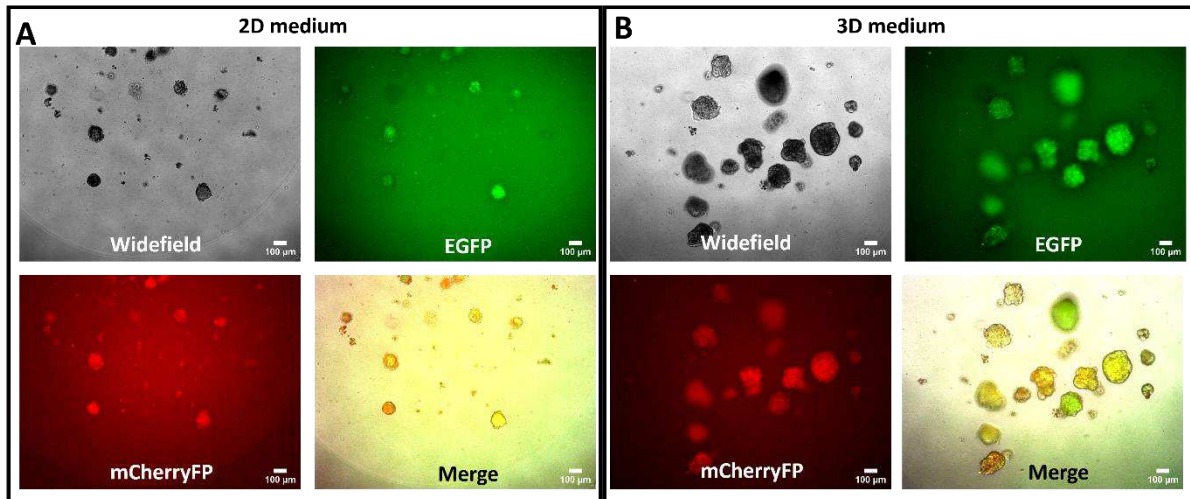


Figure 91: OVCAR4 GFP-PARP1-mCherryFP spheroids grown in differing culture media. All cells were grown for 5 days in a 3 μ L droplet of 50% BME dispensed by the Matribot. For each sample the widefield, EGFP, mCherryFP and merged images are shown. **A:** Cells grown in the conventional 2D complete medium recipe with no additives. **B:** Cells grown in 3D medium for the same duration. Imaged with a 4X objective, epifluorescence microscope. Scale bar = 100 μ m.

Herein, several different approaches were trialled and their ability to produce uniform spheroids in close proximity to the coverslip was assessed. An overview of the differences in each of these approaches is summarised in the schematic in **Figure 92**. Briefly, these involved the growth of cells in U or V-bottomed wells coated with non-adhesive coatings, or the droplet method whereby cells are embedded within a hydrogel that provides a scaffold for 3D growth.

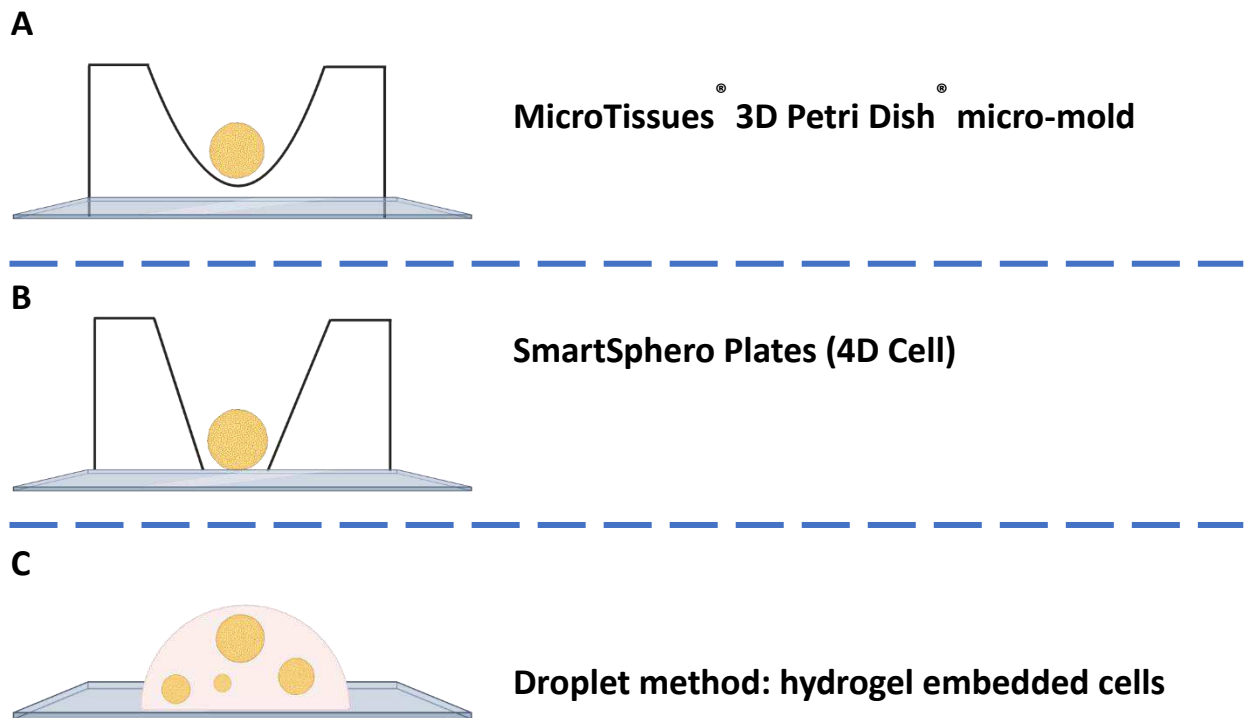


Figure 92: Different approaches trialled here for 3D cell culture. Schematics show spheroid position in the context of distance and spatial distribution from the coverslip. **A:** The micro-mold approach produces an array of single spheroid per well, separated from the coverslip by a layer of 4% agarose. **B:** SmartSphero plate uses a non-adhesive well coating forming a single spheroid directly in contact with the coverslip of each well. **C:** The overlay method produces many spheroids distributed randomly across a layer of rehydrated hydrogel (such as Matrigel). Note, spheroid sizes are not to scale.

7.3.2.1 3D Petri Dish

One method that has previously been used in HCA assays involves growing spheroids in a small, patterned agarose micro-mold that produces an array of 256 round bottomed micro-wells, termed a 3D petri dish (Microtissues Inc, 3D Petri Dish®) [83]. This non-adhesive material encourages cells that are capable of anchorage-independent growth (i.e. epithelial cells) to grow into spheroids. The process involves casting 4% agarose into the micro-mold and allowing it to set, thereby producing a non-adhesive 3D petri dish (**Figure 93A**). In short, 2×10^5 single cells were seeded directly onto the micro-mold and allowed to settle into the pockets by gravity. Within 1-3 days (depending on cell type), the cells aggregated and grew into spheroids of uniform size. Spheroids could be treated *in situ* within the micromolds as demonstrated in **Figure 93B**. These spheroids can be removed from the dish by washing, and transferred into glass bottomed imaging plates, or used for the desired downstream assay.

This methodology has several benefits, such as the production of many uniform spheroids with ease, without a scaffold material that can introduce high levels of batch-to-batch variability and further alter cell physiology. Moreover, after initially purchasing the mold, the consumable costs for this approach are close to zero, as it is simply cast with 4% agarose and can be used hundreds of times repeatedly. However, although this methodology can produce a large number of spheroids, they cannot be easily imaged *in situ* due to the large thickness of the agarose petri dish which means the spheroids are often beyond the working distance of high magnification objectives and are subject to scattering aberrations due to imaging through the agarose material. Additionally, it is technically difficult to transfer a single spheroid to each well in order to run an assay with standardised conditions. Although manual transfer is possible for some cell types such as HEK293, other cell types that form loosely associated spheroids that are dissociated during this process, meaning it is not suitable for those cell types. Moreover, this manual handling reduces the throughput and reproducibility of the approach for HCA experiments.

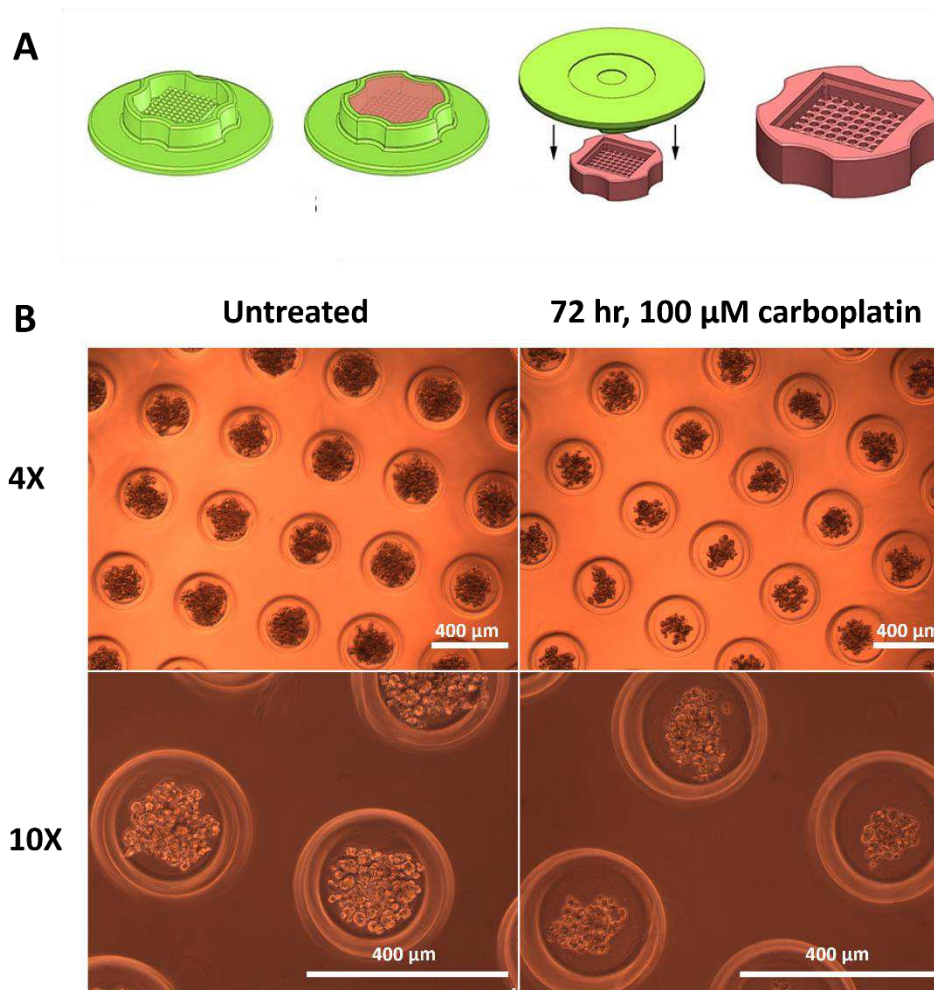


Figure 93: 3D Petri Dish approach for producing many uniform spheroids. **A:** schematic of the methodology. Liquid 4% agarose is cast into the micro-mold, which solidifies forming an array of 256 microwells. Cells are seeded into this non-adhesive material and form spheroids within 24 hr. **B:** Demonstration of culture and treatment of OVCAR4 spheroids in the micro-mold. 5×10^4 OVCAR4 cells were seeded, and 24 hr later fresh medium (untreated, left) or 100 μM carboplatin (right) medium was added for 72 hr treatment. Images were acquired at 4X (top) and 10X (bottom) magnification showing differences in size and morphology following treatment. Scale bar= 400 μm .

This approach does lend itself to the culturing of native spheroids isolated directly from patient ascites samples. The agarose petri dish can be prepared in advance and stored stably at 4°C, enabling immediate utilization upon receipt of fresh samples. To assess the viability of this method, ascites spheroids were seeded at a 1:5 dilution directly into the 3D petri dish. The cells were cultured in medium containing autologous fluid. After 24 hr the spheroids had aggregated into a single spheroid per microwell, ~200 μm in diameter (**Figure 94**). By 48 hr, the spheroids had grown to a diameter of ~300 μm . The continued cell division observed suggests this approach is suitable for the short-term growth of native patient-derived spheroids, thus mitigating the requirement for cells to be cultured in contact with plastic. Consequently, this method could be utilised for various downstream applications, such as 3D imaging or omics. However, appropriate labels must first be introduced for fluorescence imaging.

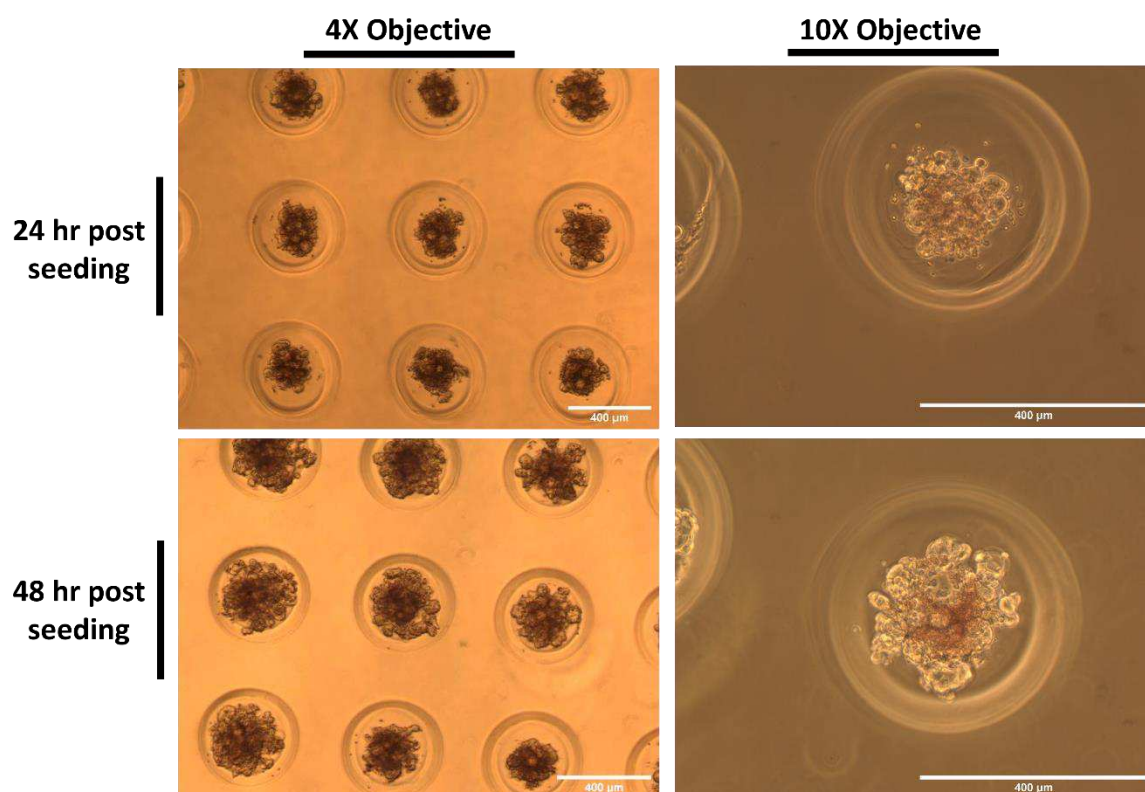


Figure 94: Culture of native spheroids from HGSC patient ascites samples (ASC22-023). Spheroids were imaged 24 hr (top row) and 48 hr (bottom row) after seeding, using widefield illumination. For an overview of spheroid reproducibility spheroids were imaged at 4X magnification (left column) and 10X magnification (right column). Scale bars = 400 μm .

7.3.2.2 4D SmartSphero Plates

A similar method using V-shaped wells is commercially available as SmartSphero plates (4DCell, France). This method successfully overcomes the requirement to transfer the spheroids into a separate imaging plate. Instead of U-bottomed shaped microwells, this method generates an array of V-bottomed micro-wells that allow the cells to directly contact the glass coverslip of the imaging well plate at the centre. The walls of the well are made of a non-adhesive hydrogel that promotes rapid self-assembly of cell aggregates into compact spheroid structures. This results in the growth of an array of 91 spheroids (**Figure 95A**) directly in contact with the glass coverslip and hence, spheroids can be cultured and imaged in the same plate, reducing variability and error introduced by the transferring step. To demonstrate this approach, primary HGSC cells (ASC20-018) were seeded into the SmartSphero plates and growth was documented upon seeding, and five days later during which time the cells went from single cells to a single spheroid $\sim 200\ \mu\text{m}$ in diameter (**Figure 95B**).

The production of a single spheroid per microwell is conducive to a (live-cell) HCA where multiple drug perturbations can be assessed across a multiwell plate with spheroids of standardised size and predictable position. Although these properties are beneficial, the product is expensive in comparison to the 3D petri dish approach, making it restrictive for resource-limited settings. Additionally, cells at

the periphery of the non-adhesive microwell pattern adhere to the glass surface in 2D, as demonstrated in **Figure 95A**. Furthermore, as the spheroids contact the coverslip, this is not a pure 3D model.

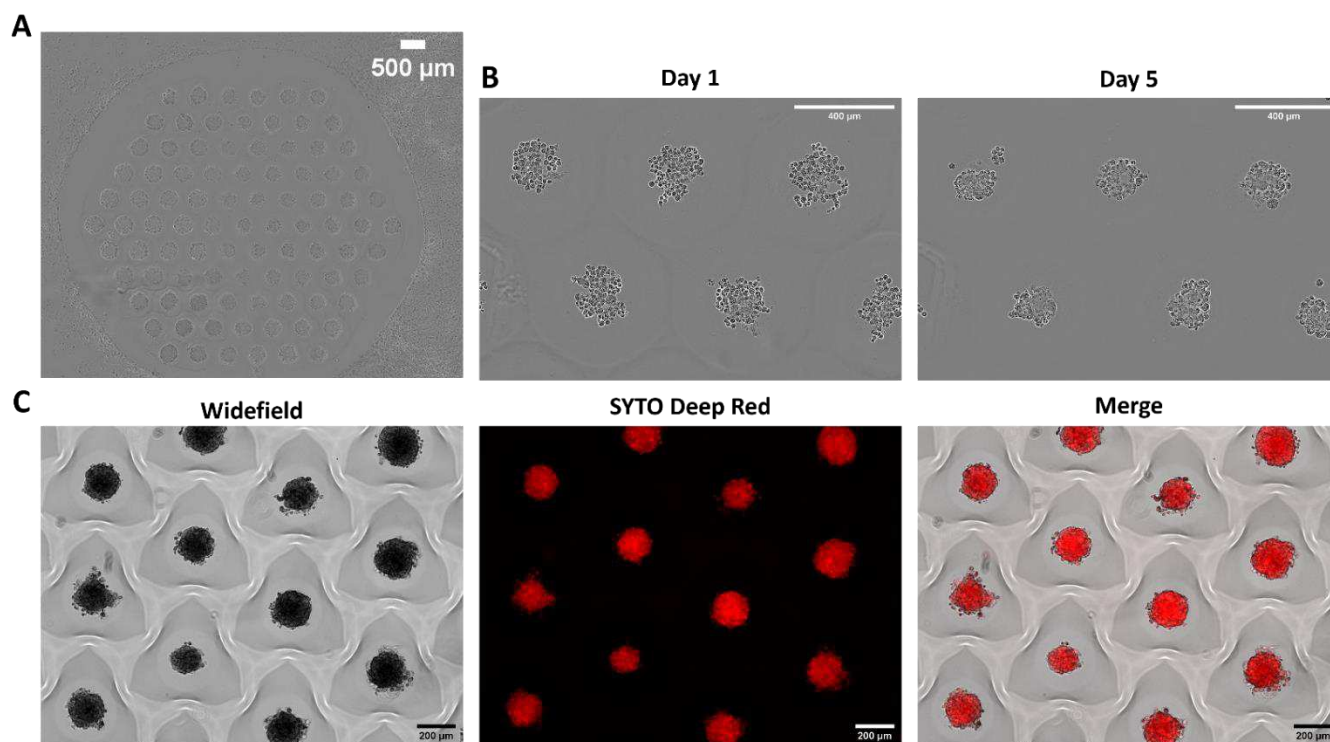


Figure 95: V-bottom wells produce an array of spheroids within a single well. **A:** Whole well overview of an array of 91 spheroids in a single well of a 6-well plate. Scale bar = 500 µm **B:** Phase images showing primary HGSC cells (ASC20-009) as single cells on day 1 of seeding that aggregate into dense spheroids by day 5. 10X Objective, scale bar = 400 µm **C:** Epi-fluorescence images of the same cells with widefield imaging and stained for SYTO Deep Red, merged image is shown. 4X Objective, scale bar = 200 µm.

7.3.2.3 PolyPico acoustic micro-dispenser

As the droplet method was previously demonstrated to be successful in producing spheroids within the working distance of a high magnification objective lens (**Figure 91**), it fulfilled one of the desired goals. However, manually dispensing small volume droplets into multi-well plates is not amenable to high-throughput assays, as it takes significant time and introduces variability in droplet position across a multi-well plate. Therefore, alternative dispensing methods were explored that could offer automated dispensing at a defined position within each well of a multiwell plate and reduce manual handling.

First, an automated picolitre dispensing system called the PicoSpotter (PolyPico) was trialled. This method utilises acoustic energy to dispense micro drops with temporal and spatial control. A single

cell suspension was resuspended in 50% BME and placed in the PolyPico cartridge and subsequently, one droplet of 200 nL per well was dispensed. By day 12, spheroids of ~100 μm had formed, embedded in the BME droplet (**Figure 96**). One technical downside of the PolyPico instrument is that it is not temperature-controlled. Thus, to work with temperature-sensitive hydrogels such as BME, the system must be operated in a dedicated cold room.

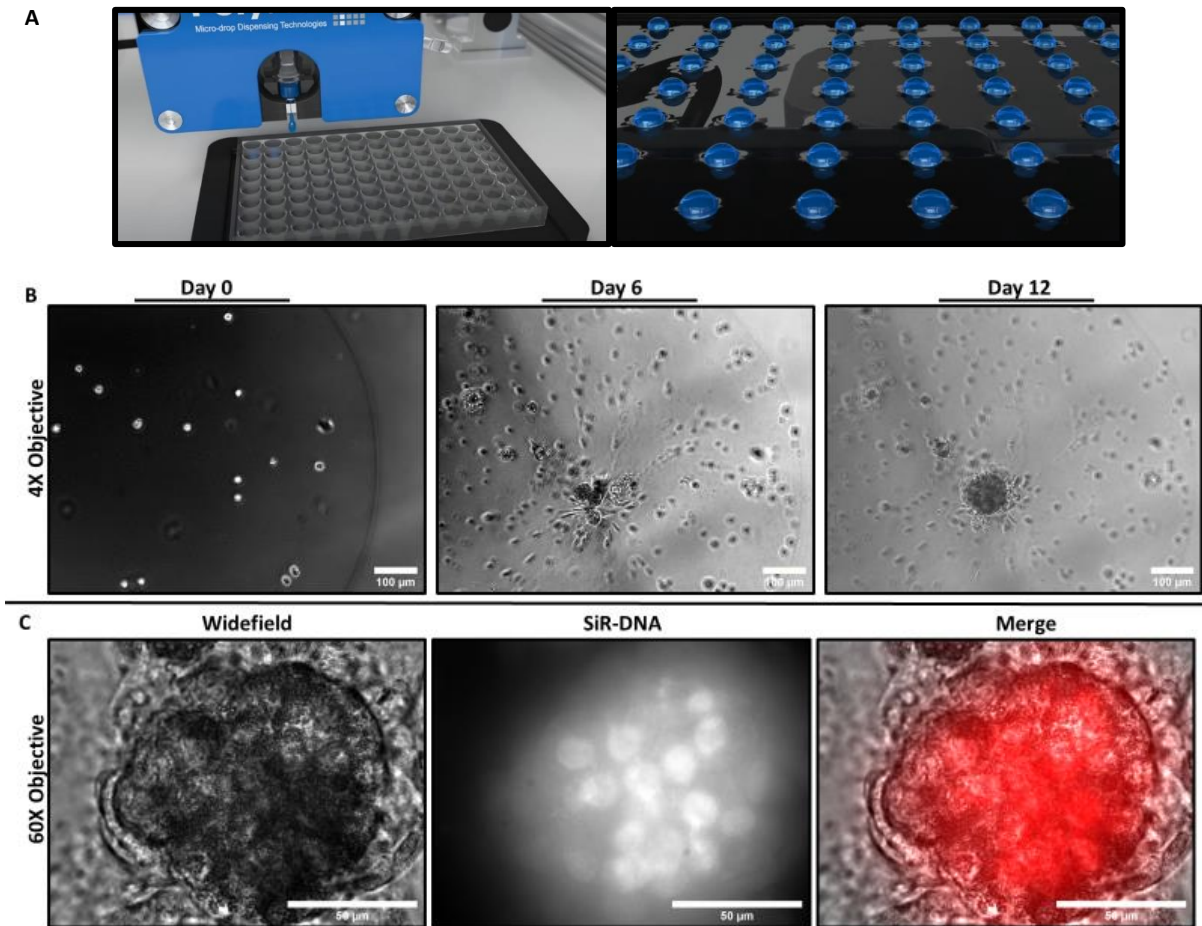


Figure 96: Seeding cells embedded in BME via an acoustic micro-dispenser. **A:** Schematic diagram of the acoustic micro-dispenser (left). 200 nL droplets of cells embedded 50% BME were dispensed as individual drops in defined coordinates within the well of an imaging plate. An array of dispensed droplets can be dispensed inside a well (right). **B:** A single cell suspension was dispensed in BME and cell growth was documented for 12 days thereafter. Single cells migrated to accumulate as dense spheroid structures around 100 μm in diameter. Scale bar = 100 μm . **C:** Cells were fixed in 4% PFA on day 12 and stained with Silicone Rhodamine DNA dye (SiR-DNA). Spheroids were imaged with a 60X objective in widefield (left) and epifluorescence (middle) images were able to resolve single nuclei within the spheroid structure (middle). Merged image shown on the right. Scale bar = 50 μm .

7.3.2.4 Matribot

The Matribot (Corning) is an automated instrument similar to the PolyPico, designed specifically for dispensing hydrogels, particularly Matrigel. The instrument incorporates a heated plate holder and a temperature controlled pipette arm, which keeps pipette tips at 4°C to prevent temperature sensitive hydrogels, such as BME, from polymerising before dispensing. Hence, various volumes of cells

resuspended in hydrogels can be directly dispensed into multiwell plates, with multiple drops per well if desired. Here, 6000 OVCAR4 EGFP-PARP1-mCherryFP cells were dispensed in 6 μ L droplets of BME, one droplet per well of a 96-well plate. The spheroids were imaged five days later, forming several spheroids per droplet that were randomly distributed both axially and laterally inside the droplet (**Figure 97A**). Importantly, the spheroids were within the working distance of the M³M microscope, allowing single-cell resolved imaging upon segmentation using mesh point clouds (**Figure 97B**). This offers a proof of concept of how these automated cell dispensing instruments can be coupled with complex microscopy systems to perform automated 3D imaging, with single-cell resolved information.

As a future step, the FLIM-FRET assays performed here could be performed in such formats, allowing the assessment of PARP trapping in the context of 3D models. This will allow factors such as drug penetrance, oxygen and nutrient gradients to be accounted for, building a more complex and physiologically relevant model of PARP trapping in HGSC. One potential drawback of this approach is that within each droplet, spheroids grow at variable axial positions from the coverslip. However, this is an essential trade off as if cells are grown in contact with the coverslip, they tend to grow in 2D. The range of differences from coverslip may be minimised using smaller droplet volumes, for which the higher precision of the PolyPico dispenser is desirable. This necessitates autofocus approaches that can optimise the focal plane to image each spheroid despite them being at varying positions. Such approaches are now being developed, often using machine learning.

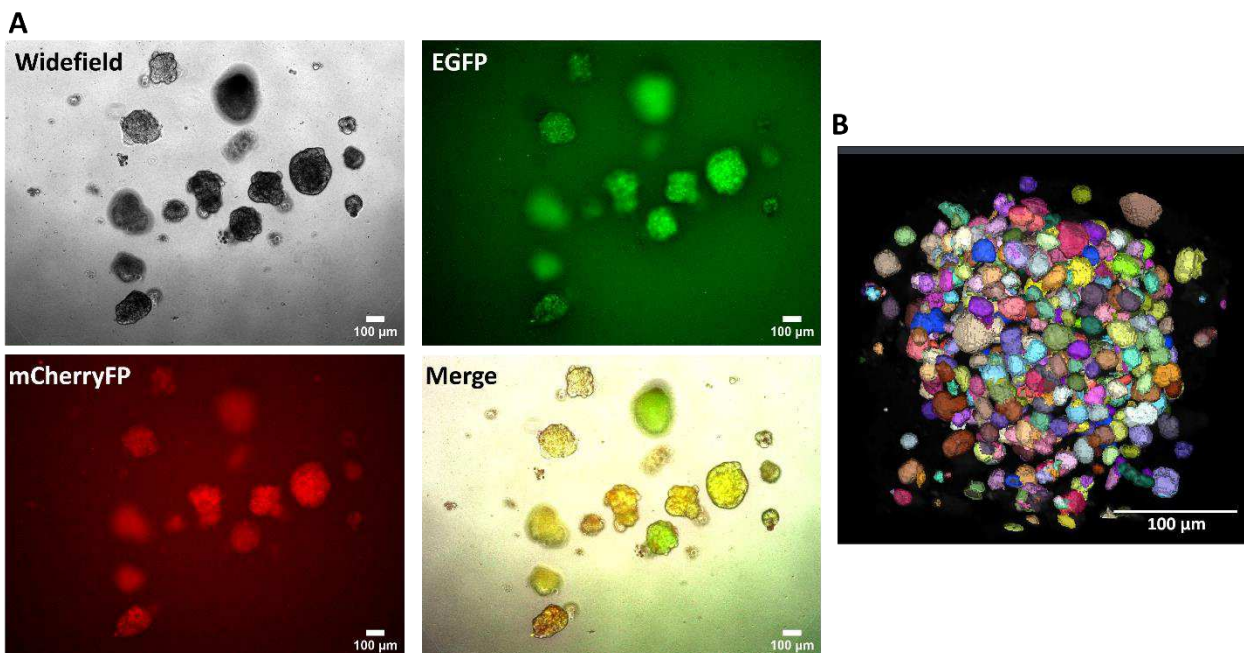


Figure 97: OVCAR4 GFP-PARP1-mCherryFP spheroids. **A:** Cells were dispensed in 3 μ L droplets of BME using a Matribot automated dispenser. A representative well is shown, imaged in widefield (top left), epifluorescence imaging of GFP (top right) and mCherryFP (bottom left) with 100 ms acquisition time. Merged image is shown (bottom right). Scale bar = 100 μ m. **B:** Mesh point cloud segmentation of a single spheroid imaged with the M3M system. Scale bar = 100 μ m.

7.3.3 Discussion

In this section, several methods suitable for culturing 3D models of HGSC were demonstrated. However, only the droplet methods (from both the Matribot and PolyPico) were amenable to *in situ* imaging of spheroids without manual handling. This approach was further developed to improve throughput by using an automated acoustic dispenser, enabling highly reproducible culture conditions across multi-well plates. In contrast, other methods often resulted in spheroid growth beyond the working distance of the high-magnification objectives required for HCA.

While numerous prior studies have produced 3D models of HGSC, they did not facilitate single-cell resolved imaging. This work successfully addresses that gap, enabling HCA drug screens in 3D models that better recapitulate physiological conditions. One drawback of this approach is the high cost of instrumentation for the automated micro-dispensers. Hence, to achieve rapid dissemination of 3D cell culture methods for HCA in low-resource settings, a trade-off between throughput and cost may necessitate the use of micro-molds.

These findings indicate that single cell resolved HCA of 3D disease models are now feasible. In the context of this thesis, culturing FRET biosensor-expressing cells in this format allows for the assessment of PARP trapping in a 3D context. This capability may provide insights into changes in trapping related to drug and nutrient gradients throughout 3D space, which are critical for understanding treatment responses and resistance development in patient tumours.

The integration of automated culture methods with advanced imaging techniques represents a significant advancement in HGSC research. Future work should focus on deploying such approaches with patient derived samples, which may offer mechanistic insights of drug resistance, and offer a route towards personalised medicine.

7.4 Chapter summary

This chapter explored various approaches to expand the assay conditions of the FLIM-FRET assay, aiming to gather complementary biological information that enhances our understanding of PARP1 binding dynamics and trapping in different contexts. The results highlight the potential to investigate PARP trapping within the cell cycle, in more complex 3D biological models, and through the label-free imaging of rucaparib to resolve drug localization and concentration.

Homologous recombination repair predominantly occurs in the late S/G2 phase of the cell cycle. By examining PARP inhibitor (PARPi) responses during and outside this critical phase, the current study may account for some of the heterogeneity previously observed in treatment responses. This

approach opens avenues for investigating the effects of cell cycle modulation, such as checkpoint inhibitors, in combination with PARPi, which could further refine therapeutic strategies.

The intrinsic fluorescence of rucaparib provides a quantifiable measure of drug localization and concentration. These data can be correlated with PARP trapping readouts on a per-cell basis, allowing for a more nuanced understanding of how drug distribution impacts PARP1 dynamics. Additionally, conducting experiments in complex biological models of high-grade serous carcinoma (HGSC) that better mimic the in vivo tumour environment will yield more clinically relevant insights into changes in PARP trapping in the context of PARPi resistance.

In summary, the preliminary work presented here demonstrates how the FLIM-FRET assay developed here can be further expanded to gain higher order information that will aid the study of the molecular mechanisms modulating PARP trapping. Future work should focus on deploying such approaches with patient derived samples, which may offer mechanistic insights into drug resistance, and offer a route towards personalised medicine.

8 Summary and future work

This chapter summarises the research presented in this thesis, outlining the key findings and broader implications of the insights gained from developing and applying a novel intramolecular FRET biosensor for studying PARP1 dynamics in ovarian high-grade serous carcinoma cells. Some limitations encountered during this research have been discussed and potential solutions proposed. Future directions that may increase the impact of this work are highlighted.

The primary objectives of this thesis research were to develop an intramolecular FRET biosensor for studying PARP1 dynamics and to investigate mechanisms of PARPi resistance. This study was divided into four aims: the development and characterization of the biosensor (**Aim 1**), application to study PARP trapping in validated models (**Aim 2**), analysis in novel models of resistance developed during the research (**Aim 3**), and the exploration of more complex models such as spheroids (**Aim 4**).

Chapter 4 outlined the successful development and validation of the FRET biosensor using CRISPR-Cas9, marking a significant methodological advancement and one of the first documented instances of endogenous dual-labelling of a gene achieved via this approach. The EGFP-PARP1-mCherryFP biosensor allows the real-time monitoring of PARP1 structural changes upon DNA binding in live cells, providing a novel tool for investigating PARP1 behaviour at a single-cell resolution.

In chapter 5, the application of fluorescence lifetime imaging (FLIM) for quantitative FRET assays enabled detailed assessment of PARP trapping and PARP1-DNA binding dynamics across various treatment conditions. The automated wide-field time-gated FLIM-FRET assay facilitated robust statistical analyses at both single-cell and population levels, allowing extensive sampling of heterogeneity in response to PARPi treatments.

Through multiple drug treatments, the study demonstrated a reproducible dose-dependent decrease in fluorescence lifetime upon PARPi treatment, indicating PARP trapping. The assay's robustness was validated by comparing the trapping potency of approved PARPi and extending it to novel models of PARPi resistance generated during this research was discussed in chapter 6. This comparative analysis provided insights into the differential PARP1 binding dynamics and trapping efficiency between sensitive and resistant cell populations. These observations are strengthened by the results acquired from negative controls, whereby no change in lifetime was observed upon drug treatment of donor only cells.

Chapter 7 presented preliminary work exploring the development of multi-parametric readouts which could complement the existing FLIM-FRET assay. The integration of machine learning and versatile quantitative phase imaging was demonstrated to resolve cell cycle stages in a label-free manner,

thereby minimising phototoxicity as normally associated with long time-lapse fluorescence imaging. Furthermore, label-free imaging of the intrinsic fluorescence of rucaparib was demonstrated, providing a potential method for quantifying drug concentration at the single-cell and intracellular levels to correlate with PARP trapping readouts.

Finally, the developed FLIM-FRET HCA approach offers a powerful tool for cancer research and therapeutic development, laying the groundwork for future studies aimed at improving PARPi treatment efficacy and potentially overcoming resistance in ovarian high-grade serous carcinoma and other cancers such as breast and prostate cancer.

This research supports the hypothesis that reduced PARP trapping may contribute to PARPi resistance in high-grade serous carcinoma. By elucidating changes in PARP trapping between sensitive and resistant populations, this thesis provides evidence from live-cell assays that reduced PARP trapping occurs in resistant cell populations. This trend is consistently observed upon acquired resistance to both rucaparib and olaparib, contributing to the broader understanding of mechanisms modulating PARP trapping.

The methodological advancement presented by the FRET biosensor enables real-time analysis of PARP1 binding dynamics, facilitating robust statistical assessments that complement multi-omics studies increasingly employed to understand complex biological systems. Clinically, the findings offer preliminary evidence that reduced PARP trapping may indeed represent a mechanism of resistance to PARPi, previously postulated based on the presence of a single PARP1 mutation (R519C) in patient tumour samples associated with lower PARP trapping levels. The *in vivo* induction of acquired resistance and the resultant changes in PARP trapping, occurring within a relatively short timeframe (~8-10 weeks), suggest that the selection pressures experienced by cancer cells in this study parallel those in patients, despite the limitations of 2D monolayer cultures.

While this research has achieved advancements in understanding PARPi resistance mechanisms and developing novel methodologies, several limitations should be acknowledged. First, although anti-PARP1 antibodies effectively detected the GFP-PARP1-mCherry tagged protein, the ability to quantitatively compare expression levels to the untagged control was limited to semi-quantitative assessments. The study primarily utilised a single heterozygous clone due to challenges associated with performing sequential CRISPR knock-ins. Achieving a homozygous knock-in would have provided more consistent expression levels but this was complicated by the polyploid nature of OVCAR4 cells, which average a ploidy of three. Future attempts to create a homozygous knock-in would require multiple rounds of CRISPR-Cas9 transfection and careful screening to ensure the absence of wild-type PARP1.

Second, information regarding the specific DNA sequences to which the biosensor binds was not obtained, restricting the contextual understanding of these interactions. Experiments were also not performed in synchronised cells, despite the importance of late S/G2 phase homologous recombination.

Third, the experiments utilised wide-field time-gated FLIM HCA, a technology available to only a limited number of research groups. Expanding the impact of this study may benefit from attempting spectral ratiometric FRET experiments, which require less specialised imaging systems. Prolonged treatment with DNA-damaging agents led to increased PARP1 expression levels, complicating FRET efficiency comparisons due to variations in donor/acceptor concentration. A lentiviral system could help address these issues by integrating the biosensor under an exogenous promoter, providing more consistent expression levels.

Furthermore, the HCA assays lacked multi-parametric data, suggesting that additional extractable information could be collected. For instance, incorporating quantitative phase contrast imaging alongside FLIM could enable label-free cell-cycle classification and clustering-based analysis, offering insights into the observed heterogeneity in single-cell FLIM data.

Looking forward, several avenues for future work can be pursued to further understanding of PARPi resistance mechanisms and enhance the methodologies developed in this thesis. Immediate efforts should focus on further validation and optimization of the FRET biosensor. Expanding the approach to additional ovarian cancer cell lines and other cancer types will establish the broader applicability of the biosensor. Refining the CRISPR-Cas9 knock-in technique to achieve homozygous expression of the biosensor in OVCAR4 cells, despite the challenges posed by polyploidy may be beneficial to reduce biological noise. Sequential rounds of CRISPR-Cas9 knock-in and meticulous screening of single-cell clones will be necessary to achieve this. Additionally, the expansion of assay conditions to include further drug treatments and combinations will likely provide novel biological information on the molecular mechanisms modulating PARP trapping. For example, exploring the effect of loss of PARG on PARP trapping, and its relationship to resistance. Loss of PARG is known to lead to an accumulation of PAR, reducing PARP trapping. Hence, exploring the effect of PARGi (to mimic the loss of PARG on PARP trapping) using the FRET biosensor developed here may offer insight into the role PARG plays in PARPi resistance.

Several methodological enhancements could be explored to improve the sensitivity and applicability of the FRET biosensor. Integrating the biosensor under a lentiviral system with an exogenous promoter could provide consistent expression levels and greater flexibility in labelling additional cell lines or

patient samples, addressing the low-throughput nature of CRISPR-Cas9 knock-in. Additionally, the changes in lifetime observed here were relatively small (on the order of 100-200 ns) and thus, an area of future work would be to optimise the FRET pair used in this biosensor. EGFP and mCherryFP have been used frequently, but it is well established that other FRET pairs offer higher quantum yields, brightness, and resistant against photobleaching. However, this is not a trivial task and changing FPs can sometimes result in a loss of signal from a biosensor, e.g., due to relative fluorophore orientation.

Expanding the scale of 3D imaging assays remains an important future direction, despite the technical challenges associated with cell culture and instrumentation. Addressing these challenges will be crucial for HCA in three-dimensional contexts, providing a more physiologically relevant assessment of PARP1 dynamics and PARPi resistance.

The genetically labelled PARP1 cell line developed in this study can be applied to a wide range of applications. For instance, micro-irradiation assays, commonly used to visualise PARP1 localization and trapping post-DNA damage, could benefit from using this cell line to observe endogenous PARP1. This would provide a more accurate representation of PARP1 behaviour in response to DNA damage, extending to other assays examining DNA repair mechanisms and drug responses.

In this thesis, automated FLIM-FRET assays were demonstrated, allowing the assessment of PARP trapping dynamics in HGSC cells. This allowed identification of a reduction of PARP trapping in resistant populations compared to sensitive cells. With further optimisation and expansion of this methodology to include multi-parametric readouts from multiple imaging modalities, this approach could potentially inform the development of novel PARP inhibitors, and the understanding of molecular mechanisms driving resistance to PARPi in HGSC.

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