

IMPERIAL

Development of a multibeam multiphoton multiwell-plate microscope (M³M) for 2D/3D FLIM FRET assays

William Flanagan

Light Community
Department of Physics
Imperial College London

Thesis submitted for the award of Doctor of Philosophy (Ph.D.)

Imperial College of Science, Technology and Medicine

Declaration of Originality

I declare that all work presented in this thesis is my own, and that any ideas, information, data, results and figures from another source have been appropriately referenced or acknowledged. No part of this degree has been submitted for another degree or qualification.

Copyright Declarations

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial-No Derivatives 4.0 International Licence (CC BY-NC-ND).

Under this licence, you may copy and redistribute the material in any medium or format on the condition that; you credit the author, do not use it for commercial purposes and do not distribute modified versions of the work.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Abstract

This thesis reports the development and application of a multibeam multiphoton multiwell plate microscope (M³M) with fluorescence lifetime imaging (FLIM) and high content analysis (HCA) capabilities based on the modular *openFrame* microscope stand. The instrument design, supported by numerical simulations, and its implementation and exemplar applications are described. As far as possible, all software components are open-source and modular hardware components can be easily interchanged or replaced. The factors impacting imaging speed are quantitatively analysed, including comparison with a conventional single excitation beam system. The integration of a novel polarisation-based differential phase contrast (pDPC) module to provide semi-quantitative phase imaging is described with its application to multiphoton FLIM HCA. Exemplar M³M HCA applications to 2D (monolayer) and 3D (spheroid) cell cultures are presented including FLIM assays of cellular autofluorescence to investigate modulation of metabolism reported by changes in NAD(P)H fluorescence lifetime. In 2D cell cultures this FLIM assay is combined with pDPC-based quantitative phase imaging to provide complementary label-free readouts that could be directly applied to primary cells. The M³M instrument was also applied to study the interaction of rucaparib, an intrinsically fluorescent anti-cancer drug, with ovarian cancer cells, including the partitioning inside cellular compartments where FLIM reported changes in local pH and provided a quantitative visualisation of rucaparib delivery in a tumour spheroid.

Author declaration

All the work presented in this thesis is my own with the following exceptions:

- Mouse melanoma spheroids in chapter 3 were supplied by Lucas Dent.
- Mouse lung tissue in chapter 3 was provided by Alix le Marois.
- Significant parts of the MultiHarp device adapter written for MicroManager were written by Sunil Kumar.
- The custom acquisition engine for MicroManager used to carry out multi-dimensional acquisitions was written by Sunil Kumar with minor modifications by myself.
- The programme used for lifetime fitting, *FLIMfit*, was previously written by Sean Warren in the group.
- The design and implementation of the pDPC modality on the microscope was aided by Huihui Liu.
- The pDPC reconstruction software was written by Huihui Liu.
- Cell culture for the rucaparib and NADH imaging experiments in chapters 6 and 7 were carried out by Edwin Garcia and Dan Marks.
- Work carried out or assisted by others is also attributed in the text as appropriate.

Acknowledgements

I'd like to say thank you to both of my supervisors for the opportunity to carry out such an exciting and interesting project. A big thanks to Paul French who provided constant guidance and encouragement, as well as freedom to find my feet in the world of microscopy research. Thanks also to Christopher Dunsby who provided stimulating and concise discussion throughout the project.

Thanks to Sunil Kumar for passing on his microscopy wisdom in an engaging and humorous manner and being kind and patient with all of my follies. Thanks also to Edwin Garcia, Dan Marks and Karishma Valand for kindly preparing biological samples and helping me understand a bit more about the world of biology.

Thanks to the many friendly and welcoming faces in the group who provided much relief in our shared microscopy-based distress & joy over the 4 years: Jonny, Connor, Leo, Sara, Zhonghe, Huihui, Meizhu, Liuba, MiQi + others.

Thank you to the workshop guys for all of their help building (and sometimes rejecting) parts for the microscope, and specifically to Martin for encouraging my artistic abilities.

A special thanks to Hugh whose sporadic karaoke sessions in the Crick lab kept me sane and alive, and for his extensive discussions on both optics and correct coffee pod etiquette, as well as Stefanos Koufidis – pray you return my Jeeves book soon.

Thanks to all of the administrative, cleaning and catering staff both Imperial and the Crick as well as the guys from Cairn who helped troubleshoot many issues.

Thanks to my friends, family and Martina who put up with me talking about microscopes significantly more in the past few years.

Funding for this research was provided by Cancer Research UK and the Physics Department of Imperial College London.

Table of contents

List of figures	- 12 -
List of abbreviations.....	- 22 -
1 Thesis overview	- 26 -
2 Fluorescence microscopy overview	- 29 -
2.1 Photo-physics.....	- 29 -
2.1.1 Fluorescence and related processes.....	- 29 -
2.1.2 Fluorescence properties.....	- 31 -
2.1.3 Photobleaching and photodamage.....	- 32 -
2.2 Fluorescence in biological samples.....	- 32 -
2.2.1 Autofluorescence	- 32 -
2.2.2 Fluorescent dyes.....	- 33 -
2.2.3 Immunolabelling	- 34 -
2.2.4 Fluorescent proteins	- 34 -
2.2.5 SNAPtag labelling.....	- 35 -
2.3 Light Microscopy.....	- 35 -
2.3.1 Brightfield microscopy	- 37 -
2.3.2 Phase contrast.....	- 37 -
2.3.3 Fluorescence microscopy.....	- 39 -
2.4 Biological sample formats	- 39 -
2.4.1 2D cell culture.....	- 40 -
2.4.2 Spheroids.....	- 40 -
2.4.3 Organoids.....	- 41 -
2.4.4 Tissue	- 41 -
2.4.5 In vivo	- 41 -
2.5 Fluorescence microscopy techniques.....	- 42 -
2.5.1 Confocal microscopy	- 42 -
2.5.2 Multiphoton microscopy	- 44 -
2.5.3 Light Sheet Fluorescence microscopy	- 46 -

2.5.4	Optical Projection Tomography (OPT).....	- 47 -
2.6	Fluorescence readouts	- 47 -
2.6.1	Intensity.....	- 47 -
2.6.2	Spectral fluorescence readouts.....	- 48 -
2.6.3	Fluorescence lifetime readouts.....	- 49 -
2.6.4	Fluorescence polarisation readouts	- 52 -
2.6.5	Readouts based on fluorescence dynamics	- 53 -
2.6.6	FRET.....	- 53 -
2.6.7	Multi-modal fluorescence microscopy.....	- 54 -
2.7	Advanced techniques	- 55 -
2.7.1	Super-resolution microscopy	- 55 -
2.7.2	Adaptive optics	- 56 -
2.7.3	High Content Analysis (HCA).....	- 57 -
3	Design and implementation of multibeam multiphoton multiwell plate microscope (M³M) with TCSPC FLIM	- 59 -
3.1	Design considerations	- 59 -
3.1.1	Motivation.....	- 59 -
3.1.2	Multiphoton beam multiplexing.....	- 61 -
3.1.3	Earlier multifocal multiphoton microscopes.....	- 63 -
3.2	Description of M ³ M excitation path.....	- 66 -
3.2.1	Laser, Power control and Beam Expansion	- 67 -
3.2.2	SLM	- 68 -
3.2.3	Scanning unit	- 71 -
3.2.4	<i>openFrame</i> microscope stand	- 74 -
3.2.5	Fibre Loom.....	- 74 -
3.2.6	TCSPC instrumentation	- 76 -
3.3	Software	- 79 -
3.3.1	Programming the SLM.....	- 80 -
3.3.2	Scan generation programme.....	- 85 -

3.3.3	MicroManager FLIM acquisition programme	- 87 -
3.4	<i>openFrame</i> components	- 90 -
3.4.1	Single photon imaging	- 92 -
3.4.2	<i>openFrame</i> microscope incubator.....	- 92 -
3.4.3	Non-descanned detection	- 93 -
3.4.4	Autofocus module	- 94 -
3.4.5	Trans-illumination.....	- 97 -
3.5	Application of M ³ M to fixed 3D biological samples	- 97 -
3.5.1	Mouse lung tissue.....	- 97 -
3.5.2	Mouse melanoma spheroids.....	- 99 -
3.5.3	Ovarian cancer spheroid segmentation	- 100 -
3.6	Summary	- 103 -
4	M³M 2nd prototype	- 104 -
4.1	Advances in M ³ M software.....	- 104 -
4.1.1	MultiHarp device adapter + MH cam	- 104 -
4.1.2	Automated acquisitions.....	- 107 -
4.1.3	Event stream interpreter	- 108 -
4.2	Advances in M ³ M excitation light path	- 110 -
4.2.1	Insight X3 Laser.....	- 110 -
4.2.2	Acousto-optic modulator.....	- 111 -
4.3	Improving throughput of excitation power	- 112 -
4.3.1	Objective lens fill factor.....	- 113 -
4.3.2	SLM diffraction efficiency.....	- 115 -
4.3.3	Impact of chromatic dispersion at the SLM.....	- 116 -
4.3.4	Collection lens magnification change	- 122 -
4.4	Optimisation of optical fibre loom.....	- 123 -
4.4.1	Multiple scattering of optical radiation	- 123 -
4.4.2	Experimental measurements of scattering in two-photon microscopy	- 125 -

4.4.3	Simulation of impact of scattering in two-photon microscopy	- 129 -
4.4.4	Construction of optical fibre loom	- 140 -
4.4.5	Cross-validation	- 141 -
4.5	Characterisation of second M ³ M implementation.....	- 142 -
4.5.1	Light throughput	- 142 -
4.5.2	Temporal performance of fluorescence detection.....	- 149 -
4.5.3	Comparison of multiple excitation beams with descanned detection and single beam excitation with non-descanned detection	- 150 -
4.6	Biological imaging.....	- 153 -
4.6.1	Live FRET construct spheroid assay.....	- 153 -
4.7	Summary	- 157 -
5	Quantitative phase imaging implementation in the M³M.....	- 158 -
5.1	Introduction to <i>pDPC</i>	- 158 -
5.1.1	Differential phase contrast (<i>DPC</i>) microscopy	- 159 -
5.1.2	<i>pDPC</i>	- 161 -
5.2	Implementation of <i>pDPC</i> in the M ³ M.....	- 164 -
5.2.1	Experimental set-up	- 164 -
5.2.2	<i>pDPC</i> reconstruction software	- 166 -
5.2.3	Exemplar M ³ M <i>pDPC</i> phase images.....	- 167 -
5.3	Exemplar applications of <i>pDPC</i> on the M ³ M	- 168 -
5.3.1	Multimodal M ³ M HCA with <i>pDPC</i> + NADH and FAD autofluorescence in OVCAR-4 cells	- 168 -
5.3.2	<i>pDPC</i> time lapse with NADH FLIM endpoint measurements.....	- 170 -
5.3.3	Analysis on <i>pDPC</i> image data	- 171 -
5.3.4	Summary	- 174 -
6	Application of the M³M to assays of metabolism.....	- 175 -
6.1	Readouts of changes in cell metabolic state.....	- 175 -
6.1.1	Intracellular NAD(P)H.....	- 175 -
6.1.2	FLIM of NAD(P)H	- 176 -

6.1.3	T2AMPKAR bio-sensor	- 177 -
6.1.4	Metabolism modulating drugs	- 177 -
6.2	Multiwell plate assay comparing NADH and T2AMPKAR readouts in cell monolayers following treatment with metabolism modulating drugs	- 178 -
6.2.1	Acquisition details.....	- 179 -
6.2.2	NADH shows greater sensitivity to metabolic changes than T2AMPKAR at 1 hour after drug treatment.....	- 180 -
6.2.3	Time response after drug application.....	- 182 -
6.2.4	pDPC morphological analysis.....	- 184 -
6.3	Multiwell plate FLIM of NADH reporting dose response of cells to phenformin in OVCAR-4 spheroids.....	- 187 -
6.3.1	Acquisition details.....	- 188 -
6.3.2	Phenformin dose response of OVCAR-4 spheroids at two time points	- 190 -
6.3.3	Variation of phenformin dose response across OVCAR-4 spheroids.....	- 192 -
6.3.4	Z-stack imaging.....	- 194 -
6.4	Summary	- 195 -
7	Application of the M3M to investigate cellular rucaparib dynamics	- 197 -
7.1	Introduction.....	- 197 -
7.1.1	Biological background	- 197 -
7.1.2	Initial rucaparib imaging experiments	- 198 -
7.2	2D rucaparib multiwell plate assay	- 199 -
7.2.1	Variation of rucaparib lifetime in different cellular compartments	- 199 -
7.2.2	Verifying lysosomal aggregation of rucaparib using bafilomycin.....	- 203 -
7.2.3	Observing the effect of olaparib in rucaparib treated cells	- 205 -
7.2.4	FRET of rucaparib to nucleic dye	- 207 -
7.3	3D rucaparib penetrance timelapse in spheroid	- 209 -
7.3.1	Experimental description	- 209 -
7.3.2	Results.....	- 210 -
7.4	Summary	- 214 -

8	Conclusions and future work.....	- 215 -
8.1	Conclusions.....	- 215 -
8.1.1	Imaging speed comparison.....	- 215 -
8.1.2	Scattering investigation.....	- 216 -
8.1.3	FLIM of rucaparib to analyse drug dynamics	- 217 -
8.1.4	pDPC and NADH FLIM readouts for analysis of cellular metabolic changes ..	- 217 -
8.2	Future work.....	- 218 -
8.2.1	Software	- 218 -
8.2.2	Automated multibeam to single beam switching	- 218 -
8.2.3	Adaptive optics for deeper imaging	- 219 -
8.2.4	Second harmonic generation imaging	- 219 -
	References	- 221 -

List of figures

Figure 1 Jablonski diagram showing the various transition routes an electron has in excitation and de-excitation. S_0 , S_1 , S_2 and T_1 are bands of vibrational and rotational energy levels associated with discrete electronic energy levels of the fluorophore.	- 30 -
Figure 2 Schematic of DNA modification used to produce fluorescently tagged protein of interest.	- 35 -
Figure 3 Infinity-corrected microscope configuration (non-telecentric).	- 36 -
Figure 4 Basic fluorescence microscope setup.	- 38 -
Figure 5 Microscope slide viewed from side and top with standard 170 micron coverslip.	- 40 -
Figure 6 Confocal microscope setup.	- 42 -
Figure 7 Multiphoton excited fluorescence confined to small volume at the focus of excitation beam.	- 45 -
Figure 8 MACH3cancer microscope development overview. The microscope developed in this project (highlighted in orange box) is intended for used on the more physiologically complex samples.	- 60 -
Figure 9 M3M multiplexed excitation/descanned detection breadboard and <i>openFrame</i>	- 66 -
Figure 10 Line and grid multifocal patterns. The line of beams allows for larger scan length in x axis compared to the grid of beams where overlapping scan regions occurs after scanning one beam spacing.	- 69 -
Figure 11 Schematic of phase ramp causing change in wavefront reflection angle on SLM ...	- 70 -
Figure 12 Beam scanning optics showing one galvo mirror. Displacing the galvo from the back focal plane of L3 causes a shift of the field at the objective pupil.	- 71 -
Figure 13 Engineering drawing of 2 axis galvo unit displaying horizontal shift between x and y galvo position.	- 72 -
Figure 14 Beam orientations along optical path showing rotation of field due to galvos, shown for vertical and pre-rotated beam array.	- 73 -
Figure 15 Setup used to image fibre output to HPMA active area in the M ³ M descanned detection path. Not to scale.	- 75 -
Figure 16 Electronics used to process pulsed laser and detector signals in a standard TCSPC setup. The green and red pulses are from the laser sync and detector outputs respectively.	- 77 -

Figure 17 Block diagram showing the different software components, and the hardware controlled by them. Colour coded by programming language.....	- 80 -
Figure 18 Flowchart indicating how the Gerchbeg-Saxton algorithm operates. As the algorithm iterates over the loop, the SLM phase ϕ_i more accurately generates the desired far-field amplitude.-	81
Figure 19 Output plots from SLM hologram programme. (a) The final phase distribution found by the algorithm, (b) the predicted intensity pattern in the far field, (c) the weights per beam used in the final iteration, (d) the intensity distribution used to illuminate the SLM, (e) the final predicted beam amplitudes and (f) the weights of each beam as a function of iteration.....	- 83 -
Figure 20 SLM control GUI in MicroManager able to shift individual beams as well as create and load a multibeam pattern.....	- 84 -
Figure 21 Labview scan control GUI used to send voltage waveforms to scanning mirrors to control their scan angles.....	- 85 -
Figure 22 X-Y scan voltages shown for (a) normal and (b) angled scans.....	- 86 -
Figure 23 (a) Line and frame clock signals for one frame with 20 X points per line, 20 lines and 10% flyback, and (b) zoomed in waveform showing 2 line markers and a frame start.....	Error!
Bookmark not defined.	
Figure 24 Steps used to fine tune scan parameters for an aligned scan field. (a) wrong angle, (b) wrong scan range, (c) correct pattern. Note that the small dark strip seen in all of the images is due to exposure time of the camera not matching scan period of the galvos exactly. Images obtained using widefield camera and fluorescent slide.....	- 88 -
Figure 25 MicroManager plug-in used to execute SymPhoTime acquisitions. GUI contains areas for (a) parameter input, (b) connecting to LabView and (c) running the full acquisition.....	- 89 -
Figure 26 Schematic of M ³ M <i>openFrame</i> -based microscope implemented for the work reported in this thesis.....	- 90 -
Figure 27 Photograph of <i>openFrame</i> microscope in a later configuration showing autofocus unit (red), incubator (orange), NDD (yellow), single photon illuminator (green), phase imaging path (cyan), slider layers (blue) and multibeam multiphoton excitation breadboard (purple).....	- 91 -
Figure 28 Cells containing Syto82 nuclear staining imaged with the single photon excitation port.	- 92 -
Figure 29 Optical setup for the non-descanned imaging path. The relay from objective pupil to NDD used a turning mirror so that the detector could be mounted to the underside of the incubator.....	- 94 -

Figure 30 Optical layout of the autofocus unit. An SLD is coupled into the unit using an optical fibre which is elliptically collimated by two cylindrical lenses. The light is then coupled into the *openFrame* where it reflects off the cover slip, after which the reflection is imaged onto a camera.- 95

-

Figure 31 Beam expansion and modified focus of autofocus system..... - 96 -

Figure 32 Orthoplots of actin labelled with phalloidin-Alexa633 (red) and nuclei labelled with CFP-YFP FRET biosensor (green) staining in mouse lung tissue acquired using the M3M system with an excitation wavelength of 800 nm. - 98 -

Figure 33 Two-colour z-stack overlay of actin labelled with phalloidin-Alexa633 (red) and nuclei labelled with CFP-YFP FRET biosensor (green) staining in mouse lung tissue imaged using the M3M system at an excitation wavelength of 800 nm. - 98 -

Figure 34 Same data as actin channel shown in Figure 28 but presented as z-slices shown in steps of 50 microns from 20 to 270 microns imaging depth, showing decreasing intensity with depth. Images displayed with the same contrast scale. FOV is 360 x 360 microns. - 99 -

Figure 35 3D M3M FLIM image of mouse cell spheroids where the histones are tagged with IRFP670. Excitation at 820 nm. The constant colour shows a constant lifetime throughout the image. 360 x 360 x 100 micron FOV with 1 micron cubed voxels. - 100 -

Figure 36 (a) single and (b) six beam images taken at a depth of 50 microns into patient derived spheroids (different spheroid in each image). This figure illustrates the similar level of image quality obtained with 6 excitation beams compared to 1 beam. The single-beam data was used to produce the 3D segmentation mask shown in Figure 34. - 101 -

Figure 37 (a) 3D render of single beam z-stack of nuclei-stained spheroid and (b) 3D segmentation mask produced using data from (a). - 102 -

Figure 38. Flowchart showing the processes of an interleaved multidimensional TCSPC acquisition. A MicroManager Beanshell script is used to switch the microscope modality between multibeam-multiphoton and brightfield modes. The MicroManager custom acquisition plug-in allows the Beanshell script to be run at z-depth, time series and XY positions specified by the user. The MultiHarp device adapter runs the appropriate MultiHarp DLL functions to carry out a FLIM acquisition. - 106 -

Figure 39 Screenshot of plug-in developed by Sunil Kumar for creating multi-dimensional acquisitions in MicroManager with the pDPC_MM script loaded. - 108 -

Figure 40 Illustration of how MicroManager plugin can be used to execute a python script which processes TTTR files into tiff files that are readable by *FLIMfit*. - 109 -

Figure 41 Average output power tuning ranges for Insight X3 and MaiTai lasers.....	- 110 -
Figure 42 Schematic showing beam path in second iteration of M3M from Insight laser to M3M breadboard via the AOM.	- 112 -
Figure 43 Plot of fluorescence signal coming from a thin 2D sheet of fluorescence, lateral resolution and power throughput normalised to max values and plotted as a function of magnification from the GBE.	- 113 -
Figure 44 Schematic of GBE convergence used to produce smaller field size on the SLM to underfill objective pupil.....	- 114 -
Figure 45 Efficiency of 1st diffracted order from a blazed phase grating for varying lateral displacements from the optical axis in the focal plane after L1. Theoretical prediction comes from equation 3 in chapter 3.....	- 115 -
Figure 46 Efficiency of power diffracted into unevened 6 beam line pattern plotted as a function of beam spacing.....	- 116 -
Figure 47 (a) PSFs plotted for 5 delta-function spectral lines for a beam diffracted 49 and 160 microns from the optical axis at the sample plane and (b) the sum of the individual wavelength PSFs. The intensity distribution is wider with a lower intensity for larger diffraction distances	- 118 -
Figure 48 Spectrally summed intensity distributions for a range of off-axis diffraction distance (d_0) values measured at the sample plane.....	- 119 -
Figure 49 (a) Sum of squares of values in PSF, (b) relative change in pulse duration and (c) resulting two-photon signal all as a function of d_0	- 120 -
Figure 50 Ratio of fluorescence in off-axis beam deflected by SLM and galvo as a function of off-axis distance measured experimentally (red) and predicted by simulation (blue).	- 121 -
Figure 51 Schematic of a photon scattering into a polar angle θ and azimuthal angle ϕ	- 124 -
Figure 52 Setup for measuring cross-talk as a function of beam-fibre distance. As the beam is shifted from the fibre using the SLM the amount of collected scattered fluorescence, shown here in a more transparent green shade, is measured.....	- 125 -
Figure 53 PMT counts as a function of beam shift at fibre plane using a fluorescent slide. Red lines indicate fibre diameter. FWHM of the beam at the fibre plane is ~ 30 μm	- 126 -
Figure 54 Fluorescence collected in 200 μm diameter optical fibre as a function of beam-optical fibre displacement and imaging depth.	- 127 -
Figure 55 Fluorescence collected in 1000 μm fibre as a function of beam-optical fibre displacement and imaging depth.....	- 127 -

Figure 56 Signals measured in 200, 600 and 1000 μm diameter fibres as a function of imaging depth for an excitation beam centred on the optical fibre detection area. (Signals for each diameter optical fibre are normalised that obtained with the 200 μm fibre at that depth).	- 128 -
Figure 57 Flowchart of the functions carried out in photon scattering simulation	- 131 -
Figure 58 Schematic showing how perceived photon position at the focal plane is altered for a photon undergoing the same series of scattering events, but excited at different tissue depths. ...	- 132 -
Figure 59 Normalised histograms of values sampled for distance travelled between scattering events and $\cos(\theta)$ with their theoretical probability distributions.	- 133 -
Figure 60 Histograms of photon polar angle after 1, 3 and 5 scatters for (a) $g = 0.9$ and (b) 0.7	- 134 -
Figure 61 Photon polar angle on escape for z depths 100 - 900 microns.	- 135 -
Figure 62 Histogram of number of scattering events per photon that exited the tissue for $z = 150$ μm and $L_s = 100$ μm and release angle of 20 degrees.	- 135 -
Figure 63 Scattered photons at fibre plane for depths of 1, 100, 200 and 300 microns. Red and green dotted circles show the 200 and 1000 micron core diameter fibres used respectively. Simulation performed using $g = 0.9$ $L_s = 100$ microns.	- 136 -
Figure 64 Simulation and experimental ratio of fluorescence captured by a 1000 μm fibre to a 200 μm diameter optical fibre using parameters of $g = 0.95$ and $L_s = 40$ μm as a function of imaging depth.	- 137 -
Figure 65 Ratio of signal collected in shifted vs centred fibre as a function of shift distance. $g = 0.95$ $L_s = 40$ μm	- 137 -
Figure 66 Heatmap showing the cross-talk % as a function of imaging depth and fibre spacing. Red outline shows regions where cross-talk exceeds 5%. $g = 0.95$ $L_s = 40$ μm	- 138 -
Figure 67 Comparison between old and new fibre looms geometries. (To scale)	- 139 -
Figure 68 Back and side view of the second iteration fibre housing unit with fibre.	- 141 -
Figure 69 Mean intensity per slice as a function of depth for the 1000 μm and 200 μm core fibres imaging mouse lung tissue and ratio of the signal from 1000 μm fibre to 200 μm fibre as a function of depth.	- 142 -
Figure 70 Power throughput for beams diffracted to three positions off the optical axis measured at L1, normalised to the power measured after the SLM.	- 143 -
Figure 71 Percentage light throughput for each component individually in the direction of propagation towards the sample together with the expected throughput from specification.	- 143 -

Figure 72 Setup used to measure fluorescence throughput through system for the 0 and 4 mm positions. The beam position is controlled by tilting the collimated laser diode module, expanding the beam and directing it into the back focal plane of the objective. Lenses shown by double-ended arrows.	- 144 -
Figure 73 Power measured through system going from sample along fluorescence path towards fibre loom.	- 145 -
Figure 74 Percentage light throughput away from sample along fluorescence path, with specification throughput.	- 146 -
Figure 75 Throughput for each fibre, and throughput to PMT with lens relay and no lens. ..	- 147 -
Figure 76 Fluorescence throughput in NDD path. Power measured was normalised to value after objective.	- 148 -
Figure 77 Ratio of NDD to descanned fluorescence throughput as function of spot position at fibre plane.	- 148 -
Figure 78 (a) Rhodamine 6g and (b) gold nanorods fluorescence decay profiles acquired using the PicoQuant Hybrid PMA40.	- 149 -
Figure 79 Intensity as a function of depth for 6-beam descanned and single-beam non-descanned configurations.	- 151 -
Figure 80 (a) Brightfield, (b) single photon widefield fluorescence image and (c) overlay of spheroid expressing the mTq2-5-mV construct.	- 153 -
Figure 81 FRET construct assay plate layout with expected lifetime trend shown by colour.	- 154 -
Figure 82 Multiwell-plate FLIM acquisition of spheroids expressing the FRET constructs, with one z-slice FOV acquired per well.	- 155 -
Figure 83 Box plot of mean lifetime per well for each FRET construct. (Outliers shown as circles)	- 155 -
Figure 84 3D imaging of wells C3 and D10 with colour showing lifetime fit for total stack.	- 156 -
Figure 85 Schematic of differential phase contrast microscopy (DPC) implemented with sequential acquisition of images using masks to provide oblique illumination by blocking half pupil plane of condenser lens.	- 159 -
Figure 86 Polarisation mask placed in the back focal plane of the condenser lens to multiplex the illumination patterns needed for pDPC microscopy.	- 161 -
Figure 87 Polarisation masks placed over every 2 x 2 pixels in the Polcam sensor.	- 162 -

Figure 88 (a) Exemplar pDPC phase image acquired on an openFrame-based microscope with (b) surface plot and (c) line profile . (Figure adapted from [153])	- 163 -
Figure 89 pDPC illumination arm following beamlets at two emission angles from the MonoLED.	- 164 -
Figure 90 Imaging side of the pDPC setup showing the light collected from one point at the sample plane.....	- 165 -
Figure 91 Settings panel on the pDPC MicroManager plug-in.	- 166 -
Figure 93 Exemplar pDPC image taken of HEK cells using the M3M. Error! Bookmark not defined.	
Figure 92 Exemplar pDPC image taken of OVCAR-4 cells using the M3M.....	- 167 -
Figure 94. M3M autofluorescence and pDPC imaging of OVCAR4 cells. (a) NADH (green) and FAD (red) multibeam multiphoton fluorescence overlay image, (b) pDPC image and (c) NADH, FAD and pDPC overlay.	- 169 -
Figure 95 Multiwell plate acquisition of OVCAR4 cells. pDPC (grey scale), NADH (green) and FAD (red).....	- 170 -
Figure 96 pDPC timelapse of OVCAR4 cells with NADH autofluorescence FLIM endpoint measurements at 0 and 15 hours.	- 171 -
Figure 97 pDPC image of HEK293 cells with manually annotated foreground (red lines) and background (blue lines) in Labkit (left) and resulting segmented image (right).	- 172 -
Figure 98 Cellpose segmented pDPC image of OVCAR-4 cells.....	- 172 -
Figure 99 Exemplar CellProfiler processing pipeline illustrated with pDPC image of HEK293 cells. An (a) input image is used for (b) object identification, which are then (c) filtered based on their size, the remaining objects are (d) filled from which final shape properties such as area and eccentricity can be measured.	- 174 -
Figure 100 Relation between predominant metabolic pathway, abundances of NADH & NAD ⁺ , and overall lifetime measured.	- 176 -
Figure 101 Plate maps for experiments carried out at 1 hour and 2 & 4 hours after drug application.	- 178 -
Figure 102 Images of unlabelled cells imaged in the NADH channel as well as active and mutant T2AMPKAR cells imaged in the mTurquoise2 channel. From the 1 hour post treatment experiment.....	- 180 -

Figure 103 Average mean weighted lifetime of NADH (rows B to C) and mTurquoise2 of T2AMPKAR FRET sensor (rows D-G) per well for the 1-hour experiment.....	- 181 -
Figure 104 Average mean weighted lifetime per column across the plate for both NADH and T2AMPK.	- 181 -
Figure 105 Average HEK293 NADH weighted mean lifetime per condition for each time point...-	182 -
Figure 106 Average HEK293 T2AMPKAR weighted mean lifetime per condition for each time point. (Data point at 2 hours for 10 uM phenformin wasn't taken due to a brief autofocus malfunction during the acquisition)	- 183 -
Figure 107 Average HEK293 T2AMPKAR mutant weighted mean lifetime per condition for each time point.	- 183 -
Figure 108 pDPC image, FLIM image and overlay of HEK293 cells in well B9 from the 1-hour experiment.....	- 184 -
Figure 109 pDPC and pDPC/FLIM overlay of HEK293 cells across multiwell plate in the 1 hour experiment.....	- 185 -
Figure 110 pDPC image for (a) untreated, (b) 0.1 uM rotenone and (c) 1 uM rotenone treated HEK293 cells (1-hour experiment).....	- 185 -
Figure 111 Original pDPC image and labkit foreground/ background segmented image.	- 186 -
Figure 112 Average phase per segmented foreground pixel in HEK293 cells plotted for each column in the 1-hour experiment.	- 186 -
Figure 113 Average phase per segmented foreground pixel plotted for each column in the 4-hour experiment.....	- 187 -
Figure 114 Plate layout for NADH FLIM phenformin dose response in OVCAR-4 spheroids.....-	188 -
Figure 115 Brightfield image of OVCAR4 spheroid used in pre-finding step. Monolayer cell colonies are seen alongside the spheroid. Red square shows multiphoton FOV.	- 189 -
Figure 116 Quantification of OVCAR-4 spheroid response to phenformin. (a) Weighted mean lifetime for 1 hour and 4 hour measurements and (b) % change in weighted mean lifetime plotted as a function of concentration on a logarithmic scale. 8 spheroids were imaged per concentration when phenformin is applied and 24 for untreated spheroids.	- 190 -
Figure 117 NADH FLIM multi-well plate acquisition of phenformin dose response in OVCAR-4 spheroids 1 (left) and 4 (right) hours after drug application.	- 190 -

Figure 118 OVCAR-4 NADH fluorescence (a) intensity for 1 hour and 4 hour measurements and (b) % change in intensity plotted as a function of phenformin concentration on a logarithmic scale. 8 spheroids imaged per concentration for phenformin treated spheroids and 24 for untreated spheroids..- 191 -

Figure 119 Average weighted mean lifetime (top) and intensity (bottom) per well across the multi-well plate for the measurement taken after 1 hour in phenformin treated OVCAR-4 spheroids... - 192 -

Figure 120 NADH autofluorescence from spheroid in well D6 suggesting difference in mean lifetime between inner and outer regions. - 192 -

Figure 121 Segmented regions found for inside (blue) and outside (green) of spheroid with rejected FOVs shown in red - 193 -

Figure 122 Difference (outer – inner) in fluorescence lifetime between segmented regions shown for both time points as a function of phenformin concentration. - 194 -

Figure 123 Z-stacks taken of spheroids in 0, 100 and 2000 micromolar phenformin conditions. Z-stacks are shown in a montage with 5 micron increments between images going from left to right and then top to bottom. - 195 -

Figure 124 M3M FLIM image of rucaparib in OVCAR-4 cells..... - 198 -

Figure 125 Multiwell plate layout used for variety of rucaparib experiments using rucaparib (ruc) in OVCR-4 cells, LysoTracker Red (lyso), SYTO Deep Red (syto), the olaparib PARP inhibitor (Olap) and a lysosome inhibitor (BF1A). 1x and 10x indicates 5 and 50 μ M concentrations respectively.- 199 -

Figure 126 Co-registered image of SYTO Deep Red nuclear staining with two-photon imaging shown in red and single-photon imaging shown in green..... - 200 -

Figure 127 OVCAR-4 cells and (a) rucaparib M3M FLIM image (b) wide-field single-photon excited LysoTracker Red image, and (c) overlay image using binarised mask of LysoTracker Red image, as well as lifetime histograms of (d) unsegmented and (e) LysoTracker mask segmented images of OVCAR-4 cells from well D10 of multiwell plate..... - 201 -

Figure 128 OVCAR-4 cells. (a) Rucaparib M3M FLIM image (b) wide-field single-photon excited SYTO Deep Red image, (c) overlay image using binarised mask of SYTO Deep Red image, as well as lifetime histogram of (d) unsegmented and (e) SYTO Deep Red mask segmented images of OVCAR-4 cells from D9 of multiwell plate. - 202 -

Figure 129 M3M FLIM of rucaparib treated OVCAR-4 cells (a) with and (b) without bafilomycin with their respective lifetime histograms across all 8 images shown in (c) and (d). The addition of bafilomycin removes the low lifetime regions seen in the rucaparib only images. - 203 -

Figure 130 FFT of rucaparib imaged in cells treated with and without bafilomycin.	- 204 -
Figure 131 Rucaparib intensity with and without the presence of lysosome inhibitor bafilomycin. - 205 -	
Figure 132 Intensity and weighted mean tau box plots for 5 microM rucaparib treated OVCAR-4 cells with and without prior 50 microM olaparib treatment.	- 207 -
Figure 133 Weighted mean tau histograms OVCAR-4 cells treated with (a) rucaparib and (b) rucaparib with SYTO Deep red nuclear dye, with (c) overlay of the histograms showing rucaparib in orange and rucaparib + SYTO in green. Histograms generated from 8 FOVs per condition.	- 207 -
Figure 134 Emission spectra of SYTO deep red dye plotted with the transmission spectra of the emission filter used for rucaparib imaging.	- 208 -
Figure 135 Box plots of weighted mean lifetime of rucaparib per well for cells treated with and without SYTO deep red.	- 208 -
Figure 136 Spectral overlap of the emission spectra of rucaparib and excitation spectra of SYTO Deep dye.	- 209 -
Figure 137 OVCAR-4 spheroid with inner and outer segmentation regions shown.	- 210 -
Figure 138 (a) Intensity and (b) weighted mean lifetime in rucaparib treated spheroid shown at 10 second intervals for inner and outer segmented regions.	- 211 -
Figure 139 Average rucaparib intensity in OVCAR-4 spheroids as a function of time for the inner and outer segmented regions over 6 minutes.	- 212 -
Figure 140 OVCAR-4 spheroids treated with rucaparib. Intensity as a function of time shown until 3 minutes with intensity of inner and outer regions shown normalised to their maximum value..	- 213 -
Figure 141 OVCAR-4 spheroids treated with rucaparib. Weighted mean lifetime as a function of time for inner and outer regions.....	- 213 -

List of abbreviations

- **ADC** – Analog-to-Digital Converter
 - **ADP** – Adenosine Diphosphate
 - **AF** – Autofocus
 - **AMP** – Adenosine Monophosphate
 - **AMPK** – AMP-Activated Protein Kinase
 - **AOM** – Acousto-Optic Modulator
 - **ATP** – Adenosine Triphosphate
-
- **BFP** – Back Focal Plane
 - **BME** – Basement Membrane Extract
-
- **CDPC** – Colour Differential Phase Contrast
 - **CFP** – Cyan Fluorescent Protein
 - **CFD** – Constant Fraction Discriminator
 - **CMOS** – Complementary Metal-Oxide Semiconductor
 - **CRUK** – Cancer Research UK
 - **CRISPR** – Clustered Regularly Interspaced Short Palindromic Repeats
-
- **DAQ** – Data Acquisition
 - **DAPI** – 4',6-Diamidino-2-Phenylindole (DNA stain)
 - **DIC** – Differential Interference Contrast
 - **DNA** – Deoxyribonucleic Acid
 - **DLL** – Dynamic Link Library
 - **DPC** – Differential Phase Contrast
 - **DVI** – Digital Visual Interface
-
- **ETC** – Electron Transport Chain

-
- **FAD** – Flavin Adenine Dinucleotide
 - **FCS** – Fluorescence Correlation Spectroscopy
 - **FCCP** – Carbonyl Cyanide-p-Trifluoromethoxyphenylhydrazone (*mitochondrial uncoupler*)
 - **FLIM** – Fluorescence Lifetime Imaging
 - **FLIRR** – Fluorescence Lifetime Redox Ratio
 - **FP** – Fluorescent Protein
 - **FRAP** – Fluorescence Recovery After Photobleaching
 - **FOV** – Field of View
 - **FRET** – Förster Resonance Energy Transfer
 - **FWHM** – Full Width at Half Maximum
 - **FFT** – Fast Fourier Transform
-

- **GFP** – Green Fluorescent Protein
 - **GS** – Gerchberg Saxton
 - **GUI** – Graphical User Interface
 - **GBE** – Galilean Beam Expander
 - **GPU** – Graphics Processing Unit
-

- **HCA** – High Content Analysis
 - **HEK** – Human Embryonic Kidney (cells)
 - **HPMA** – Hybrid Photomultiplier Assembly
 - **HIV** – Human Immunodeficiency Virus
 - **HWP** – Half Wave Plate
-

- **IRF** – Instrument Response Function
 - **IRFP** – Infrared Fluorescent Protein
-

- **LCOS** – Liquid Crystal on Silicon
- **LED** – Light Emitting Diode
- **LP** – Linear Polariser
- **LSCM** – Laser Scanning Confocal Microscopy

-
- **MAPMT** – Multi-Anode Photomultiplier Tube
 - **MM** – Multibeam Multiphoton

-
- **NADH** – Nicotinamide Adenine Dinucleotide
 - **NADPH** – Nicotinamide Adenine Dinucleotide Phosphate
 - **NA** – Numerical Aperture
 - **NDD** – Non-Descanned Detection

-
- **OPL** – Optical Path Length
 - **OPT** – Optical Projection Tomography
 - **OPM** – Oblique Plane Microscopy
 - **OVCAR** – Ovarian Cancer (cell line)

-
- **PBS** – Phosphate Buffered Saline
 - **PARP** – Poly (ADP-ribose) Polymerase
 - **PARPi** – PARP Inhibitor
 - **PDPC** – Polarisation-Differential Phase Contrast
 - **PMT** – Photomultiplier Tube
 - **PSF** – Point Spread Function

-
- **QPI** – Quantitative Phase Imaging

-
- **SHG** – Second Harmonic Generation

- **SLM** – Spatial Light Modulator
 - **SMLM** – Single Molecule Localization Microscopy
 - **SPAD** – Single-Photon Avalanche Diode
 - **SLD** – Superluminescent Diode
 - **SIM** – Structured Illumination Microscopy
 - **STED** – Stimulated Emission Depletion
 - **STORM** – Stochastic Optical Reconstruction Microscopy
-

- **TAC** – Time-to-Amplitude Converter
 - **TCSPC** – Time-Correlated Single Photon Counting
 - **TTTR** – Time-Tagged Time-Resolved
-

- **YFP** – Yellow Fluorescent Protein

1 Thesis overview

Automated multiwell plate microscopy is a powerful tool for drug discovery and biology research, enabling rapid interrogation of arrays of samples prepared with consistent methodology and systematic manipulations (e.g., drug treatments, gene knockouts, etc.) while enabling repeats of experimental measurements to provide statistically robust readouts integrating data from many fields of view (FOV). When implemented with subcellular resolution, this approach is described as high content analysis (HCA). As well as enabling investigations at throughput, single cell resolved time-lapse HCA of live cells can reveal heterogeneity in response to drugs or other manipulations. This may provide insights into mechanisms of disease progression or drug resistance. To date, most HCA instrumentation is limited to relatively basic microscopy techniques such as fluorescence microscopy with spectral or polarisation-resolved imaging, with the commercial state of the art being quasi-confocal spinning disc imaging. In recent years, however, there is considerable interest in assays of disease models of increasing complexity to better recapitulate the in vivo context. These include 3D cell cultures such as spheroids or organoids that can be grown and imaged in multiwell plates or tissue slices, as well as small organisms such as *C. elegans*. Such samples can compromise quantitative intensity-based assays and can benefit from advanced techniques including fluorescence lifetime imaging (FLIM) for more quantitative readouts and multiphoton microscopy for deeper imaging in heterogenous and scattering biological samples, but these capabilities are typically not available in commercial HCA instruments.

The research in this thesis aimed to address the need for HCA instruments that could combine advanced imaging modalities including multiphoton excitation, FLIM, and quantitative phase imaging (QPI), including for single-cell-resolved time-lapse assays. Commercial HCA instruments are already beyond the budget of most laboratories and present proprietary barriers to being upgraded. If such advanced instruments were offered commercially, they would be even more expensive than the current state of the art, further limiting their accessibility. Accordingly, our laboratory at Imperial is developing a modular microscope platform (www.openScopes.com) that users can use to configure a range of instruments using open-source software with flexibility to modify or upgrade functionality as and when needed. Within this context, this thesis describes the development and application of an automated multibeam multiphoton multiwell plate microscope (M³M) with FLIM and QPI capabilities based on a modular *openFrame* microscope stand that can enable microscopes to be assembled from a basic component cost of £10,000 to < £150,000 component cost for the M³M (excluding excitation laser).

Since a key motivation was to enable single-cell-resolved time-lapse assays with 3D resolution over multiple hours (cell cycles), an important consideration was to minimise phototoxicity. While multiphoton microscopy results in lower photobleaching/phototoxicity than single-photon excited confocal microscopy when imaging a volume (z-stack), the higher intensity results in greater photobleaching/toxicity in a single optical section. Further, the degree of phototoxicity increases

nonlinearly with excitation intensity faster than the quadratic intensity dependence of multiphoton excitation [1]]. We therefore designed a multibeam multiphoton microscope so that the peak excitation intensity required to image at a given rate would be reduced due to the multiplexed excitation beams. Conversely, this would also enable the imaging rate to be increased for a given degree of phototoxicity with the imaging rate scaling with the number of excitation beams. When a single assay can take many hours, even a modest reduction of total image data acquisition can be essential to make it practically achievable.

We implemented time-correlated single photon counting (TCSPC) to detect the fluorescence photons using (high quantum efficiency, large area) hybrid photomultipliers and enable FLIM with high photon detection efficiency. This microscope should thus facilitate 2D and 3D multiphoton FLIM assays, providing biologists with practical advanced assay capabilities applicable to more physiologically complex samples. Thus, the M³M is intended to make HCA with advanced microscopy modalities available (and affordable) for the first time at Imperial and the Francis Crick Institute. The cost-effective open-source software/open hardware approach was intended to provide accessibility to the wider research community.

The following text gives an overview of the contents of each chapter:

Chapter 2 introduces the photophysics of fluorescence, the properties and use of fluorophores and the standard fluorescence imaging modalities typically utilised in microscopy, including FLIM and techniques for optical sectioning in 3D samples. An overview of QPI is also provided and the use of optical microscopy techniques in HCA is explored.

Chapter 3 describes the first implementation of the multibeam multiphoton microscope on an *openFrame* microscope stand. The considerations for a multibeam multiphoton microscope are discussed in the context of other multibeam multiphoton instruments that have been reported. A description of the optical components and subsystems used for the 6-beam multiphoton excitation is given, with an overview of the software used to control these components and a discussion of how FLIM TCSPC data is obtained and processed in the M³M. The modular *openFrame*-based microscope is presented outlining the capabilities it offers for live cell multiwell plate imaging, including a novel prototype modular microscope incubator that can be configured for multiple imaging modalities. Some exemplar images of volumetric (z-stack) FLIM of fixed spheroids and a tissue slice are presented, illustrating the potential of the M³M for 3D multiwell plate assays.

Chapter 4 reports the development of the M³M after it was transferred to the Francis Crick Institute. New MicroManager software was written to enable efficient multibeam, multiphoton TCSPC FLIM data acquisition and processing, as well as providing a live 6-beam multiphoton view. Optimisation of the multibeam multiphoton microscope for deeper imaging is discussed, noting that we were excitation power-limited at larger imaging depths and that optical scattering would degrade the collected

fluorescence signals. Experiments and simulation are carried out to quantify the effect of scattering when imaging deeper into a biological sample, informing a new design of the optical fibre configuration used to collect more fluorescence in the 6 parallel channels when imaging scattering samples. The excitation and fluorescence light throughput of the microscope was characterised and the performance of the 6-beam set-up was compared to a conventional single excitation beam multiphoton microscope. Finally, an exemplar 3D FLIM assay using this new M³M configuration is presented.

Chapter 5 describes the implementation of QPI using the polarisation resolved differential phase contrast (pDPC) technique on the microscope. pDPC is introduced and the adaptation required to implement this on the M³M is discussed. A multiwell plate assay interleaving QPI with multibeam multiphoton fluorescence imaging of NADH and FAD is described, as well as a time lapse QPI assay with FLIM data acquired at the endpoints. Label-free image segmentation is a key application of QPI for HCA and some open-source analysis software tools are applied to the pDPC phase image data.

Chapter 6 describes the application of the M³M to fluorescence lifetime imaging of NADH in 2D and 3D cell cultures to illustrate its capability for label-free readouts of the metabolic functioning of cells under a set of drug treatments. Autofluorescence lifetime readouts for NADH were compared to readouts of a genetically expressed FRET biosensor, T2AMPKAR, that reports AMPK activity. For the 2D assay, pDPC phase image data was used to segment cells and to quantify cell rounding during apoptosis under drug treatments. An image data processing pipeline to elucidate lifetime differences in the inner and outer regions of spheroids is also described.

Chapter 7 presents the M³M applied to investigate the intracellular behaviour of an innately fluorescent anticancer drug: rucaparib, which is a PARP inhibitor. The fluorescence lifetime of the drug was found to vary throughout the cell and multiwell plate imaging of 2D cultures of ovarian cancer cells were undertaken to explore this heterogeneity. The dynamics of rucaparib permeating into a spheroid was also evaluated using the M³M.

Chapter 8 summarises the progress made and findings during this PhD thesis research and discusses future prospects for the M³M, including how the instrument can be improved and extended.

2 Fluorescence microscopy overview

This section will introduce the process of fluorescence and how it occurs in a biological context. The basic methods of light microscopy are discussed as well as the formats used to mount biological samples. Further microscopy techniques focussing on 3D imaging are then considered, as well as a discussion of the type of fluorescence readouts often acquired. Finally, some advanced microscopy techniques are explored.

2.1 Photo-physics

The basis for fluorescence microscopy is spontaneous emission from stochastic decays of excited electrons in molecules that are generically described as fluorophores. This section lays out the physics involved in fluorescence and reviews some of the properties of commonly used fluorophores.

2.1.1 Fluorescence and related processes

Fluorescence is the process in which a molecule (fluorophore) first absorbs a photon that excites an electron from its ground state to a higher energy level, from which the electron can then dissipate energy in a variety of ways, finally relaxing from a lower excited state back to the ground state, emitting a photon of a lower energy than the initial excitation photon. For this process to occur the excitation photon must have the appropriate energy to excite an electron from the ground state to an available excited state of the fluorophore. The range of appropriate excitation photon energies is described as the excitation spectrum.

Upon absorption of a photon, a molecule initially in the lowest electronic state S_0 , usually the only occupied state at room temperature, can be promoted to any of the higher electronic energy levels S_n and higher vibrational modes of the molecule. The vibrational excitation of the molecule can relax, e.g. through collisions with the solvent, leaving the molecule in the lowest vibrational mode of the excited electronic state. The molecule may then transition to a lower electronic energy level via a process called internal conversion; here the rate of transition is inversely proportional to the energy difference between levels, meaning it is more rapid for closely spaced energy levels. Internal conversion would then be followed by vibrational relaxation to the lowest vibrational level of the new electronic state. Both of these processes allow a molecule to release energy without emitting a photon and are known as non-radiative transitions. As these non-radiative transitions occur at a much higher rate than fluorescence, they outcompete the fluorescence pathways for all energy levels until the lowest vibrational state of the electronic energy band, S_1 , is reached. At this point, the internal conversion rate is similar to that of the fluorescence rate due to the larger energy gap between S_1 and S_0 , meaning both processes can occur. These considerations result in Kasha's rule, which states that the majority of fluorescence from an excited molecule will be emitted from its lowest excited level, i.e., the lowest

vibrational level of the S_1 state, due to the efficient internal conversion to this state. These processes are shown in Figure 1 [2].

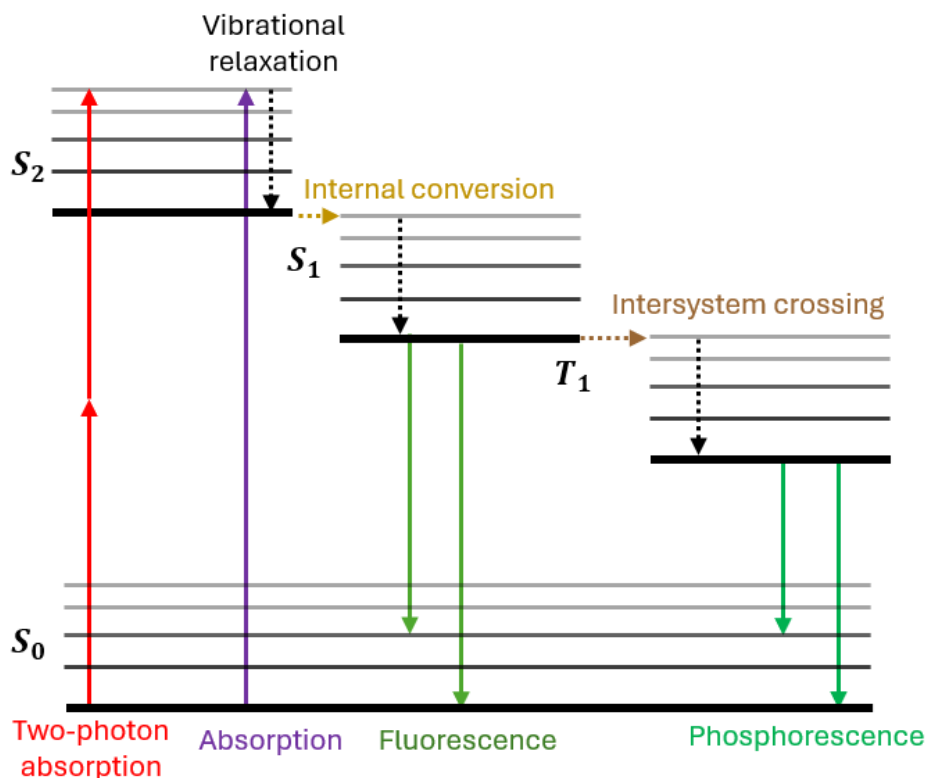


Figure 1 Jablonski diagram showing the various transition routes an electron has in excitation and de-excitation. S_0 , S_1 , S_2 and T_1 are bands of vibrational and rotational energy levels associated with discrete electronic energy levels of the fluorophore.

Another possibility for the molecule is that it undergoes intersystem crossing, making a transition from a singlet state S - where all electron spins are paired up and down, to a triplet state T - where one of the electron spins is flipped due to spin-orbit coupling. As two electrons in the same energy level must have opposite spins according to the Pauli exclusion principle, de-excitation of the electron from the triplet state is spin-forbidden, and therefore occurs over much longer time scales than fluorescence. This radiative mechanism from the triplet state to the ground state is known as phosphorescence. The probability of intersystem crossing to the triplet state ($S_1 \rightarrow T_1$) is relatively low (with a lifetime of microseconds) but over time fluorophores can accumulate in the triplet state, which can reduce the fluorescence intensity, e.g., during a measurement.

Excitation of a fluorophore can also occur when light of half of the excitation energy gap illuminates the molecule. This happens when two photons interact with the molecule simultaneously and the sum of the energies of both photons is sufficient for excitation. This phenomena was first

predicted in 1931 by Maria Göppert-Mayer, after whom the two-photon excitation cross-section is named [3], and observed experimentally in 1961 [4].

2.1.2 Fluorescence properties

The relationship between the excitation or emission wavelength, λ , and the photon energy associated with excitation or emission of an electron across an energy gap ΔE is given by

$$\text{Equation 1} \quad \lambda = \frac{hc}{\Delta E}$$

where h is Planck's constant and c is the speed of light. As the molecule loses some energy whilst in the excited state and after fluorescence emission through vibrational relaxation, the photon it emits upon de-excitation is of a slightly lower energy, and therefore longer wavelength ($\lambda_{exc} > \lambda_{em}$). This is known as the Stokes shift and is usually of the order of 50 nm.

Given that molecules may decay through radiative and non-radiative processes, it is useful to define the quantum yield, Q , of a molecule, this is defined by

$$\text{Equation 2} \quad Q = \frac{k_r}{k_r + k_{nr}}$$

where k_r and k_{nr} are radiative and non-radiative decay rates respectively. Molecules with high quantum yields make for better fluorophores in a microscopy context as they decay mostly through radiative pathways and are therefore brighter.

The fluorescence lifetime of a fluorophore indicates how long on average it will stay in the excited state after excitation, it is defined according to

$$\text{Equation 3} \quad \tau = \frac{1}{k_r + k_{nr}}$$

Fluorescence lifetime can be sensitive to a fluorophore's local environment, changing with pH levels, temperature, local refractive index, and various other parameters[5].

As fluorescence can be considered to be emitted by oscillating dipoles in the fluorophores, the emission will have a polarisation that is dependent on the orientation of this oscillating dipole and the excitation efficiency will depend on the polarisation of the excitation field. The degree of polarisation of fluorescence emission is often described by the anisotropy factor r ,

$$\text{Equation 4} \quad r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of light measured with polarisers orientated parallel and perpendicular to the polarisation of the excitation field. After excitation from a polarised field, fluorophores with dipole moments aligned with the field will be preferably excited. If the fluorophore is stationary the emitted light will be aligned with the excitation. However, between excitation and emission a fluorophore can rotate, removing the alignment between the excitation and emission fields. This indicates that more rapidly rotating fluorophores show lower degrees of anisotropy. Binding events can therefore be inferred due to increases in anisotropy, as the bound molecule rotates more slowly than the unbound.

2.1.3 Photobleaching and photodamage

Photobleaching is the process by which fluorophores stop emitting fluorescence due to intersystem crossing to the triplet state. This can result in permanent changes in the structure of the fluorophore due to reactions of oxygen with the fluorophore excited triplet state [6] and this can lead to a build-up of reactive singlet oxygen, which is toxic to cells. Photobleaching and the resulting phototoxicity places limits on biological imaging, especially for long time-lapse and high-resolution imaging. The decrease in fluorescence signal due to photobleaching over time can be detrimental when attempting to make quantitative comparisons between fluorescence signals in different spectral channels as one must consider potential differences in photobleaching rates.

Photodamage and phototoxicity are terms used to refer to the negative effects light dose can have on the viability and functioning of biological cells. On the extreme end of this spectrum, high enough intensities can ablate cells leaving them completely unfunctional. However, lower light intensities can still cause damage via the singlet oxygen generated from fluorophores in the excited state, and higher energy (ultraviolet) photons can break bonds, e.g., leading to damage of DNA. A number of studies have quantified safe light dose levels for healthy cell proliferation [7] [8].

2.2 Fluorescence in biological samples

Fluorescence occurs naturally in biological tissue as well as being artificially induced through fluorescent labels to allow the investigation of dynamics of interest. This section will discuss the autofluorescence properties of cells and the methods by which components of the cell are labelled with fluorescent markers to allow studies of specific processes.

2.2.1 Autofluorescence

Among the many different molecular species found within cells, there are a plethora of naturally fluorescing molecules whose emission is described as autofluorescence. This is often unwanted when specific elements of the cell are being studied – typically using fluorescent labels – and other fluorescence contributions present background noise. However, it can also be exploited to obtain information on cellular processes. Two of the main molecules used in autofluorescence imaging of cells are Nicotinamide Adenine Dinucleotide (NADH) and Flavin Adenine Dinucleotide (FAD), both of which play crucial roles in metabolic processes within a cell [9]. Both enzymes can exist in bound and unbound states, with each presenting a different fluorescence lifetime for both molecules. The proportion of bound and unbound populations can be an indicator of the metabolic state of the cell, meaning that autofluorescence lifetime images of cells can provide information on the metabolic functioning of cells [10].

Other common autofluorescent species found in biological media are collagens, which make up part of the extra-cellular matrix of biological tissue. There is increasing interest in investigating the relationship between tumour growth and the properties of the microenvironment surrounding cancer cells, of which collagen is a key constituent. It has been shown that certain types of collagen can be linked to the development of a tumour into a metastatic state [11]. Collagen may also be studied using second harmonic generation microscopy due to its long repeating structure. This technique can elucidate information on collagen fibre organisation, another property that can be linked to the health of tissue [12]. Other autofluorescent molecules such as tryptophan and tyrosine have been shown to be useful in distinguishing between normal and cancerous cells [13] and inferring information on protein conformational dynamics [14] respectively.

Most autofluorescent molecules in the cell are excited within the UV range, meaning that caution should be exercised due to the high potential for photodamage. Often multiphoton excitation can provide a less harmful method to observe autofluorescence of the same molecular species, as it reduces out-of-focus fluorescence excitation and damage.

2.2.2 Fluorescent dyes

Fluorescent dyes are small organic molecules that can be bound to specific targets, enabling detailed visualization of cellular structures and processes. Some dyes exhibit high specificity for particular cellular compartments; for instance, DAPI selectively binds to DNA, producing a bright fluorescence that highlights the nucleus in cell imaging. Furthermore, DAPI is “fluorogenic”, meaning that its fluorescence increases significantly when bound to its target (DNA) such that the target molecules are visualised with high contrast [15]. However, achieving precise localization of dyes to other specific sites within the cell may require additional labelling techniques, such as conjugating the dye to a targeting molecule like an antibody or ligand. Because organic dyes are typically small

molecules – DAPI, for example, has a molecular weight of only 350 Daltons – they can easily penetrate cellular membranes, making them well-suited for intracellular staining.

2.2.3 Immunolabelling

Immunostaining uses antibodies to label specific targets (epitopes) within a cell with fluorophores that are conjugated to the antibody. Antibodies bind their targets with high specificity according to their function within the immune system and new antibodies can be generated to bind to specific proteins using the immune system of a host animal (e.g., mouse, rabbit, goat, llama). The most common method of antibody staining uses both primary and secondary antibodies. First a primary antibody is applied which binds to the protein of interest, then secondary antibodies conjugated to fluorescent labels are introduced, several of which can bind to one specific primary antibody, providing amplification of the fluorescence signal associated with the specific protein of interest. Antibodies are most commonly used for labelling components on the surface of live cells as their size (in the range of ~100 kDa) precludes them crossing the cell membrane unless the membrane is permeabilised. In fixed cells permeabilisation enables immunolabelling of most proteins in the cell.

2.2.4 Fluorescent proteins

Labelling with fluorescent proteins (FP) started following the discovery and isolation of GFP [16]. Having determined the specific DNA sequence that encodes GFP, it is possible to produce GFP in cells coupled to specific target proteins by introducing the relevant DNA sequence into the cell. This can be done transiently, e.g. by introducing DNA containing plasmids into a cell, or by editing the DNA of a cell, e.g., using CRISPR/Cas9. Directly editing the cell genome creates stable cell lines that express fluorescent proteins labelling their endogenous target proteins. Plasmid transfection is transient as the new DNA is inserted into the cell but not incorporated into the cell genome. Initially the desired construct (target protein linked to FP) is over-expressed, which can compromise the normal cell biology, and over time the FP-labeled protein diminishes with each cell division.

Genetic engineering with FP, e.g., using plasmid transfection or gene editing, is a powerful approach to label proteins for live cell imaging since it provides the highest possible specificity and avoids issues with toxicity of dyes. However, they are generally less bright and less photostable than dyes and their relatively large size (GFP is 27 kDa) means that normal biological processes can be perturbed when the relevant proteins are attached to fluorescent proteins, so one must consider this when designing experiments. Since their discovery, many new fluorescent proteins have been engineered to provide brighter fluorophores across many spectral channels with improved photostability

[17]. A schematic showing how the combination of DNA used to encode the protein of interest and the fluorescent proteins can produce a fluorescently tagged protein of interest is shown in Figure 2.

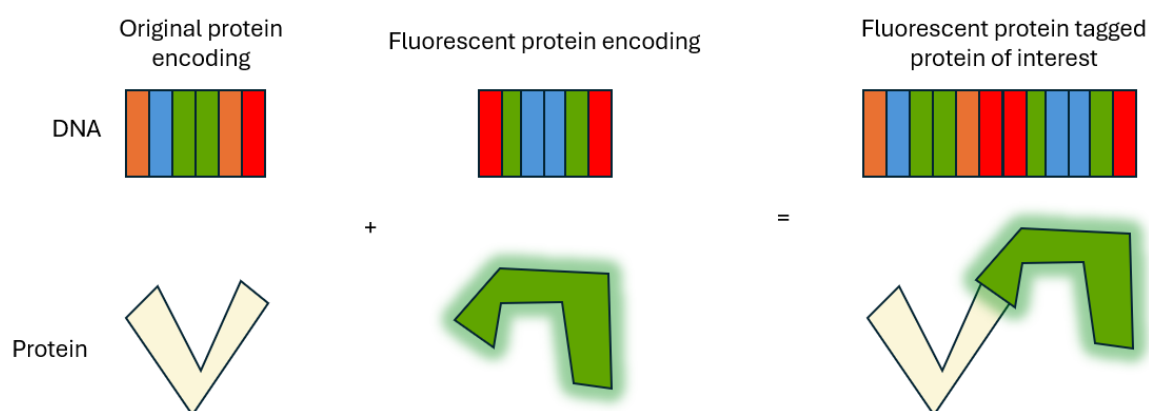


Figure 2 Schematic of DNA modification used to produce fluorescently tagged protein of interest.

2.2.5 SNAP-tag labelling

A SNAP-tag is a small tag that can be attached to one end of a protein of interest using the same genetic engineering techniques as for fluorescent proteins. Once cells are expressing the protein of interest coupled to the SNAP-tag, fluorogenic fluorescent dyes can be introduced to the cell that will attach to the SNAP-tag and enable live cell fluorescence imaging of intracellular proteins. Thus, this approach provides a way of highly specific labelling of proteins with (bright, photostable) dyes without using antibodies [18]. The technique can also be used for specific site targeting of drugs introduced into cells. SNAP-tag labels are of the order of 20 kDa so they potentially present a reduced perturbation of normal cell function compared to fluorescent proteins. Similar techniques using different binding substrates such as HaloTags and CLIP-tags also exist.

2.3 Light Microscopy

Microscopes facilitated groundbreaking biological discoveries in the 18th century by pioneers such as Antonie van Leeuwenhoek [19] and Robert Hooke [20]. This section introduces light microscopy to provide context for the fluorescence microscopy techniques that are now ubiquitous in the study of cell biology. In most light microscopes, lenses are used to magnify the illuminated object and most modern microscopes use an infinity-corrected configuration, where the lens placed nearest to the object collimates the incoming rays from its focal plane and a second lens focusses these rays to produce a real image in its back focal plane, where a camera can be placed for digital image acquisition. This

configuration is illustrated in Figure 3. It has the advantage that planar optical components placed in the region between the two lenses, known as the infinity space, cause minimal aberrations as the rays are not being focussed to form an image.

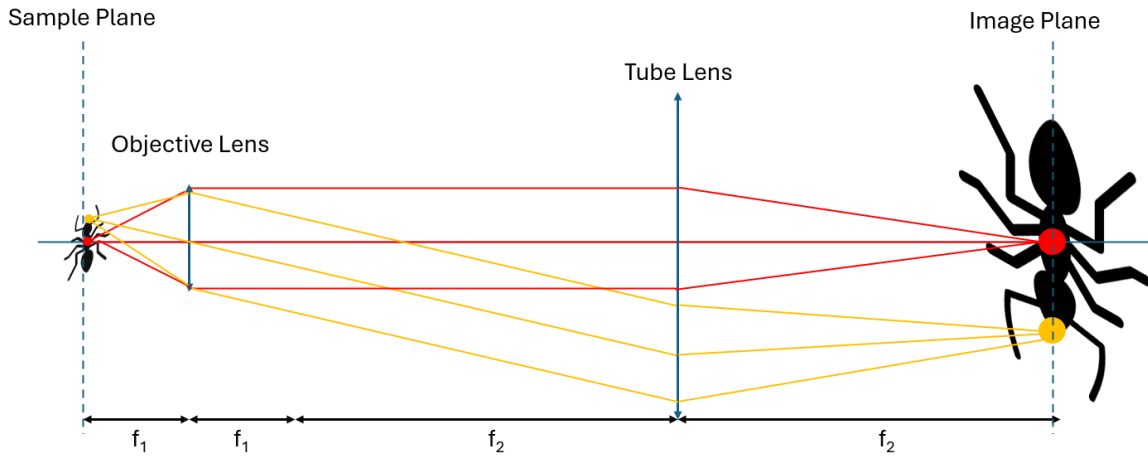


Figure 3 Infinity-corrected microscope configuration (non-telecentric).

The lens nearest the object is named the objective lens, and the second lens used to focus the rays to the image is known as the tube lens. The lens placements seen in Figure 3 are known as a 4-f configuration, where the first lens is placed its focal length from the sample plane, the distance between the lenses is the sum of their focal lengths, and an image is formed of the object at the focal plane of lens two. When the stop is at the back focal plane of the objective, 4-f systems are telecentric in both object and image space, meaning that the centres of the ray bundles collected from the object and the centres of the ray bundles focussed to the image are parallel to the optical axis. The magnification of this set up is given by

$$\text{Equation 5} \quad M = -\frac{f_2}{f_1}$$

where f_2 is the focal length of the second lens and f_1 the focal length of the first lens, in direction of light travel, meaning as the focal length of the objective lens is decreased, the magnification increases. The short focal length of objective lenses means that they are the most heavily engineered lenses in optical systems, as shorter focal lengths require higher curvatures of the lens surfaces, meaning aberrations are more significant and must be mitigated with complex multi-element designs. Microscope manufacturers have settled on combinations of a tube lens around 200 mm focal length, matched with the appropriate objective lens needed for the desired magnification (in the case of a 20x system, a 10 mm focal length objective would be required). The field of view (FOV) depends on the sensor size placed at the image plane of the tube lens, provided that the FOV of the intermediary optics is larger. By demagnifying a sensor of linear dimension x back to the sample plane it is clear that a FOV

of $\frac{x}{M}$ will be achieved - usually on the order of 100s of microns for a camera sensor on the scale of 10 mm.

An important parameter in microscopy is the numerical aperture, NA, of a lens which is defined as

$$\text{Equation 6} \quad NA = n \sin(\theta)$$

where θ is the maximum acceptance angle from the optical axis of the lens and n is the refractive index of the media into which the light is being focussed or accepted. This angle is increased by having a smaller focal length lens with a larger aperture. The diffraction limited resolution achievable by a microscope is related to the NA of the objective lens by

$$\text{Equation 7} \quad R = \frac{0.61 \lambda}{NA}$$

when using the Rayleigh criterion [21]. If one considers the image of an infinitely small point object, called the Point Spread Function (PSF), this would be described by an Airy function with a full width at half maximum of $\frac{0.51 \lambda}{NA}$ [22] and the Rayleigh criterion corresponds to a pair of such point objects being considered resolved when the maximum of one Airy function (PSF) is located at the first zero of the adjacent Airy function. This value falls in the range of hundreds of nanometres when imaging biological samples in a standard imaging system where the maximum achievable NA is ~ 1.5 . Traditionally this placed a fundamental limit on the ability to resolve small structures with microscopy. However, there are now techniques to image with resolution beyond the diffraction limit as will be discussed later in this chapter.

2.3.1 Brightfield microscopy

Light microscopy was initially dominated by brightfield microscopy, which relies on the attenuation of light by a sample when trans-illuminated to produce contrast upon viewing. In bright field microscopy, the sample must present sufficient contrast to resolve the features of interest, and in histopathology this is achieved by staining fixed samples with strongly absorbing dyes such as hematoxylin and eosin.

2.3.2 Phase contrast

Brightfield microscopy images the attenuation of light by the sample, which for unstained cells can be very weak. Phase contrast microscopy was developed to improve visualisation of near transparent biological samples by converting the phase modulation of light transmitted through a sample (due to variations in optical path length) to an intensity modulation of the image. The earliest technique to do this was Zernike phase contrast (winning the 1953 Nobel Prize in Physics), where an annulus is placed

in the back focal plane of the condenser lens used to illuminate the sample, and a phase ring is placed in the back focal plane of the objective lens [23]. The general principle is that a phase object will scatter some of the incident light which will be phase delayed. The unscattered light will pass through the phase ring where it will be phase delayed such that it constructively interferes with light scattered by the sample. This results in intensity contrast between regions of background and brighter regions where the sample has scattered the light to produce constructive interference, allowing visualisation of near-transparent samples, including live cells. Following the success of Zernike phase contrast microscopy, the technique of differential interference contrast (DIC) microscopy was invented by Nomarski [24]. This illuminates the sample with two orthogonally polarised beams that are displaced (sheared) at the sample plane (e.g., using a Wollaston or Nomarski prism) and recombined at the image plane. The interference of the two beams at recombination is sensitive to their optical path difference and so an intensity image of phase gradients is recorded. Zernike and Nomarski DIC microscopy provide qualitative phase images that allow thin transparent objects to be visualised, but their features cannot be directly related to structure in the sample.

Phase microscopy techniques have since been developed to provide more quantitative readouts of optical pathlength across the FOV, this is referred to as a quantitative phase imaging (QPI). Some exemplar techniques are differential phase contrast [25] and Fourier ptychography [26] which use precise mathematical models to allow calculation of the sample phase distribution from one or more intensity images. The quantitative nature of these measurements allow more precise comparisons between data sets, and also allow evaluation of more relevant biological parameters such as the dry mass of the cell, an indicator of the amount of protein within a cell.

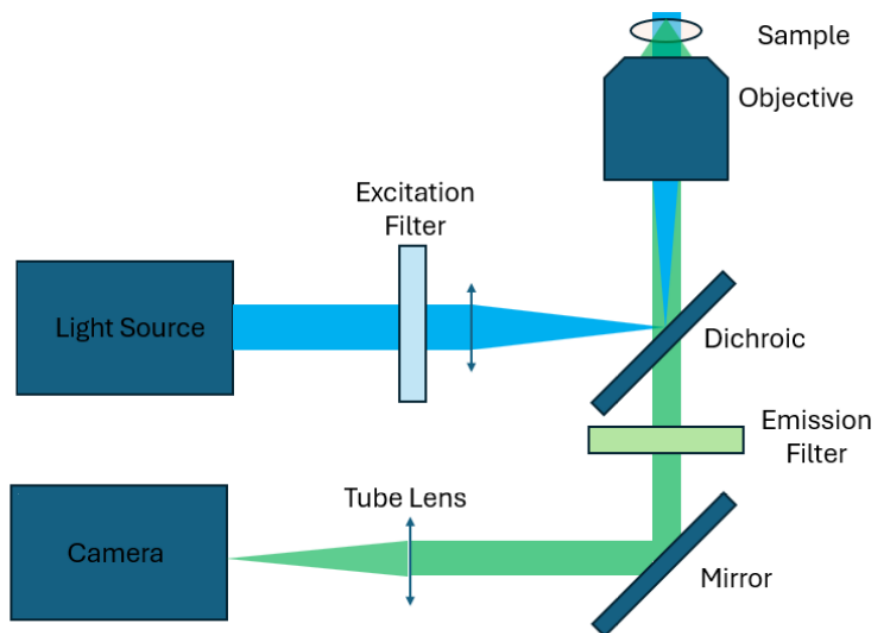


Figure 4 Basic fluorescence microscope setup.

2.3.3 Fluorescence microscopy

Fluorescence microscopes can use similar optical configurations as brightfield microscopes except that, instead of acquiring transmitted light images, fluorescence is excited within the sample, and imaged using the microscope. Besides the infinity-corrected microscope set-up, a fluorescence microscope requires a light source that must be able to excite the desired fluorophores; today this is usually a laser or LED emitting near the wavelength needed to excite the fluorophore used. An excitation filter can be used to further define the excitation spectrum. The excitation light is coupled into the microscope by reflecting it at a dichroic beamsplitter, which is selected to preferentially reflect light at the excitation wavelength and to transmit the longer wavelength (due to the Stokes shift) fluorescence emission. An emission filter is placed after the dichroic that transmits the fluorescence and blocks any excitation light. This basic layout can be seen in Figure 4. When the objective lens is used to relay both the excitation and emission to the sample, this is known as an epi-illumination set-up. The combination of dichroic beamsplitter and emission filter provide a high signal to background ratio such that emission from a single fluorophore molecule can be detected using even relatively modest cameras.

2.4 Biological sample formats

This section will discuss the most common formats of biological samples imaged by light microscopy. These include “thin” samples such as histological tissue section or “2D” cell cultures on glass or plastic substrates, and extended sample formats including “bulk” tissue samples ranging from ex vivo tissue slices to whole organisms imaged *in vivo*, and 3D cell cultures such as spheroids, organoids, and engineered tissues (e.g., “organ-on-a-chip”). Samples may be imaged “live” (at the cellular level), e.g., in fresh or perfused tissue slices), or they may be fixed. The specific format impacts the choice of the type of microscopy and labelling approaches that are used.

A key consideration is that a conventional wide-field microscope will form an image of the sample (from light emanating from the objective lens focal plane for an infinity-corrected microscope) but the wide-field detector (camera) will also detect light from outside the focal plane. This out-of-focus light typically presents a blurred background that degrades image contrast. The principal approaches to mitigate this challenge are to contrive that the sample format is thin ($\ll$ depth of field of the objective lens), such that all the light incident at the detector has interacted only with sample at the focal plane and so contributes only to an in-focus image, or, as discussed below, the microscope is configured to provide optical sectioning, such that only light from near the focal plane is incident on the detector. As bulk (tissue) samples increase in size and complexity, they may present more challenges for imaging, including absorption, scattering and refraction leading to optical aberrations that can degrade image contrast.

2.4.1 2D cell culture

“2D” monolayers of cells on flat surfaces provide an ideal thin sample format, allowing for convenient preparation, imaging and manipulation of cells and 2D cell cultures, and are the most widely used format in cell biology and drug discovery. However, while this format provides outstanding experimental convenience, the simplified environment does not replicate the physiological complexity found *in vivo*, and cell biological processes studied may present differently compared to the *in vivo* context. Typically, cells are cultured in a flask or petri dish and deposited on a microscope slide for imaging, as depicted in Figure 5, although they can also be grown directly on the imaging substrate. Modern microscopy most commonly uses a 170-micron thick sheet of glass called a coverslip to sandwich the cells on the microscope slide and the cells are imaged through the coverslip in an epifluorescence microscope. The optical design of many higher NA objective lenses is therefore optimised to image through this thickness of glass.

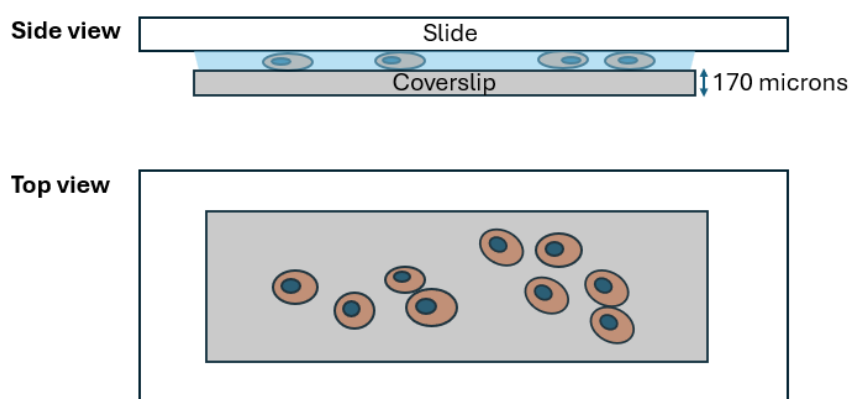


Figure 5 Microscope slide viewed from side and top with standard 170 micron coverslip.

2.4.2 Spheroids

Spheroids are 3D cell cultures that are grown in a way that allows them to aggregate and form (typically) spherical structures. These spheroids better mimic the architecture and microenvironment of the *in vivo* context [27], allowing for some characteristics of solid tumours such as gradients of nutrient availability and oxygen levels. This sample format is being actively explored for studying 3D cellular behaviour, tumour biology and drug responses. Cells can either naturally aggregate in suspension (e.g., in a hydrogel) to form spheroids or can be encouraged to grow into spheroids by culturing them in a well with curved bottom and coated to prevent adhesion that leads to aggregation. For imaging, spheroids are often transferred to a multiwell plate that comprises an array of chambers with a 170-micron thick glass bottom and so is directly compatible with standard microscope objectives. Also used

with 2D cell cultures, multiwell plates allow similarly prepared samples to be arrayed for imaging, enabling repeats of experiments in parallel and comparisons of outcomes between different conditions or treatments.

2.4.3 Organoids

Organoids are more complex, three-dimensional cellular structures, often derived from stem cells that can self-organize into miniature organ-like tissues. Compared to spheroids, they more faithfully replicate the cellular architecture and functionality of real organs, improving their relevance for studying development, disease, and drug responses. Different organoid types can be grown by adjusting the culture conditions of stem cells, mimicking different tissue types. Cells taken from a patient can be used to make patient derived organoids that may behave similarly to cells within the patient's body, potentially making them useful, e.g., for testing drug responses. However, the increased physiological relevance to human biology, comes at the cost of increased complexity in their preparation and more challenges with respect to imaging. Organoids are typically grown to be larger than spheroids, but many can still be arrayed for imaging in multiwell plates on a microscope.

2.4.4 Tissue

After organoids, tissue slices can be considered as a next step towards the physiological complexity of the *in vivo* context. Tissue can be imaged *in vivo*, *ex vivo* soon after excision or can be maintained with perfusion of water and nutrients, etc., to sustain biological functions. Typically, on a mm scale, tissue slices can be arrayed in multi-chamber slides or multiwell plates for imaging and are often maintained in an imaging chamber integrated with microfluidics for perfusion. Imaging tissue slices presents many of the challenges associated with intravital microscopy although providing much greater experimental convenience.

2.4.5 *In vivo*

In vivo imaging refers to techniques used to visualize biological processes within a living organism, providing real-time insights into physiological and pathological conditions. *In vivo* imaging of animal models can be useful to study disease progression, to evaluate treatment efficacy, and to explore complex biological interactions in their natural physiological context, while intravital microscopy or endoscopy in humans can be used for clinical diagnosis and monitoring of therapy, as well as for biomedical research subject to ethics approval. Because optical absorption and scattering render

biological tissue opaque beyond a few 100 μm depth, surgical preparations are often required to image internal tissues in animal models of disease. Ethical considerations strongly encourage minimal use of animals in research and this is part of the drive to explore 3D cell cultures as disease models.

2.5 Fluorescence microscopy techniques

The basic epifluorescence microscope outlined above is well suited to image thin samples such as histological sections or 2D cell cultures. As discussed, however, when imaging thicker samples, it is necessary to implement optical sectioning and to mitigate the deleterious effects of optical scattering and aberrations. This section will discuss the most widely used optical sectioning techniques, as well as optical projection tomography, which is an alternative approach to 3D imaging of bulk samples. techniques that have been developed and the benefits they offer for imaging.

2.5.1 Confocal microscopy

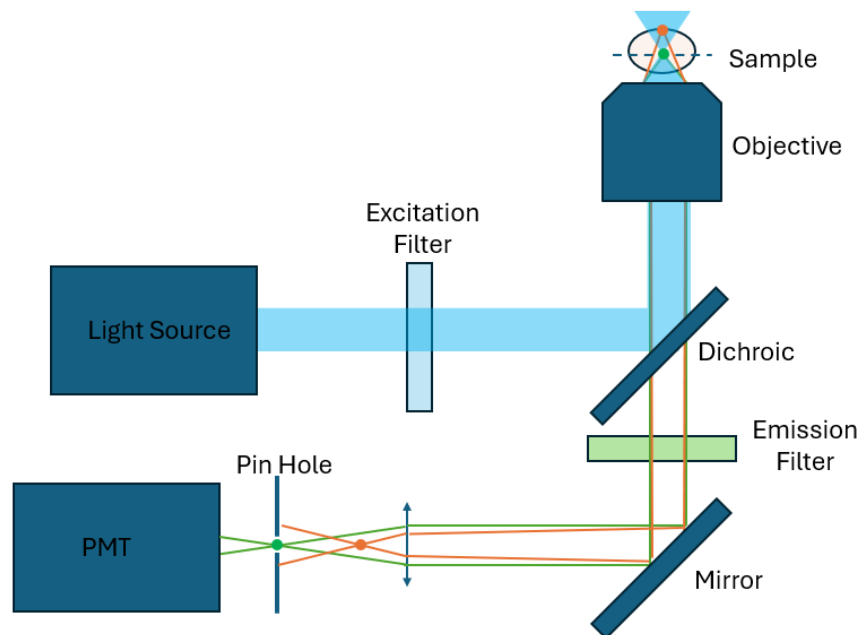


Figure 6 Confocal microscope setup.

As discussed, the standard epi-illumination widefield fluorescence microscope works well with 2D biological samples but fluorescence from outside the focal plane results in a blurred background signal superimposed on the image of the sample in the objective lens focal plane. The confocal microscope mitigates this issue by using an optical set-up that blocks the majority of the out of focus fluorescence, such that fluorescence from the focal plane dominates the signal reaching the detector [28]. The set-up uses a light source (usually a laser) that is focussed to a diffraction limited excitation spot at the sample

plane. As the excitation light travels through the sample, it will excite fluorescence emanating from out of the focal plane, as shown in Figure 6 in orange, as well as fluorescence from the focal plane, shown in green. A pinhole is placed on the optical axis in the image plane (where the camera was located in the widefield set-up), resulting in light originating from outside the focal plane being defocussed at the pinhole and mostly blocked. Fluorescence from the excitation spot on the focal plane is focussed through the pinhole and subsequently collected on a “single pixel” detector such as a photomultiplier (PMT). As the fluorescence collected emanates from a single point in the sample plane, it is necessary to scan the sample or the excitation beam to build up an image. Typically, the excitation spot is scanned along the x and y axes using a pair of scanning mirrors. As a point scanning technique, confocal microscopy is a slower imaging modality than widefield imaging. However, it enables a volumetric fluorescence image to be built up by acquiring a z-stack of optical sectioned images as the objective lens is moved relative to the sample. It also provides improved image contrast due to the suppression of out of focus light. If the pinhole was to be set to be infinitely small, the PSF of the confocal microscope is the square of the PSF of the widefield microscope with a lateral FWHM of $0.37 \lambda/NA$ [22], and so provides enhanced later resolution as well as axial sectioning with an axial PSF FWHM given by

$$\text{Equation 8} \quad AFWHM_{conf} = 0.64 \frac{\lambda}{n - \sqrt{n^2 - NA^2}}$$

This is larger than the lateral PSF FWHM and typically $>0.5 \mu\text{m}$ for visible light. In practice, the detected signal scales with the confocal pinhole size and usually the lateral resolution is comparable to a wide-field microscope when the pinhole is set to provide a reasonable signal [29], although some optical sectioning is retained with pinhole sizes up to few times the width of the PSF [22].

One method to increasing the speed of a scanning confocal microscope is using a spinning disc array of pinholes that parallelise the confocal imaging [30]. In this approach a disc arrayed with spirals of pinholes in is illuminated with a light source and imaged onto the sample such that each pinhole produces an excitation spot from which the fluorescence is imaged back to the disc pinhole array. Thus, each pinhole in the disc both creates the point exaction and serves as the confocal pinhole to block the out-of-focus fluorescence. The fluorescence transmitted back through the disc is imaged onto a wide-field detector (camera) and the image is built up as the spinning disc moves the pinhole array to map out the whole field of view. There is a trade-off with respect to the size of the pinholes, since smaller pinholes block more out of focus fluorescence and also block more unwanted scattered fluorescence excited by light from neighbouring pinholes but reduce the fluorescence detection efficiency. This unwanted “cross-talk” between the parallel confocal imaging channels decreases with increased spacing of the pinholes but the reduced pinhole density increases the image acquisition time and also decreases the fraction of incident excitation light that is transmitted through the pinhole. This latter issue can be

ameliorated by adding an array of microlenses aligned with the pinholes to efficiently focus an incident laser beam through the disc pinhole array [31].

Laser scanning confocal microscopy (LSCM) has also been parallelised in line-scanning configurations using line illumination in conjunction with a confocal slit detector, e.g., [32], and this approach is the basis of the slit scanning ophthalmoscope. More recently the use of a rolling shutter of an sCMOS camera has been used to replace the detection slit for the rejection of out of focus fluorescence [33]. Line-scanning confocal microscopy offers comparable performance to LSCM and spinning disc confocal microscopy with slightly inferior depth resolution compared to LSCM and degradation of performance in scattering samples where interpixel cross-talk along the illumination/detection line increases with depth.

2.5.2 Multiphoton microscopy

Multiphoton microscopy makes use of the nonlinear intensity dependence of multiphoton excitation. When two photons of half the energy required to excite a fluorophore are incident at a sample within a narrow time window ($\sim 10^{-16}$ s), then the fluorophores can be excited through two photon absorption. The probability of two photons arriving at the fluorophore within this time window is small, and so high intensities are required for two photon excitation of fluorescence to be practically useful and the observed two photon fluorescence signal is intensity-dependent – proportional to the square of the excitation intensity [34]. This effect is utilised in multiphoton microscopy by illuminating the sample with ultrashort optical pulses through a high NA objective lens such that the excitation intensity is at its maximum in the focal plane and decreases away from the focal plane. Ultrashort pulses are used to provide high excitation intensities with low average power to minimise thermal damage of the sample. It is possible to set the excitation power such that appreciable fluorescence is only excited within a small focal volume, as shown in Figure 7.

As multiphoton excitation produces negligible out of focus fluorescence, no confocal pinhole is needed to realise optical sectioning. Thus, a laser scanning multiphoton microscope can be configured similarly to an LSCM but with no detection pinhole, and all of the excited fluorescence can be detected over the detection area of the photomultiplier providing greater fluorescence collection efficiency – particularly when imaging in scattering samples such as biological tissue. A further benefit of this technique is that it uses longer wavelength light to excite a given fluorophore compared to LSCM and so the excitation light incurs less absorption and scattering, enabling deeper imaging in biological samples. Two photon excitation of visible fluorophores typically requires radiation in the near infrared ($\sim 700\text{-}1200\text{ nm}$) which is the transmission window of biological tissue where absorption and scattering are minimised. It is also possible to excite visible fluorophores with three photon excitation (i.e., with 3 photons arriving almost simultaneously at the sample) [35] in which case the excitation is in the longer (short wave infrared, $\sim 1050\text{-}1800\text{ nm}$) region where attenuation is even lower and so even deeper imaging is possible and has been exploited *in vivo* [36].

It is generally preferable to use an ultrashort pulsed laser source for multiphoton excitation, as this provides higher peak power for a given average power. The optimum pulse duration depends on the specific requirements: in general, the multiphoton signal increases with shorter pulse durations and higher excitation intensities but so does nonlinear photodamage. Using longer pulses and lower peak powers requires higher average power [37] or lower duty cycles [38] but may increase cell viability under prolonged multiphoton excitation. A further issue is the dispersive broadening of ultrashort pulses, which increases with the spectral width of the pulses. Longer pulses with narrow spectra will be less impacted by group velocity dispersion in the optical train of the microscope. Where pulses $< 100\text{ fs}$ are used, it is common to use pre-dispersion compensators to maintain the short pulse durations at the

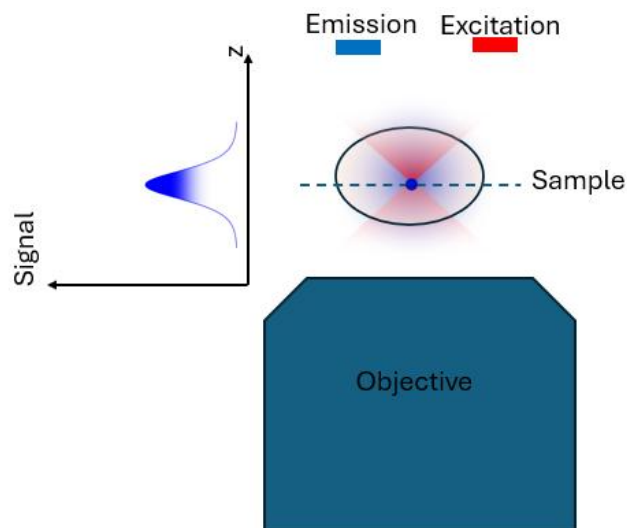


Figure 7 Multiphoton excited fluorescence confined to small volume at the focus of excitation beam.

sample. The number of photons absorbed per fluorophore per pulse in terms of pulsed laser properties is given by [34]:

$$\text{Equation 9} \quad n = \frac{p^2 \delta}{\tau f^2} \left(\frac{NA^2}{2\hbar c \lambda} \right)^2$$

where c is the speed of light, $\hbar$ is the reduced Planck's constant, δ the two-photon absorption cross-section, p is average incident power, NA is the numerical aperture of the objective lens, τ is the pulse duration, f is the repetition rate of the laser and λ is the wavelength of the light. This equation indicates the importance of using a high NA objective lens and illustrates how the two-photon fluorescence signal will scale with average power, pulse duration and pulse repetition rate.

Multiphoton microscopy is usually implemented in laser scanning microscopes and, since the excitation cross-sections are lower than those for single photon excitation, the imaging rate is slower than LSCM. Various efforts have been made to speed up acquisition times including wide-field [39] and line-scanning [40] multiphoton microscopy enhanced using temporal focussing, as well as multibeam scanning approaches, e.g., using a rotating microlens array [41], a multibeam splitter array [42], a diffractive optical element [43], and a spatial light modulator [44] to generate parallel excitation beams.

2.5.3 Light Sheet Fluorescence microscopy

In light sheet fluorescence microscopy the sample is illuminated with a sheet of excitation light in the sample plane to be imaged, so that the optical axis of the camera is at an angle (usually 90°) to the plane of the light sheet [45]. Usually, two objective lenses are used, one to focus the excitation light onto the sample and the other, at an angle to the first, used to detect the fluorescence. This configuration provides intrinsic wide-field optical sectioning and minimal photobleaching/damage [46]. since only the sample volume to be imaged is illuminated (in the absence of scattering). Combined with high frame rate cameras, this makes light sheet microscopy especially useful for rapid 3D fluorescence imaging [47].

One issue found with the technique is that the need for two objective lenses to provide orthogonal illumination and imaging axes is not compatible with standard biological sample formats or microscope frames. Oblique plane microscopy (OPM) combines oblique illumination and imaging through a single objective lens combined with remote focussing to correct aberrations and facilitate rapid scanning [48]. This approach, which can be implemented using a galvo mirror to provide lateral scanning of the illumination and detection plane in a method known as SCAPE (Swept, Confocally Aligned Planar Excitation) [49] can be implemented on standard epifluorescence microscopes and applied to standard

biological sample formats, including in multiwell plates, meaning that the technique can be extended more easily to high throughput imaging[50].

2.5.4 Optical Projection Tomography (OPT)

OPT is a tomographic technique analogous to X-ray computed tomography that works by imaging the sample at multiple angles and applying filtered back projection techniques based on the Radon transform to reconstruct a 3D volume from the acquired fluorescence projection images. The technique is well suited to larger “mesoscopic” transparent samples in the mm – cm range. For such samples, optical clearing, is often required to make the samples transparent, as was demonstrated with the first OPT fluorescence imaging experiment [51].

2.6 Fluorescence readouts

This section will discuss the main readouts used in fluorescence microscopy to provide information on the distribution of different fluorophore species (and therefore the proteins they label) and on the use of fluorophores as sensors to report on variations in fluorophore environment.

2.6.1 Intensity

Fluorescence intensity is the most commonly used readout, which is proportional to the number of fluorescence photons detected and therefore the (local) concentration of fluorophores. Thus, fluorescence intensity can be used to map concentrations of the target molecules to which fluorophores are bound. Fluorescence intensity images map the spatial distribution of fluorophores and their targets to reveal spatial structures and dynamics, e.g., of cellular processes.

Quantitative fluorescence intensity measurements are extremely challenging, requiring knowledge of incident and detected optical powers, excitation and emission cross-sections, attenuation factors, quantum efficiencies of fluorophores and detectors, any background contributions to the detected signal and any environmental factors that may perturb the fluorophore characteristics.

One approach is that the illumination and detection efficiency are assumed to be uniform across the field of view, or their variations are calibrated, and then the relative intensities of fluorophores across a field of view can be used to infer the relative distribution of fluorophore concentrations. In some situations, it is possible to use a known fluorescence intensity reference standard to calibrate quantitative fluorescence readouts.

Where measurements of fluorophore quantum efficiency are required to probe changes in fluorophore environment, it is also necessary to know the fluorophore concentration. Since this is

usually not known, it is often necessary to instead use spectroscopic readouts that can be insensitive to fluorophore concentration.

2.6.2 Spectral fluorescence readouts

Spectrally resolved fluorescence readouts can be used to distinguish emission from different fluorophore species or to provide quantitative information about fluorophores that is independent of potential artefacts due to unknown variations in concentration or attenuation of excitation or emission radiation. If the emission spectra of fluorophores are known, one can distinguish between multiple fluorophores in complex samples, enabling multiplexed analysis of several fluorescently labelled biological targets in the same assay, or one can determine relative concentrations of different fluorophore species through spectral unmixing. At a simple level, fluorescence intensity images resolved in different spectral bands can be overlaid and their respective spatial distributions displayed using different colours to visualise their associations, from which interactions may be inferred.

The acquisition of separate spectral channels is often done sequentially, with different fluorophore species being distinguished according to differences in their excitation and/or emission spectra through the use of spectral filters. By judicious choice of the excitation wavelength, or the use of multi-line or broadband excitation sources, it may be possible to excite multiple fluorophores simultaneously and separate their emission signals using dichroic beamsplitters or using a hyperspectral imaging set-up, e.g., using a spectrograph that maps emission wavelength to a spatial dimension on a camera allowing simultaneous acquisition over a broad spectral range. With hyperspectral image data, some form of spectral unmixing may be used to separate contributions from different fluorophores. An important emerging application of multispectral or hyperspectral imaging is cell painting, which is a technique to stain and image multiple components of cells – DNA, mitochondria, membrane etc. – simultaneously using different dyes. Because cell painting can be applied to live cells [52], the fluorescence readouts can be used to phenotype cell responses to drugs, gene silencing and other manipulations.

For quantitative readouts of fluorophore environment – where the fluorophore is used as a sensor, e.g., of pH, interaction with other molecules, etc., it is possible to design robust readouts if the spectral profile of the fluorophore changes in a known way as a function of the environmental variation. Typically, this is implemented using wavelength ratiometric imaging, i.e., by measuring the fluorescence intensity for two different excitation or emission wavelengths and calculating their ratio, which can be independent of fluorophore concentration and for which attenuation of excitation or emission radiation can be assumed to be the same or determined *a priori*. Wavelength ratiometric imaging is the basis of several small molecule dye-based reporters and of genetically engineered biosensors utilising fluorescent proteins used to sense analytes such as calcium, e.g., [53], [54] or physical

properties such as membrane tension [55] and is also a common readout of Förster resonant energy transfer, as discussed below.

2.6.3 Fluorescence lifetime readouts

Fluorescence lifetime readouts entail measuring characteristics of the decay profile of the spontaneous emission of a population of fluorophores over time following excitation due to the relaxation of the electrons in the S_1 excited state. The characteristic fluorescence lifetime, τ , of a fluorophore species is the average time taken for the fluorescence intensity to reduce to $\frac{1}{e}$ of the initial intensity after instantaneous excitation. For most organic fluorophores, this is usually in the range of nanoseconds. When fluorescence lifetime measurements are made at each pixel across a field of view, this is known as fluorescence lifetime imaging (FLIM).

Fluorescence lifetime measurements can be used to distinguish different fluorophore species and, since they are independent of a fluorophore's concentration, can provide quantitative measurements of changes in the local fluorophore environment, such as pH, viscosity, and interactions with other molecules, that impact the photophysics of the fluorophore. This can be exploited to study molecular dynamics, binding events, and conformational changes of the fluorophores themselves or of proteins to which they are attached. There are multiple approaches to fluorescence lifetime measurements and FLIM; these can broadly be split into time and frequency domain techniques.

Time domain techniques generally utilise excitation by periodic trains of ultrashort optical pulses with pulse durations shorter than the fluorescence lifetime being measured (typically femtosecond or picosecond pulses). The measured decay data can be used to directly estimate fluorescence lifetimes, or they can be fitted to models that can represent mixtures of fluorophores producing the experimental fluorescence decay profiles. The most widely used time domain techniques are time correlated single photon counting (TCSPC) and wide-field time-gated FLIM.

In TCSPC the excitation power is set sufficiently low such that, on average, less than one fluorescence photon is detected for each excitation pulse and so their arrival times with respect to the excitation pulse can be measured using fast electronics, allowing a histogram to be constructed whose envelope corresponds to the fluorescence decay profile [56]. TCSPC is usually implemented in laser scanning confocal or multiphoton microscopes where a single pixel detector such as a single photon counting photomultiplier is used.

Wide-field time-gated FLIM uses ultrashort pulsed excitation across the whole FOV and the resulting periodic fluorescence decay profiles are sampled using a gated image intensifier in front of a camera that acts like an ultrafast shutter with a resolution of ~ 100 ps [57]. By varying the delay between the excitation pulse and the time gate, the fluorescence decay profile can be measured. As TCSPC measures each excited photon, but wide-field time-gated FLIM discards photons outside of the intensifier window, it is fair to say the former yields a higher signal per excited photon. However, as wide-field time-gated FLIM collects photons across the entire FOV simultaneously, it offers a greater signal per unit time. Both time domain techniques will have a finite system response time to an instantaneous photon signal, this is known as the instrument response function (IRF), for which shorter IRF widths allow more precise lifetime calculations, additionally by measuring the IRF one can deconvolve the measured data, allowing more accurate analysis.

Frequency domain FLIM approaches originally utilised sinusoidally modulated excitation that result in sinusoidally modulated fluorescence emission that is shifted in phase with respect to the excitation signal. By measuring this phase shift and/or the change in modulation depth between the excitation and emission signals, parameters related to the fluorescence lifetime can be measured. Frequency domain FLIM can be implemented in wide-field microscopes, e.g., using sinusoidally modulated image intensifiers or cameras, or in laser scanning microscopes where the sinusoidal “demodulation” can be implemented electronically after the fluorescence photons are detected. With the wide availability of ultrafast (mode-locked) lasers, including in multiphoton microscopes, today frequency domain FLIM is usually implemented with ultrashort pulsed excitation using the first Fourier component of the periodic pulse train to effectively provide the sinusoidal excitation signal.

Each fluorophore species nominally presents a characteristic monoexponential decay profile, but where samples contain multiple fluorophores, or fluorophores in multiple states, in the same pixel, the measured fluorescence decay profiles will be more complex. These can be treated as a continuous range of fluorescence lifetimes with some assumed distribution, e.g., a stretched exponential decay profile [58] or can be modelled by two or more discrete lifetime components according to:

$$\text{Equation 10} \quad I(t) = \sum_{i=1}^n a_i \exp\left(\frac{-t}{\tau_i}\right)$$

where a_i is the intensity of the i^{th} exponential component at time $t=0$ and n is the number of exponential decay components. When displaying FLIM images in systems with multiexponential decays the intensity weighted mean lifetime per pixel is often displayed, which is calculated as:

$$\text{Equation 11} \quad \tau_{WM} = \frac{\sum_{i=1}^n a_i \tau_i^2}{\sum_{i=1}^n a_i \tau_i}$$

For a monoexponential fluorescence decay profile, a few hundred detected photons are required to provide an accuracy of $\sim 10\%$ [59]. Reliable fitting to a larger number of decay components requires many more detected photons and it is often not practical to acquire these before photobleaching or phototoxicity compromise the experiment. It is therefore best practice to fit FLIM data to models with the minimum number of lifetime components that provide a useful analysis. When the number of detected photons is limited, e.g., for live cell imaging, it is common to fit complex fluorescence decay profiles to a monoexponential decay model and comparisons of the lifetime value obtained can provide a semi-quantitative readout. For more rigorous analysis of complex fluorescence decay profiles in FLIM experiments it is possible to increase the effective number of detected photons used in the fitting through global analysis techniques where either all the pixels in a region of interest are assumed to have the same fluorescence decay profile and so their respective FLIM data can be combined to fit to a complex model (“global binning”) or it is assumed that, although each pixel can have a different fluorescence decay profile, it always results from a mixture of spatially invariant lifetime components such that all the FLIM pixel data can be fitted simultaneously (“global fitting”) [60]. Global fitting enables fitting to double or triple lifetime component models with a similar number of detected photons per pixel as is required for fitting a pixel to a monoexponential decay.

An alternative approach to handle FLIM of complex decay profiles is phasor analysis, which is a fit-free graphical approach to analysing FLIM data by calculating the first Fourier harmonic components from the decay profiles to yield two parameters, $\mathbf{S}$ and $\mathbf{G}$, that represent the sine and cosine components of the decay profile respectively, enabling the FLIM data to be visualised on a polar plot [61]. Mono-exponential lifetime decay profiles are mapped to points along a semicircle, while two-component (bi-exponential) decay profiles appear along a chord connecting the two points on the semicircle corresponding to each of the lifetime components. When all the FLIM data from a field of view are plotted on the phasor ($\mathbf{G}$, $\mathbf{S}$ polar) plot, clusters may appear that can be attributed to individual lifetime components or mixtures of lifetime components according to their position. A key advantage of the phasor approach is that it eliminates the need to predefine a fitting model, allowing for an initially unbiased analysis. If there are more than two lifetime components present in a pixel, the phasor analysis can be extended to higher Fourier harmonics of the decay profiles.

Two key applications of FLIM data fitted to a two component model are FRET (discussed below) and FLIM of cellular autofluorescence attributed to nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), two coenzymes active in cell metabolism processes. FAD and NADH are autofluorescent and each exhibit different lifetimes according to whether they are bound or unbound to other molecules. For FAD the free molecule has a longer lifetime than the bound, and vice-versa for NADH. The proportion of free and bound FAD and NADH within a cell is commonly

interpreted as being dependent on the dominant process of energy production active within the cell, i.e., glycolysis or oxidative phosphorylation [62], although this is a simplified model and the actual dependence is more complicated. If the short and long lifetimes are designated as a_{short} and a_{long} respectively, then during glycolysis the ratio $\frac{a_{short}}{a_{long}}$ increases for NADH, and decreases for FAD. It is possible to calculate a metric from such FLIM data known as the fluorescence lifetime redox ratio, which is calculated as:

$$\text{Equation 12} \quad FLIRR = \frac{\left(\frac{a_{long}}{a_{short} + a_{long}} \right)_{NADH}}{\left(\frac{a_{short}}{a_{short} + a_{long}} \right)_{FAD}}$$

where it can be seen that the sum $a_{short} + a_{long}$ gives the total fluorescence for that channel, meaning that this ratio is independent of intensity and relative abundancies of NADH and FAD [63].

2.6.4 Fluorescence polarisation readouts

When a population of fluorophores are excited with polarised light, the fluorophore dipoles suitably aligned with that polarisation are preferentially excited and the resulting fluorescence exhibits a net polarisation.

By resolving the polarisation state of fluorescence emission resulting from polarised excitation light, it is possible to map variations in fluorophore dipole orientation, which in turn can provide information about orientation of nanostructure. For example, polarisation-resolved fluorescence imaging of collagen can reveal information about the orientation of the collagen structure..

In many samples, including live cells, the fluorophores may have the freedom to rotate over the timescale of their fluorescence lifetime. This allows the fluorescence dipole orientations to become randomised after excitation resulting in the fluorescence emission being less polarised. This is parameterised by a quantity called the fluorescence anisotropy. Fluorophores bound e.g, to proteins with restricted freedom to rotate will exhibit a high fluorescence anisotropy compared to fluorophores with greater rotational freedom. The rate of rotational dephasing (and decrease of fluorescence anisotropy) also depends on the viscosity of the environment and on the size of the entity undergoing Brownian motion. Measuring the rotational dephasing time can be useful for studying molecular interactions and conformational changes in proteins, as these events can affect the rotational mobility of attached fluorophores [64].

2.6.5 Readouts based on fluorescence dynamics

Fluorescence correlation spectroscopy (FCS) is a technique that measures fluctuations in fluorescence intensity over time to provide insights into molecular dynamics within cells [65]. In the classic FCS set-up, a tightly focussed beam illuminates the sample and confocal detection is used to detect the fluorophores in a femtoliter volume. Fluorophores passing in and out of this volume cause fluctuations in the detected fluorescence intensity and these fluctuations can be mathematically modelled such that the diffusion coefficient can be calculated. This coefficient is dependent on the mobility of the fluorophore and therefore changes depending on its size (which may change due to molecular binding), and on local viscosity changes. The method has been used to investigate drug target engagement [66]. As the fluctuations in intensity occur over sub-nanosecond time scales, detectors with a high enough time resolution are necessary, which are similar to those used for TCSPC FLIM. FCS is less often implemented as an imaging modality as the time taken to collect sufficient data from each point (focal volume) is significantly longer than that needed for fluorescence lifetime imaging. However, there are image scanning FCS modalities such as line scanning FCS, e.g., [67].

Fluorescence recovery after photobleaching (FRAP) is another technique that can provide information on diffusion dynamics in the cell. Here the fluorophores in a specific region are intentionally photobleached irreversibly using high intensity light. As time passes, non-bleached fluorophores from the surrounding region will diffuse into the photobleached area such that fluorescence emission is restored and by measuring the recovery of fluorescence intensity as a function of time one can learn about dynamics in the cell such as the mobility of nuclear proteins [68]

2.6.6 FRET

Förster resonance energy transfer (FRET) is a technique used to study molecular interactions and proximity at the nanometer scale. It occurs when energy is transferred non-radiatively from an excited “donor” fluorophore to an “acceptor” fluorophore in close (~ 10 nm) proximity when the excitation spectrum of the acceptor overlaps with the emission spectrum of the donor fluorophore. The efficiency of energy transfer is dependent on the orientation between the donor and acceptor, the degree of overlap between the donor emission and acceptor excitation spectra and is inversely proportional to the sixth power of the distance between them, with the relationship $E_{FRET} \propto \left(\frac{R_0}{r}\right)^6$ defining R_0 , the Förster distance at which the FRET efficiency between the donor and acceptor is 50%. E_{FRET} is very sensitive to changes in intermolecular distance and R_0 is on the order of nanometres for a typical FRET pair of fluorophores [69].

FRET provides a mechanism to optically readout when donor and acceptor fluorophores are within 10 nm of one another and therefore provides a means to detect and map molecular interactions. This is a common approach to study protein interactions in cell signalling pathways where cells are genetically engineered to express the proteins of interest labelled with fluorescent proteins, e.g., CFP and YFP or GFP and mCherryFP serving as donor and acceptor respectively. Another key application is to engineer FRET biosensors that are molecular constructs with a FRET pair of fluorophores typically linked at each end of a sensing protein domain whose conformation changes when it binds an analyte, thereby changing the separation and/or relative angle of the donor and acceptor fluorophores and therefore the FRET efficiency. One example of a FRET biosensor is the AMPKAR biosensor that is sensitive to the ratio of key metabolites (AMP + ADP)/ATP and therefore to the metabolic status of the cell [70].

FRET can be detected and mapped using spectral or lifetime modalities and there are many techniques in use (see e.g. [71]). In the spectral domain, the most common approaches excite the donor and ratio the intensity of the acceptor fluorescence to the intensity of the donor fluorescence. However, all spectral FRET measurements are prone to artefacts due to direct excitation of the acceptor as well as spectral bleed-through between the donor and acceptor channels [72] and it is challenging to make quantitative measurements of FRET efficiency using spectral methods.

FRET can also be detected and quantified using lifetime measurements, as an increase in the FRET efficiency causes a decrease in the donor fluorescence lifetime as the excited state donor population loses energy more quickly. By fitting the donor fluorescence decay profile to a two component decay model, it is possible to determine the lifetimes of the FRETing and non FRETing donors and also to determine the population fraction of donor fluorophores that are undergoing FRET. This can be used for quantitative assays of protein interactions and FRET biosensors, e.g.[73], and today FLIM has become the gold standard for quantitative FRET measurements, but it does require more detected photons to make a measurement compared to intensity-based wavelength ratiometric measurements of FRET [74]. FLIM FRET is a particularly convenient tool to implement in multiphoton microscopes since they inherently have the ultrashort excitation pulses to enable FLIM and the use of multiphoton excitation is well suited to study cell biology in 3D cell cultures, e.g., [75]

2.6.7 Multi-modal fluorescence microscopy

Multi-modal microscopy involves using different imaging techniques on the same sample, with each modality providing complementary information to enhance the overall analysis. Any of the aforementioned fluorescence readouts can be used together and in conjunction with brightfield or phase contrast techniques to gain a more complete picture, e.g., of biological processes in a cell or tissue. For example, phase contrast and FLIM have been combined to provide morphological information with

complementary functional readouts from FLIM of autofluorescence to study changes in cell metabolism [76]. At the state of the art, multiphoton microscopy can be combined with other nonlinear microscopy techniques including second and third harmonic generation to study intravital biology [77], and this approach can be further extended with simultaneous near-infrared fluorescence imaging and reflectance confocal microscopy as well as two-photon excitation, second harmonic generation and FLIM modalities [78],

2.7 Advanced techniques

This section will briefly review some advanced techniques that extend the capabilities of light microscopy but which I did not use for the work reported in this thesis.

2.7.1 Super-resolution microscopy

As previously mentioned, there is a fundamental limit to the lateral spatial resolution of a diffraction-limited light microscope of $r = \frac{0.51\lambda}{NA}$, which usually falls in the range of 100s of nanometres. Obtaining resolution beyond the diffraction limit has been a thriving area of research, with two approaches being recognised by the Nobel Prize in Chemistry in 2014 [79].

The first of these approaches was stimulated emission depletion (STED) microscopy [80]. In this extension of laser scanning confocal microscopy, the sample is illuminated with two laser beams, one conventional excitation beam focussed to a diffraction limited spot to populate the S_1 excited state of the fluorophores, and a second annular shaped beam used to deplete the excited state population through stimulated emission, switching off the fluorescence except at the centre of the beam. The result is a central population of excited fluorophores that is much smaller than the illumination spot size, such that higher resolution can be achieved. The size of the central region of fluorescence depends on the degree to which the excited fluorophore population is depleted and so depends on the power of the stimulated emission depletion (STED) beam. In principle this can be increased arbitrarily to further enhance the resolution but the high intensity illumination will eventually cause photobleaching/photodamage with detrimental effects to biological samples. In practice, resolutions below 30 nm have been achieved in fixed cells using a STED microscope, e.g., [81].

The second approach to realise super-resolved microscopy encompasses a broad range of techniques that can be described as single molecule localisation microscopy (SMLM). A specific SMLM technique in wide use is dSTORM [82], which is an evolution of stochastic optical reconstruction microscopy (STORM) [83] and has achieved resolutions below 10 nm [84]. The technique also relies on switching of emission from fluorophores that can be induced to transition stochastically between fluorescent and non-fluorescent states. A sequence of fluorescence images of the field of view of “blinking” fluorophores is acquired in which only a sparse subset of the fluorophores

are emitting during each image frame. This reduces the overlapping of the PSFs making it possible to centroid each one and determine the position of the fluorophore to a precision beyond the diffraction limit that depends on the signal to noise ratio. By compiling a localisation table of all the emitting fluorophore positions, a super-resolved image can be reconstructed from the acquired image sequence data.

A further technique to improve the resolution of a microscope is structured illumination microscopy (SIM) [85], [86]. This method illuminates the sample with a set of spatially varying excitation intensity patterns, resulting in spatially modulated fluorescence images that contain information about higher spatial frequencies present in the sample in a manner analogous to Moire fringes. Using the appropriate Fourier analysis, one can demodulate the acquired image data to obtain a final image with twice the standard lateral and axial resolution of the widefield microscope, e.g., [86]. Although not providing true super resolution, SIM surpasses the conventional diffraction limit.

2.7.2 Adaptive optics

Adaptive optical elements are components that can control the amplitude or phase of a wavefront. A classic use of adaptive optics in fluorescence microscopy is to correct distortions of wavefronts occurring when light propagates through aberrating optical systems such as refractive index interfaces (e.g. at the sample/coverlip/immersion fluid interfaces) and biological tissue. In laser scanning microscopy, optical aberrations can broaden the spot size of the focussed excitation beam generated in the sample, and also broaden the spot size of the fluorescence signal returning to the confocal pinhole, degrading the 3D imaging [87]. Adaptive optical elements can be programmed to pre-distort the wavefront in the exact opposite manner to the distortion caused by the aberrated imaging process, meaning that the focussing beam is closer to the ideal spherical wavefront needed to achieve the minimum focal volume in the sample. This is especially important for two photon microscopy as the fluorescence signal is proportional to the square of the peak intensity [88]. There are a range of methods to determine the appropriate correction to apply to the adaptive optical element shaping the incident wavefront. Sensor based adaptive optics directly measures aberrations in the wavefront, for example by using a Shack-Hartmann wavefront sensor to measure a reflected wavefront from the excitation light, which can then be used to calculate a correction [89]. Alternatively, an image-based metric such as sharpness or total intensity may be used as a measure of success, where iteratively applying varying amounts of known aberrations, e.g. a set of Zernike polynomials, to the wavefront and measuring the metric allows an optimum wavefront modulation to be found. This is known as sensorless adaptive optics, as no direct wavefront sensor is used; this technique has recently been making use of machine learning to create more accurate guesses for the wavefront correction [90]. The two most commonly used adaptive optics elements are spatial light modulators (SLM) and deformable mirror devices

(DMD), which can be programmed to manipulate the amplitude or phase of the wavefront on a pixel-wise basis.

2.7.3 High Content Analysis (HCA)

Light microscopy is a ubiquitous tool in cell biology, biomedicine and drug discovery. Given the variability found in biological samples, and the range of conditions certain experiments can demand, taking images of multiple FOVs is often desired. This process can be laborious, requiring human attention for several hours, and also contributes to the increased possibility of human error or bias entering into the experiment. A solution to this is automated imaging of multi-well plates, which grants the ability of collecting vast amounts of data without the need for human control, a technique often referred to as HCA. Not only does HCA improve the amount of data that can be collected, but due to the automated nature of the control and acquisitions, reproducibility of experiments is also improved. This allows biologists to carry out experiments with greater statistical power, revealing weaker readouts through averaging of many samples. HCA can be carried out on fixed samples, providing an end-point readout, or on live cells where an incubator must be incorporated into the microscope frame allowing readouts at multiple time-points.

A common HCA experiment will take in a multi-well plate containing cells in each well, with a set of conditions each with some number of repeats used across the well. Repeat measurements can be made within a well or between wells, with the latter accounting for variability in the pipetting process. A position list dictating the locations where the automated sample stage will visit must first be made. The positions may be simply calculated, e.g. a centred on the middle of each well, or found automatically during the experiment. Some HCA systems may have a pre-find procedure which scans each well and uses some metric to determine which regions of the well are of interest. If images using multiple modalities are desired at each position, the components required to switch modalities must also be automated and scripts written to automatically change the configuration settings of the components. As multiple wells are visited within an experiment, it is vital that the labelling of images contains the well in which an image is taken. Often this is saved in the image metadata that can be read off at a later date to determine the conditions present for that well. Once data is collected specialised image processing pipelines are often used to compress the large amount of image data into the readouts relevant to the experiment carried out. Image segmentation can also be used so that statistics can be calculated on a per cell basis. Once the relevant information is collected, figures indicating how readouts vary over the multi-well plate provide an easy method of viewing the outcome of the experiment. HCA is very effective in drug discovery programmes. The HCA process described is carried out with different drugs applied across the plate, rapidly increasing acquisition time and therefore the range of compounds that are tested compared to manual image acquisition [91].

Due to the increased complexity of automation and larger amounts of data to process, HCA is mainly limited to intensity imaging. Cell painting is a morphological profiling assay that uses readouts from multiple spectral channels to observe the change in shape of various components of the cell. This assay has been carried out using genetic and chemical perturbations across multi-well plates [92]. The HCA format has been used with ratiometric FRET to study protein-protein interactions [93] and with anisotropy measurements for investigations of antibiotic binding [94]. HCA has been demonstrated with more advanced microscopy techniques, for example work done in the group at Imperial used FLIM FRET assays to study the oligomerisation of the HIV-1 Gag protein [95]. Super-resolution HCA has also been realised using STORM to image cell focal adhesions in a multi-well plate [96] and HCA QPI has been applied to non-invasive monitoring of live-cell population biomass [97]. 3D FRET has also been studied in HCA ratiometrically using a light-sheet system [98]. Despite these examples, the uptake of advanced microscopy techniques in HCA is generally low.

The aim of the microscope described in this thesis is to create a system that is both HCA compatible and allows advanced techniques such as 3D imaging, FLIM-FRET and QPI. In order to improve the imaging speed of the multiphoton 3D FLIM-FRET imaging, a multibeam configuration is used, allowing higher throughput for assays.

3 Design and implementation of multibeam multiphoton multiwell plate microscope (M³M) with TCSPC FLIM

This chapter describes the initial design and implementation of a multi-point-scanning multiphoton TCSPC FLIM microscope implemented for automated imaging of samples arrayed in multiwell plates. This chapter reports the first design and implementation, with some preliminary results imaging biological samples to demonstrate its potential for multiphoton high content analysis. After testing, this M³M HCA instrument was further refined, as discussed in the following chapter.

3.1 Design considerations

Here the motivation to build the microscope will be discussed, as well as specific considerations for implementing beam multiplexing in multiphoton microscopy. I will also review some previously reported multibeam multiphoton microscopes to provide context and motivation for the specific design considerations for this M³M instrument.

3.1.1 Motivation

This microscope was developed as part of the MACH3Cancer CRUK Accelerator project (www.mach3cancer.org) that aims to develop automated microscopy instrumentation to systematically investigate heterogeneity in response to cancer drugs in 3D cancer models. These 3D models represent a bridge between conventional 2D cell-based assays that enable high throughput and *in vivo* studies in animal models. This entails facilitating single-cell resolved time-lapse assays, which may run over multiple cell cycles, of more physiologically complex cancer models such as spheroids, organoids and tissue slices. For such long time-lapse assays in 3D disease models, minimal photobleaching/phototoxicity and optical sectioning are critical issues. Within the MACH3Cancer Accelerator, two optically sectioning fluorescence microscope approaches had already been implemented for automated multiwell plate imaging namely a widefield time-gated FLIM microscope with a spinning disc confocal scanner and an oblique plane microscope (OPM) system, both of which are routinely used for HCA in our project. However, spinning disc confocal microscopy is limited by interpixel cross-talk that increases with imaging depth due to light scattering and OPM is also limited by light scattering and is not yet developed to provide rapid FLIM capabilities. In order to realise deeper imaging, and also to benefit from the ability of multiphoton microscopy to excite cellular autofluorescence and facilitate FLIM, a third optically sectioned single-cell resolved microscopy approach was explored with the intention to apply it to thicker, more physiologically complex biological samples, including for HCA. This approach is termed “**multibeam, multiphoton, multi-well plate microscopy**” (M³M). Figure 8 shows a schematic of the MACH3 Cancer Accelerator project and the instruments implemented. Given the lower excitation cross section for multiphoton microscopy, it is

expected to be slower than the single-photon excited spinning disc confocal and OPM techniques but to be able to image deeper into complex biological samples recapitulating the tissue context.

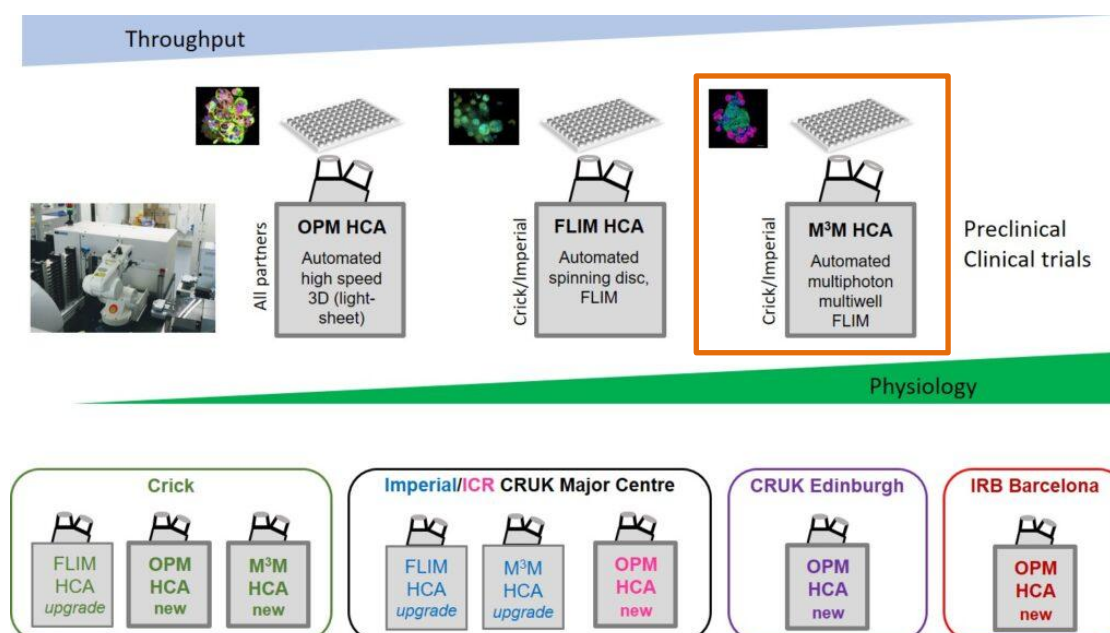


Figure 8 MACH3cancer microscope development overview. The microscope developed in this project (highlighted in orange box) is intended for used on the more physiologically complex samples.

The goal for this microscope was to investigate 3D tumour spheroids, organoids and tissue with single cell resolution up to depths of $\sim 200 \mu\text{m}$ – the practical limit for OPM in scattering tissue, whilst minimising phototoxicity/maximising imaging speed and providing fluorescence lifetime readouts. The motivation for even modest increases in imaging speed is to increase throughput in HCA, enabling more biological conditions to be probed within a given time-frame, rather than to resolve fast biological processes. A high content multiwell plate assay can take many hours and so even a factor of 2 is important to make an assay more practical to undertake. Fluorescence lifetime readouts can be used for quantitative FRET measurements and for assays utilising cellular autofluorescence, e.g., of NAD(P)H and/or flavoproteins to probe changes in cellular metabolic state. Compared to intensity measurements, fluorescence lifetime readouts are relatively robust in the presence of optical scattering and are the effective gold standard for quantitative measurements of fluorescence biosensors, but lifetime readouts require significantly more photons per measurement than intensity-based readouts – necessitating longer data acquisition times or higher excitation powers.

To meet these aims, two-photon fluorescence excitation was chosen as it is an effective means to achieve optical sectioning and the ultrafast excitation pulses can be exploited for FLIM. The broad 2-photon excitation spectra also make it relatively easy to excite a wide range of fluorophores, including those that would otherwise require phototoxic ultraviolet excitation. Phototoxicity is a critical issue for

time-lapse live cell imaging and, while 2-photon excitation has the advantage that out-of-plane fluorophores are not excited, the photobleaching/photodamage associated with the in-plane nonlinear excitation is greater than that of single-photon excitation and scales nonlinearly with peak power with a higher exponent than the quadratic dependence of the 2-photon fluorescence signal [99]. To minimise phototoxicity and also to speed up image data acquisition, we chose to multiplex the excitation laser beams such that multiple points at the sample are excited simultaneously. This decreases the photodamage for a given image data acquisition time and can increase the imaging speed if sufficient excitation power is available.

3.1.2 Multiphoton beam multiplexing

Most multiphoton microscopes entail scanning a single excitation beam on the sample and are frequently optimised for deep imaging, where a large detector placed close to the sample is used to collect as much fluorescence as possible in a “non-descanned” mode. Where high speed multiphoton imaging is desired, e.g., in neuroscience to map fast voltage transients [3], image data acquisition times are typically minimised by only acquiring data from regions of interest within the field of view. Such “smart scanning” techniques may first take a coarse overview image and then select specific regions to scan, allow quicker acquisition of regions of interest by avoiding scanning areas without fluorescence. One example is “random access scanning” [100]. For very bright samples (e.g., dye-based fluorophores) where phototoxicity is not a prime consideration, fast resonant scanners have been used to realise video-rate two photon imaging, however, fluorescence saturation can start to become an issue [4]. Since phototoxicity inevitably increases with peak excitation power, it can only be reduced while maintaining imaging rate by dividing the excitation power between multiple excitation foci at the sample. This approach to increase imaging speed by scanning multiple excitation beams in parallel can be described as excitation beam multiplexing and is usually implemented in a descanned imaging mode, where the fluorescence captured by the objective lens passes back through the scanning mirrors such that it presents a stationary intensity distribution at the detection plane. There has been at least one attempt to realise a non-descanned multifoci multiphoton microscope [5] but this entailed significant experimental complexity and required multiple image acquisitions per FOV of a moving sample. Our ambition was to make a practical instrument that could be easily replicated by the community using easily obtainable components and the MACH3Cancer project was funded to explore using parallelised excitation and descanned detection.

There are some important considerations for multiplexed beam excitation in a multiphoton microscope. The power one can use to excite a sample in two-photon microscopy is limited by phototoxicity to some maximum, P_0 . Dividing P_0 between n beams results in a total two-photon fluorescence signal proportional to $n \cdot \left(\frac{P_0}{n}\right)^2$, which is a factor of n less than the fluorescence signal

produced if all the excitation power was in one beam. If it is not possible to increase the power of the multiplexed beams back to the original P_0 per beam, then the signal acquisition rate will be decreased. This can easily happen when attempting to image deep in attenuating samples where the exponential decrease of the excitation light intensity with depth needs to be balanced by increasing input excitation power (up to a limit, e.g., imposed by surface thermal damage). For example, excitation powers in the range of 100s of mW are required to image down to > 500 microns in mouse tissue [101] but most multiphoton excitation lasers provide less than 2 W average excitation power and there are significant losses before the excitation light reaches the sample. This include the optical system for dividing the excitation laser into multiple beams and any losses in the objective lens – noting that many do not offer high transmission efficiency in the near infrared. This means at a certain imaging depth when the power cannot be increased to the photodamage limit, it starts to become less preferable to have multiplexed beams. Therefore, the M³M instrument was designed to be flexible with respect to the number of parallel exaction beams – and to provide the option of a single scanned excitation beam with non-descanned detection, e.g. for use when imaging as deep as possible.

There are also significant losses in the descanned imaging path. One key benefit of multiphoton microscopy is the lack of a need for a pinhole to reject out of focus fluorescence, as the excitation properties already provide the optical sectioning. This allows a single beam multiphoton microscope to use a large detector collecting all of the emitted fluorescence, i.e. scattered and un-scattered photons, with confidence that they originated from the focus of the excitation beam. When multiplexing excitation beams the detectors must now collect photons from distinct spatial regions in the FOV, meaning that the collection area must be smaller, therefore collecting less scattered light. More strongly scattering samples therefore favour single beam excitation with the higher collection efficiency of the larger detector. Also, in a multibeam configuration one expects losses on the order of 30% along the descanned path compared to a non-descanned path due to optical surfaces and apertures [102], further diminishing the collected signal for multibeam vs. single beam modalities. There can also be crosstalk where fluorescence excited by one beam is scattered such that it is detected in another channel. This cross-talk increases as detectors are brought closer together. As more scattering occurs when imaging deeper into a sample, the collection efficiency of scattered photons decreases due to the finite detector size and cross-talk increases for multiplexed detectors, meaning at some point it becomes preferable to switch to the single beam non-descanned modality.

3.1.3 Earlier multifocal multiphoton microscopes

The first multifocal multiphoton microscopes used a microlens array, [41], [103] beamsplitter [42] or a diffractive optical element [43] to generate multiple beams for multiphoton excitation and then implemented non-descanned detection with a camera – or a gated image intensifier for FLIM [104], [105] [106], [107]. While this approach works reasonably well for imaging thin samples, the performance is severely degraded with imaging depth in a scattering medium due to interpixel cross-talk. For deeper imaging, e.g., in tissue or 3D cell cultures, it is desirable to use fewer excitation beams spaced adequately to minimise cross talk and with sufficiently large detection areas to be able to collect a significant fraction of the scattered fluorescence photons. In this regime, it is desirable to benefit from the high signal to noise of sensitive photon-counting detectors – where TCSPC can be utilised for FLIM.

This section briefly reviews three distinct approaches to multifocal multiphoton microscopy and sets out key factors in the design of a multibeam multiphoton system. These are the beam multiplexing technique, collection area of individual detectors, distance between individual detectors and detector properties. It is convenient to consider the demagnified detection unit at the sample plane comparing its dimensions with respect to the $\sim 0.5 \mu\text{m}$ FWHM PSF one expects to find for a multiphoton beam focus.

The first of these systems, reported in 2007 by K. Kim [108], utilised an 8×8 array of square microlenses to produce an excitation beam array producing foci spaced by 45 microns at the sample plane. The use of square micro-lenses allows a higher light throughput compared to their circular counterpart. However, microlens arrays can suffer from power variations per beam due to uneven (Gaussian beam) illumination of the array or lower throughput if the array is over-filled by an expanded beam. For parallelised descanned detection, these authors used a multi-anode photomultiplier tube (MAPMT) array consisting of an 8×8 grid of 2 mm by 2 mm anodes to detect the emitted fluorescence, with a 45x magnification from sample to detector plane. This results in detectors of 90x the PSF size, spaced by the same factor. The MAPMTs used had a typical dark count of 100 counts per second (cps) and 15-20% quantum efficiency across the visible spectral range. They reported maximum data transfer rates of 8 MHz using a specially designed multichannel photon counting card [109]. This system was not configured for FLIM.

The second system reported in the same year by S. Kumar [110] (at Imperial) used a 50/50 beam splitter mirror array, incorporated in a commercial microscope system (TriMscope, LaVision Biotec GmbH) for beam multiplexing and a 16-stripe MAPMT for TCSPC detection for FLIM. The anode stripes were 0.8 mm x 16 mm with 1 mm spacing between stripes. A 400x magnification from detector to sample plane meant that the spacing between stripes was 5 PSFs, and the detector size was 4 PSFs in height and 80 PSFs in width. They reported maximum count rates of 2.4 MHz for the whole detector array. The MAPMT had a sensitivity of <30% at 450 nm. This system was partially descanned – the fluorescence beams moved along the anode stripes during each image acquisition.

A more recent approach to realise multifoci multiphoton FLIM microscopy reported by S. Poland et al [44], used an SLM to diffract an incoming beam into an 8 by 8 array with a 12.5 micron spacing at the sample plane, the fluorescence from which was detected by an 8 x 8 array of 6 micron diameter SPADs, spaced by 50 microns. Magnification from sample to detector was 4x, meaning that the SPAD diameter at the sample plane was 3 PSFs, and the spacing 25 PSFs. They state their maximum count rate per detector was 0.5 MHz, resulting in a total count rate of 32 MHz. The SPADs had 28% detection efficiency at 500 nm.

The M³M system reported in this chapter also used an SLM for beam multiplexing and the detection aperture was provided by optical fibres of core diameter 200 microns, spaced by 3.6 mm. There was a 60x magnification from fibre to sample plane, meaning the detection diameter was 6.7 PSFs and detector spacing 120 PSFs. The detectors used each allowed a maximum count rate of 8 MHz, giving a total of 48 MHz with 6 detectors, with 45% efficiency at 500 nm.

System	Beam multiplexing method	Detector diameter/ PSF FWHM	Detector spacing/ PSF FWHM	Max count rate/ MHz
Kim et al [6]	Micro-lens	90	90	8
Kumar et al [8]	50/50 beam splitter mirror array	4	5	2.4
Poland et al [9]	SLM	3	25	32
Flanagan (1 st iteration)	SLM	6.7	120	48
Flanagan (2 nd iteration)	SLM	25	180	48

Table 1 Comparison of detection geometry parameters for existing multibeam multiphoton systems and the system designed in this work for both the 1st and 2nd iterations.

From Table 1, one can draw conclusions about the potential effectiveness of these systems for deeper imaging, where increased scattering of fluorescence is a factor. Detection systems with larger detector diameters are more likely to capture scattered light. Additionally, a smaller detector spacing and larger detector diameters increases the likelihood of cross-talk. The intention of our system is to be applicable to both thin “2D” cellular samples and thicker scattering samples including 3D cell culture and tissue slices. We want to realise multiplexed imaging without incurring high levels of cross-talk in

scattering samples and so we aim to have a low detector diameter to spacing ratio. The combination of detectors and TCSPC electronics used in our system also allows for a high max count rate and quantum efficiency at visible wavelengths. In particular we used an array of hybrid photomultipliers since these are the best available detectors for TCSPC, providing high quantum efficiency (maximum 50%) and low after-pulsing. The size of these detectors does not allow for them to be placed together perpendicular to the optical axis, so optical fibres were used to collect and deliver the fluorescence to them, a technique previously used in our laboratory [111] . An SLM was chosen for producing the multiple beams as it could be programmed for fine tuning of the positions of the excitation beams with respect to the optical fibres, whilst also being able to work with the broad range of wavelengths covering the tuning range of our excitation laser (690- 1040 nm) enabling the excitation of a wide range of fluorophores.

This M³M system was intended to be applicable to different sample formats and so was designed such that the number of excitation beams could be easily changed (programming the SLM) and could also be switched between multibeam descanned detection and single-beam non-descanned detection. Further flexibility was conferred by implementing the M³M on the *openFrame*-based microscope platform [112] that originated in our laboratory and was co-developed with Cairn Research Ltd. This includes an optical autofocus module to enable time-lapse and multiwell plate imaging and can be configured with a range of excitation and detection options, here including both wide-field single photon excitation and (spectrally-resolved) detection, multiplexed scanned multiphoton excitation and detection, and scanning single beam excitation with non-descanned detection. It was also adapted for wide-field brightfield and quantitative phase imaging and could be adapted for other modalities including polarisation-resolved imaging and harmonic microscopy - including with transmitted light detection. This M³M microscope also incorporated a novel microscope incubator constructed using optical breadboards that can enable a range of transmitted light sources and detectors to be mounted.

3.2 Description of M³M excitation path

The key components of the first M³M instrument implemented and described in this chapter are represented in Figure 9, with Table 2 providing a key for the optical components. These are the femtosecond excitation laser, the power control unit, beam expander, SLM, scanning mirrors, *openFrame* microscope stand, optical fibres used for detection and the array of hybrid photomultiplier assemblies (HPMA – incorporating focusing lens and photomultiplier) and TCSPC electronics. The components for excitation beam multiplexing and scanning/descanned detection are mounted on an enclosed breadboard that is coupled to the (*openFrame*) microscope stand.

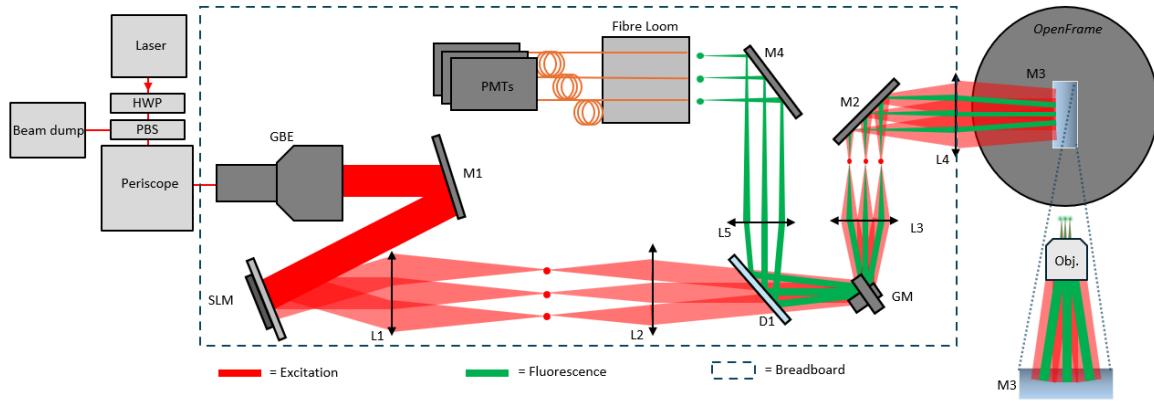


Figure 9 M³M multiplexed excitation/descanned detection breadboard and *openFrame*.

For this M³M implementation the output beam of a femtosecond near infrared laser system (Spectra Physics, MaiTai HP) was directed through a (halfwave plate/linear polariser) power control unit into the breadboard module where the beam is expanded onto an SLM (Santec, LCOS-200) whose function is to diffract the incoming beam into 6 collimated outgoing beams upon reflection. These beams were scanned across the sample using a set of XY galvanometric scanning mirrors (Thorlabs QS7XY-AG - Dual-Axis). The fluorescence emitted by the sample was descanned passing back through the galvanometric scanning mirrors and reflected at a long pass dichroic beamsplitter to be directed to the optical fibre loom. This comprised a line of six 200 micron core multimode optical fibres that delivered the light to a set of HPMAs that are connected to the TCSPC electronics.

Label	Component	Details
GBE	Beam expander	10x
SLM	Spatial light modulator	Creates multiple beams
L1	Relay Lens	F = 300 mm
L2	Relay Lens	F = 150 mm
D1	Dichroic	Long Pass
GM	Galvo Mirrors	Scans beams
L3	Scan Lens	F = 50 mm
L4	Tube Lens	F = 200 mm
L5	Collection Lens	F = 150 mm
Fibre Loom	Fibre Loom	Six 200 micron fibres
Obj.	Objective lens	20x water immersion 0.95 NA

Table 2 Components in M3M excitation/ descanned detection optical path

3.2.1 Laser, Power control and Beam Expansion

The Spectra Physics MaiTai HP laser used for multiphoton excitation provided <100 fs pulses at 80 MHz repetition rate and a tuning range of 690 – 1040 nm with an average power of >2.5 W at 800 nm [113]. The output laser beam was polarised > 500:1 horizontal: vertical with a diameter <1.2 mm ($1/e^2$) and beam divergence <1.2 mrad.

To provide electronic control of the output power of the laser, a rotatable HWP followed by a polarising beam splitter (PBS) was used. Using the HWP, the initial horizontal polarisation of the laser output could be rotated to any linear polarisation angle. The PBS placed after the HWP then transmits a proportion of light according to

$$\text{Equation 13} \quad I = I_0 \cos^2(\theta)$$

Where I and I_0 are intensities after and before the linear polariser respectively and θ is the angle between the polarisation after the HWP and the transmission axis of the PBS. With this setup, power can be

controlled whilst maintaining a constant output polarisation from the power control unit. The reflected axis of the PBS was sent to a beam dump which could absorb the unused optical power.

A 10x Thorlabs GBE was used to magnify the beam onto the SLM: this magnification was chosen such that the beam out of the MaiTai would slightly overfill the smaller axis of the SLM (9.6 mm) given the beam output diameter of <1.2 mm. A shear plate was used to ensure a collimated output from the GBE.

3.2.2 SLM

A Santec LCOS-200 phase only SLM was used to diffract the incoming light from the GBE and produce the multiple focussed beams that were scanned across the sample [114]. The active face contains 1920 x 1200 pixels (width x height) with each pixel measuring 7.8 microns x 7.8 microns. There is 0.2 microns dead space between pixels, meaning a distance of 8 microns between pixel centres. The total panel size is therefore 15.36 mm x 9.6 mm (width x height). It had a 10-bit bit-depth and an operating wavelength range of 450-1600 nm with a 60 Hz refresh rate. The SLM could be controlled in MicroManager through a DVI cable, treating the SLM as another monitor for the PC.

An LCOS SLM is controlled through the voltages applied across the array of pixels. At each pixel the optical axis orientation of a layer of nematic liquid crystal is altered by the applied voltage. The orientation of the liquid crystal changes the refractive index for light propagating in that location, meaning that by controlling the pixel voltage, the output phase of light from that pixel can be varied. The far field distribution of light is given by the Fourier transform of the field at the SLM; thus, the intensity distribution at the far field – and therefore in the sample plane - can be controlled by varying the SLM phase modulation.

As each beam will be translated by the same amount as the galvo mirrors are scanned, ideally over a rectangular FOV, only a line and grid pattern of evenly spaced beams is practical. A line of 6 foci was chosen for the first pattern generated by the SLM as it is simpler than a grid, and has the added advantage that one may scan further in one axis, as opposed to a grid where scanning the beams by more than one beam spacing unit would overlap them with a previously scanned area, as is shown in Figure 10. A FOV of 360 x 360 microns was chosen, intended to be sufficient to image larger spheroids and organoids. The foci were spaced by 60 microns in one axis at the sample plane, corresponding to a spacing of 3.6 mm at the image plane between L1 and L2, with the outermost beams positioned at +/- 9 mm away from the optical axis at this plane. Given the focal length of L1 was 300 mm, each 3.6 mm shifted away from the optical axis corresponds to 12 mrad angular subtense from the SLM.

Lenses L1 and L2 are used to relay the SLM to the midplane of the galvo-scanning mirrors with a magnification of 0.5x. This allows the 9.6 mm diameter field to fit onto the 7 mm aperture galvo mirrors with some room to spare to avoid vignetting. The 150 mm focal length of L2 also allowed for sufficient space between the lens and the galvo scanners to insert a dichroic beamsplitter for diverting the fluorescence signal towards the detection fibres.

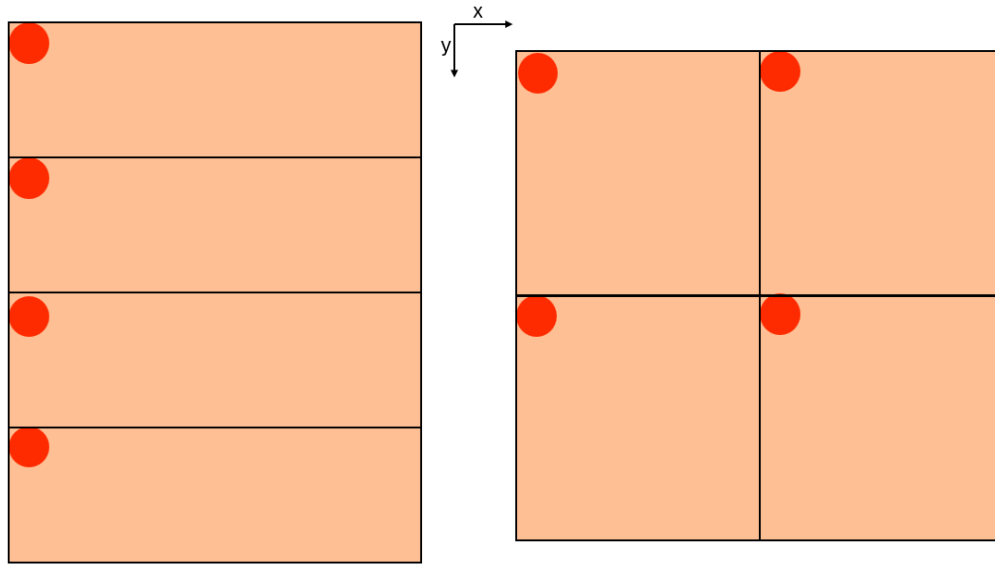


Figure 10 Line and grid multifocal patterns. The line of beams allows for larger scan length in x axis compared to the grid of beams where overlapping scan regions occurs after scanning one beam spacing.

3.2.2.1 SLM parameters

To appreciate the range of physical parameters required to programme the SLM, we can consider the lateral distance a beam is shifted at the focus of a lens when a phase ramp is applied. By increasing the phase of the pixels on the SLM, one is effectively adjusting the delay of the reflected light across it. By applying a phase of 2π radians to a pixel, the light is retarded by a full cycle, or λ spatial delay upon reflection; for an arbitrary phase change P , the delay distance is $\frac{P\lambda}{2\pi}$.

A phase change across the SLM at a constant rate in one direction is known as a phase ramp. Once the phase ramp reaches a value of 2π radians one can “wrap” the phase by setting it back to 0 and repeating the ramp, as shifting the phase of a wave by 2π leaves the wave back in phase with its starting state. A phase ramp with one wrap is seen in Figure 11. If the phase ramp has been applied over a distance A , it is evident that the angle of reflection when the wavefront exits the SLM is given by $\tan(\theta) = \frac{P\lambda}{2\pi A}$. Given the reflection angles in most scenarios will be small one can assume $\tan(\theta) = \theta$

and so $\theta = \frac{P\lambda}{2\pi A}$. If a lens of focal length f is placed after the SLM, this will create a focus at a lateral distance from the optical axis of $x = \frac{FP\lambda}{2\pi A}$.

Another way of looking at the phase ramp on the SLM is to consider over how many pixels, N , a full 2π phase ramp is covered. By calculating $\frac{\text{total \#pix}}{N}$ one acquires the number of 2π phase ramps, so that $P = 2\pi \frac{\text{total \#pix}}{N}$ and the previous equation becomes $x = \frac{F\lambda \text{ total \#pix}}{AN}$ whereby finally substituting $d_{\text{pix}} = \frac{A}{\text{total \#pix}}$ for the size of the pixel, one obtains

$$\text{Equation 14} \quad x = \frac{F\lambda}{d_{\text{pix}} N}$$

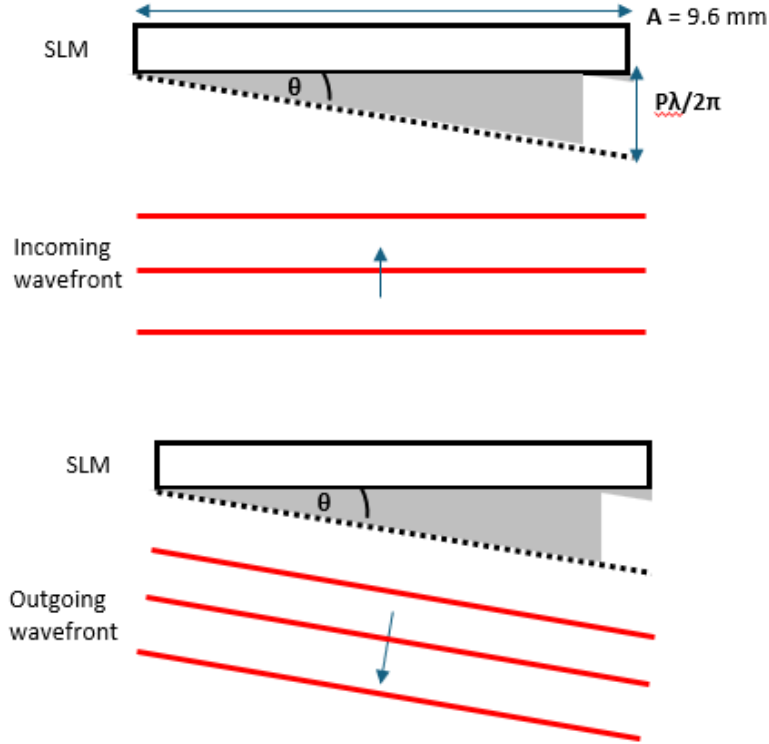


Figure 11 Schematic of phase ramp causing change in wavefront reflection angle on SLM

The diffraction efficiency for the first order of a blazed grating with N pixels per 2π radians is given by [115]

$$\text{Equation 15} \quad \eta = \text{sinc}^2\left(\frac{1}{N}\right)$$

Substituting $\lambda = 850 \text{ nm}$, $F = 300 \text{ mm}$ and $d_{pix} = 8 \text{ microns}$ into Equation 14 yields $N = \frac{32 \text{ mm}}{x}$ which can be substituted into Equation 15 to determine the diffraction efficiency as a function of lateral beam displacement. The phase patterns used to make multiple beams are more complex and will be discussed in a later section.

3.2.3 Scanning unit

3.2.3.1 Galvo scanning mirror set-up

The six beams created by the SLM must be scanned over the sample for a full image to be constructed. A Thorlabs QS7XY-AG - Dual-Axis scanner [116] was used to scan the beams in x and y, deflecting each of the six beams in step simultaneously, a schematic showing one of the scanning mirrors placed at the BFP of the scan lens is shown in Figure 12.

The small angle step response time of the galvo scanners was $250 \mu\text{s}$. This is the time it takes after for the galvos to settle to within 10% of the commanded angle after a small change in angle. The standard optical step size used between pixels in this M³M system was 0.02 degrees, and typically a scan speed of 5 kHz was used. The aperture size of the scanner unit was 7 mm diameter, allowing a 5 mm diameter field to pass through the unit with minimal (5%) attenuation. Larger aperture scanner units are available; however, they suffer from longer step response times.

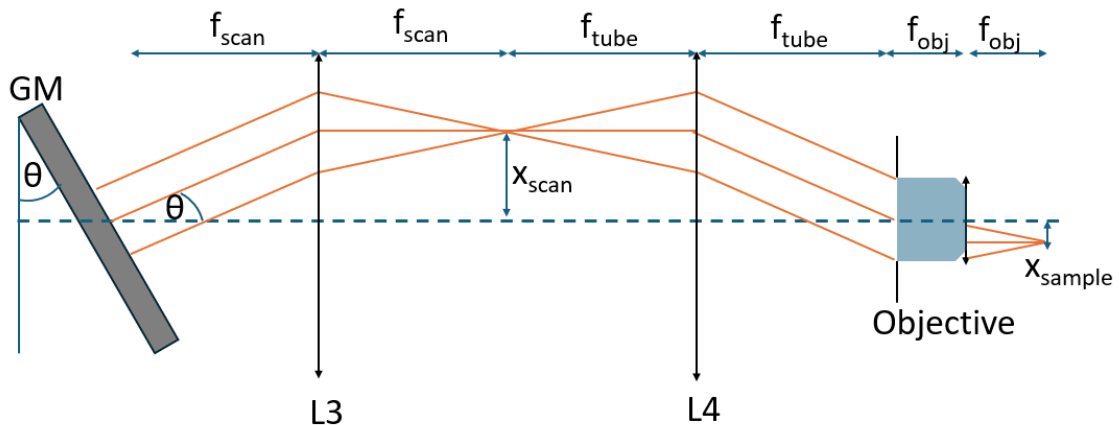


Figure 12 Beam scanning optics showing one galvo mirror. Displacing the galvo from the back focal plane of L3 causes a shift of the field at the objective pupil.

The galvo scanning mirrors were positioned such that the front focal plane of L2 and the back focal plane of L3 were located midway between the two scanning mirrors (4 mm from both). This is not ideal as it means neither mirror is exactly at the back focal plane of the scan lens and therefore, as the mirrors scan the beams, the field location on the back aperture of the objective will also move [117]. This could result in non-uniformity of illumination power and PSF when scanning at large angles. To quantify the extent of lateral shift of the field at the BFP of the objective lens, it is clear that the centre of the field will have moved by 4θ mm when it reaches the plane conjugate to the BFP of L3 after reflecting off the first galvo, where θ is the optical scan angle of the galvo. This distance is then magnified by L3 and L4 by a factor of 4, resulting in a shift of 16θ mm at the objective BFP. With the FOV size used, the line of beams must be scanned by $360\text{ }\mu\text{m}$ in the sample plane along one axis, and $60\text{ }\mu\text{m}$ in the other axis to make a square FOV. A $\pm 180\text{ }\mu\text{m}$ shift at the sample plane is caused by $\pm 70\text{ mrad}$ optical rotation of the mirrors, causing a 1.1 mm shift of the field at the BFP of the objective lens for one axis. As the pupil of the objective is 19.2 mm in diameter it shouldn't affect imaging quality significantly, especially when the back aperture is overfilled.

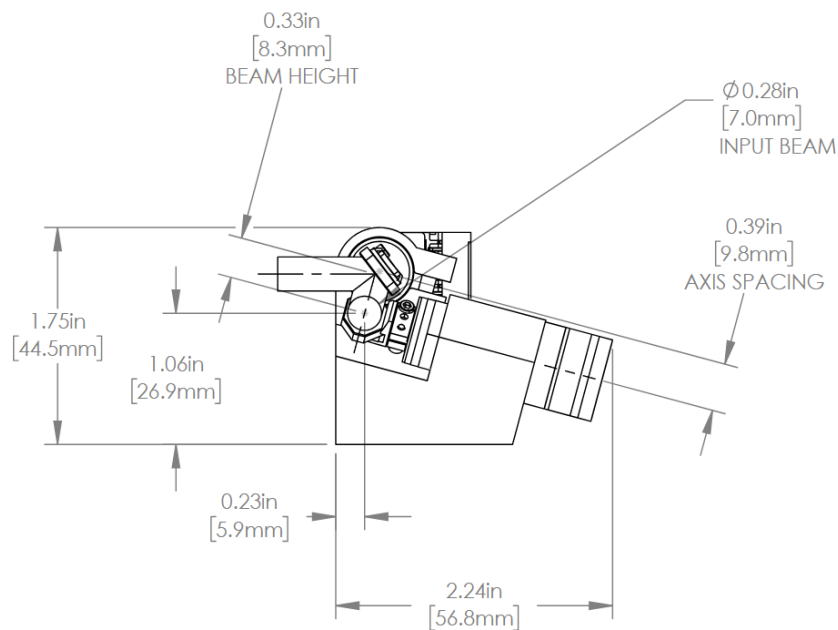


Figure 13 Engineering drawing of 2 axis galvo unit displaying horizontal shift between x and y galvo position.

The two mirrors constituting the scanning system were not aligned directly vertically above one another, as seen in Figure 13. This results in a rotation of the input field by an angle of $\theta = \arccos\left(\frac{8}{8.3}\right) = 15.5\text{ deg}$, where 8.3 mm is the distance between galvo centres and 8 mm is the vertical component of that distance. This rotation means that a vertical set of beams input to the galvos will be rotated upon exiting the unit. Therefore, beams that originally would have been scanned at 90 degrees

from the beam spacing axis, must now be scanned at 105.5 deg to produce a rectangular, rather than rhomboidal, field. The angle scan voltages could be produced by applying a standard rotational transform for a specified angle to the original X-Y scan pattern. As the scan axes are no longer parallel with the axes of motion of the microscope stage, a rotation mount was bought for the fibre loom such that the line of detection optical fibres could be rotated. As depicted in Figure 14, this allowed us to pre-rotate the SLM hologram such that the beams from the scanner would be arranged along the microscope stage axes when at the sample plane, and the fibre loom could then be rotated to match the descanned beam array orientation. This enables straightforward image tiling.

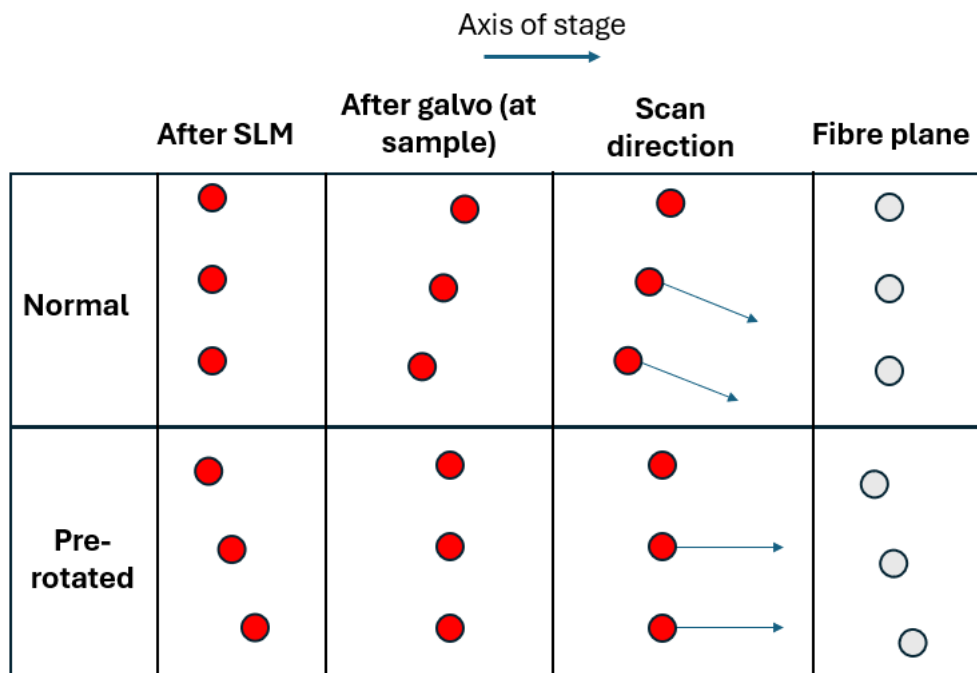


Figure 14 Beam orientations along optical path showing rotation of field due to galvos, shown for vertical and pre-rotated beam array.

3.2.3.2 Scanner mirror control

An NI USB-6363 DAQ box was used to set the voltage applied to the scanner mirrors that sets the rotation angle [118]. The maximum angle of rotation for the mirrors is ± 22.5 degrees (optical), for voltages of ± 5 V, yielding $\theta = 4.55$ degrees/V. By propagating the field forwards through the system, we can predict the lateral shift of a beam for a given voltage applied to the scanner mirrors. A beam image will be made at the focus of L3, where it can be deduced that $\tan(\theta) = \frac{x_{scan}}{f}$, where x_{scan} is the lateral displacement of the beam and f is the focal length of the scan lens. This image is magnified by L4 and the objective by a factor of M , after that we may then substitute in the relation between θ and V and apply the conversion of θ into radians to yield,

$$\text{Equation 16} \quad x_{sample} = 4.55 f M V \frac{\pi}{180}$$

Upon substituting the appropriate values for f and M , we find $x_{sample} = 0.2 \left(\frac{mm}{Volt} \right) V$, meaning that to scan a $360 \mu m \times 360 \mu m$ field, voltage ranges of $\pm 0.9 V$ and $\pm 0.15 V$ must be swept along the axes perpendicular and parallel to the 6 beam line array respectively.

3.2.4 *openFrame* microscope stand

The light coming from the scanning unit was coupled into the excitation layer of the *openFrame* microscope stand via a scan and tube lens. Within the *openFrame* layer, a mirror cube directs the light vertically upwards through the frame towards the sample which is held on a motorised X-Y stage. The excitation beams were focussed onto the sample using a 20x water immersion Nikon 0.95 objective lens [119] which was mounted on a piezo controlled automated Z stage. The ability to electronically control XYZ positions from software enables automated 3D assays. The mirror cube used for descanned imaging could be switched to a short pass dichroic allowing observation of two-photon fluorescence signal with a camera. Various other modalities could be implemented on the modular *openFrame* microscope stand, which will be discussed later in this chapter.

3.2.5 Fibre Loom

The fluorescence from the sample is directed back through the system along the scanning mirrors, after which the descanned beams are stationary as the galvo scanning mirrors are rotated. A dichroic beamsplitter is used to reflect the fluorescence towards a final lens and mirror, L5 and M4 that direct the fluorescence to the optical fibre loom via an electronic filter wheel. The optical fibre loom comprised a series of 6 multimode optical fibres equally spaced by 3.6 mm along a line. The loom was constructed by first cutting in half three 200 μm core multimode optical fibre patch cables with SMA connectors (Thorlabs M25L05) and stripping a region of ~ 70 mm at the cut ends. An indent was machined across the length of a metal block to house the cut optical fibre ends, which were glued in place using an ultraviolet curing epoxy adhesive (Araldite). Once the epoxy was hardened, another metal block was placed on top, leaving the ends of the fibres slightly exposed for subsequent polishing such that they were all coplanar. The connectorized ends of these optical fibres were routed inside the excitation/descanned detection enclosure to their respective photomultipliers in the HPMA. The optical fibre loom was mounted to the excitation breadboard such that its height could be adjusted. The correct plane at which to locate the loom could be initially determined by placing a camera at the loom plane and exciting a point of fluorescence at the sample plane. The camera could then be adjusted axially until a

minimum was seen in the FWHM of the fluorescence spot observed on the camera. The loom would then be placed at the same plane.

Using the 20x 0.95 NA Nikon objective with an over-filled back aperture, one would expect a fluorescence emission PSF of 0.5 micron FWHM laterally using two-photon excitation. Magnifying this to the fibre plane gives 30 micron FWHM. This indicated that using 200 μm fibres should collect all the energy from an unscattered PSF.

3.2.5.1 Optical fibre to HPMA relay

The ends of each optical fibre from the fibre loom were routed to their respective HPMA entrance apertures. The engineering drawing of the hybrid photomultiplier indicated that the closest distance the end of the fibre could be positioned to the 3 mm diameter active detection area was 17 mm owing to the c-mount flange on the front of the detector. The 200 μm core fibres had an NA of 0.22, meaning that at a distance of 17 mm the beam will have spread laterally to 7.5 mm in total - much larger than the active area of the HPMA. In practice the NA of the fluorescence exiting the fibres will not be the full NA of the fibre, as the light entering the fibres is that of the focussing NA into the fibres, which is $0.95/60\times = 0.016$ (considering the NA of the objective lens and magnification from sample to loom). However, this emerging fluorescence NA may be increased if the light is not focussed perfectly on axis when entering the optical fibres, and some light could be coupled into higher order modes due to bending of the fibres. Therefore, to increase efficiency of signal collection, the end of each fibre was imaged onto each photomultiplier active area using an 8 mm focal length lens. The adapter needed for the lens meant that the closest position it could be placed to the HPMA active area (to minimise the

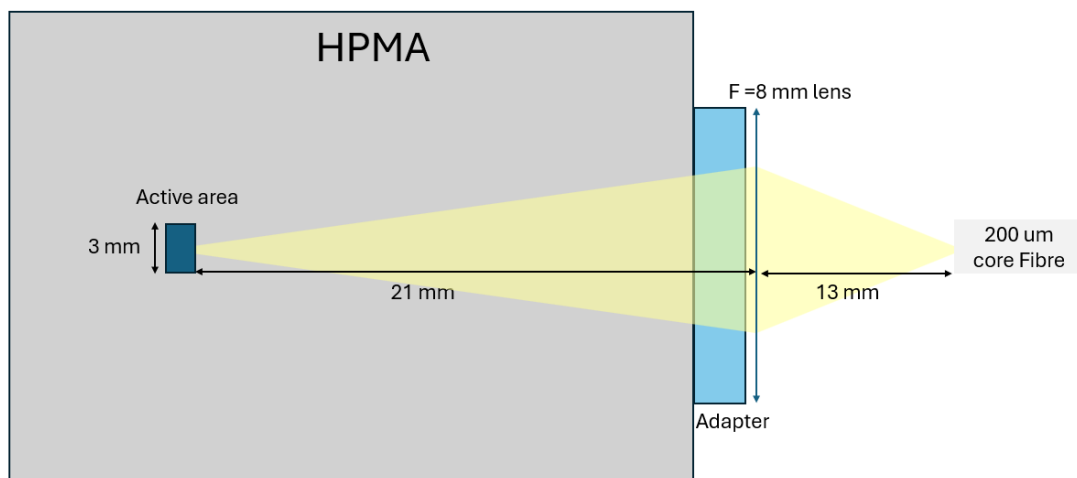


Figure 15 Setup used to image fibre output to HPMA active area in the M³M descanned detection path. Not to scale.

magnification from the fibre to detector) was 21 mm. For this lens location a 13 mm spacing from the fibre to the lens produces a 0.35 mm diameter focus at the HPMa active area - well within the 3 mm diameter. This set-up is shown in Figure 15. Using the full NA of the fibre, the maximum spread from the optical fibre to the lens will be 5.7 mm full lateral extent, which is smaller than the 9 mm diameter of the lens, meaning the lens won't cause any vignetting. It was noted that when using the fibre-lens relay, much more stray laser light diffracted by the SLM was also collected by the input ends of the fibres, so that additional light shielding needed to be added to the input area of the fibres.

3.2.6 TCSPC instrumentation

3.2.6.1 Time-correlated single photon counting

TCSPC is a method of acquiring time-domain FLIM information. First an ultrashort pulsed laser is used for excitation, which can be a mode-locked laser pulsing at 80 MHz repetition rate. The emission photons are incident on the active area of a single photon counting detector with an appropriate spectral response. After successful detection of a photon, the detector outputs an electrical pulse that is connected to the input of the TCSPC electronics. The spread in transit times of the electrons through the detector determines the width of the electrical pulse. Following detection of a pulse, the detector is typically unable to respond to another pulse for a few nanoseconds, and this is referred to as the detector deadtime.

The standard electronics setup for signal processing is shown in Figure 16. A constant fraction discriminator (CFD) is used to reduce the timing imprecision caused by pulse-to-pulse variations in amplitude. In the simplest configuration, the output pulse from the laser CFD starts a time to amplitude converter (TAC) whilst the detector CFD signal stops the TAC. (In practice this is reversed to allow higher trigger rates.) The TAC outputs a voltage proportional to the time difference between the start and stop pulses, which is sent to an analogue to digital converter (ADC). The resulting digital timing information is then stored in memory, allowing the construction of a histogram. The ADC process also takes some time during which the electronics cannot handle another photon detection event and this TCSPC dead time is generally on the order of 100 ns. It is important if using multiple detectors and one TCSPC electronics unit that the total maximum output rate of the detectors can be processed by the electronics.

In FLIM it is important to know the 'macro' time scale of detected photons to allow the positions of photons in the image to be recorded as well as the information on the decay 'micro' time scale. This is often done using time-tagged time-resolved measurements (TTTR) in which timing signals as well as photons are recorded as events. The additional markers, usually consisting of line starts/ends and frame starts, can then be used to reconstruct the full FLIM image.

The TCSPC deadtime typically limits the photon detection rate to 1 photon in 100 excitation pulses. Once measurements exceed this rate, some photons aren't measured and this leads to inefficiency in the system. As the TCSPC system is unable to respond to a second photon once a first photon is detected within in any laser repetition period, there is also a bias in measurements towards shorter lifetimes as the later arriving photon will go unmeasured.

The system used in this work (MultiHarp150, PicoQuant) does not use a TAC, and provides timing information on multiple photons per laser repetition period, therefore allowing higher count rates to be achieved [120].

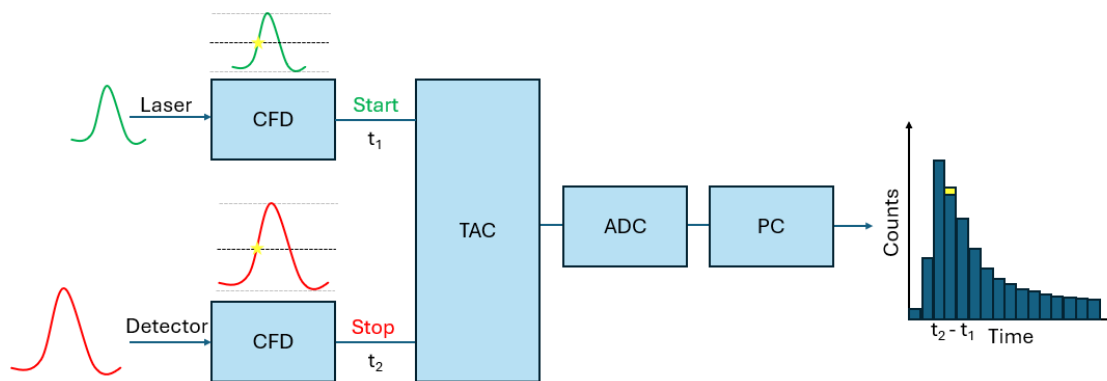


Figure 16 Electronics used to process pulsed laser and detector signals in a standard TCSPC setup. The green and red pulses are from the laser sync and detector outputs respectively

3.2.6.2 M³M TCSPC hardware

A commercial (PicoQuant GmbH, Multiharp150) TCSPC unit was used for FLIM data acquisition [121]. The unit has multiple inputs: 8 ports for photon counting photomultiplier signals, a sync signal input and a port for a variety of clock signals, for which we used line start, line end and frame start. The excitation laser used provided an electronic output at the pulse frequency that was connected to the sync signal input, 6 of the photon counting input ports were used for the descanned HPMAs, and one used for the non-descanned HPMA. The HPMAs used were each limited to a maximum count rate of 8 MHz, giving a total of 48 MHz with 6 detectors, with 45% efficiency at 500 nm.

3.2.6.3 Event stream interpretation

The Multiharp works by recording arrival times of photon signals or timing signals detected at each input port in a TTTR modality. 4 bytes of information are allocated for each detection event, the division of this information amongst different categories is shown in Table 3.

Photons, clocks and overflows are the three types of event recorded by the Multiharp. A photon event is when a photon arrives at any of the detectors. The special bit is 0 for photon events, the channel indicates at which HPMA the photon was detected, the Dtime gives the time elapsed since the last sync until the photon arrived and Nsync gives the number of syncs since the last overflow. Clock signals are recorded with a 1 for the special bit, 1, 2 or 4 for the channel information, for line start, line end and frame start respectively. Dtime is 0 for clock markers as it isn't needed, and Nsync again records the

Event Name	Bits	Usage
Special	1	Photon/ not photon
Channel	6	Channel of HPMA/ type of clock
Dtime	15	Arrival time of photon after last sync
Nsync	10	Number of syncs since last overflow

Table 3 TTTR record format and bit allocation for Multiharp150.

number of syncs since the last overflow. While sync signals are sent to the Multiharp the Nsync counter increases. As Nsync has 10 bits allocated, after 1024 sync signals (12.8 μ s) the Nsync entry will return to 0. For this to be recorded the Multiharp saves an 'Overflow' event to the event stream every time this happens, so that in later processing the correct times for photon and clock arrivals can be determined. This event is recorded with a special bit of 1, channel is set to 32, and Dtime and Nsync are set to 0. The absolute time of the nearest sync signal that happened before a photon event is known as the macro time and can be calculated by

$$\text{Equation 17} \quad T_{macro} = \#OFs * 12.8 \mu s + Nsync * T_{rep}$$

Where #OFs is the number of overflows seen before the event, T_{rep} the pulse period of the laser (12.5 ns for 80 MHz) and Nsync is given in the photon event information.

The minimum resolution for Dtime was 10 ps, defining the smallest histogram bin width possible. With the 15 bits available in this slot, this allowed events to be recorded over up to 32 ns, much larger than the 12.5 ns pulse period, meaning we were comfortably able to use the smallest resolution and still measure the total decay profile.

3.2.6.4 Fluorescence lifetime image construction

By reading and processing the event fluorescence lifetime image can be displayed. This is done by first creating an $n \times m$ array where n and m are the number of pixels in x and y used in the scan. Once a frame start clock is received, the programme will begin allocating photons to the $n \times m$ array at every line end clock. The time taken between line end and line start can be found by calculating the difference in the macro time between the two events: $T_{line} = T_{line\ end} - T_{line\ start}$. The dwell time of each pixel is then given by $T_{pixel} = \frac{T_{line}}{n}$. For each photon event, the elapsed macro time after the most recent line start is divided by T_{pixel} and rounded down to give the pixel number corresponding to that photon.

$$\text{Equation 18} \quad x\ pixel\ \# = \left\lfloor n \frac{T_{photon\ macro} - T_{line\ start}}{T_{line}} \right\rfloor$$

Photon events recorded between the line end and line start aren't recorded as these occur during the time of the scan flyback. The y pixel # is given by the amount of line starts received since the last frame start. Over multiple scans, photons are accumulated in the correct array entries from which an image of photon arrival times can be constructed. Sub-images from multiple beams can then be concatenated to produce a full image.

For quantitative analysis of fluorescence lifetime information, the Dtime value for each photon is recorded and a histogram is constructed for each pixel from this information. The full decay dataset then contains $n \times m$ pixels each with a set of time bins. FLIM data sets of 256×256 pixels and 1250 time bins at 16 bit depth corresponds to a size of 160 MB per image. Depending on the number of collected photons in an acquisition, the photon stream file can be considerable smaller than this in practice, e.g., of the order of 10s of MB. One way to decrease the size of the full image file below 160 MB is to bin the time dimension. This is reasonable if the experiment is not required to resolve very short lifetimes, and the time bin size is still sufficiently small to adequately sample the system temporal IRF.

3.3 Software

Here I review the various software components that I wrote with help from Sunil Kumar to allow practical functioning of the M³M system in a user-friendly manner. Figure 17 shows an overview of the software components used and the hardware they control.

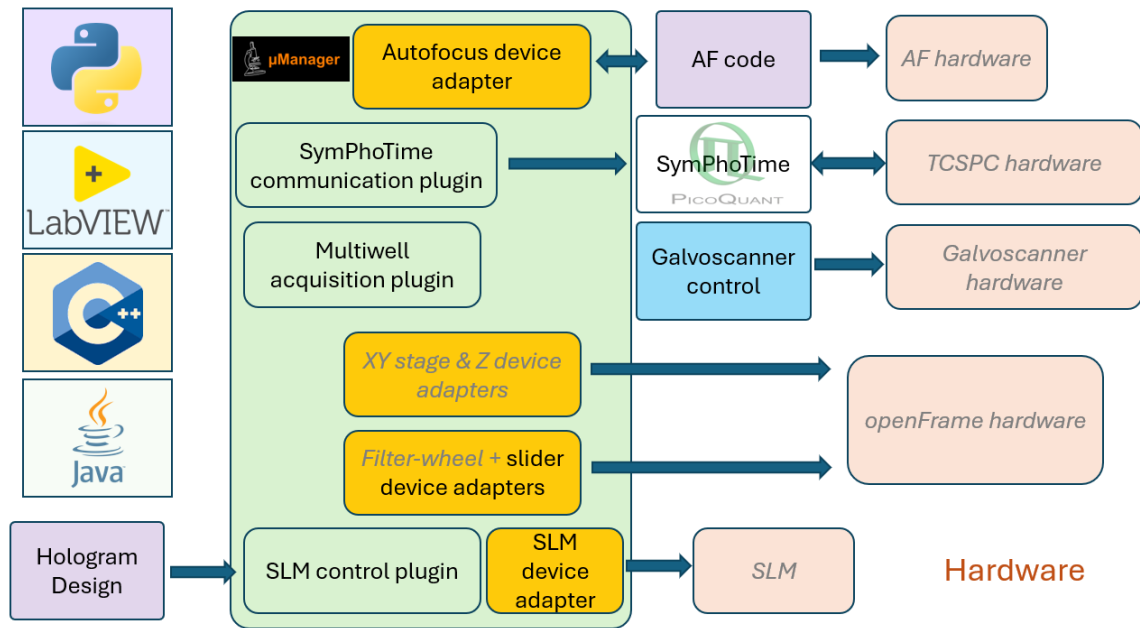


Figure 17 Block diagram showing the different software components, and the hardware controlled by them. Colour coded by programming language.

3.3.1 Programming the SLM

It was necessary to programme the SLM to apply phase ramps for beam-scanning and to make fine adjustments to each fluorescence beam position at the optical fibre loom.

3.3.1.1 Hologram design programme

To construct the phase hologram required the generation of 6 excitation beams, a Gerchberg-Saxton (GS) algorithm [122] was implemented in Python using Cupy, a GPU accelerated Python library [123]. A 30 iteration GS run could be carried out in under 10 seconds. The algorithm works by first initialising an array representing the phase and amplitude values for each pixel of the SLM. The phase is initially set to a random distribution and the amplitude is typically set to be uniform. One could input a Gaussian amplitude profile that would more accurately match the real illumination distribution but it was found that this didn't make much difference to the outcome and so a uniform amplitude was used. From this starting point, one can calculate the FFT to yield the amplitude and phase of the field at the focal plane of a lens placed after the SLM. This far field amplitude is then replaced by the desired amplitude; in our case this would be a set of 6 points. An inverse FFT is then applied to this far-field phase and amplitude to yield the amplitude and phase at the SLM plane. The SLM amplitude is again replaced by

the known input distribution and the process is repeated. Upon each iteration the phase calculated at the SLM plane gets closer and closer to a pattern which produces the desired amplitude in the far field, a flowchart showing how this works is shown in Figure 18 where the phase on the i^{th} iteration at the SLM and far field are referred to as ϕ_i and Ψ_i respectively.

Calculating an FFT of an image with 1920×1200 pixels can be time consuming so we took advantage of the fact that our desired pattern consisted of only 6 foci. Given this, only those 6 pixels would contribute to the field on which we apply the inverse FFT seen on the bottom row in Figure 18, as everywhere else the amplitude is set to 0. Therefore, none of the other pixels need to be calculated, considerably reducing calculation times.

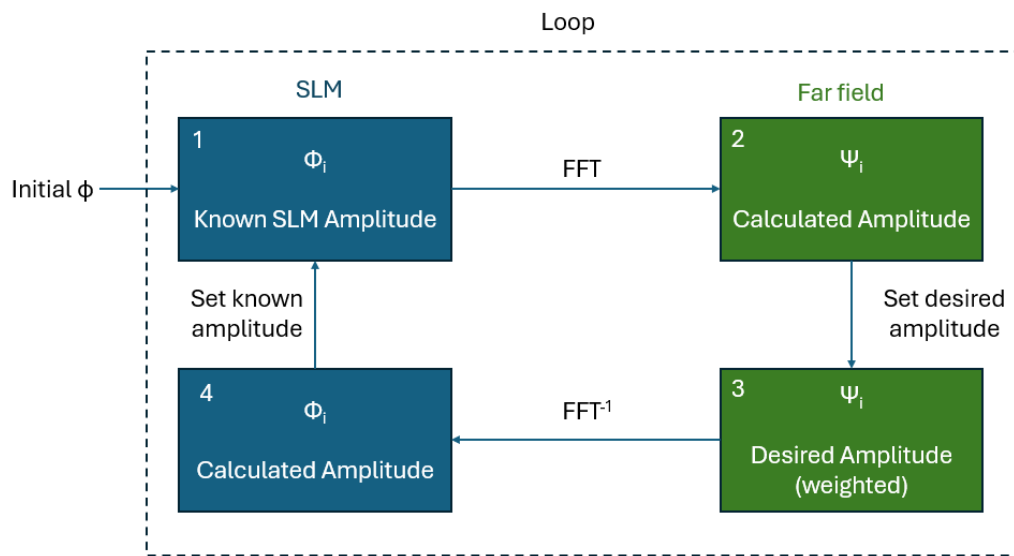


Figure 18 Flowchart indicating how the Gerchberg-Saxton algorithm operates. As the algorithm iterates over the loop, the SLM phase ϕ_i more accurately generates the desired far-field amplitude.

On the inverse FFT step the k^{th} beam is weighted on the j^{th} iteration by a factor $w_{j,k}$ according to:

$$\text{Equation 19} \quad w_{j+1,k} = w_{j,k} \cdot \left(\frac{\langle A \rangle_j}{A_{j,k}} \right)^{s_j}$$

where $\langle A \rangle_j$ is the average of the beam amplitudes, $A_{j,k}$ gives the k^{th} beam amplitude on the j^{th} iteration and $w_{0,0}$ is initialised as 1. The effect of this weight is to overshoot the correction when transforming the field back to the SLM plane, in hopes of arriving at an accurate hologram sooner. This is caused by the factor $\frac{\langle A \rangle_j}{A_{j,k}}$ being large for beams with a smaller calculated amplitude, and vice versa. The exponent

s_j , given by $s_j = \left(1 - \frac{j}{\text{max iterations}}\right)^{\frac{1}{3}}$, is used to control the extent of this overshoot by allowing the $\frac{\langle A \rangle_j}{A_{j,k}}$ term to have an effect early on in the iterations, meaning the algorithm will overshoot and take more aggressive steps to an accurate hologram at the start, but the term will have less effect when j is closer to the max iterations, meaning the algorithm will stop overshooting and settle on a stable hologram. The exponent of $\frac{1}{3}$ was heuristically found to provide a good balance between these two effects. Initially the weighting was chosen without the recursive $w_{j,k}$ term included, but after trying the form in Equation 19 observed in [124], the holograms were seen to settle to a more accurate distribution and do so faster.

After the hologram is calculated the beam amplitudes may still be uneven, meaning they must be balanced. This can be due to imperfections in the SLM which can effectively low pass filter the hologram as well as preferential transmission of beams throughout the system. Beam evening can be done using two methods in the programme. The first method is to include the low pass filtering applied by the SLM in the GS algorithm by applying a Gaussian blur to ϕ_i in step 1 of Figure 18. This is done so that the iterations done in the algorithm optimise for a hologram that has been low passed. The final output phase is not low-passed as this will be done by the SLM physically. The result of this is for the hologram to add more power to the beams further away from the optical axis, as their power has been diminished by the low-pass filter on each iteration. The second method of evening out the beams can be applied to individual beams, it is achieved by modifying Equation 19 so that the $\frac{\langle A \rangle_j}{A_{j,k}}$ term is multiplied by a new factor B_k which can be unique for each beam. A larger B_k for a beam with respect to the other beams means that beam is given a larger weight on step 3 to 4 of the algorithm, and ends up with a larger final amplitude. Other work has shown live feedback mechanisms of measured fluorescence to find an iterative algorithm finding B_k values which progressively even out the beams further [125]. In this work no algorithms were used and the appropriate values were found manually.

Plots were produced so that the output of the programme could be checked, one could view the plots on each iteration or just at the end of the total iterations, with the latter allowing the programme to run much faster. Plots of the final hologram, predicted beam output, final beam weights and amplitude, illumination intensity and beam weights as a function of iteration could be plotted and are shown in Figure 19.

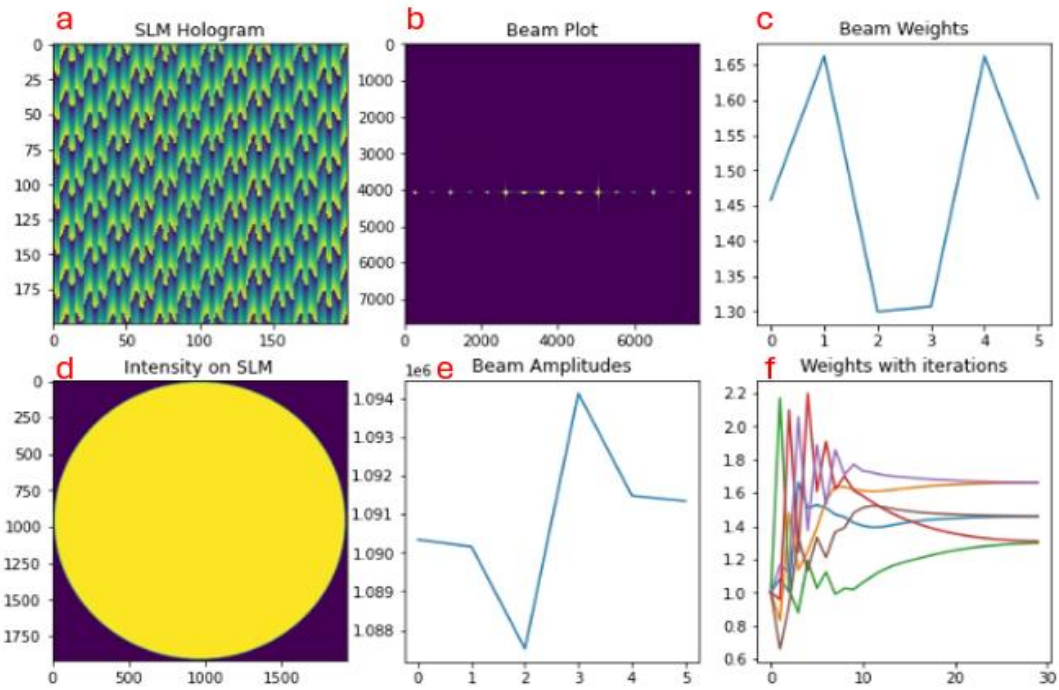


Figure 19 Output plots from SLM hologram programme. (a) The final phase distribution found by the algorithm, (b) the predicted intensity pattern in the far field, (c) the weights per beam used in the final iteration, (d) the intensity distribution used to illuminate the SLM, (e) the final predicted beam amplitudes and (f) the weights of each beam as a function of iteration.

Metric	Formula	Ideal Value
Zero-order	$\frac{E_{zero}}{E_{tot}}$	0
Efficiency	$\frac{E_{beams}}{E_{tot}}$	1
Uniformity	$\left(1 - \frac{E_{max} - E_{min}}{E_{max} + E_{min}}\right)$	1

Table 4 SLM hologram output metrics with associated formulae and ideal values.

The predicted beam output was produced by applying an FFT to the phase hologram. From this plot one could calculate the energy in each beam and in the zero order, and subsequently calculate the values seen in Table 4 where E_{zero} is the energy in the zero order of the diffraction pattern, E_{tot} is the energy incident on the SLM, E_{beams} is the total energy in the desired beams and $E_{max/min}$ is the energy in highest and lowest intensity beams respectively. These were useful metrics to observe how changing certain parameters could affect the output of the programme.

3.3.1.2 MicroManager control programme

To have flexibility in moving the excitation beams a MicroManager plug-in was created that allows blazed gratings to be written to the SLM. The user inputs the lateral displacement to which they want the beam set at the focal plane of L1 and Equation 14 then allows calculation of the appropriate period of the blazed grating. Arrow buttons were added with customisable shift distances to allow one to quickly find the optimal position of the beam with respect to detection in the fibre loop as seen in Figure 20 (b). Beam positions could be stored, and the active beam could be switched meaning one could easily switch between beams for troubleshooting purposes, this is done in region (a) of Figure 20. The relative amplitude balance factors could be altered within the GUI allowing correction of uneven beam intensities, this is done in region (c). Once the positions and intensities are set, clicking ‘Make Pattern’ runs the python GS algorithm described previously and saves the hologram. Once the hologram code has finished, the name of the saved hologram BMP file is loaded into the ‘Load Pattern’ text entry area, it can then be applied to the SLM by clicking the load pattern button. The file name for the hologram contains information necessary to deduce beam positions, relative intensity weightings used and the design wavelength, meaning that if the hologram needs to be altered at some point in the future it is easy to determine the settings used to produce the hologram. These buttons are seen in region (d).

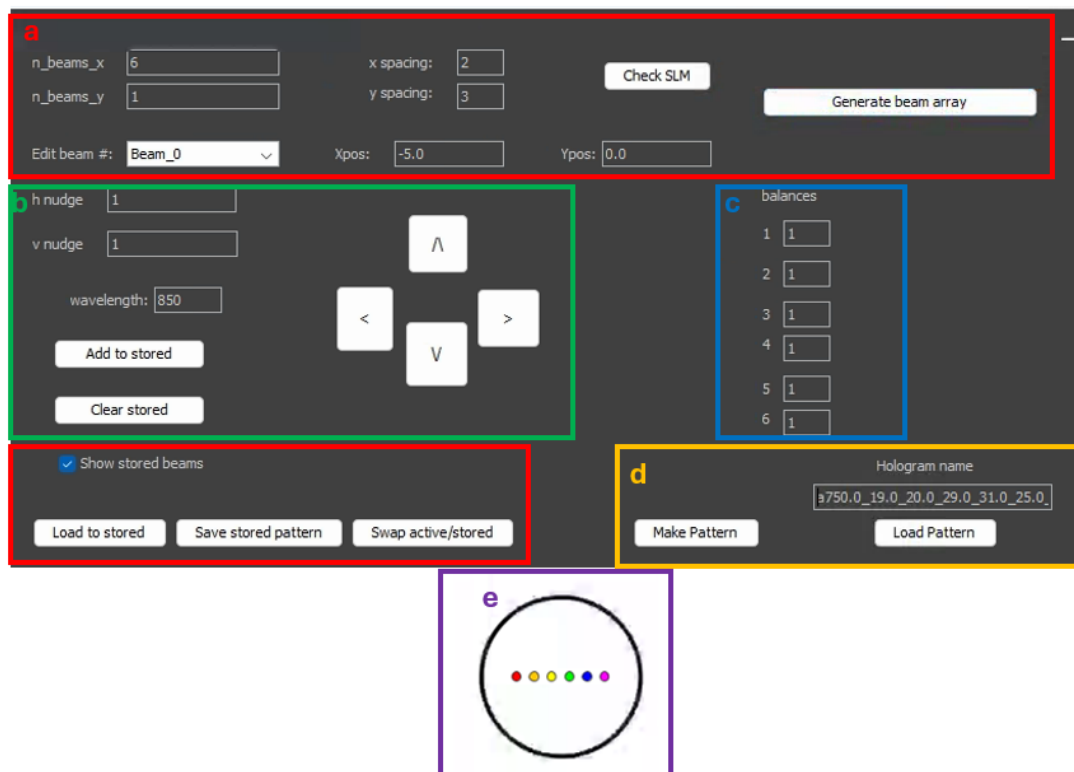


Figure 20 SLM control GUI in MicroManager able to shift individual beams as well as create and load a multibeam pattern.

In (e) the current beam pattern is visualised to make it clear when there is a systematic bias in beam positions indicating the loom could be mounted at a slanted angle.

3.3.2 Scan generation programme

A DAQ box controlled in LabView was used to set the voltages for the galvo scanners, as well as send line and frame clock signals to the TCSPC box. The number of pixels, voltage swept through in x and y, scan rate, flyback fraction, scan angle and # of scans to complete were controllable in the Labview GUI as seen in Figure 21. A voltage waveform was produced for each galvo scanning mirror. First, two waveforms were made for a non-angled scan using the parameters provided. Given the inputs in Figure 21 this would equate to a range of -0.38 V to 0.38 V with 120 steps for the X galvo and -0.23 V to 0.23 V with 80 steps for the Y galvo. These waveforms are then concatenated ‘# Scans’ number of times. The angle can then be applied to the scan pattern according to

$$\text{Equation 20} \quad \begin{bmatrix} x_{angle} \\ y_{angle} \end{bmatrix} = \begin{bmatrix} \cos(\theta) & -\sin(\theta) \\ \sin(\theta) & \cos(\theta) \end{bmatrix} \begin{bmatrix} x \\ y \end{bmatrix}$$

to produce the correct voltages for an angled scan pattern. Figure 22 shows forms of the x and y scan positions for a normal and angled scan from -1 to 1 volt.

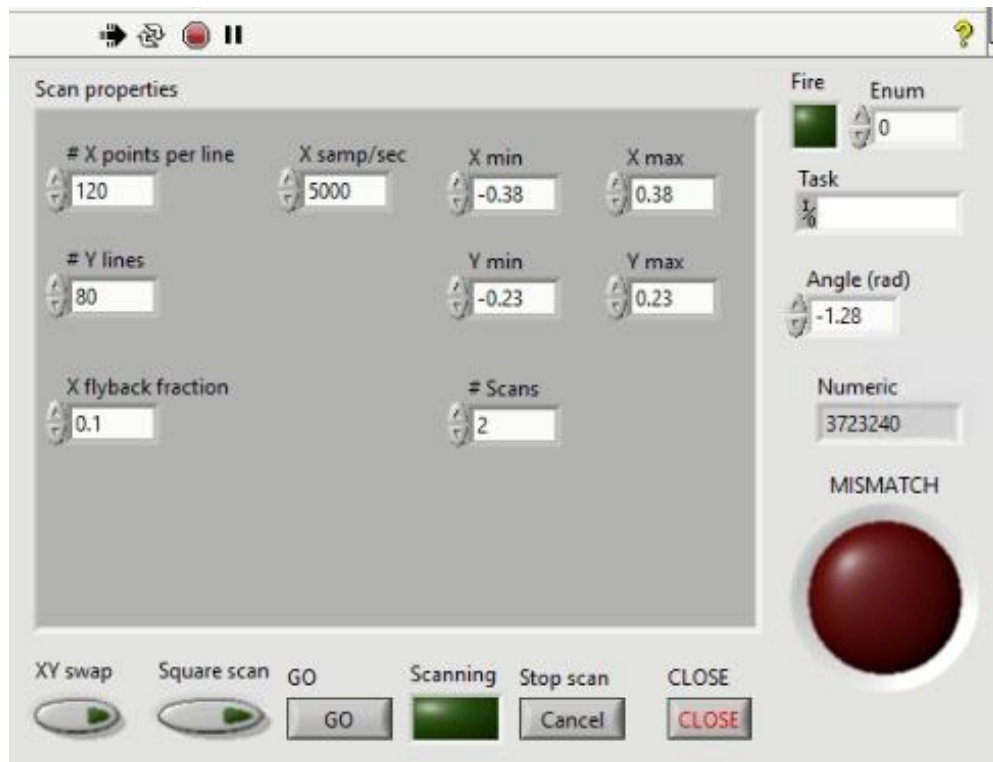


Figure 21 Labview scan control GUI used to send voltage waveforms to scanning mirrors to control their scan angles.

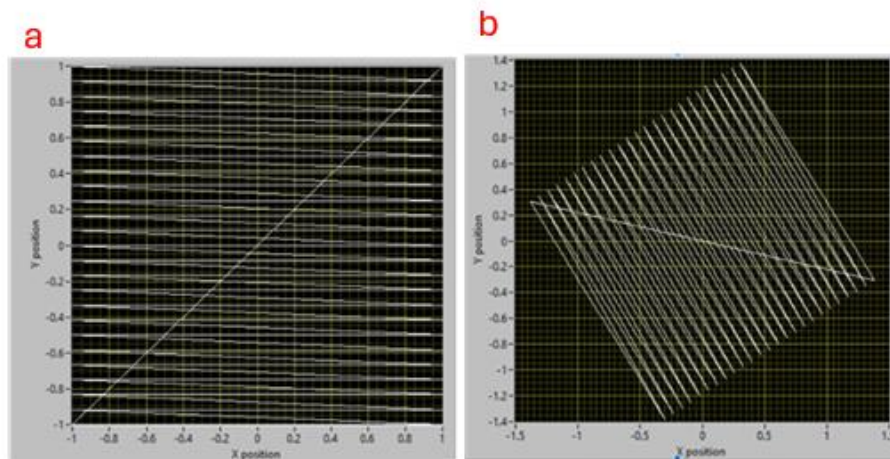


Figure 22 X-Y scan voltages shown for (a) normal and (b) angled scans.

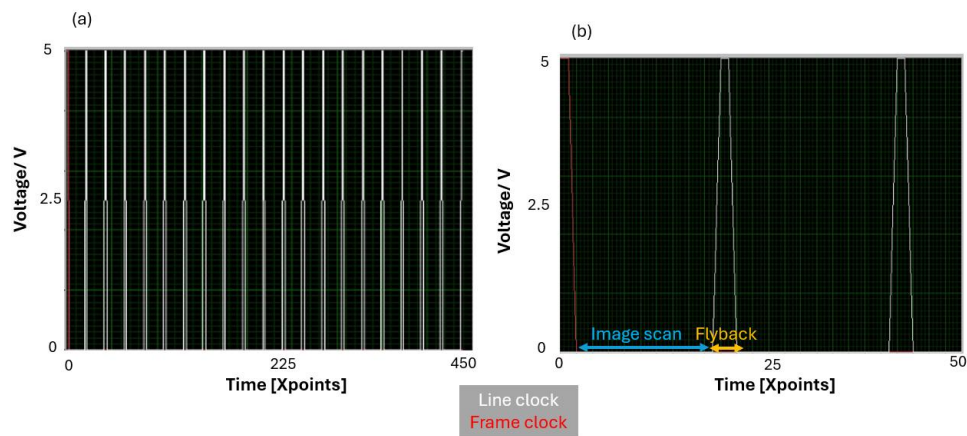


Figure 23 (a) Line and frame clock signals for one frame with 20 X points per line, 20 lines and 10% flyback, and (b) zoomed in waveform showing 2 line markers and a frame start.

Another two outputs from the DAQ were sent to the MultiHarp to record line and frame clocks, seen in **Error! Reference source not found.** The line clock signal rose to 5 V at the end of a line and dropped back to 0 V at the start of a line, with the flyback region in-between. This signal was split into two and connected to both the line start and line end inputs of the MultiHarp. The MultiHarp line start input triggered on a falling edge, and the line end input triggered on a rising edge. **Error! Reference source not found.** (b) shows how this configuration results in a certain portion of the scan designated as flyback.

A socket module was implemented in the LabView programme so that it could communicate with MicroManager. The protocol was for a particular number to be sent at the start of a message from MicroManager to LabView indicating whether the message was to update scan settings, start a scan or stop a scan. If scan settings were being sent, they were transmitted in JSON format, which could then be unpacked in Labview.

The LabView control was the main software component that was not open source, so a MicroManager replacement using loaded scan patterns and an Arduino micro-controller have been discussed.

The scan range could be theoretically calculated, but due to small offsets of lenses in the system it was easier to use a fluorescent slide and alter the scan parameters until a uniform fluorescence field was excited. This process is shown in Figure 24, where a 2 x 3 grid of beams is being used. In image (a) the wrong scan angle is used, meaning the individual scan regions don't align with the rotation of the SLM hologram caused by the galvos. In image (b) this has been corrected, but as the scan range isn't large enough there are areas between the sub-regions left unilluminated. Finally image (c) shows the aligned scan pattern.

3.3.3 MicroManager FLIM acquisition programme

Imaging using the M³M was initially done using the supplied PicoQuant software 'SymPhoTime' and using a mixture of MicroManager and SymPhoTime. Software was later developed to carry out lifetime acquisitions without using SymPhoTime, this is covered in chapter 4.

SymPhoTime allowed a user to view a live lifetime image, perform a FLIM acquisition and carry out various types of analysis on acquired lifetime data. As one of the intentions of this project was to produce an open-source system, the use of SymPhoTime wasn't ideal, however for initial data acquisition and testing it was very useful. Another issue with the programme is that it was not designed to handle multibeam data, separate detectors were assumed to be from different spectral channels showing the same field of view, so would be overlaid on top of each other by default.

The programme came with a remote access sub-module, this allowed a user to execute SymPhoTime commands using a port connection within the PC. As the code was provided for the remote access sub-module it could be altered so that upon executing the programme it automatically sent commands to SymPhoTime, rather than a user having to click the commands on the GUI. The executable was programmed to take in a set of arguments dictating the imaging parameters, meaning that an acquisition could be executed from the command line if SymPhoTime was already open.

Since most system components were controlled using MicroManager, it was advantageous to integrate FLIM acquisitions into MicroManager as well. With the capability to initiate acquisitions in SymPhoTime through the command line and a recently developed package by Huihui Liu in the group that allowed the execution of command-line arguments within MicroManager, a functional approach emerged for managing the entire image acquisition process through MicroManager. However, the method had limitations due to overhead times associated with launching the executable file, initiating scans, and repositioning the stage. These delays varied with the size of the photon stream file generated in SymPhoTime, necessitating longer pause intervals to ensure synchronization between automated acquisition commands and their execution. Figure 25 shows the Micromanager plugin used for the FLIM acquisitions. One needed to first set the appropriate parameters and send them to the Labview scanning programme over a socket, then ensure SymPhoTime was open and click on the ‘Run SPT’ button to start the automated acquisition.

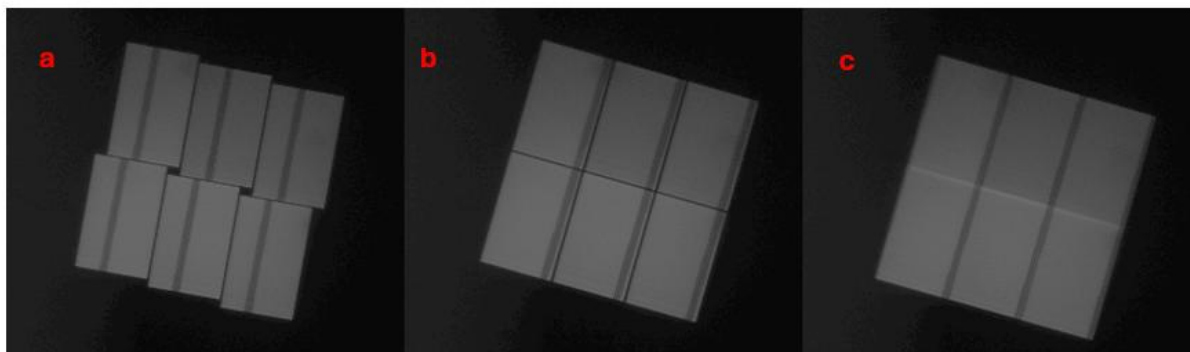


Figure 24 Steps used to fine tune scan parameters for an aligned scan field. (a) wrong angle, (b) wrong scan range, (c) correct pattern. Note that the small dark strip seen in all of the images is due to exposure time of the camera not matching scan period of the galvos exactly. Images obtained using widefield camera and fluorescent slide.

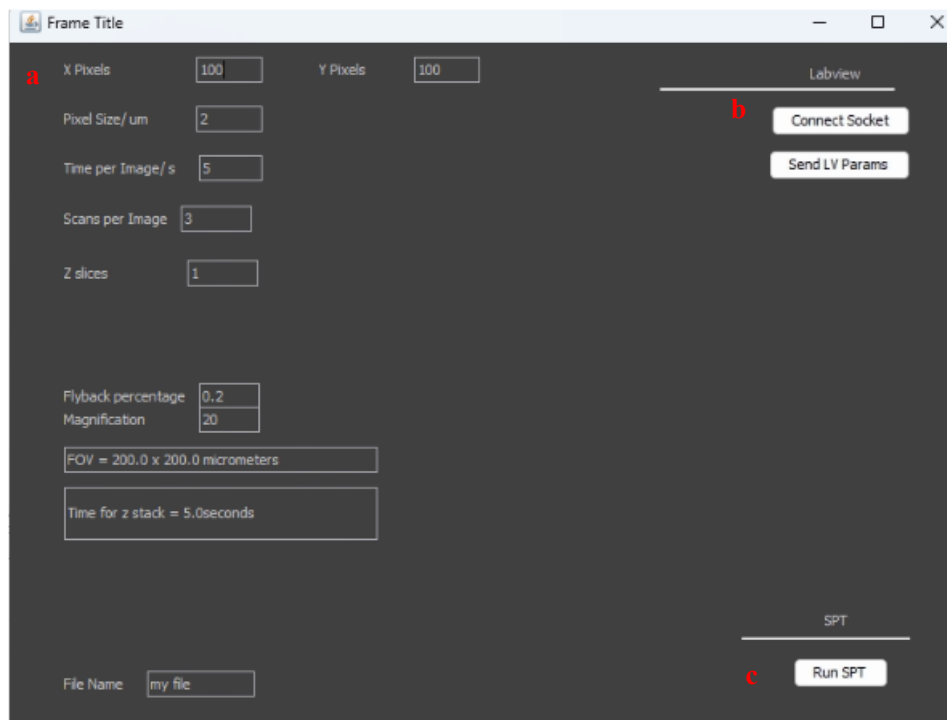


Figure 25 MicroManager plug-in used to execute SymPhoTime acquisitions. GUI contains areas for (a) parameter input, (b) connecting to LabView and (c) running the full acquisition.

3.4 *openFrame* components

The modular *openFrame* microscope stand [112] is assembled from layers with specific functions. This approach makes it straightforward to add more capabilities, such as additional imaging or excitation ports. In this M³M instrument, some layers contain electronic sliders, enabling multiple cubes to be switched in and out of the beam path depending on the intended imaging modality. This can be controlled using MicroManager such that multiple imaging modalities can be applied to each FOV, allowing complementary information to be acquired, e.g. during HCA assays.

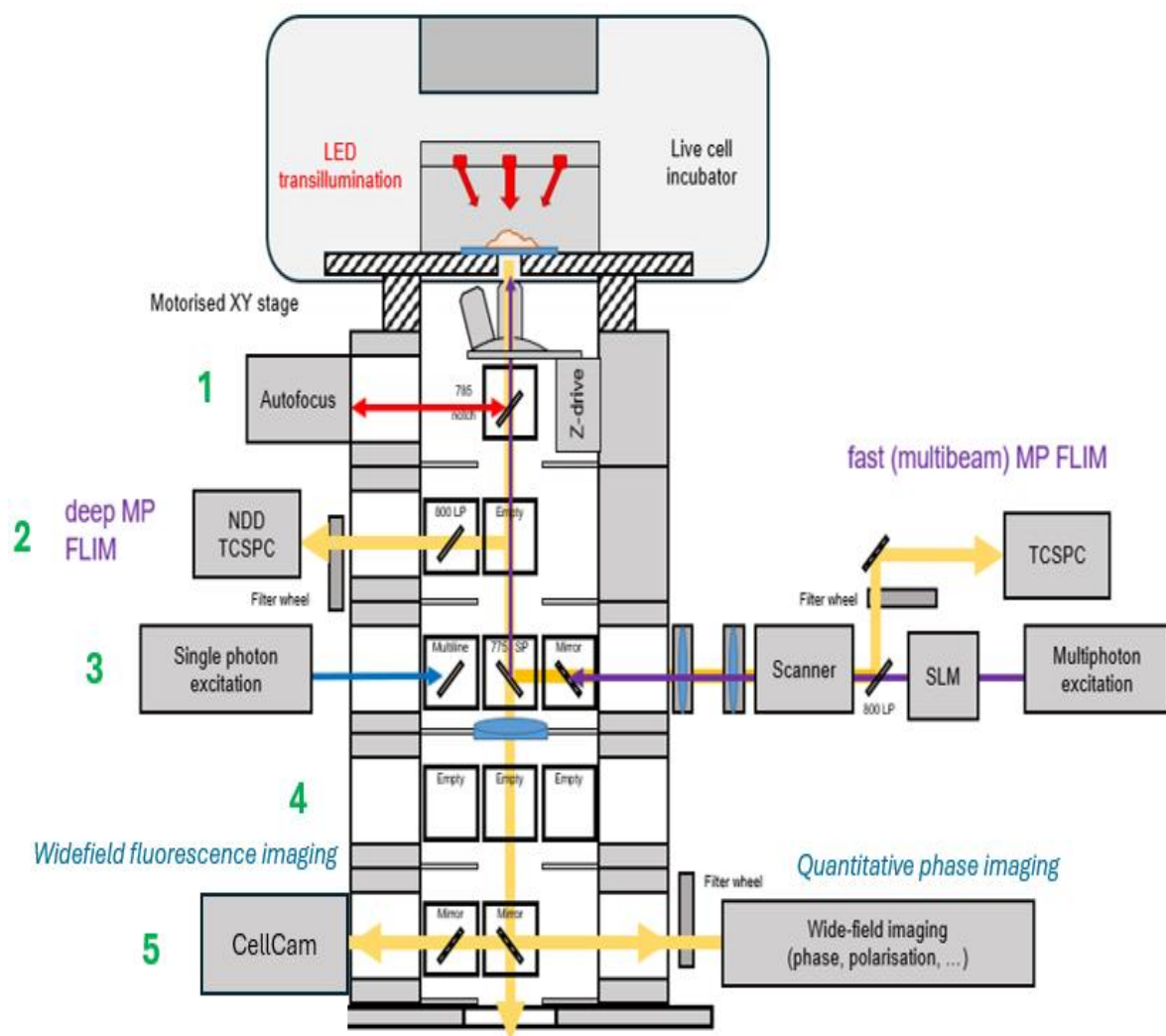


Figure 26 Schematic of M³M *openFrame*-based microscope implemented for the work reported in this thesis.

Figure 26 shows the schematic for a the *openFrame* configuration used with the M³M, with a stage-top incubator for live cell imaging and five layers labelled 1-5 from the top. Level 1 is the objective focussing unit that also contains an autofocus module that utilises infrared light centred at 795 nm –

coupled into the imaging path via a notch dichroic beamsplitter. Layer 2 contains a filter cube slider that can either present a long-pass dichroic beamsplitter to couple out 2-photon excited fluorescence for non-descanned imaging or presents an empty (pass-through) cube for all other imaging modalities. Layer 3 provides 3 switchable filter cubes: a standard fluorescence cube that can couple in a single photon excitation source from the left-hand side, a plane mirror to couple in the multibeam, multiphoton excitation beams from the descanned imaging unit described previously on the right, and a cube with a short-pass dichroic beamsplitter to allow wide-field multiphoton imaging using the cameras on level 5. The short-pass dichroic is also used for transmitted light imaging. The right-hand side of level 4 is not



Figure 27 Photograph of *openFrame* microscope in a later configuration showing autofocus unit (red), incubator (orange), NDD (yellow), single photon illuminator (green), phase imaging path (cyan), slider layers (blue) and multibeam multiphoton excitation breadboard (purple).

used because of lack of space due to the breadboard used for multiplexed multibeam excitation/descanned imaging and the left side port is currently not in use. Level 5 contains a slider with plane mirrors that can switch the imaging path between two cameras, a TE-cooled CMOS camera (Cairn Research Ltd , CellCam Kikker 100MT) for widefield single-photon fluorescence imaging and a polarisation-resolving CMOS camera (Clearview Ltd BFS-U3-%1S5P-C, with a Sony, Polarsens™ sensor) which is used for quantitative phase imaging. A photograph of the M³M *openFrame* in a later configuration is shown in Figure 27.

3.4.1 Single photon imaging

An LED light source (CoolLED, pE-4000) was mounted opposite to the multibeam excitation port on level 3 for widefield single-photon epi-illumination fluorescence imaging, as shown on the left side of Figure 26. This modality provided a convenient means to find fluorescent samples - rather than using the multiphoton excitation modality - and could be used as a cross-modality verification of sample brightness. Figure 28 shows an exemplar image of some OVCAR4 cells with a Syto82 nuclear staining. A Cellcam Kikker 100MT was used for imaging of single-photon fluorescence. The camera contains 4128 x 2808 pixels of size 4.6 microns, giving it a total FOV under a 20x magnification of 950 x 645 microns and sampling of 230 nm/pixel.

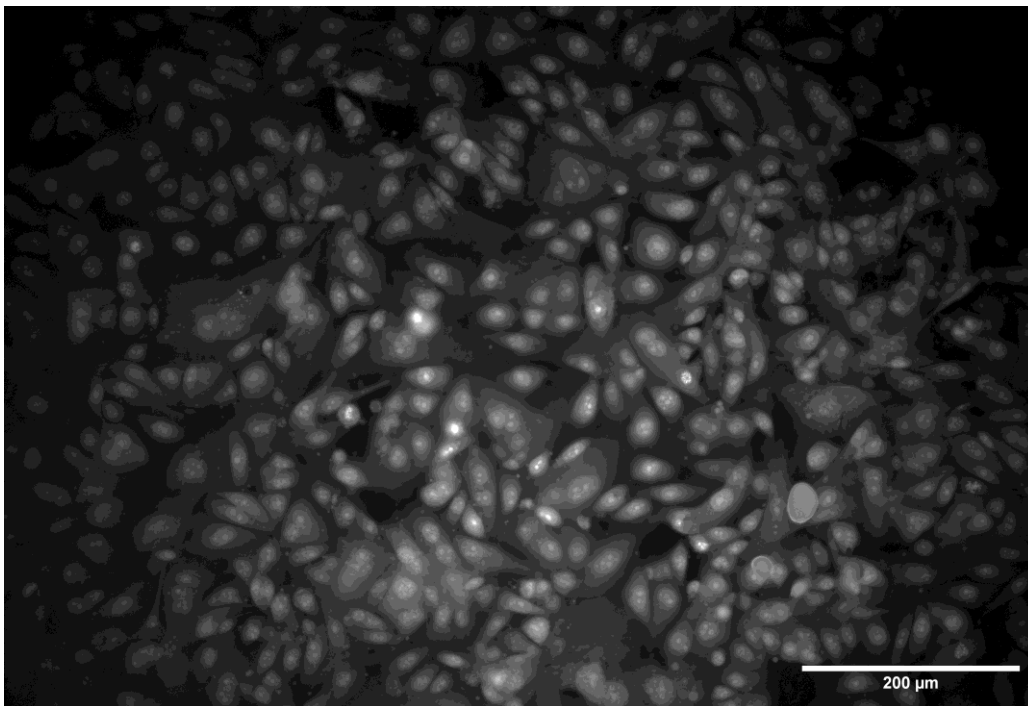


Figure 28 Cells containing Syto82 nuclear staining imaged with the single photon excitation port.

3.4.2 *openFrame* microscope incubator

To enable live cell imaging an incubator must be installed around the sample imaging area to maintain the sample at 37 degrees Celsius and with an atmosphere of 5% CO₂ gas. In conjunction with Cairn Research [126] we designed an incubator based on two tapped breadboards forming the top and bottom of the chamber, allowing for further optomechanical customisation in the future. The incubator dimensions allowed full movement of the motorised XY stage with room for attaching the humidifier and water trap on the side wall. The side panels of the incubator could be removed making them easy to modify, which was necessary to install the XY stage power cable and gas cable.

An OKO labs temperature and gas control unit was used to supply the appropriate conditions for the incubator. The temperature control system consisted of a control box from which two thermistors are connected, one to detect the temperature inside the incubator and one that can be dipped into a well in a multiwell plate to provide a readout closer to the actual temperature of the sample. The control unit is connected to a heating unit which has two cold and two hot outputs that are connected to the back of the incubator. Depending on the temperature read out by the thermistors, the control unit can heat or cool the incubator appropriately. The standard way of using the system is first to calibrate the temperature between the thermistor inside the multiwell plate and the thermistor inside the incubator, and then to use the incubator thermistor for the temperature control.

The gas control unit takes in CO₂ gas from a supply and air from a provided OKO Labs air pump, mixes the gases to the input mix percentage, and outputs the gas at a flow rate decided by the user. The gas exits the mixer and enters through the side panel of the incubator through a gas cable. Inside the incubator the gas is passed through a humidifier to reduce evaporation effects in a multiwell plate, and a water trap that collects any back propagating water droplets when the system is turned on and off, which otherwise may cause blockages in the gas cables. A cable delivers the gas from the output of the water trap to the K-frame unit, a small enclosure with a sliding top able housing the multiwell plate and containing the delivered gas.

3.4.3 Non-descanned detection

As an alternative to the 6-beam multiplexed descanned set-up, a non-descanned detection (NDD) arm was implemented on the *openFrame* microscope stand. The light could be directed towards the non-descanned detector using a translatable mirror cube containing a long pass dichroic beamsplitter. In a non-descanned setup the fluorescence does not pass back through the scanning mirrors and therefore moves as the mirrors are scanned. A common approach to non-descanned imaging is to image the pupil of the objective lens to the active area of the detector. As the pupil remains stationary so does the fluorescence at the active detector area, with only the angle of incidence changing as the scan angle is varied. An HPMA with a 5 mm diameter active area was used (compared to 3 mm diameter for the descanned detectors). Given the pupil size of 19.2 mm and active area of 5 mm, a 0.25x magnification or less was needed between the objective pupil plane and the detector active area. The distance from the active area of the HPMA to the nearest point one could place a lens was 21 mm, so lenses of focal length 200 mm and 30 mm were used to relay the fluorescence to the NDD, providing a 0.15x magnification when used in a 4-f configuration. A 30 mm turning cube was used in the imaging path due to the spatial constraints within the lab, and the HPMA was attached to the underside of the incubator using its M6 tapped holes. A filter wheel was placed between the two lenses to allow spectral selection of fluorescence, shown in Figure 29.

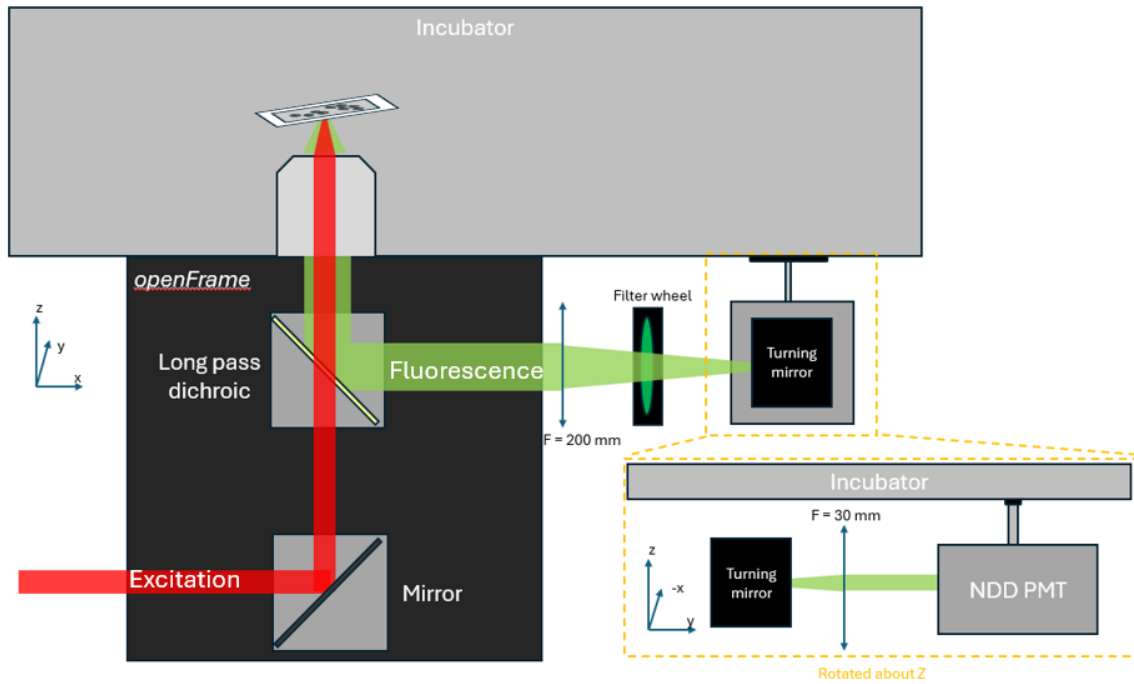


Figure 29 Optical setup for the non-descanned imaging path. The relay from objective pupil to NDD used a turning mirror so that the detector could be mounted to the underside of the incubator.

3.4.4 Autofocus module

An optical autofocus (AF) was installed on the system so that the distance from the objective lens to the coverslip could be monitored and adjusted to maintain focus. This is important when imaging a multiwell plate as the coverslip distance can change between wells. The AF unit had been developed previously in the group [127] for use in a STORM system using an oil objective lens.

The system operates by first coupling a super-luminescent diode (SLD) into the AF unit using a single-mode optical fibre. An SLD was used instead of a standard laser diode as its larger bandwidth means it has a shorter coherence length, which decreases unwanted interference with back reflections

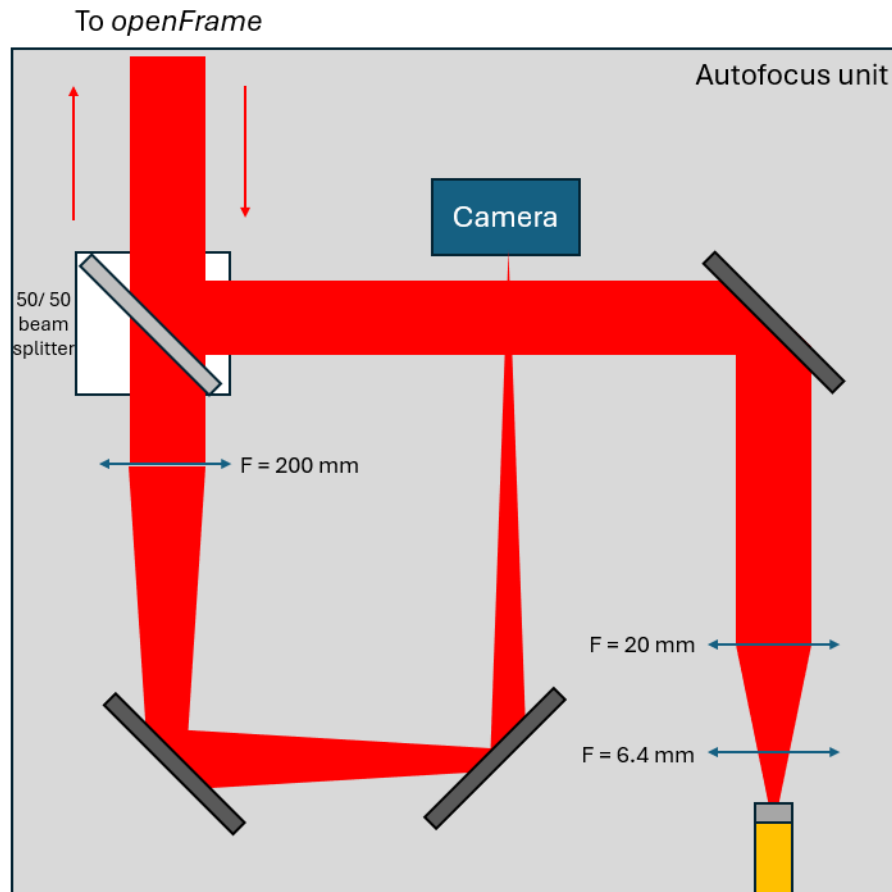


Figure 30 Optical layout of the autofocus unit. An SLD is coupled into the unit using an optical fibre which is elliptically collimated by two cylindrical lenses. The light is then coupled into the *openFrame* where it reflects off the cover slip, after which the reflection is imaged onto a camera.

in the autofocus unit that can degrade the autofocus performance. Two cylindrical lenses are placed their focal distances away from the optical fibre output such that a collimated elliptical beam output is produced. This beam is coupled into the microscope frame using a 50/50 beam splitter in the AF unit, then towards the sample via a notch dichroic beamsplitter. The autofocus beam is focused onto the coverslip by the objective lens. Since a water immersion objective is used, reflections occur at both the water-glass interface (from immersion fluid to coverslip) and the glass-liquid interface (from coverslip to sample), resulting in two distinct reflected beams - in contrast to an oil immersion lens that results in only one reflected beam due to the closer refractive indices of oil and glass compared to water and glass. Both reflected beams are relayed by the objective lens to the AF unit via the notch dichroic beamsplitter and partially transmitted through the 50/50 beam splitter before being imaged onto an autofocus camera. The optical layout of the unit is shown in Figure 30. When one surface of the coverslip is in the focal

plane of objective lens, the beam at the autofocus camera is also focussed to a point. By quantifying the beam's shape as a function of defocus, a lookup table can be created and used to determine the defocus of the coverslip relative to the focal plane of the objective lens.

The reason for producing the elliptical collimated autofocus beam is to provide different orthogonal confocal parameters – one providing greater precision and the other providing greater range of operation [22]. Additionally, one of the cylindrical lenses is deliberately offset from optimal collimation to introduce astigmatism that enables the system to determine the polarity of the defocus.

The system was initially designed for an objective lens with a smaller back aperture diameter than the objective we used, which meant the beam filled less of the objective NA. Since the coherence functions of the reflected beams from both sides of the coverslip overlapped significantly, a 3x beam expanding telescope was implemented between the autofocus unit and the objective lens to increase the autofocus NA and reduce its confocal parameter. This separated the coherence functions of the two reflected beams and the system was set to use the reflection from the bottom of the coverslip, as reflections from the top surface could be more affected by scattered light in the sample. This was achieved by displacing the second lens in the relay forward slightly, such the beam was caused to converge as it entered the objective lens. This setup is displayed in Figure 31.

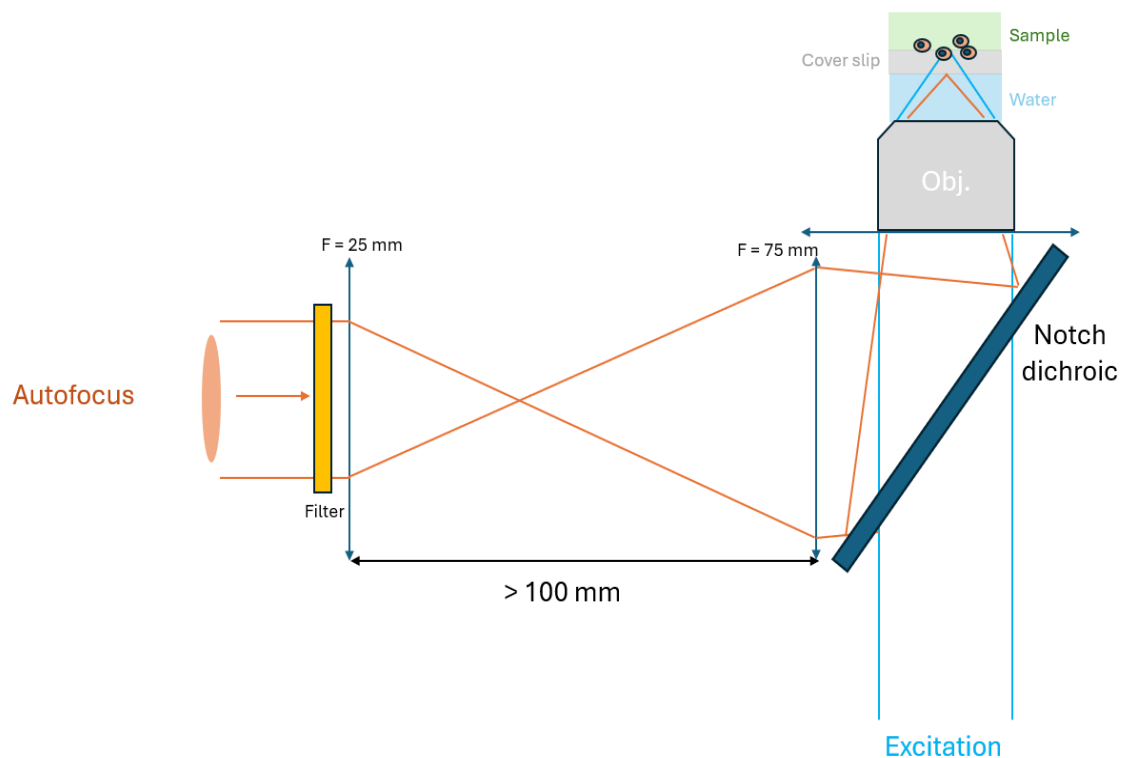


Figure 31 Beam expansion and modified focus of autofocus system.

A clean-up filter was placed at the entrance of the AF unit so that stray autofocus light would not be incident on the camera. Later in the development of the AF, a diffuser was placed after the second cylindrical lens, causing the light at the coverslip to present a speckle interference pattern. This reduced the impact of coverslip imperfections or scattering in the sample. The AF setups were tested with and without the diffuser, with the diffuser found to perform better.

3.4.5 Trans-illumination

A transillumination pillar was mounted below the top breadboard of the incubator, allowing illumination from above whilst maintaining the sealed atmosphere in the incubator. A Cairn MonoLED was used as a light source with the CellCam used for transmitted light detection. In later development the transillumination arm was modified to provide quantitative phase contrast imaging using a polarisation sensitive camera, as will be discussed in Chapter 5.

3.5 Application of M³M to fixed 3D biological samples

This section describes the application of this first implementation of the M³M instrument to 3D imaging of biological samples to explore the system performance. More significant 3D multiphoton HCA assays are described in Chapters 6 and 7. The FLIM data presented here were acquired using the linear 6-fibre loom described above using SymPhoTime + MicroManager software together as described in this chapter.

3.5.1 Mouse lung tissue

A slice of mouse lung tissue with small tumour nodules was obtained by injecting a mouse via a tail vein with PC9 cells, a human non-small cell lung cancer cell line, and later harvesting the lung from the mouse. The tissue was prepared by Alix le Marois at the Francis Crick Institute for a different study and I was able to take advantage of an unwanted tissue section. The actin had been stained with phalloidin-Alexa633 and the nuclei were expressing a CFP-YFP FRET biosensor. Two-photon excitation of 800 nm was used to excite both fluorophores with a 643/50 emission filter used for the actin imaging channel and a 493/20 filter used to image the nuclei. z-stacks were acquired for each spectral channel using the M³M imaging set-up already described with 6 beams spaced by 60 microns in a line at the sample plane with the beam intensities having been equalised. 360 x 60 image pixels were acquired by each beam and then concatenated to produce a 360 x 360 pixel image, with 1 micron/pixel. 20 mW per excitation beam was used for imaging in each channel at depths to 300 and 200 microns deep for the actin and nuclei respectively, with 1 micron voxel size in z achieved by

stepping the objective by 1 micron between acquisitions. Image acquisition times were 10 and 20 seconds per x-y frame for actin and nuclei respectively. Figure 32 shows an orthoplot and Figure 33 shows a 3D representation of the data rendered using ICY, actin is shown in red and nuclei shown in green for both figures. The tissue was mounted on a 24 well plate. After placing it in the well, phosphate

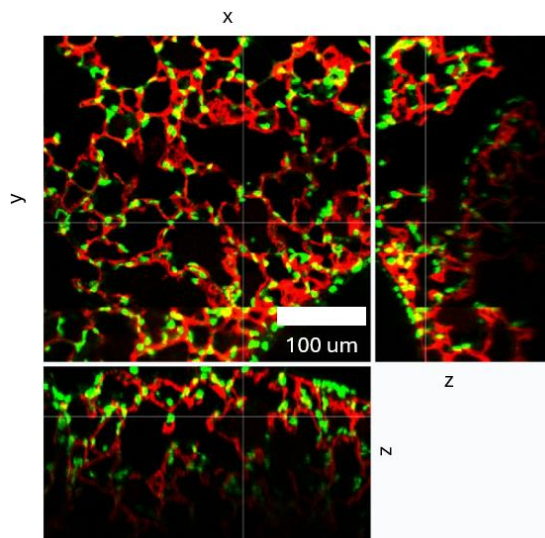


Figure 32 Orthoplots of actin labelled with phalloidin-Alexa633 (red) and nuclei labelled with CFP-YFP FRET biosensor (green) staining in mouse lung tissue acquired using the M3M system with an excitation wavelength of 800 nm.

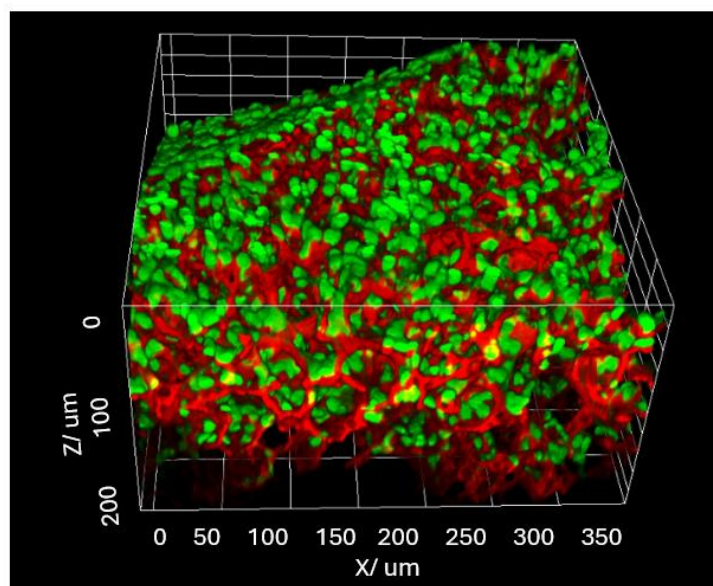


Figure 33 Two-colour z-stack overlay of actin labelled with phalloidin-Alexa633 (red) and nuclei labelled with CFP-YFP FRET biosensor (green) staining in mouse lung tissue imaged using the M3M system at an excitation wavelength of 800 nm.

buffered saline (PBS) was applied to make it moist, with care taken not to add excessive amounts that could cause the tissue to float and therefore undergo more movement during imaging.

Figure 34 shows how the signal acquired for the same excitation power and acquisition time decreases as the imaging depth increases. This is due mostly to the increased effect of aberrations on the excitation spot and scattering of emission light for deeper imaging. Approaches to reduce the effect of scattering are discussed in the following chapter.

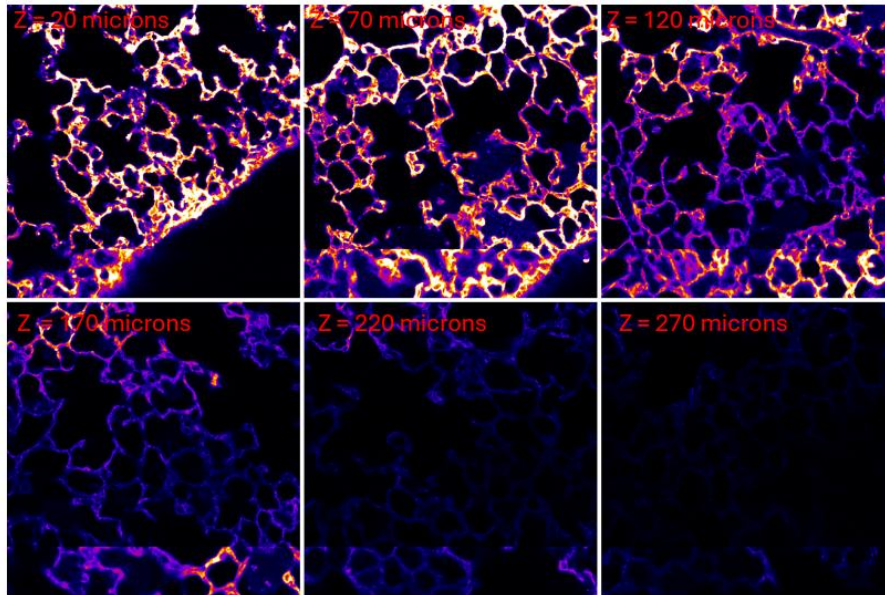


Figure 34 Same data as actin channel shown in Figure 28 but presented as z-slices shown in steps of 50 microns from 20 to 270 microns imaging depth, showing decreasing intensity with depth. Images displayed with the same contrast scale. FOV is 360 x 360 microns.

3.5.2 Mouse melanoma spheroids

This implementation of the M³M was also applied to image spheroids with IRFP670 histone labelling. The spheroids were formed from mouse melanoma cells expressing an NRASG12D mutation, which were fixed and arrayed in a standard 384 well plate by Lucas Dent at the ICR. The spheroids were of the order of 70 microns diameter, so that multiple spheroids were present in a 360 x 360 x 100 μ m FOV. Images were acquired at 1 μ m per pixel resolution in all axes, 10 seconds image data acquisition per x-y plane, and 10 mW per beam. Excitation light at 820 nm was used with an emission filter at 643/50 nm. Although FLIM data was acquired, only a single fluorophore was present in the emission channel imaged and with no environmental factors affecting the fluorophores, the same

lifetime was observed throughout the sample. To show both intensity and lifetime information in the same image, ImageJ was used to load the data into a Hue-Saturation-Value (HSV) stack, with intensity used as the Value and lifetime used as the Hue. This was applied to each z-slice and rendered as a 3D image in Icy, which is shown in Figure 35.

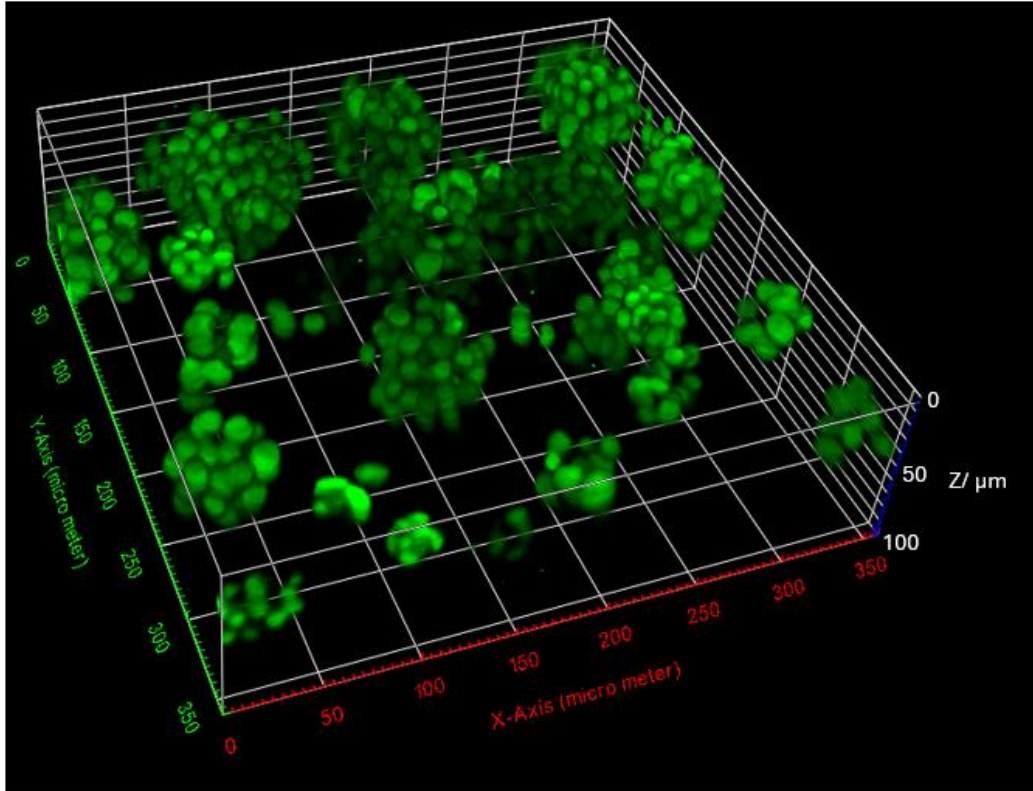


Figure 35 3D M3M FLIM image of mouse cell spheroids where the histones are tagged with IRFP670. Excitation at 820 nm. The constant colour shows a constant lifetime throughout the image. 360 x 360 x 100 micron FOV with 1 micron cubed voxels.

3.5.3 Ovarian cancer spheroid segmentation

To explore the efficacy of the M³M instrument to deliver single-cell segmented volumetric image data, it was applied to image larger spheroids of ASC22-018 cells, grown from primary cells from a patient with ovarian high-grade serous carcinoma in hydrogel-based microwell structures in 4D cell SmartSphero 24 well plates, and fixed for imaging. These well plates had an adhesive bottom surface to anchor the spheroids, preventing movement and loss of structure in manipulations such as media

exchange or drug application. The 3D segmentation programme used was developed within the accelerator by Matthew De Vries at the Institute of Cancer Research and utilised machine learning trained on previously annotated 3D nuclei data for ground truth.

Descanned multiphoton imaging was undertaken with 6 excitation beams, and with a single excitation beam as shown in Figure 36. Excitation at 842 nm was used to excite the SYTO Deep Red nuclear staining with 5 seconds per z-slice and 5 mW per beam. As the nuclear segmentation machine learning model was still being developed, the single-beam data was used as an input for the segmentation mask since it was expected to produce better results and this is shown in Figure 37. Figure 36 shows the 3D rendered 6-beam excited image alongside the 3D segmentation mask made from the single beam excited data. The similarity in image quality between single and 6 beam data suggests that the 6-beam data would produce similar segmentation output results. This (3D) single-cell segmentation can be used to provide (fluorescence readout) statistics on a per nucleus basis.

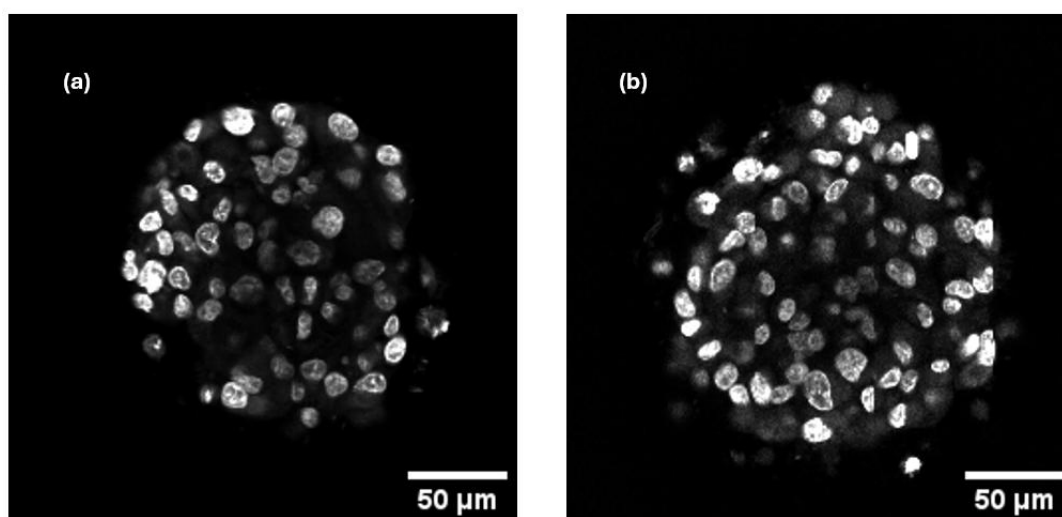


Figure 36 (a) single and (b) six beam images taken at a depth of 50 microns into patient derived spheroids (different spheroid in each image). This figure illustrates the similar level of image quality obtained with 6 excitation beams compared to 1 beam. The single-beam data was used to produce the 3D segmentation mask shown in Figure 34.

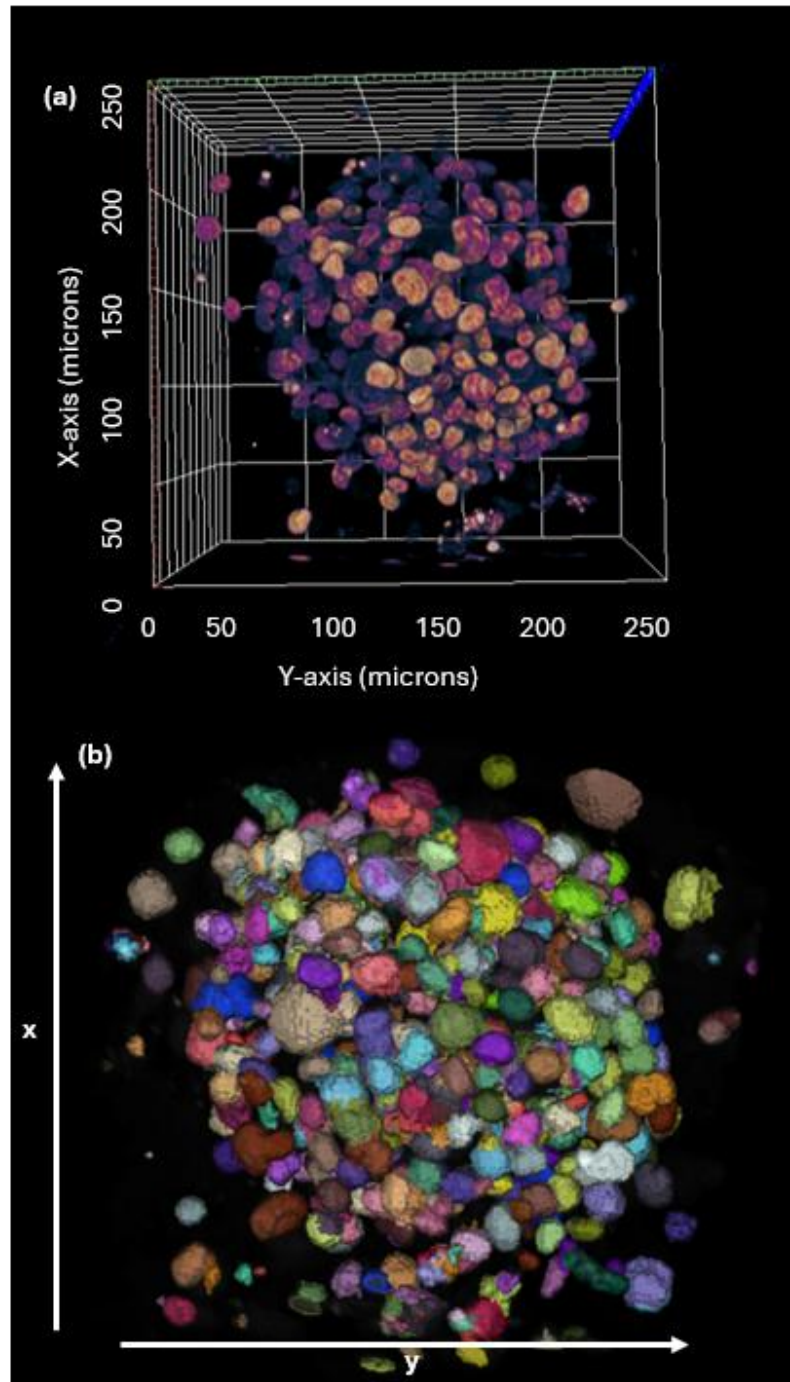


Figure 37 (a) 3D render of single beam z-stack of nuclei-stained spheroid and (b) 3D segmentation mask produced using data from (a).

3.6 Summary

This chapter has presented a description of the design and implementation of a multibeam, multiphoton, multiwell plate microscope (M³M) with TCSPC FLIM capabilities. An introduction was first given to motivate the design of the instrument as well as provide descriptions of some other multibeam multiphoton systems. A description of the components used in the optical path of the excitation and detection light for the multibeam, multiphoton modality was then given as well as the software used to control them. The *openFrame* microscope frame is then presented alongside the additional modalities it has allowed the implementation of. Some fixed 3D biological imaging was then shown indicating the application of the microscope to high content analysis of 3D samples.

4 M³M 2nd prototype

Having demonstrated the first M³M prototype in the Physics Department at Imperial, the instrument was moved to the Francis Crick Institute to be used to provide multiphoton excited HCA for assays of 2D and 3D cancer models. This modular instrument was reassembled, and a number of improvements were implemented. In line with the goal to make the M³M an open-source instrument, I worked to replace the commercial TCSPC software (SymPhoTime, PicoQuant GmbH), previously used with the TCSPC data acquisition hardware (MultiHarp, PicoQuant GmbH), with a device adapter that would enable the TCSPC data acquisition to be controlled with MicroManager. This also entailed writing new software to interpret and represent the acquired TCSPC data (“event streams”). This leaves only the LabVIEW programme controlling the scanner as commercial rather than open-source software in the M³M data acquisition software.

The other improvements were focussed on the excitation path, with a view to increasing the flexibility of the instrument and its ability to image at depth in 3D cancer models. First, the excitation laser was changed from a femtosecond Ti:Sapphire (Spectra Physics, MaiTai) laser providing ~100 fs pulses tunable over 690–1040 nm with a femtosecond laser-based excitation source (Spectra Physics, InSight X3 Dual) with dispersion pre-compensation that provided <120 fs pulses tunable from 680–1300 nm with up to 2 W average output power (at 900 nm). The output power from this laser was attenuated using a computer controlled acousto-optic modulator. To maximise the multiphoton excitation efficiency, the design of the excitation light path was reviewed, particularly in terms of the extent to which the objective pupil was filled – noting that this determines a balance between spatial resolution and peak excitation power. The detection light path was also reviewed, particularly with respect to the optical fibre loom. The initial design with six 200 µm core fibres spaced at 3.6 mm in a line was modified to better accommodate detection of fluorescence from deeper imaging depths in scattering samples.

4.1 Advances in M³M software

MicroManager communicates with hardware through device adapters. To improve the usability of the M³M instrument and to simplify future automated workflows, a device adapter was written for communication between *MicroManager* and the TCSPC unit.

4.1.1 MultiHarp device adapter + MH cam

A dynamic linked library (DLL) was provided with the MultiHarp TCSPC hardware that includes functions that can be used to send and receive data to the unit, as well as some exemplar C++ code using these functions. These functions remove the need to delve into the intricacies of communication

protocols and specific command sequences, providing a convenient bridge between the device adapter and the TCSPC hardware. Using this DLL, a device adapter was written that allowed MultiHarp acquisitions to be executed from MicroManager and the data collected to be displayed. This device adapter also enabled the MultiHarp settings used in fluorescence lifetime data acquisitions, such as the delay applied between PMT signals, trigger levels used and timing resolution, to be adjusted. The steps required for a MultiHarp TCSPC data acquisition are as follows (also shown in the yellow box in Figure 38):

1. Create a file to which the acquired data will be written.
2. Run the *MH_StartMeas* function to initiate an acquisition with a given acquisition time; once this time has elapsed a flag is set.
3. Start a while loop.
4. Run *MH_ReadFIFO* to extract the events acquired by the MultiHarp and place them in the buffer that is written to the file stream and/or to a live camera view.
5. Check for flags with *MH_GetFlags* and exit the while loop once a flag is set to indicate the end of the acquisition time.
6. Run the *MH_StopMeas* function.
7. Close the file.

Using this new device adapter, the existing MicroManager camera device adapter could be modified to take in information from the MultiHarp unit and provide real-time viewing of the multibeam multiphoton fluorescence channel in the MicroManager GUI window. This is beneficial for optimisation procedures such as wavelength tuning or selecting a FOV and/or focal plane to be imaged. To create the live view, the user inputs the number of beams in the x and y axes, and the number of pixels in a scan sub-region. Using the new 2 x 3 optical fibre loom layout discussed below, these parameters were set to 2 in x and 3 in y, usually with a 120 x 80 pixel sub-region, creating a 240 x 240 pixel total image. A 240 x 240 array is then created, into which the photon events saved into the buffer in step 4 of the process above can be entered. When using the live camera view, it may not be practical to save every image, so a “save enabled” option is available, for which steps 1 and 7 are removed.

In the current state of the system, the desired scanner parameters are set manually in LabVIEW; however, work is going on to automatically send the current scan properties from MicroManager to LabVIEW whenever a scan is initiated.

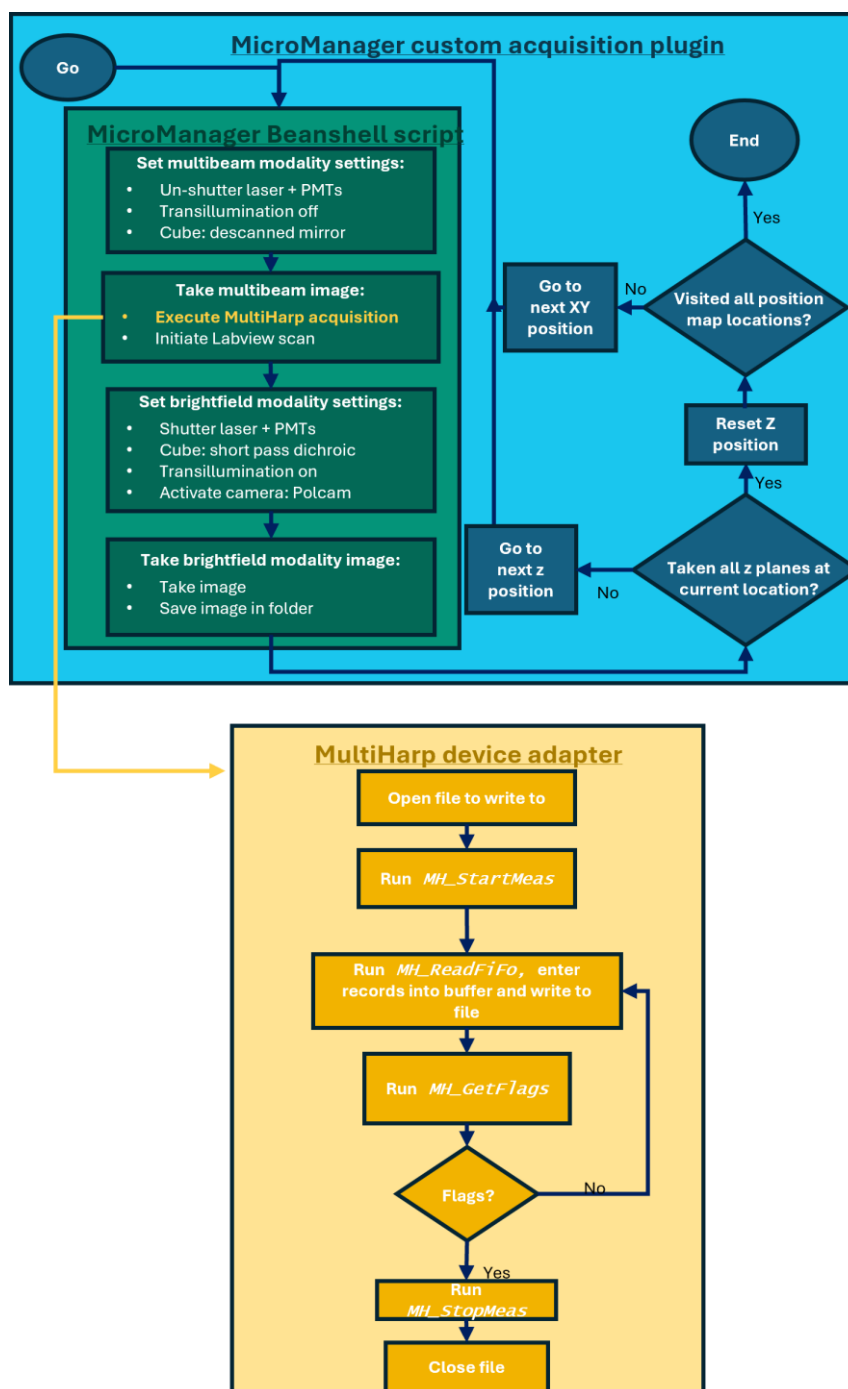


Figure 38. Flowchart showing the processes of an interleaved multidimensional TCSPC acquisition. A MicroManager Beanshell script is used to switch the microscope modality between multibeam-multiphoton and brightfield modes. The MicroManager custom acquisition plug-in allows the Beanshell script to be run at z-depth, time series and XY positions specified by the user. The MultiHarp device adapter runs the appropriate MultiHarp DLL functions to carry out a FLIM acquisition.

4.1.2 Automated acquisitions

Using this device adapter, a MultiHarp TCSPC data acquisition could be executed by setting the '*MultiHarp status*' value in the micromanager device property browser to '*Start*', after which the status shows '*Running*' until the acquisition is over. This allows acquisitions to be inserted into a standard MicroManager script, where they can be coupled with an existing custom MicroManager image acquisition engine written by Sunil Kumar that enables multi-dimensional acquisitions to be executed at specific times, locations or z-positions. Further commands could be added to this script, such as a changing the filter settings for the multiphoton acquisition or acquiring image data using another modality such as phase contrast.

A key command for the MultiHarp TCSPC data acquisition is the '*start scanning*' command sent to LabVIEW over a socket connection. This required that LabVIEW was open with the scan settings required for the acquisition already entered into the LabVIEW GUI. A flowchart showing how MicroManager script, custom acquisition plug-in and MultiHarp device adapter work together is shown in Figure 38, for interleaved FLIM and brightfield image data acquisition over multiple z-depths and XY positions. Figure 39 shows the GUI for the MicroManager custom image acquisition engine plug-in. The active script - *pDPC_MM* - is shown in the script control panel; this script controls interleaved brightfield and multibeam multiphoton imaging, the details of the script are shown in the green box in Figure 38.

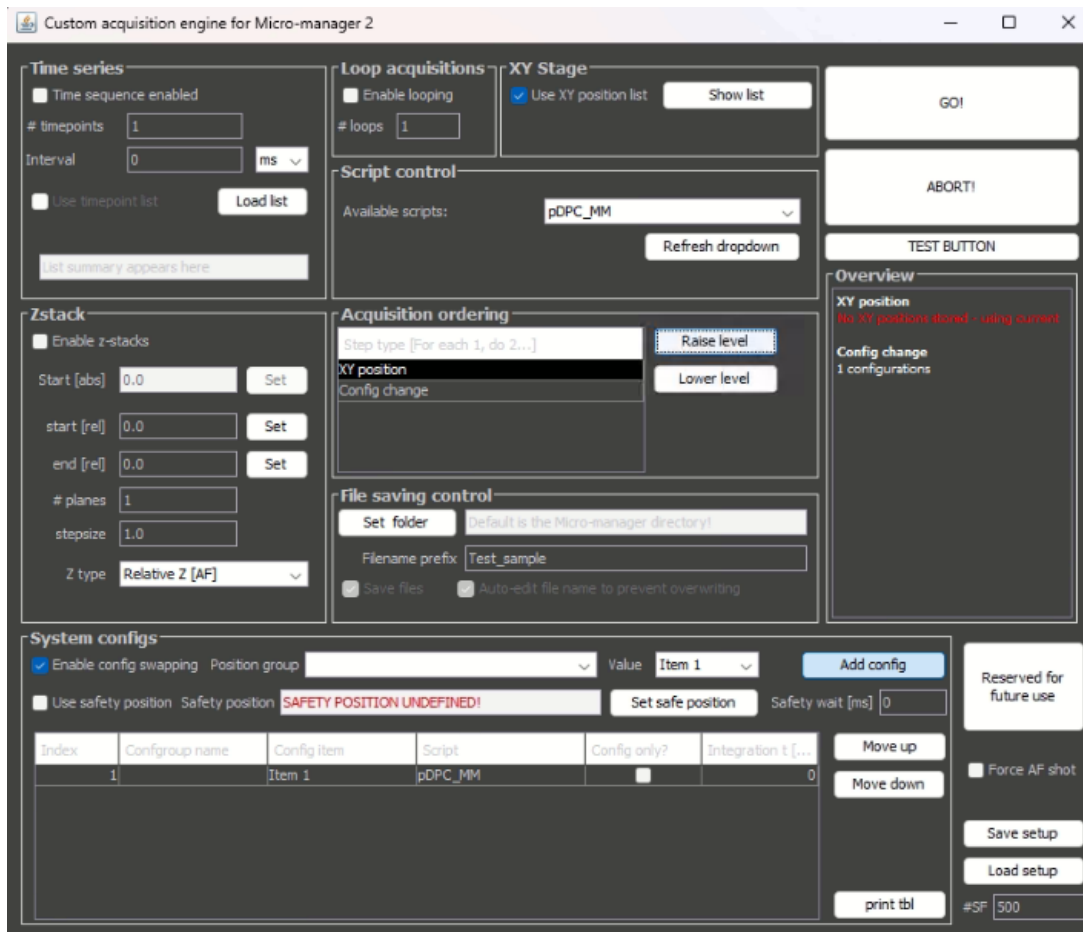


Figure 39 Screenshot of plug-in developed by Sunil Kumar for creating multi-dimensional acquisitions in MicroManager with the pDPC_MM script loaded.

4.1.3 Event stream interpreter

The event streams saved during a MultiHarp TCSPC data acquisition had to be processed to produce images that could be opened in image processing software tools such as *ImageJ* or *FLIMfit* - a fluorescence image data lifetime analysis programme created in the group. A python script “*event-stream interpreter*” was written, adapting work from [128], which would read the event stream, interpreting the events to create an image. Sub-images were made for each detector that then had to be appropriately concatenated in the correct orientation in order to form the full (fluorescence lifetime) image. The programme could also be used to produce segmented images of the data using the *cellpose* [129] software and could apply binning to the lifetime data, as the default 10 ps time bins in the MultiHarp event stream produce large file sizes and this temporal resolution is not always necessary.

Further TCSPC image manipulation and analysis could be done in this python script, however, given the expertise using the *FLIMfit* software in the laboratory, this software was preferred for FLIM data analysis. A key function of the python script was therefore to save a tiff file of the lifetime data such that it could be correctly interpreted by *FLIMfit*, as the current version of *FLIMfit* does not support MultiHarp event stream data, or multibeam data. Care had to be taken to adjust the lifetime metadata appropriately such that upon loading the tiff file into *FLIMfit* the correct values on the time axis are displayed.

FLIMfit has the capability to interpret a tiff file name to assign data to specific wells in a multiwell plate. Options were added to the python script to input the start and end well, as well as the number of repeat measurements per well. As the programme cycles through the event streams to be processed, it saves the tiff file with the appropriate file name, indicating the well index to be read by *FLIMfit*.

A plug-in was written to enable this python script to be executed from MicroManager, allowing for a smoother user experience. The plugin processed the event streams in the directory to which the MicroManager device adapter saved them. The row and column data could be entered into this plug-in along with the plate scan path, which could either be snaked or non-snaked. The repeats per well could also be entered. The desired folder name could be set within the plug-in, such that the programme then creates a TCSPC data folder with that name within a specific user-defined M³M data folder. Figure 40 shows the GUI of this plug-in, which was incorporated into the existing SLM control plug-in to reduce the number of windows that are needed to be open simultaneously when using the system.

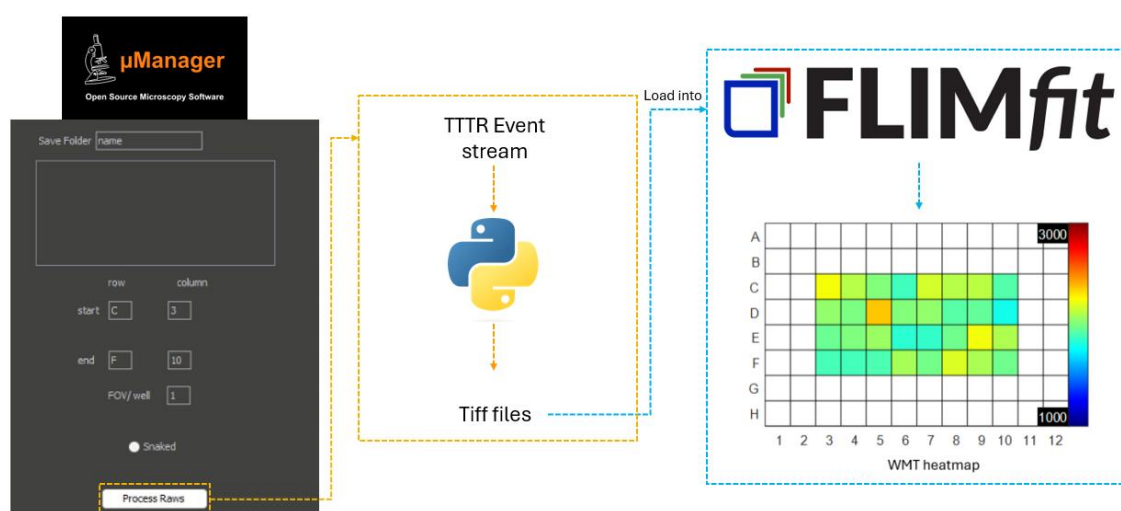


Figure 40 Illustration of how MicroManager plugin can be used to execute a python script which processes TTTR files into tiff files that are readable by *FLIMfit*.

4.2 Advances in M³M excitation light path

4.2.1 Insight X3 Laser

A more broadly tuneable multiphoton excitation laser with dispersion pre-compensation (Spectra Physics, Insight X3) was installed at the Francis Crick institute for use with the M³M instrument. Compared to the Ti:Sapphire laser (Spectra Physics, MaiTai) used for the work presented in the previous chapter, this laser provided a larger tuning range, as shown in Figure 41, allowing excitation of a wider set of fluorophores. In addition, the Insight X3 laser incorporated a dispersion pre-compensation unit (Spectra Physics, DeepSee) that can apply negative group velocity dispersion to each pulse such that the pulse broadening in the optical components in the excitation path is counteracted to reduce the excitation pulse duration at the sample. Without this dispersion pre-compensation, the

Feature	MaiTai	Insight X3
Pulse Width	<100 fs from laser	<120 fs from laser
Tuning Range	690-1040 nm	680 – 1300 nm
M ²	<1.1	<1.2
Dispersion pre-compensation	No	Yes

Table 5 Comparison of MaiTai and Insight X3 excitation lasers.

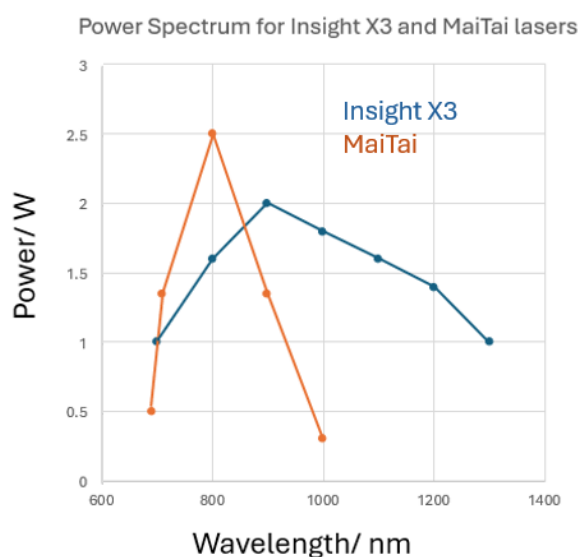


Figure 41 Average output power tuning ranges for Insight X3 and MaiTai lasers.

significant pulse broadening would decrease the multiphoton excited fluorescence signal by a factor of ~ 2 . A table comparing some differences between the two lasers is shown in Table 5.

4.2.2 Acousto-optic modulator

To control the excitation power into the microscope, the half-wave plate/PBS set-up used with the T:Sapphire laser was replaced by an acousto-optic modulator (AOM, model AA Opto-Electronic MT110-B69A1 with a MPDSnC digital synthesiser driver). The AOM has a rapid update rate of the order of ~ 100 ns, meaning that the power can be modulated more quickly than with the half-wave plate/linear polariser set-up. This can be exploited to block the laser on flyback of the scan so that no fluorescence is excited during this time, therefore eliminating unnecessary photobleaching or photodamage.

An AOM functions by generating an acoustic wave that propagates across a crystal through which the laser beam passes. The sound wave creates local modulations in the refractive index of the crystal on the scale of the optical wavelength that diffract energy from the incident (zero order) laser beam. The amplitude of the sound waves controls the amount of light coupled into the diffracted order and the modulation frequency determines the optical wavelengths diffracted with high efficiency. For light to be diffracted efficiently, Bragg's law must be satisfied meaning that $2\Lambda \sin(\theta_B) = \lambda$, where λ is the light wavelength, Λ is the wavelength of the acoustic wave in the crystal and θ_B is the Bragg angle for the light incident on the crystal. For small angles this results in a Bragg angle of $\theta_B = \frac{\lambda}{2\Lambda}$. The angle between the undiffracted 0th order and 1st order output of the AOM is then twice this angle, therefore given by

$$\text{Equation 21} \quad \theta = \frac{\lambda F_c}{v}$$

where F_c is the frequency of the sound wave and v the velocity of the sound wave in the crystal, listed as 4200 m/s in the specification sheet for our AOM unit. Often manufacturers list a specific angle that they have optimised the AOM for which should be used as the Bragg angle, in our case this was $\theta_B = 23.4$ mrad. This means there is a simple relationship between the wavelength used and the frequency needed to be applied to the crystal of $F_c = \frac{\theta_B v}{\lambda}$, using the values of v and θ_B provided by the manufacturer. A diagram showing the beam path from the Insight laser to the excitation breadboard through the AOM is shown in Figure 42.

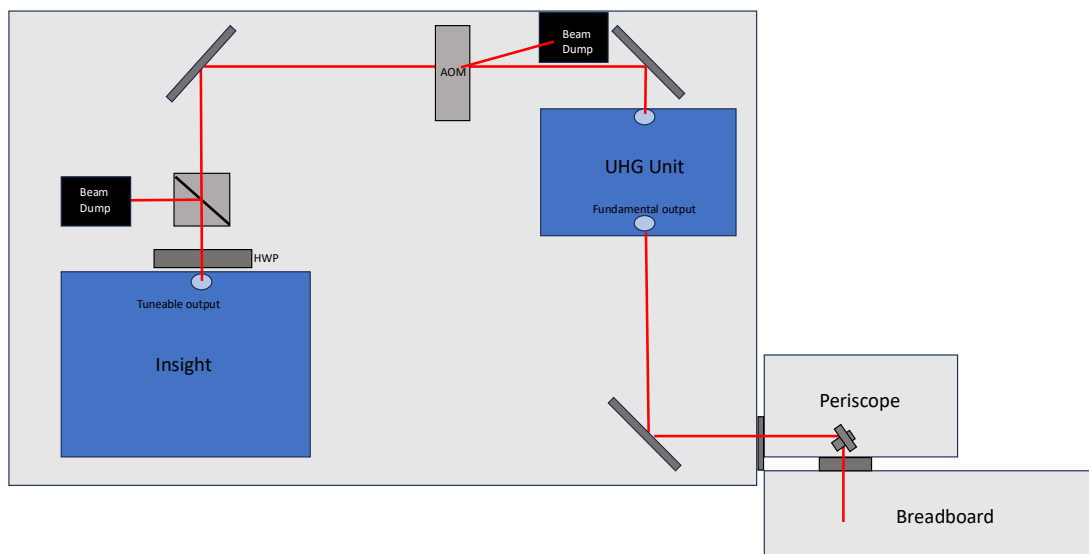


Figure 42 Schematic showing beam path in second iteration of M3M from Insight laser to M3M breadboard via the AOM.

4.3 Improving throughput of excitation power

Since the multiphoton excitation efficiency depends on the peak intensity at the sample, it is important to maximise the average power reaching the sample and to minimise the focal spot size and the pulse duration. The first M³M implementation provided 10% total transmission efficiency of 850 nm light through the optical components in the light path from the laser to the sample plane when using the linear 6-beam excitation. This means a maximum of 33 mW average power per beam is possible at the sample plane for a laser input of 2 W. While this is reasonable for (superficial) imaging with minimal light scattering, deeper imaging requires higher powers per excitation beam to counteract light absorption and scattering. One way to transmit more power from laser to sample is to underfill the back aperture of the objective lens. This means more power is transmitted through the objective, albeit at the cost of increasing the focal excitation spot size and therefore diminishing the resolution and excitation efficiency. To gauge the trade-off between these two effects a simulation was carried out.

4.3.1 Objective lens fill factor

Due to the Gaussian distribution of propagating light, it is difficult to apply a uniform illumination to the pupil of an objective lens, although this would maximise image resolution. It is therefore common practice to magnify the Gaussian beam such that it overfills the back aperture (pupil) of the objective lens, reducing the variation in intensity across this aperture. This can be beneficial where the power lost in overfilling is not an issue. However, given the need to maximise excitation intensity at the sample, we explored adjusting the fill factor of the objective lens pupil by changing the illumination field size at the SLM, initially determined by the 10x magnification of the Galilean beam expander (GBE) of an expected 1 mm FWHM input beam from the Insight laser source. This 10x magnification was originally chosen to just overfill the 19.2 mm diameter pupil. However, a measurement made of the beam diameter at the entrance of the M³M breadboard found that the input beam FWHM was 1.4 mm, due to beam divergence along the ~2 m beam path from the femtosecond laser output to the M³M optical breadboard.

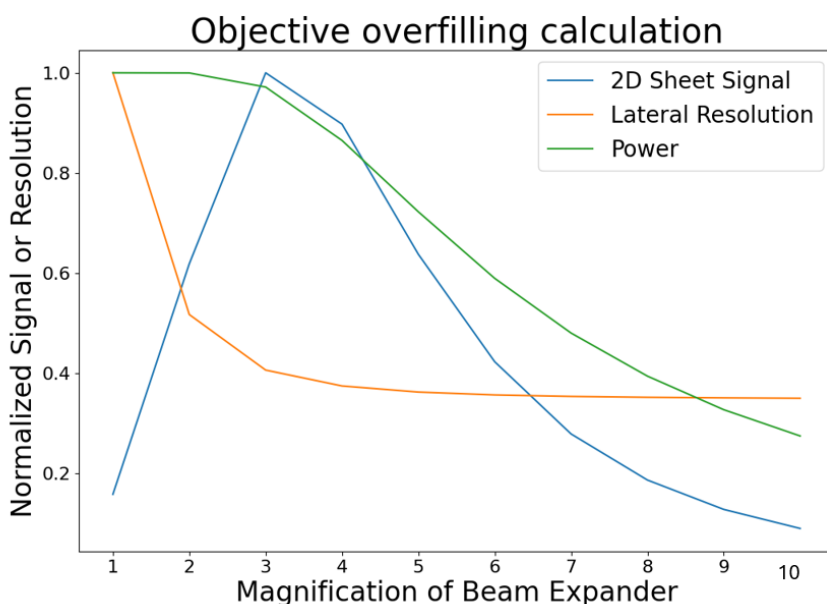


Figure 43 Plot of fluorescence signal coming from a thin 2D sheet of fluorescence, lateral resolution and power throughput normalised to max values and plotted as a function of magnification from the GBE.

A simulation was carried out to help optimise the fluorescence signal and image resolution as a function of magnification of the 1.4 mm diameter input laser beam. The parameters calculated were the power throughput to the sample, the 2-photon fluorescence signal excited from a thin sheet of fluorophores and the lateral resolution. Figure 43 shows how these parameters vary as a function of the magnification applied at the GBE. This simulation confirmed that we could lower the excitation beam fill factor at the objective lens pupil to increase the excitation power at the sample without significantly degrading the resolution. This is shown by the region on the plot in which one can obtain an increase in

signal when decreasing the magnification from 10x but minimally increase the minimum lateral resolution.

Rather than replacing the GBE, we reduced the diameter of the light distribution on the SLM by making use of the collimation setting on the GBE to implement a slightly converging exit beam. We then adjusted lens L2 such that the output beam from L2 was collimated, meaning sharply focussed points were still formed at the focal plane of the objective. The beam diameter at the SLM could be decreased further by increasing the distance between the mirror M1 and the GBE. This adjustment changes the diameter of the field at the objective pupil, underfilling the objective such that the transmitted excitation power increased by 70% with no obvious degradation of image resolution. From the simulation data, this improvement in power is estimated to be due to the magnification decreasing from 10x to 6x, producing a 16.8 mm FWHM beam at the objective pupil compared to the previous 28 mm FWHM beam. A diagram of the scheme used is shown in Figure 44.

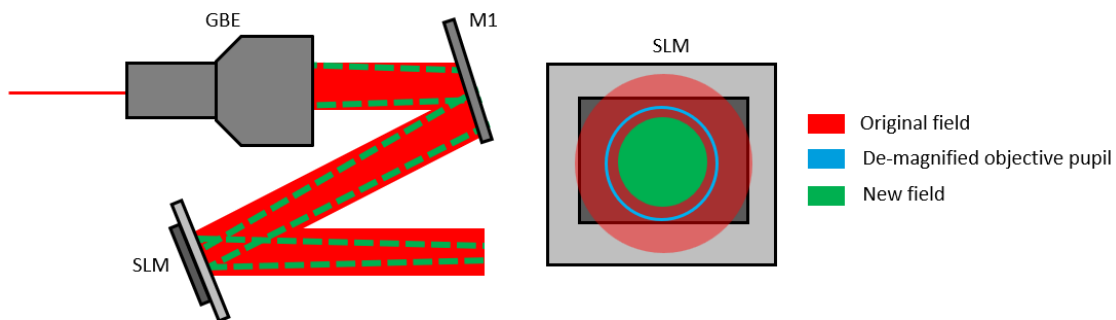


Figure 44 Schematic of GBE convergence used to produce smaller field size on the SLM to underfill objective pupil.

4.3.2 SLM diffraction efficiency

The SLM produces a significant reduction in average power in the excitation path and so its diffraction efficiency as a function of output beam pattern was studied. To establish the diffraction efficiency as a function of output beam displacement using a phase ramp on the SLM, a pinhole was placed at the focus of L1 on the optical axis and a particular diffracted order was directed through the pinhole using the kinematic mount adjustments of the SLM. This meant the power of the desired diffracted order could be measured without contributions from other orders. This wasn't an ideal setup as diffraction efficiencies would usually be measured for a constant angle of incidence, however as the maximum rotation of the mount was 3 degrees (for the lateral displacement of 16 mm), we were happy this wouldn't impact the experiment significantly. The relative efficiency given by the power in this beam relative to the average power measured for a beam diffracted by the minimum angle needed to be separated from the zeroth order is plotted in Figure 45 as a function of the lateral displacement that would be caused by the phase ramp displayed on the SLM. A theoretical calculation of this efficiency matches the experimental measurements reasonably well until lateral displacements exceed 9 mm. This can be attributed to an effect in LCOS SLMs whereby the nematic liquid crystals cannot change their orientation over arbitrarily small distances. This results in an effective low pass in the frequencies of the phase hologram displayed.

Now using 6-beam holograms, the total power in all 6 diffracted beams was measured by applying the appropriate hologram to the SLM and sequentially measuring the power in each of the 6 diffracted beams using the pinhole to isolate them in turn. Figure 46 shows the summed power of the 6 diffracted beams for the line configuration as a function of the beam spacing for beam spacings from 1

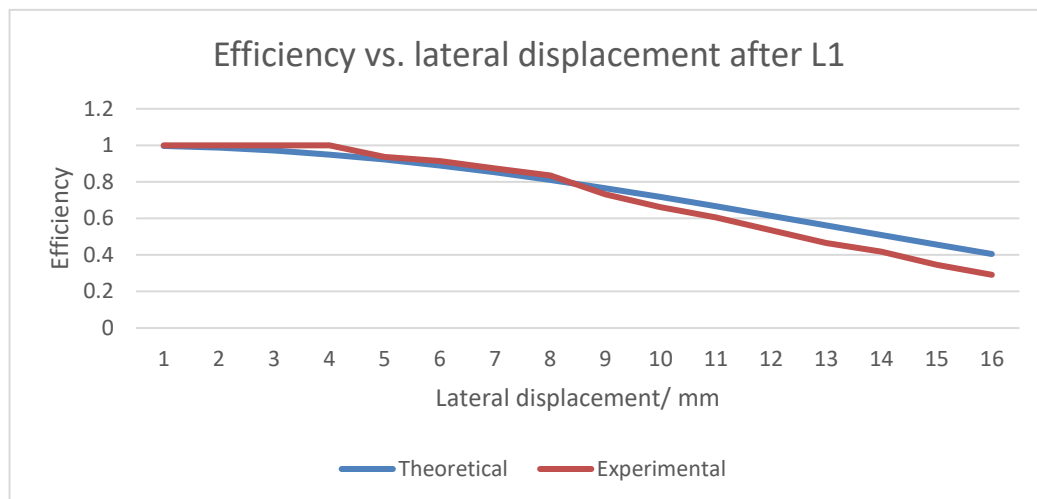


Figure 45 Efficiency of 1st diffracted order from a blazed phase grating for varying lateral displacements from the optical axis in the focal plane after L1. Theoretical prediction comes from equation 3 in chapter 3.

mm to 6 mm. The beam intensities were not evened in this experiment. The spacing used for the line pattern initially used in the system was 3.6 mm, for which the relative efficiency has a value of ~80%, the process of evening out the beam intensities was found to decrease the efficiency further by a few percent.

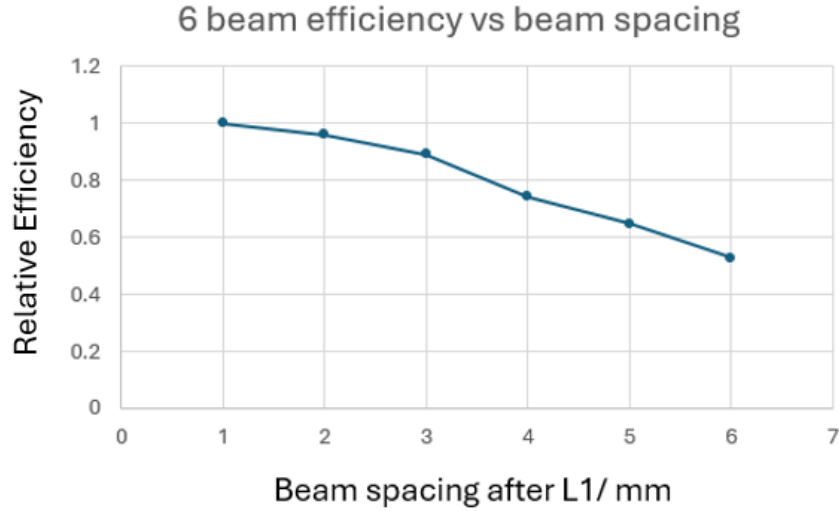


Figure 46 Efficiency of power diffracted into unevened 6 beam line pattern plotted as a function of beam spacing.

It is clear that to increase the excitation power available at the sample, it is helpful to reduce the spacing of the beams diffracted off the SLM. As well as higher SLM diffraction efficiencies, this also results in less chromatic dispersion of the beams. Doing this requires the adjustment of the focal length of L5 such that the different beam spacing is still mapped onto the fibre loom.

4.3.3 Impact of chromatic dispersion at the SLM

Diffraction at the SLM is a wavelength dependent process, meaning that for a given hologram displayed on the SLM, different wavelengths will be diffracted at different angles. The spectral bandwidth in Hz of an ultrashort laser pulse with a Gaussian intensity profile can be estimated using the time-bandwidth product:

$$\text{Equation 22} \quad \Delta f = \frac{0.44}{\Delta T}$$

where Δf is the frequency bandwidth and ΔT is the duration of the pulse. Furthermore, the spectral range in terms of wavelength for a given frequency bandwidth can be calculated using the equation

$$\text{Equation 23} \quad \Delta \lambda = \frac{\lambda_0^2 \Delta f}{c}$$

where λ_0 is the central wavelength. Substituting Equation 22 into Equation 23 and assuming a pulse duration of 100 fs with central wavelength of 850 nm, gives $\Delta\lambda = \sim 10$ nm.

If we consider a hologram designed to diffract light of wavelength λ_0 to a position d_0 in the focal plane of L1, then a wavelength component λ will be diffracted to a position d according to,

$$\text{Equation 24} \quad d = \lambda \frac{d_0}{\lambda_0}.$$

This chromatic dispersion leads to spreading of the excitation spot focussed at the sample plane. This dispersive spreading of the excitation beam increases with distance d_0 in the sample plane, with the 10 nm bandwidth pulses at 850 nm centre wavelength spreading by 700 nm for a diffraction distance d_0 of 60 microns, a diffraction distance on the order of those used in the line patterns we had been using. Thus, this chromatic dispersive spreading may be significant in our setup. As the two-photon fluorescence signal is proportional to the square of the intensity, spreading the excitation radiation causes emission from a larger area, but with a much lower intensity, decreasing the overall fluorescence signal. In addition to this reduced intensity, the spatial dispersion of wavelengths results in a narrower frequency bandwidth at each point in the smeared PSF, which increases the effective pulse duration and reduces the peak excitation power, further diminishing the fluorescence signal. This effect has been noted previously in the design of a different multibeam multiphoton microscope [43], although the magnifications and beam separations used in their system resulted in a negligible chromatic spreading effect.

A simulation was used to estimate the impact of chromatic dispersion in the M³M microscope. The simulation first initialised a set of 5000 delta-function spectral lines spaced by 2 pm over the 10 nm linewidth of the laser. This 2 pm line spacing corresponds to the 80 MHz spacing between modes of the excitation laser and the amplitudes of each wavelength were set to be equal, modelling the spectrum of the laser pulses as a top-hat function. The PSF formed by each wavelength at the sample plane was calculated and summed to produce the full PSF of the total frequency spectrum. This process is shown for two d_0 positions in Figure 47 where only the signal from 5 delta-function lines are displayed in (a) for ease of viewing. The simulation shows that the peak intensity decreases, and the spatial distribution gets wider as d_0 increases; this is shown for a range of d_0 values in Figure 48.

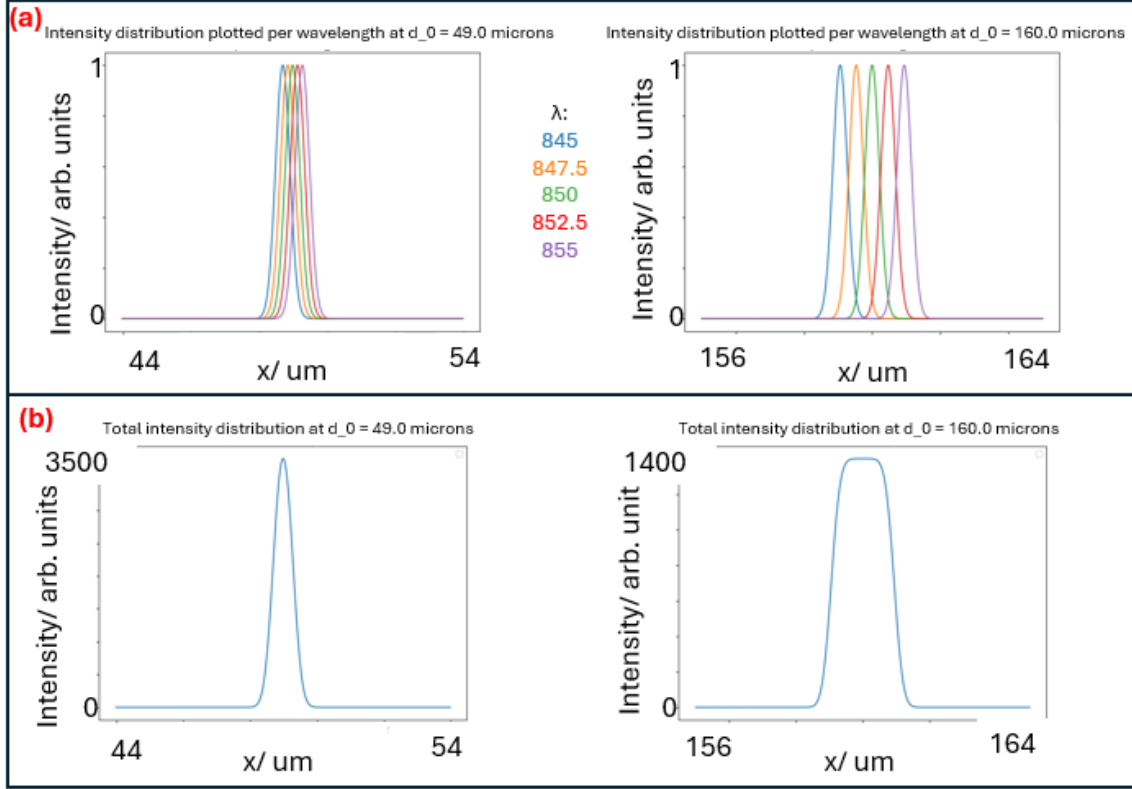


Figure 47 (a) PSFs plotted for 5 delta-function spectral lines for a beam diffracted 49 and 160 microns from the optical axis at the sample plane and (b) the sum of the individual wavelength PSFs. The intensity distribution is wider with a lower intensity for larger diffraction distances

The two-photon fluorescence obtained from the PSF is proportional to the square of the total intensity at each point summed over all points within the PSF, according to $F \propto \sum I^2$. To quantify the amount of temporal spreading of the pulse, the range of wavelengths found at the centre of the PSF was calculated by determining the change in wavelength necessary to laterally shift the beam by the FWHM of the PSF using Equation 24. This wavelength range was then used to calculate the pulse duration using Equation 22 and Equation 23. This change in wavelength would be smaller for larger d_0 values, therefore leading to smaller bandwidths and longer pulses.

This method of calculation ignores effects at the edge of the PSF. The inverse of the pulse length is proportional to the amount of two-photon fluorescence, according to $F \propto \frac{1}{T}$. Combining both spatial and temporal pulse broadening due to chromatic dispersion, the fluorescence signal will vary according to

$F \propto \frac{\sum I^2}{T}$, therefore this ratio was calculated as a function of d_0 . The values of $\sum I^2$, $\frac{1}{T}$ and F are shown plotted as a function of d_0 in Figure 49.

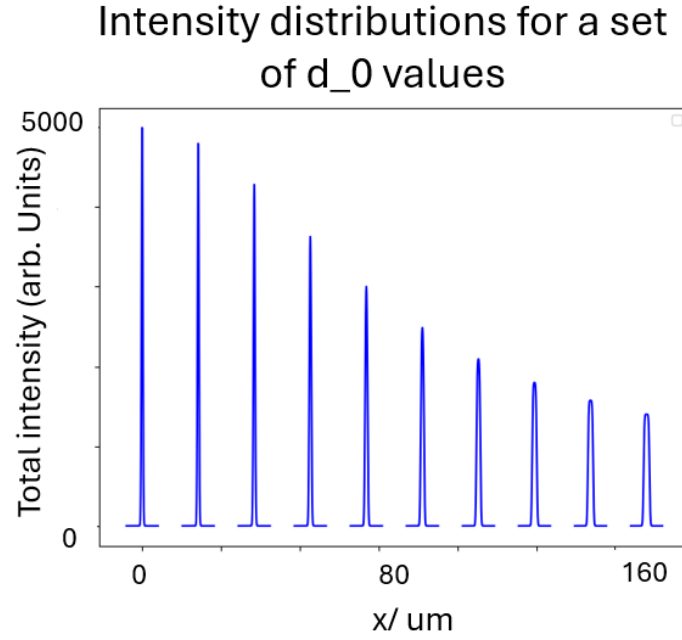


Figure 48 Spectrally summed intensity distributions for a range of off-axis diffraction distance (d_0) values measured at the sample plane.

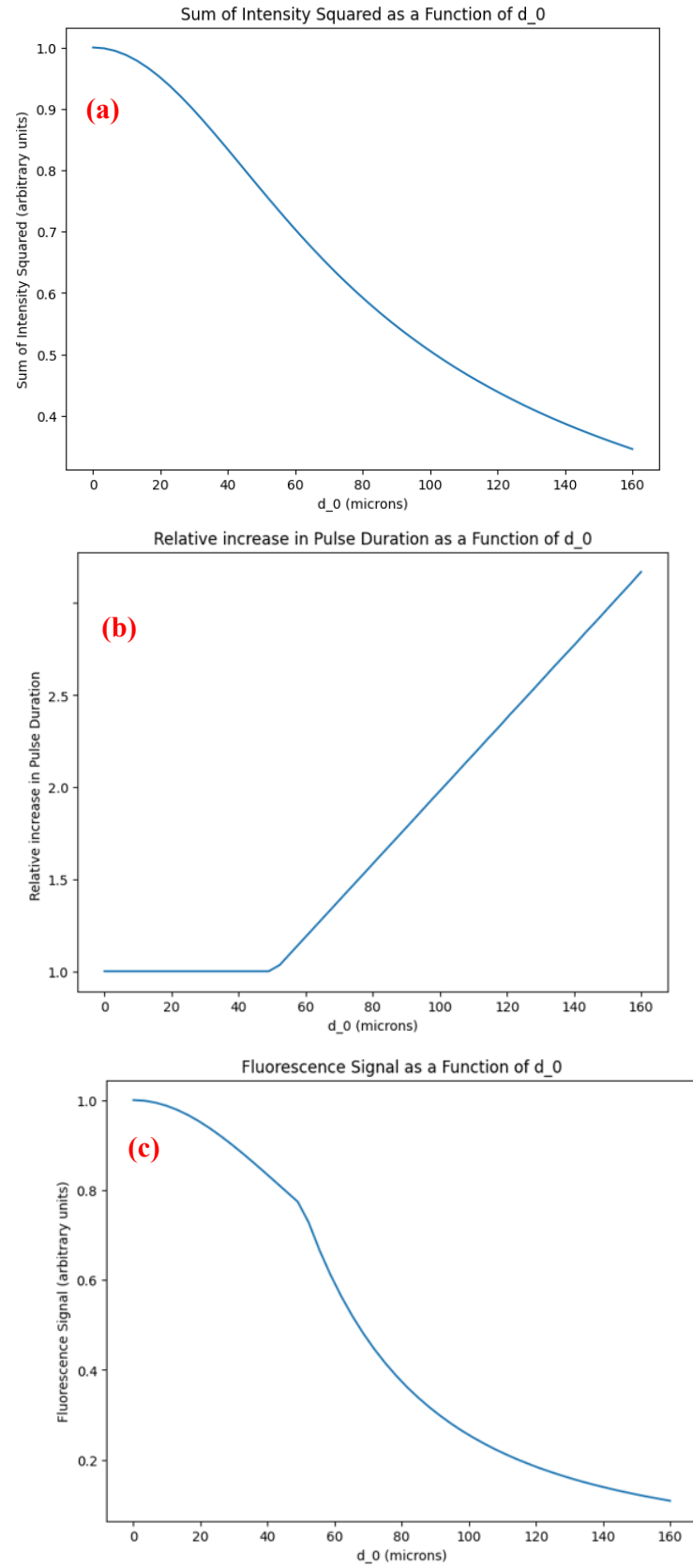


Figure 49 (a) Sum of squares of values in PSF, (b) relative change in pulse duration and (c) resulting two-photon signal all as a function of d_0

An experiment was carried out to test whether the fluorescence signal loss matched the predictions of the simulation. In the experiment a beam was displaced by a set of distances from the optical axis using first the SLM, and then the galvo scanners, and the ratio of the fluorescence signals was determined. We reasoned that any reduction in excitation efficiency resulting from the beam moved away from the optical axis would manifest in the galvo-deflected signal and the ratio would reveal losses resulting from diffraction at the SLM. The SLM fluorescence signal was also corrected by dividing by the square of the diffraction efficiency factor previously found to account for the fact there would be less power in the diffracted beam away from the optical axis. This corrected fluorescence signal is plotted as a function of distance d_0 from the optical axis in Figure 50 alongside the prediction from the simulation. The theory matches the experiment well, indicating that the dispersion effect was indeed the cause of signal loss.

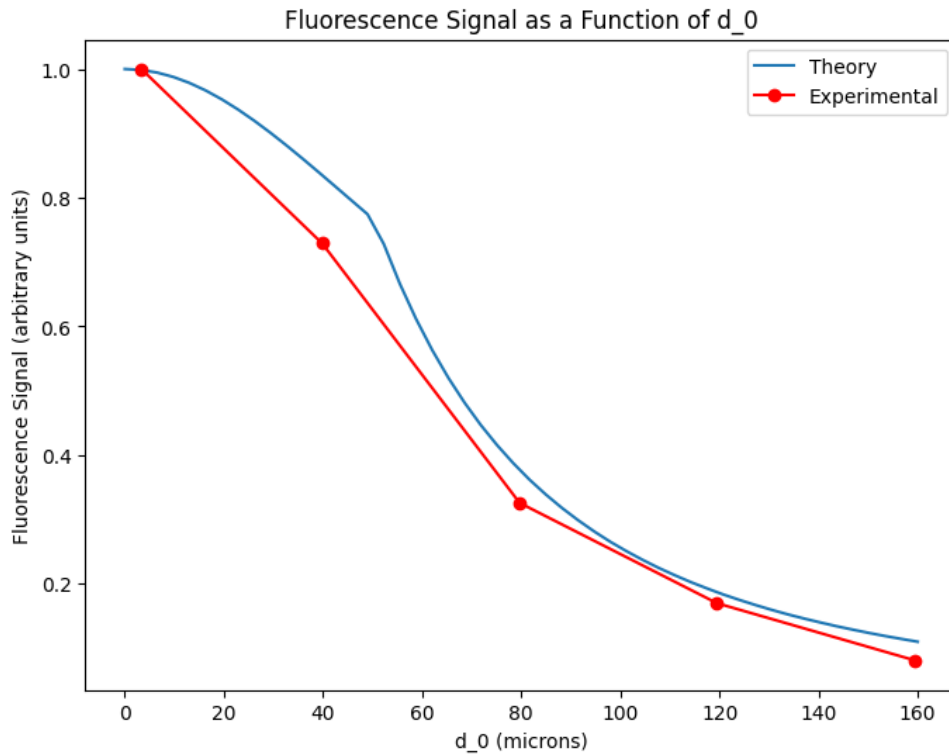


Figure 50 Ratio of fluorescence in off-axis beam deflected by SLM and galvo as a function of off-axis distance measured experimentally (red) and predicted by simulation (blue).

This simulation was undertaken to estimate the order of magnitude of the impact of chromatic dispersion. A more rigorous calculation could consider the complete electric field variation in space

and time for each wavelength component using Fourier analysis. However, the model of chromatic spatial dispersion used, which built on work reported in [130], is consistent with the measured reduction in fluorescence signal.

However, a key issue for the time-lapse multiphoton excited assays for which the M³M instrument is intended, is the phototoxicity that accompanies the fluorescence imaging. Whilst chromatic dispersion decreases the fluorescence signal due to diffracting multiple beams from the SLM, the phototoxicity is also decreased. Previous studies investigating the relationship between the sample photodamage rate the intensity of two-photon excitation light used [1] found a scaling of $D \propto \langle \int dV I^{2.5}(x, y, z, t) \rangle$, where D is damage rate, $I(x, y, z, t)$ is the instantaneous intensity of the pulse at a given point in space and time, V is the excitation volume and $\langle \rangle$ indicates taking a time average. Other studies have also found the same 2.5 exponent dependence [131]. Given that the two-photon excited signal scales as $F \propto \langle \int dV I^2(x, y, z, t) \rangle$, it is clear that by confining the same excitation power into a tighter region in space and time, the ratio of $\frac{F}{D}$ would decrease due to I being raised to a higher power in its proportionality to damage compared to fluorescence. Thus, by spreading a pulse in space and time, one reduces the fluorescence but also the phototoxicity - and by a greater factor. If one could increase the illumination power, one could increase the fluorescence signal obtained for the original phototoxicity rate D . The authors of [1] calculate the proportionality of two-photon fluorescence when changing only the pulse duration τ or the radius of illumination at the back aperture r , whilst remaining at constant D , to be $F \propto \frac{\tau^{0.2}}{r^{0.8}}$. This indicates that by using longer pulses or under-illuminating the objective back aperture while maintaining the same phototoxicity rate, one can obtain more fluorescence signal for a given damage rate. The authors note, however, that this analysis is heavily dependent on the exponents found in the fitting of their data, whose errors are not insignificant. They note that other studies have shown constant fluorescence whilst elongating pulses and maintaining power at constant damage threshold [7].

4.3.4 Collection lens magnification change

Given the results found on the SLM diffraction efficiency and chromatic dispersion, one way to increase power throughput and the amount of excited fluorescence per unit power was to demagnify the beam pattern therefore requiring lower diffraction angles. As the fluorescence must still be mapped onto the fibre loom, a greater magnification of the lens pair L5 and L4 is necessary, which can be produced by increasing the focal length of L5. This was implemented by using a 200 mm lens for L5 rather than the original 150 mm lens, increasing the magnification to $\frac{4}{3}$ from the plane after L1 to the fibre loom plane. The new beam spacing had a 20% higher diffraction efficiency from the SLM and a 30% increase in signal due to less dispersion. Overall this results in a 87% increase in signal. The

smaller beam spacing also reduces the FOV to 270 microns x 270 microns, however this was still sufficient to image spheroids of a size used in cancer assays [132]. Increasing the magnification also increases the spatial extent of the scattered photons at the loom plane, meaning cross-talk effects are increased, this is analysed further in the following section.

4.4 Optimisation of optical fibre loom

After demonstrating the first M³M prototype, we considered whether the design of the optical fibre loom could be improved with a view to maximising the imaging depth in samples such as biological tissue that would present significant multiple scattering of photons. We reasoned that using larger diameter core optical fibres would collect more scattered fluorescence, but this could come at the cost of incurring significant crosstalk between the excited fluorescence beams that could cause significant image degradation. From the previous section it is also clear that using beams that are diffracted at a lesser angle by the SLM increases the diffraction efficiency and reduces dispersion effects, allowing for greater signal. However, decreasing the inter-beam distance also contributes to cross-talk. Experiment and simulations were therefore undertaken to design an improved optical fibre loom. The following section introduces some basic concepts in light scattering that informed these experiments and simulations. Simulation and experiments were done using the changes made in section 4.3, namely a lower fill factor of the objective and a greater magnification of the beams onto the loom.

4.4.1 Multiple scattering of optical radiation

As light travels through a medium with inhomogeneous refractive index, photons can be scattered into different trajectories. Since the various components of biological cells and tissue have different refractive indices, fluorescence photons emitted from the excitation volume of a multiphoton microscope may be scattered multiple times as they propagate through a biological sample to the objective lens. This can result in loss of signal if the scattered photons do not reach the appropriate detector and crosstalk between multiple descanned detectors can decrease the signal to background ratio of the image and also decrease the resolution.

The scattering properties of biological media can be represented by the scattering mean free path, L_s , and the anisotropy factor, g . The scattering mean free path is the average distance a photon travels between scattering events and is usually around 50-100 μm in biological tissues. From the Beer-Lambert law one may use this parameter to construct the equation for the probability, $p(x)$, of the distance x travelled by a photon between scattering events:

$$\text{Equation 25} \quad p(x) = \frac{1}{L_s} \exp\left(-\frac{x}{L_s}\right)$$

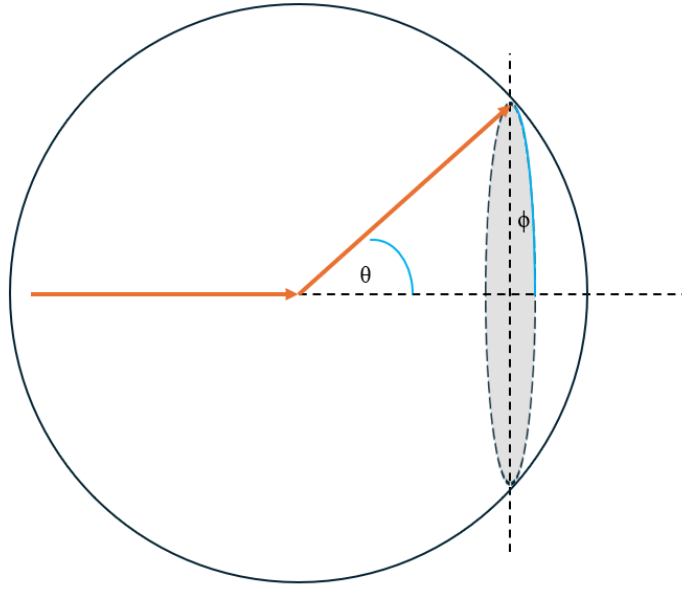


Figure 51 Schematic of a photon scattering into a polar angle θ and azimuthal angle ϕ .

where $\langle x \rangle = \frac{\int_0^\infty xP(x)dx}{\int_0^\infty P(x)dx} = L_s$. The scattering coefficient, μ_s , is related to the scattering mean free path (SMFP) L_s by $\mu_s = \frac{1}{L_s}$ which is more commonly used in photon scattering analysis. On each scattering event the photon is scattered at an angle θ from the axis of its original direction of travel, and an azimuthal angle ϕ , this is shown in Figure 51.

The anisotropy factor, g , is defined as the average cosine of the polar scattering angle θ , which is given by [133]

$$\text{Equation 26} \quad g = \int_0^\pi p(\theta) \cos(\theta) 2\pi \sin(\theta) d\theta = \langle \cos(\theta) \rangle$$

where $p(\theta)$ is the probability distribution of θ , and the factor $2\pi \sin(\theta)$ comes from integrating over the sphere. For a scattering medium that predominantly scatters photons at angles close to their original trajectory, g will be close to 1, while for isotropic scatterers $g = 0$, and for strongly backscattering media, g will be close to -1. The probability distribution for ϕ is usually considered to be uniform and doesn't significantly impact photon transport. The exact form of $p(\theta)$ can be determined experimentally, or modelled with Mie scattering theory, but a commonly used model is given by the Henyey-Greenstein scattering function. This function produces a $p(\theta)$ for a specific value of g , and is given by [134]:

$$\text{Equation 27} \quad p(\theta) = \frac{1}{4\pi} \frac{1-g^2}{(1+g^2-2g\cos(\theta))^{\frac{3}{2}}}$$

Equation 25 and Equation 27 can be utilised in simulations of photons propagating through a scattering medium. When constructing a model to describe the fluorescence emanating from a scattering sample that is measured at a detector, one typically considers unscattered and scattered components; the unscattered light will propagate as predicted using standard wave optics, while the scattered term will present a lower peak intensity and larger spatial extent.

4.4.2 Experimental measurements of scattering in two-photon microscopy

Experiments were carried out using OVCAR4 cellular spheroids to study the impact of scattering on the desired fluorescence signal and crosstalk as a function of optical fibre core diameter, imaging depth, and distance between excitation spot and collection area of the detection optical fibre.

The SLM was used to systematically laterally displace a single excitation spot away from the position on the sample corresponding to the centre of a specific detection optical fibre and the number of counts detected from that optical fibre were measured as a function of this displacement. The measurement indicates the amount of cross-talk occurring for a given fibre spacing. As depicted in Figure 52, this was done whilst scanning a lateral region larger than the spheroid. This meant that as the beam was shifted the entire spheroid cross-section would still be scanned, and therefore no changes in fluorescence intensity would be caused by the spheroid being out of the FOV. A low excitation power (of ~5 mW) was used to minimise any photobleaching over the total acquisition time. The lateral

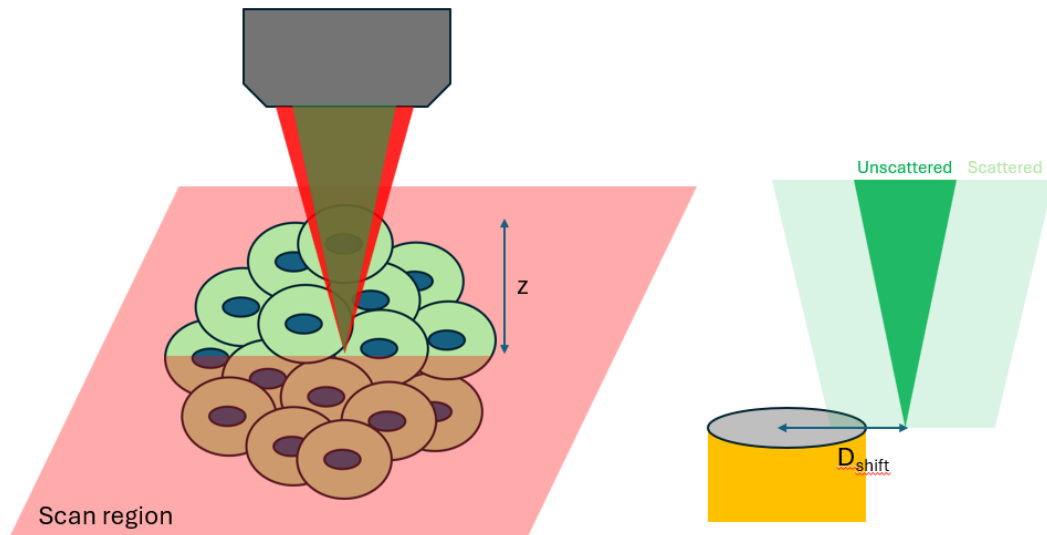


Figure 52 Setup for measuring cross-talk as a function of beam-fibre distance. As the beam is shifted from the fibre using the SLM the amount of collected scattered fluorescence, shown here in a more transparent green shade, is measured.

displacements applied were sufficiently small (< 3 mm at L1) that no change in SLM diffraction efficiency was expected. When considering the results of this experiment with a single excitation spot



Figure 53 PMT counts as a function of beam shift at fibre plane using a fluorescent slide. Red lines indicate fibre diameter. FWHM of the beam at the fibre plane is ~ 30 μm .

and detection optical fibre, one must remember that with a full 6 beam excitation pattern and optical fibre loom there would more than one neighbouring beam that could contribute to crosstalk.

This experiment was first carried out using a fluorescent slide, for which the results obtained using the $600\text{ }\mu\text{m}$ diameter optical fibre are shown in Figure 53. The signal decreases as the excitation spot was moved to the edges of the detection area corresponding to the optical fibre as expected. The beam spot size of $\text{FWHM} \approx 30\text{ }\mu\text{m}$ at the fibre plane accounts for the finite distance over which the signal fades away at the edges of the fibre.

The average intensity across a $400 \times 400\text{ }\mu\text{m}$ scanned FOV was then measured as a function of excitation beam displacement from the optical fibre detection area at z depths ranging from 0 to 200 microns; the plots for these are seen in Figure 54 and Figure 55 for the $200\text{ }\mu\text{m}$ and $1000\text{ }\mu\text{m}$ fibres respectively. The beam shift was varied up to $2000\text{ }\mu\text{m}$ for the $200\text{ }\mu\text{m}$ fibre and to $2500\text{ }\mu\text{m}$ for the $1000\text{ }\mu\text{m}$ fibre at the deepest imaging depth of $200\text{ }\mu\text{m}$. The values plotted on the y axis are the average intensity over the field of view scanned, background subtracted and normalised for each z depth.

As expected, the measurements show that more scattered fluorescence is collected by the $1000\text{ }\mu\text{m}$ compared to the $200\text{ }\mu\text{m}$ diameter optical fibre as the beam is displaced further away from the optical

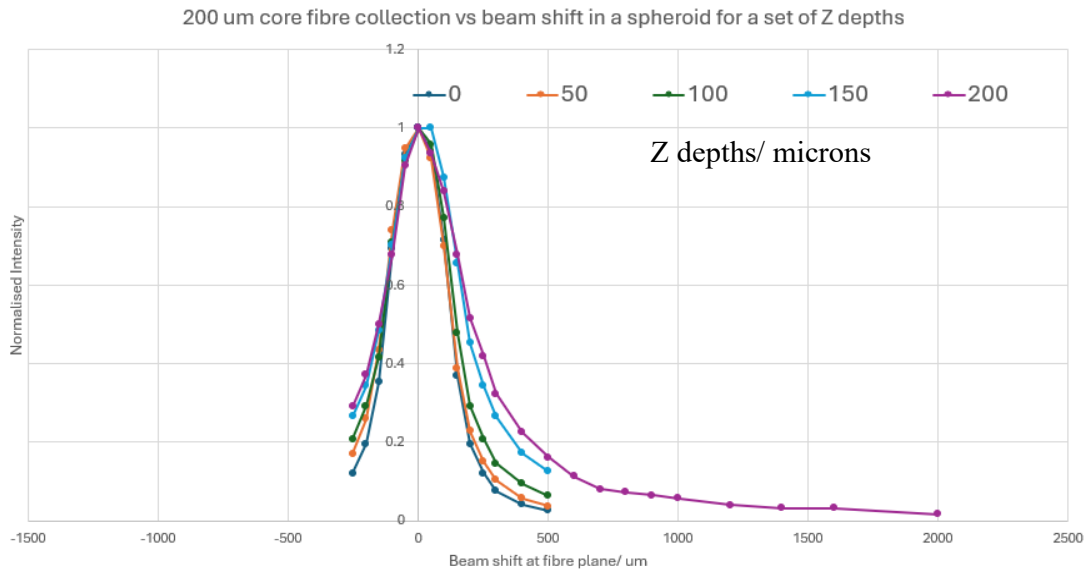


Figure 54 Fluorescence collected in 200 μm diameter optical fibre as a function of beam-optical fibre displacement and imaging depth.

fibre detection area due to its larger collection area. For reference, in the initial optical fibre loom the fibres were separated by 3600 μm at the fibre plane, which is beyond the range explored here. However, the data shows that for this spacing of 200 μm optical fibres, the amount of crosstalk would be negligible and that switching to larger diameter optical fibres at this spacing could allow non cross-talk degraded images up to depths of the order of 100 microns.

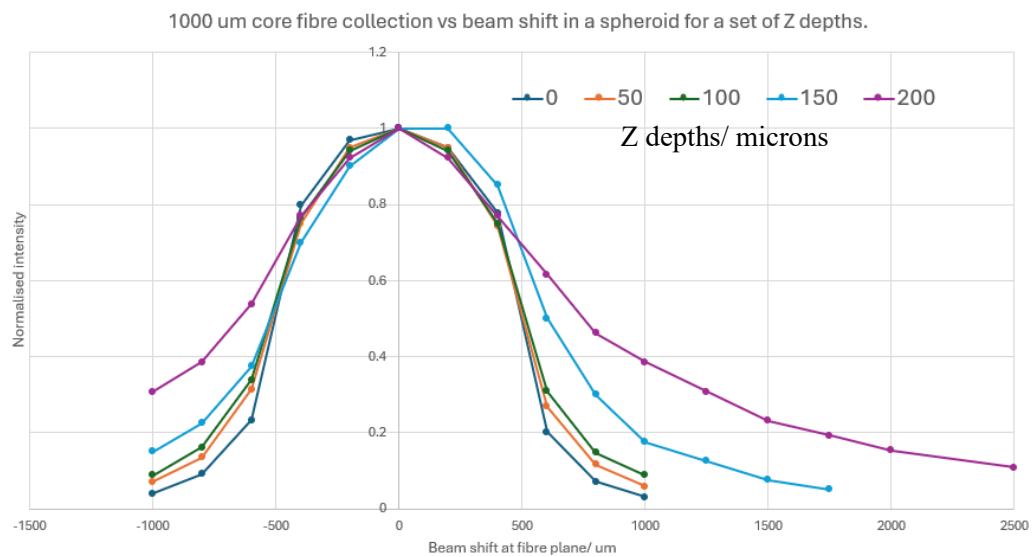


Figure 55 Fluorescence collected in 1000 μm fibre as a function of beam-optical fibre displacement and imaging depth

The signal detected using the 200, 600 and 1000 μm optical fibres was compared as a function of imaging depth by plotting in Figure 56 the average background-subtracted fluorescence intensity across a scanned field of view for each optical fibre at three imaging depths and normalising the values plotted to the signal obtained for the 200 μm diameter optical fibre at that depth. For these measurements the excitation beam was centred on the optical fibre detection area, which was on the microscope optical axis.

Figure 56 shows how the collected signal increases for larger optical fibre diameters at greater z depths. At 0 μm depth, theory would predict that the fluorescence is unscattered and each fibre collects

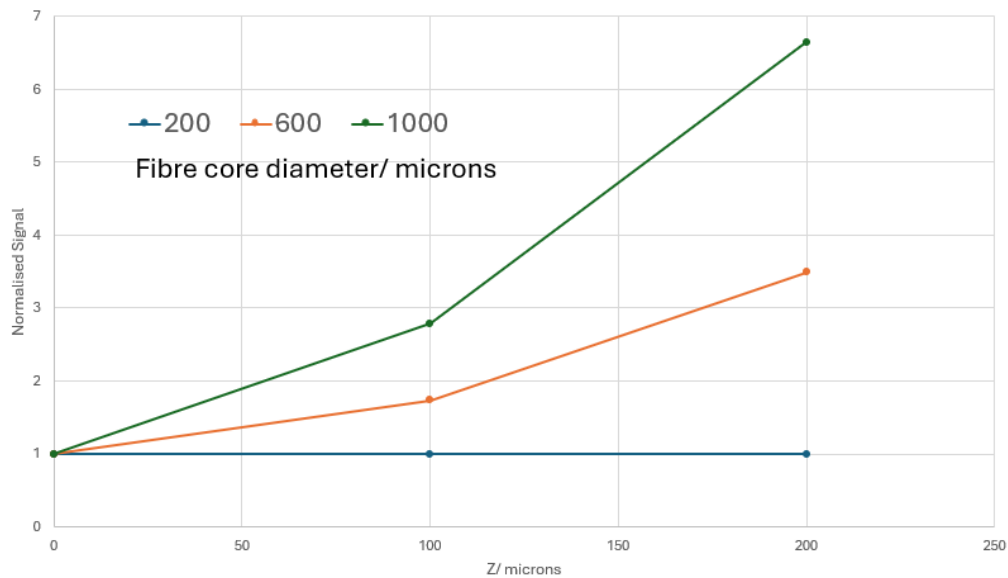


Figure 56 Signals measured in 200, 600 and 1000 μm diameter fibres as a function of imaging depth for an excitation beam centred on the optical fibre detection area. (Signals for each diameter optical fibre are normalised that obtained with the 200 μm fibre at that depth).

all of the available fluorescence. At greater imaging depths the larger diameter optical fibres can collect more of the scattered fluorescence. For reference, the airy unit using 200 mm lens for L5 is roughly 20 microns in diameter, meaning the smaller and larger fibres are 10 and 50 airy units respectively.

4.4.3 Simulation of impact of scattering in two-photon microscopy

A simulation was written to support the design of improved optical fibre looms for the multibeam multiphoton detection. The code was written using CuPy, a GPU enhanced Python library, which allowed significant acceleration as the simulation worked by applying the same operations to every photon on each iteration, a process which is sped up with GPU enhancement.

4.4.3.1 Simulation description

The programme was inspired by previous Monte Carlo type simulations in which photons travel through a medium and scatter according to defined probability distributions until they either scatter too many times (according to some user-defined cutoff) or exit the tissue [135]. Given a defined probability distribution for a variable, e.g., Equation 25, one can generate instances of this variable which conform with a known probability distributions using Inverse Transform Sampling. This works by first finding the cumulative probability distribution $F(x) = \int_a^x p(x')dx'$ where a is the lower bound on the variable x' . One then applies the inverse of this function to a random variable, U , picked from a uniform distribution between 0 and 1, such that the sampled variable is given by $x_{sample} = F^{-1}(U)$ [136], [137].

The simulation used two main functions. The first function, *simulate_photon_emission*, simulated photons travelling and scattering through tissue, recording their positions and velocities at each scattering event. The second function, *get_perceived_positions*, extracted the perceived position of each photon traced back to the focal plane of the excitation volume at a specified depth in the tissue. This was done so that for one run of *simulate_photon_emission*, information could be extracted for various z depths by applying *get_perceived_positions* for different z values. This reduced the run time significantly as simulating the photon scattering was the most time-consuming task of the programme. A selection of the input parameters for the simulation are seen in Table 6.

The first function, *simulate_photon_emission*, started by initialising random positions p_0 for N photons emitted from within an excitation volume with a specified lateral and axial FWHM. The photons were also assigned an emission direction vector v_0 , which was constructed to uniformly sample the objective-side hemi-sphere surrounding the excitation volume, given we had made the assumption of isotropically emitted fluorescence. A hemisphere was selected for the simulation because including the full sphere had a negligible impact on the output. This simplification was justified by the high g value expected in biological tissue, which ensures that nearly all photons in the lower hemisphere fail to exit the tissue. A for-loop was then implemented to scatter the photon S times. Different values of S were tested with the intention of producing a representative simulation without excessively long computation times, as more photon scattering events must be calculated for larger values of S . A value

of $S = 20$ appeared to provide a good balance of these two factors. To propagate the photons, the distance until the next scattering event was first sampled using Equation 25. Using this distance and the initial direction vector, the position of the first scattering event could be determined. A polar scattering angle θ was then sampled using Equation 27, which allowed the direction vector to be updated accordingly. From this point, the loop restarts with the new position and direction. The output of this function was a $3 \times N \times S$ array representing both the positions and velocities of N photons in x , y and z , for S scattering events.

Variable	Meaning	Usual value
N	# of photon simulated	1,000,000
S	# of scatters simulated	20
μ_s	Scattering coefficient	20 mm^{-1}
g	Anisotropy factor	0.9
Z_{max}	Depth of excitation below tissue surface	0 - 400 μm
x_{fibre}	Spacing between neighbouring fibres	1 – 10 mm
d_{core}	Fibre core diameter	100 – 1000 μm

Table 6 Input parameters used in scattering simulation

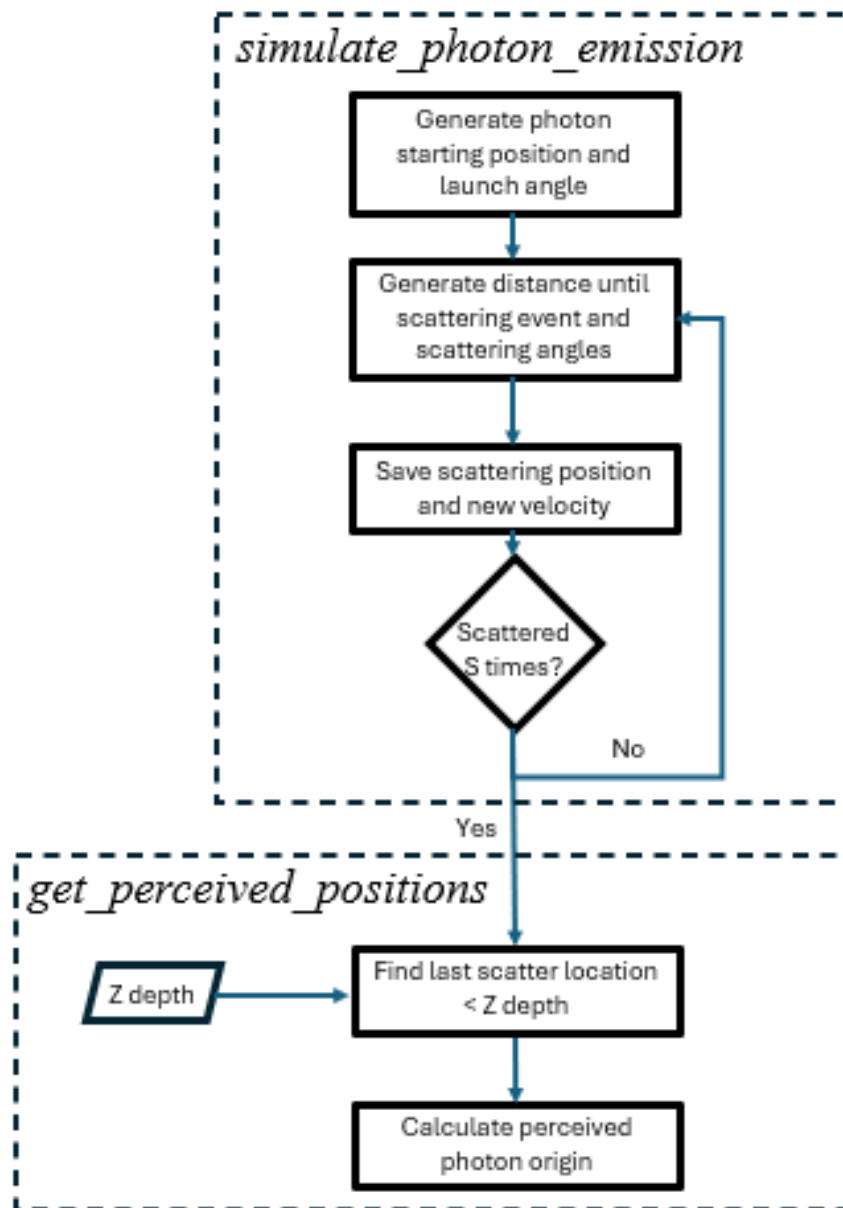


Figure 57 Flowchart of the functions carried out in photon scattering simulation

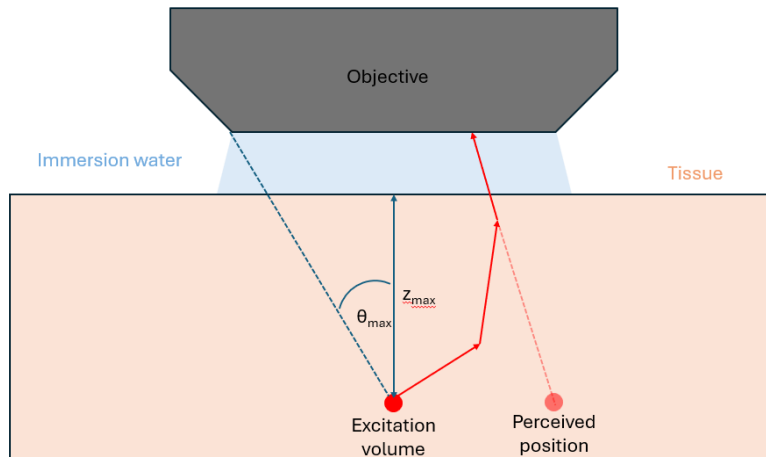


Figure 58 Schematic showing how perceived photon position at the focal plane is altered for a photon undergoing the same series of scattering events, but excited at different tissue depths.

Using these two arrays one can then apply the second function, *get_perceived_positions*, to calculate the perceived position, labelled r_{scat} , of each photon excited at a specific depth in the tissue. A flowchart showing how the two functions link together is shown in Figure 57, these functions are applied to each of the N photons simultaneously. The code calculates the point at which a ray exits the scattering medium at z_{max} . One can then use the position and direction of each photon exiting the tissue to trace back its trajectory and calculate the perceived position of the photon at the focal plane of the objective lens. A ray initially leaving the excitation volume but being traced back to a different point on the focal plane due to photon scattering is shown in in Figure 58. By propagating this position through the system one can determine whether the photon would be collected or not by different sized fibres.

The magnification from the optical fibre plane can be applied to find the size of the optical fibre detection area (the projection of the optical fibre core) at the sample plane. Photons with a r_{scat} value within the projected optical fibre core would be collected as long as the exiting photon is within the objective lens NA (0.95). To study the impact of crosstalk, the centre of the optical fibre collection area could be displaced within the simulation whilst observing the number of photons entering the fibre.

4.4.3.2 Testing the simulation

To verify that the simulation was working as expected, some initial tests were run to establish whether the variables for scattering mean free path and polar scattering angle being sampled were following the expected theoretical distribution. Figure 59 shows that the values sampled for distance travelled between scattering events and angle polar scattering angle found using inverse transform sampling matched the probability distributions described in Equation 25 and Equation 27

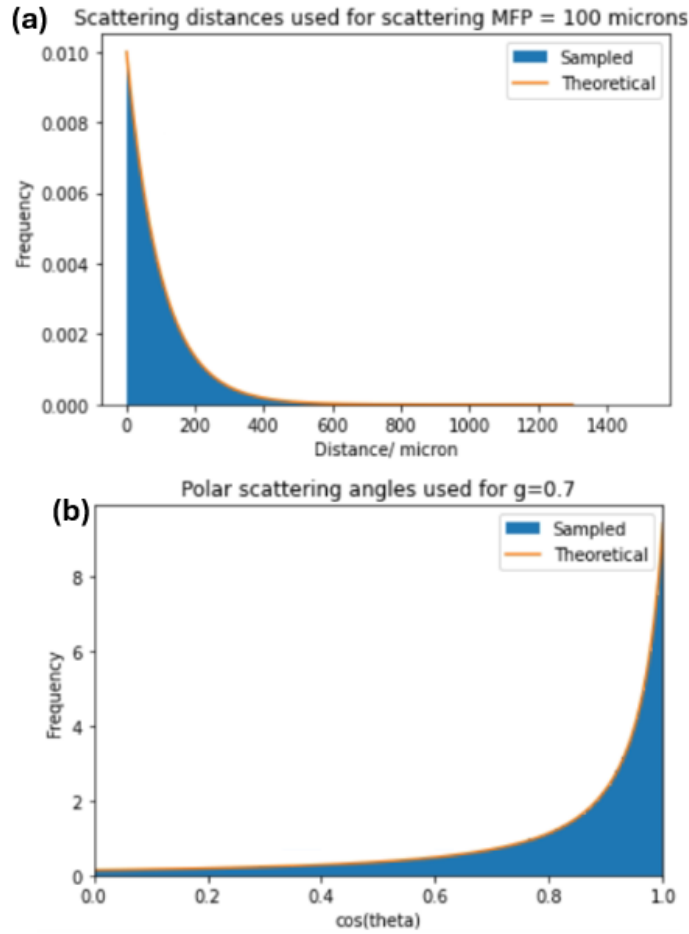


Figure 59 Normalised histograms of values sampled for distance travelled between scattering events and $\cos(\theta)$ with their theoretical probability distributions.

Figure 60 presents histograms of the photon polar angle after 1, 3 and 5 scattering events plotted for two values of g . These figures show the expected behaviour of the polar angle, as the photons are only released into the upper hemisphere the distribution starts with all angles less than $\frac{\pi}{2}$. As the photon undergoes more scattering events, the distribution in angles spreads out. The two plots in show how this

angular spreading of scattering trajectories is increased when using a lower value of g , as now upon each scatter the photon has a higher chance of scattering into larger angles.

Figure 61 shows histograms of the polar angle for photons on escaping the tissue for 5 different (z) depths. Photons must be travelling in the positive z direction to escape, hence no polar angles exceed $\frac{\pi}{2}$ radians. It is apparent that fewer photons escape from greater depths as there is a higher probability that they are retained within the tissue after S scattering events. Events to the right of the red vertical line would not be detected as they exceed the NA of the objective lens used.

Figure 62 presents a histogram of the number of scattering events undergone by each photon together with the theoretical Poisson distribution calculated for the appropriate mean number of scattering events. This was simulated for a small launch angle so the photons roughly travelled directly out of the

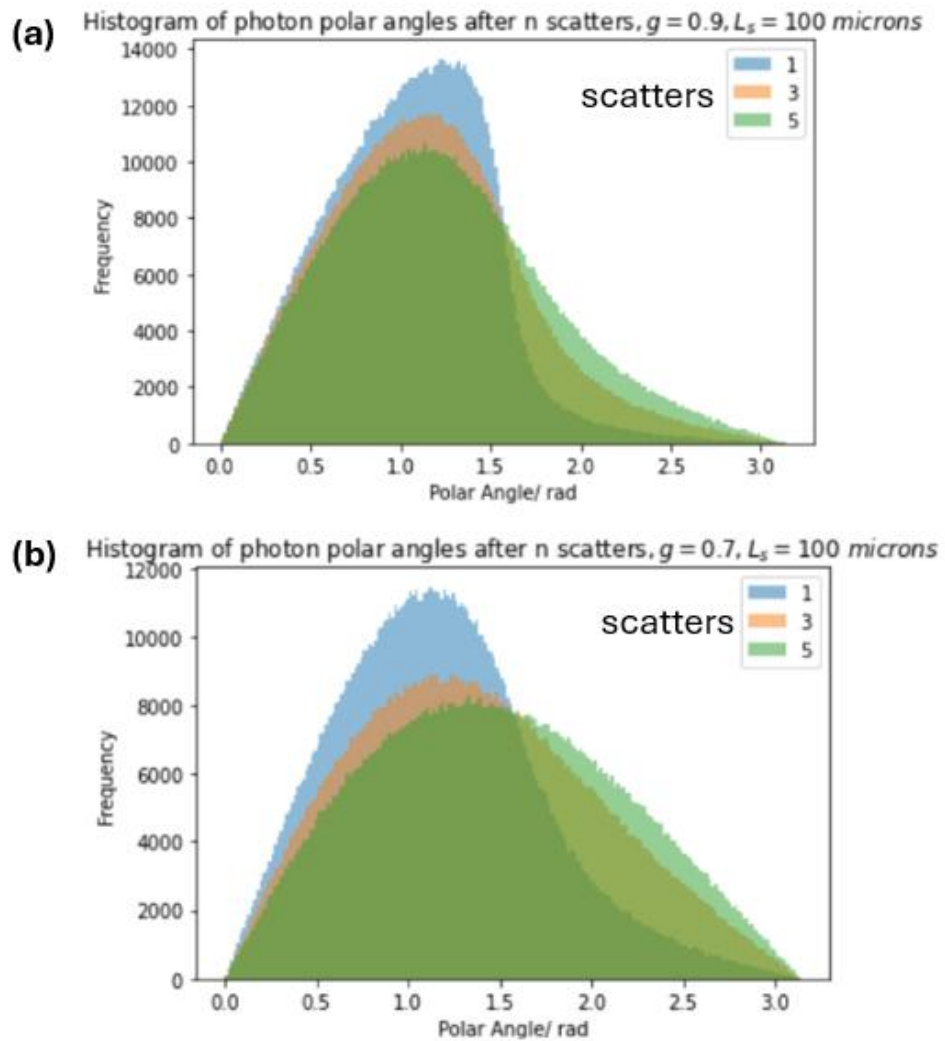


Figure 60 Histograms of photon polar angle after 1, 3 and 5 scatters for (a) $g = 0.9$ and (b) 0.7 .

tissue. The figure shows the number of scattering events matches the Poisson distribution closely as expected.

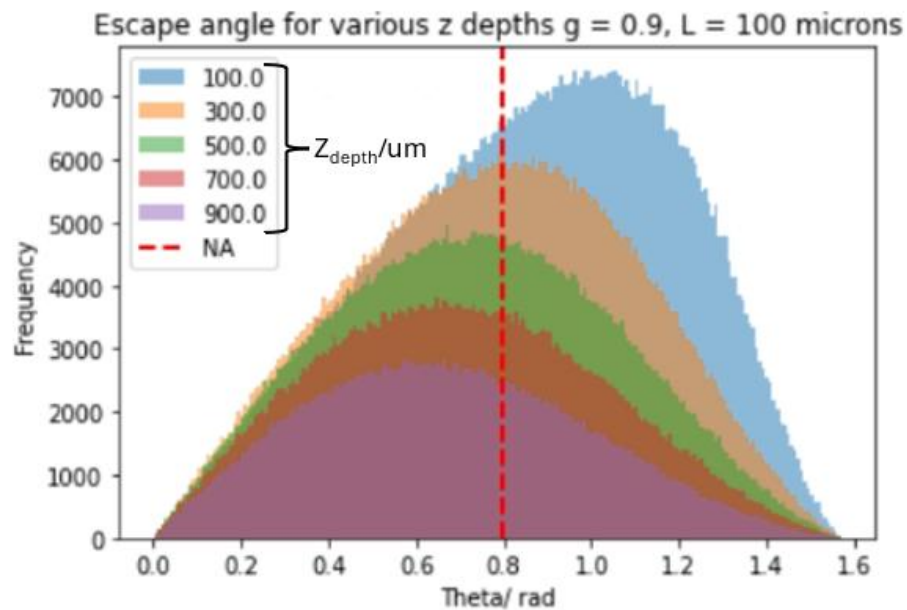


Figure 61 Photon polar angle on escape for z depths 100 - 900 microns.

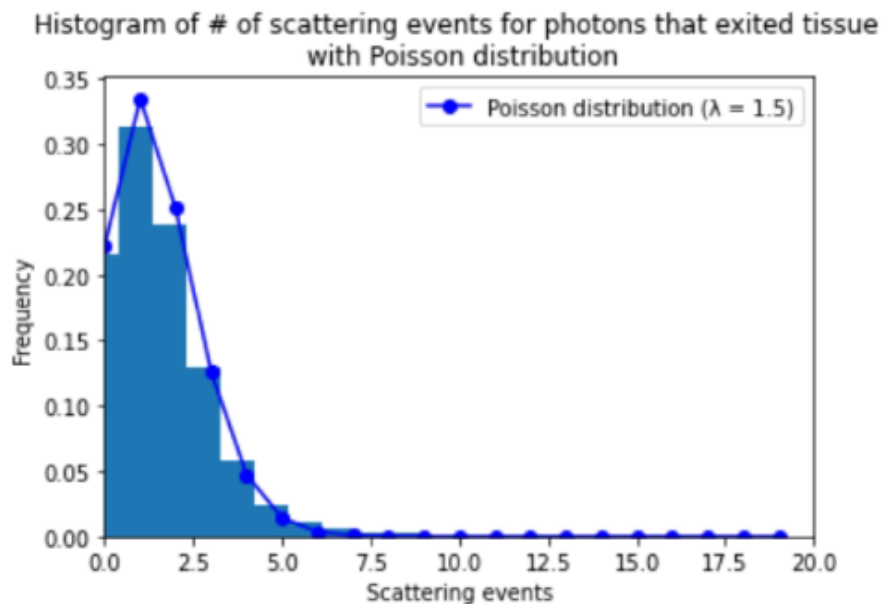


Figure 62 Histogram of number of scattering events per photon that exited the tissue for $z = 150$ μm and $L_s = 100$ μm and release angle of 20 degrees.

4.4.3.3 Results

2D histogram plots were produced to provide visualisations of the distribution of scattered photons at the optical fibre detection plane. Figure 63 shows heatmaps of the scattered photons (unscattered photons are excluded) for four Z positions. As the fluorescence excitation volume is placed deeper into the tissue, fewer photons reach the optical fibre detection plane, as they are more likely to have scattered more than S times, or may have been scattered outside of the objective NA. The photons that do arrive are more spread out, meaning that at a depth of 1 micron most of the scattered photons are collected by the 200 micron diameter optical fibre, but at a depth of 300 microns many fewer are detected. Using this information, the ratio between the number of photons collected by different sized optical fibres could be calculated.

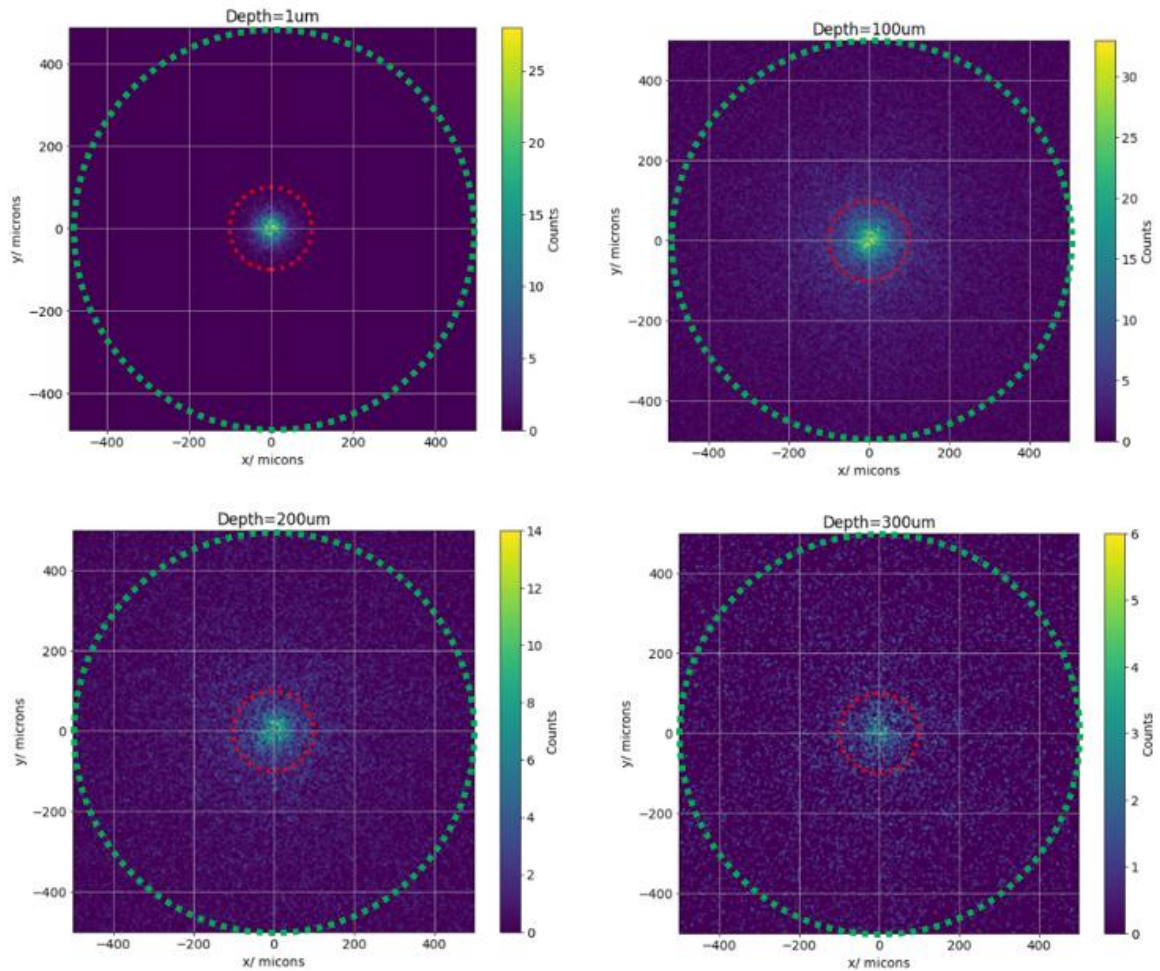


Figure 63 Scattered photons at fibre plane for depths of 1, 100, 200 and 300 microns. Red and green dotted circles show the 200 and 1000 micron core diameter fibres used respectively. Simulation performed using $g = 0.9$ $L_s = 100$ microns.

Figure 64 shows the ratio of the signal collected in the 1000 μm diameter to that of the 200 μm diameter optical fibre as a function of imaging depth for the simulation and the experiment described previously. The parameters used in the simulation to match the experimental data were $g = 0.95$ and $L_s = 40 \mu\text{m}$, which are within the range of typical values found in the literature [138].

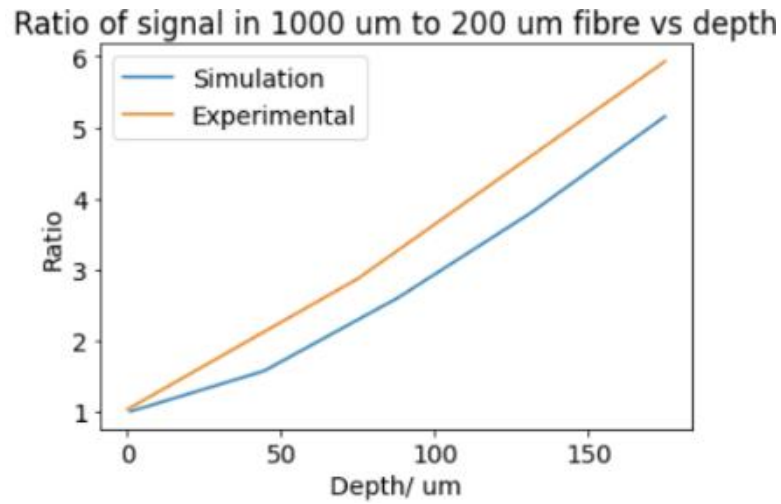


Figure 64 Simulation and experimental ratio of fluorescence captured by a 1000 μm fibre to a 200 μm diameter optical fibre using parameters of $g = 0.95$ and $L_s = 40 \mu\text{m}$ as a function of imaging depth.

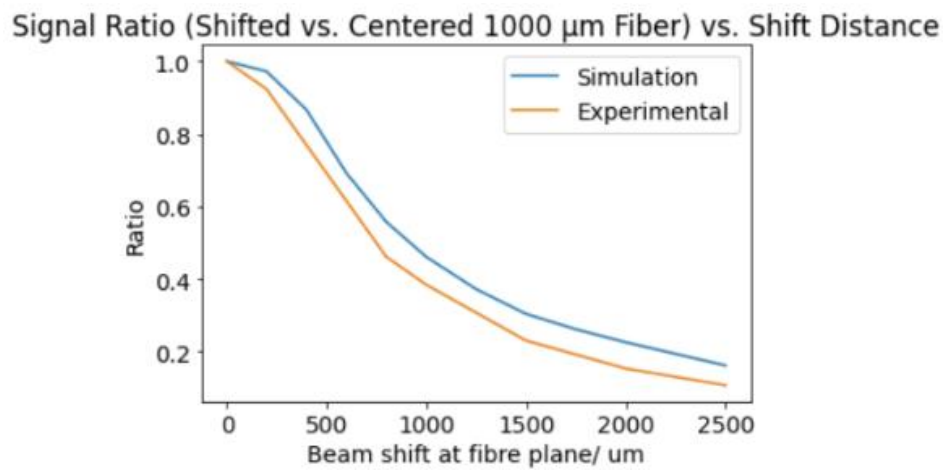


Figure 65 Ratio of signal collected in shifted vs centred fibre as a function of shift distance. $g = 0.95$ $L_s = 40 \mu\text{m}$

The signal collected in the 1000 μm diameter optical fibre as a function of beam displacement was then calculated. Figure 65 shows the simulation and experimental values for the ratio of signal - including unscattered fluorescence - collected by a fibre of 1000 μm core diameter for $z = 200$ micron imaging depth displaced from the beam with respect to that collected when the fibre is centred on the excitation beam. The simulation used the same values found to match the experimental data in Figure 63, namely $g = 0.95$ and $L_s = 40$ μm . Using these values Figure 65 shows that the simulation fits the experimental data reasonably well. As the simulation data from Figure 64 and Figure 65 matches the respective experimental data using the same set of parameters, the use of the simulation tool to predict the performance of further detection configurations for multiphoton fluorescence imaging in scattering media is supported.

Heatmaps can be produced using the simulation to explore the parameter space for different imaging configurations. Figure 66 shows a heatmap displaying the percentage crosstalk, calculated as the ratio of signal entering a displaced vs a centred optical fibre detection area, as a function of beam displacement and imaging depth for $g = 0.95$ and $L_s = 40$ μm . The regions with a red border indicate where the cross-talk is above 5% - arbitrarily defining a limit where cross-talk degrades the image. Similar heatmaps plotted against L_s and g could provide guidance on the amount of crosstalk one would expect to incur when imaging different tissue types in a multibeam configuration, noting that L_s and g can vary between tissues.

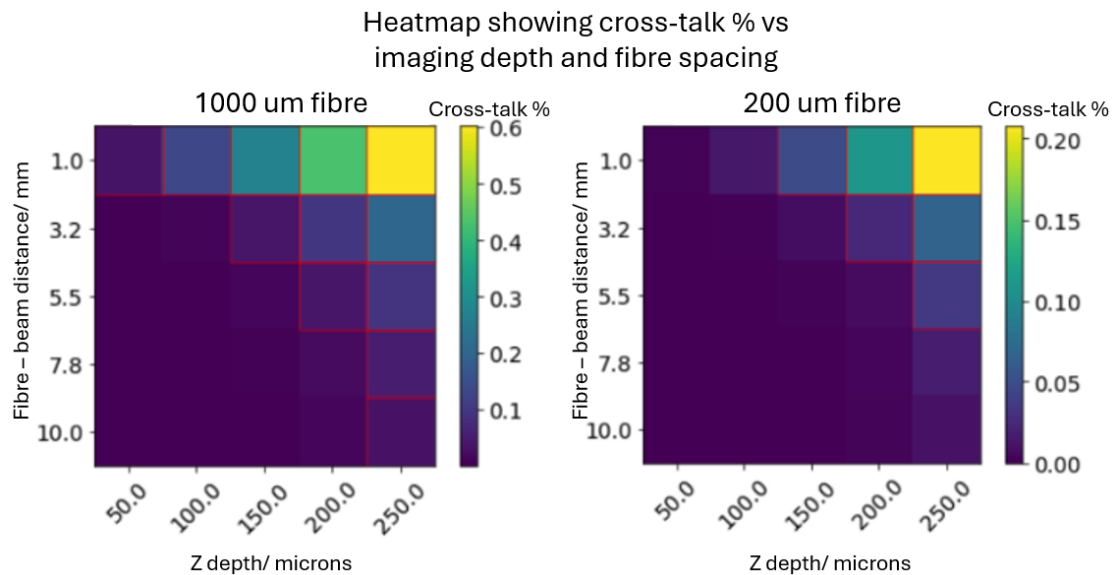


Figure 66 Heatmap showing the cross-talk % as a function of imaging depth and fibre spacing.

Red outline shows regions where cross-talk exceeds 5%. $g = 0.95$ $L_s = 40$ μm

Based on experiments and simulation, it was decided that a loom of 1000 μm core diameter optical fibres should be constructed but, in order to minimise crosstalk at imaging depths up to 250 μm , the inter-fibre spacing would be increased while maintaining the same FOV by arranging the optical fibres in a 2 x 3 grid instead of a line. This increases the optical fibre spacing from 3.6 mm to 10.8 and 7.2 mm along the two axes respectively. The layout of the initial and second optical fibre looms are presented in Figure 67.



Figure 67 Comparison between old and new fibre looms geometries. (To scale)

4.4.4 Construction of optical fibre loom

The construction method for this second optical fibre loom was modified as the previous technique of clamping two pieces of metal together would not work as easily for a pattern with two columns. The 1 mm optical fibres had dimensions shown in Table 7.

A metal block of 22 mm diameter and length 25 mm was used to house the optical fibres, for each of which an entry hole of 4 mm diameter was cut into the metal half way down the length to house the outer jacket of diameter 3.8 mm. The diameter was then decreased to 1.1 mm to house the cladding of 1.035 mm diameter. A glass plate was placed over the end of the metal block with 6 holes cut to hold the optical fibres where they were fixed in place using uv-curing epoxy glue (Araldite), and to provide a stable surface that could be polished down such that the optical fibre end faces would be in the same plane. Back and side views of the optical fibre loom housing are shown in Figure 68. One further benefit of this optical fibre loom design was that it could easily be attached to the existing cage rod system used on the excitation breadboard. The cylindrical loom was fixed inside a mount (using 3 grub screws) that was then screwed into a (Thorlabs) cage plate that was attached to the cage rods coming from the final turning mirror M4.

Fibre Part	Size/ mm
Core	1
Cladding	1.035
Coating	1.4
Jacket	3.8

Table 7 Dimension for the 1 mm core fibres used in the second iteration fibre loom.



Figure 68 Back and side view of the second iteration fibre housing unit with fibre.

4.4.5 Cross-validation

The 200 and 1000 μm core optical fibres were used to take z-stacks of the mouse lung tissue sample (described fully in the previous chapter) in order to confirm that the increase in signal seen with the spheroids would be present in another sample type. The background-subtracted mean intensity per image slice is seen plotted as a function of imaging depth in Figure 69(a) for both the 1000 μm and 200 μm fibres, with the ratio of these signals plotted in Figure 69(b). The figure shows that the signal ratio for larger vs smaller optical fibre core diameter again increases as a function of imaging depth. The anomalous spike in intensity seen for the 1000 micron fibres at a z depth of 135 microns is attributed to unwanted stray light entering the fibre during the measurement.

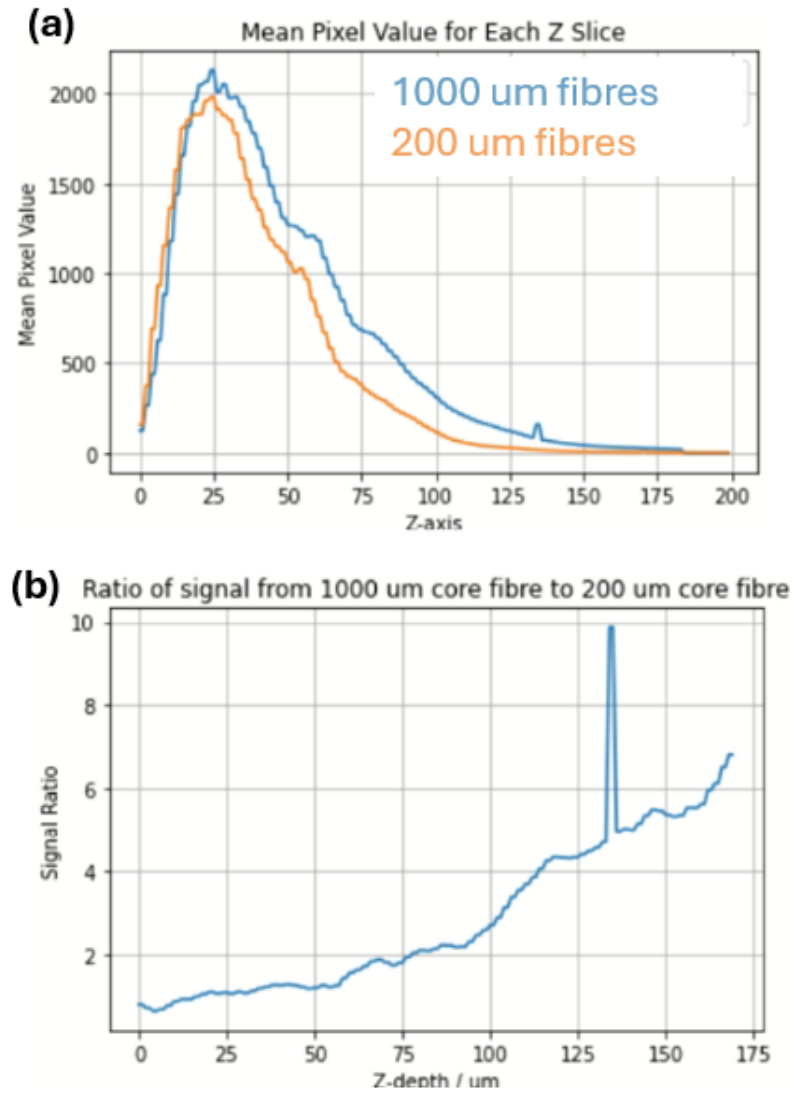


Figure 69 Mean intensity per slice as a function of depth for the 1000 um and 200 um core fibres imaging mouse lung tissue and ratio of the signal from 1000 um fibre to 200 um fibre as a function of depth.

4.5 Characterisation of second M³M implementation

4.5.1 Light throughput

The transmission efficiencies for both the excitation and emission light were measured with the aims of maximising throughput from excitation laser to sample to enable deeper imaging and to maximise throughput of fluorescence to acquire images at the highest practical rate. These measurements were carried out with the changes made from section 4.3 and 4.4.

4.5.1.1 Excitation light throughput to the sample

The light throughput was measured for excitation beams displaced by 0, 4 and 8 mm from the optical axis at the focal plane of L1 through the system. Light at 850 nm was used as this was a representative wavelength used for two-photon excitation. Since the SLM's diffraction efficiency varies with the lateral displacement of the beam, the plot was normalized for each displacement to the power measured at the focus of L1 in order that the light throughput measurement excluded the impact of SLM

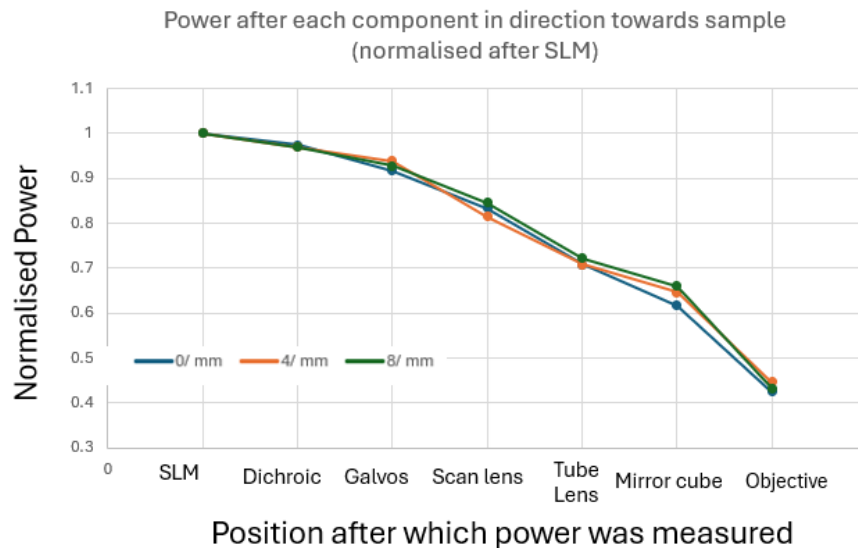


Figure 70 Power throughput for beams diffracted to three positions off the optical axis measured at L1, normalised to the power measured after the SLM.

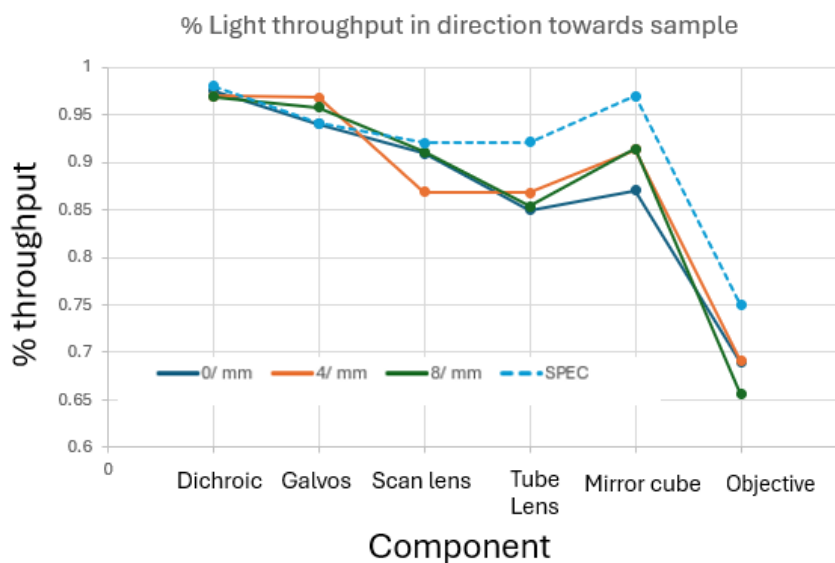


Figure 71 Percentage light throughput for each component individually in the direction of propagation towards the sample together with the expected throughput from specification.

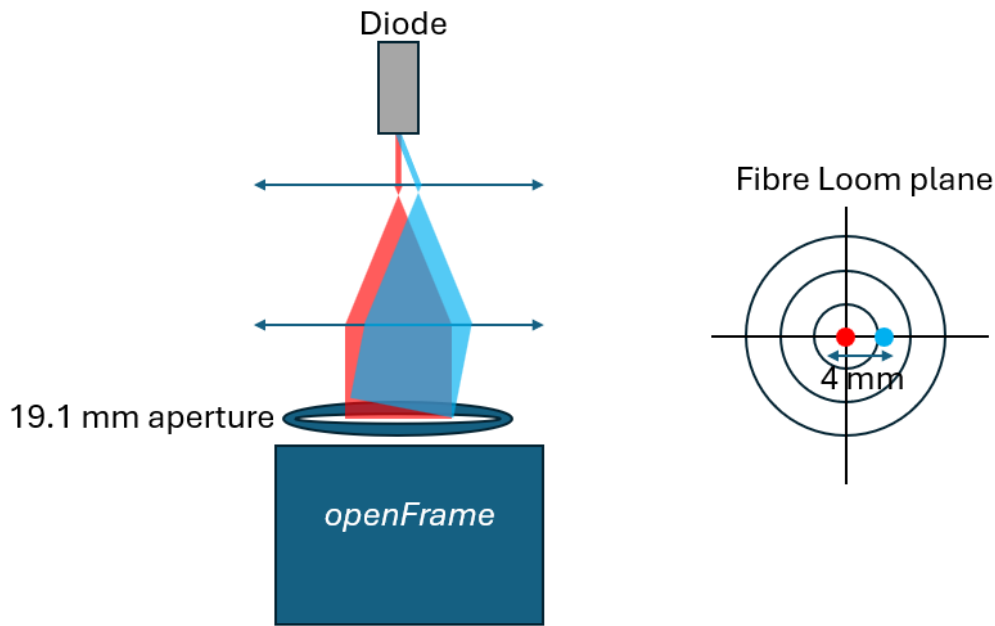


Figure 72 Setup used to measure fluorescence throughput through system for the 0 and 4 mm positions. The beam position is controlled by tilting the collimated laser diode module, expanding the beam and directing it into the back focal plane of the objective. Lenses shown by double-ended arrows.

diffraction efficiency variations. Figure 70 shows the power measured through the system, with the lateral beam displacements shown in different colours. The throughput is fairly uniform across the set of lateral beam offsets, indicating that no significant vignetting is occurring. The x-axis of the plot indicates the component after which the power measurement was taken, to highlight the effects of individual components.

In Figure 71 this was plotted in terms of the percentage throughput between measurement locations – i.e. the transmission of each element – alongside the predicted % throughput from quoted transmission values in the specifications of the components in the light path. Care was taken to include all of the components along the optical path between two measurements when calculating the expected optical transmission; for example the measurement taken after the tube lens also includes another turning mirror. Figure 71 indicates that the performance is below expected for the specified throughput of the tube lens, mirror cube and objective lens, but not by a drastic margin.

4.5.1.2 Fluorescence throughput to detectors

The power throughput from the sample plane to the optical fibre loom plane was then measured. As the fluorescence signals excited are weak compared to excitation signals, it was impractical to

measure the fluorescence using a standard power meter. In addition, when measuring the power in the double pass region of the descanned optical path, the excitation light needed to produce the fluorescence would be blocked. Therefore, a collimated laser diode module at 635 nm was used to mimic fluorescence by expanding and collimating this beam and directed back through the system from the sample plane with the objective lens removed. A 19.2 mm aperture was placed at the end of the telescope used to expand the diode laser output to mimic the back aperture of the objective lens. The set-up does not provide an exact replica of the fluorescence signal emanating from a sample, as the light from the diode is polarised; however, it will give a reasonable indication of the transmission efficiencies through the system. The lateral position of the beam at the fibre plane was altered by changing the output angle from the collimated laser diode module and using an alignment target to confirm the lateral offsets corresponding to the changes in angle. This setup is shown in Figure 72.

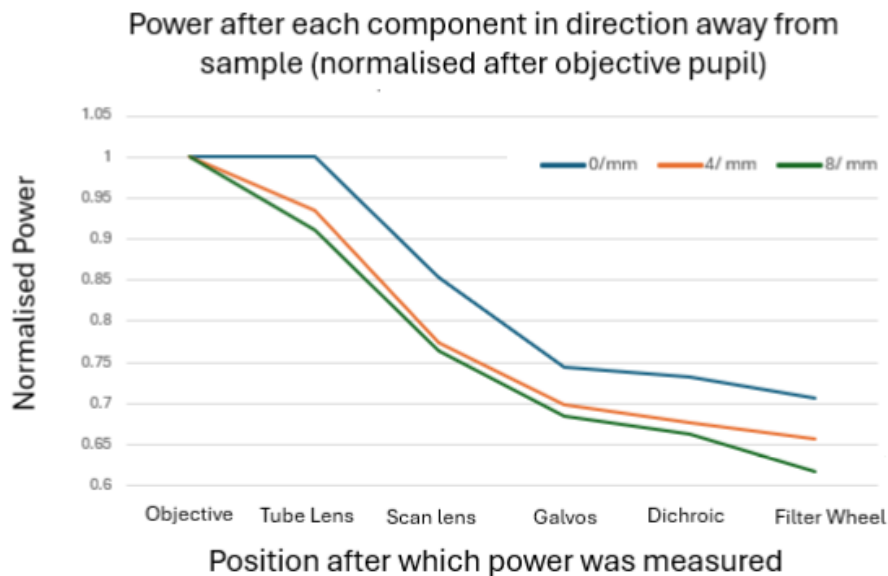


Figure 73 Power measured through system going from sample along fluorescence path towards fibre loom.

The measurements from this experiment are shown in Figure 73. The power was normalised for each displacement of the effective source to that of the measurement made after the objective aperture, as the laser output power varied slightly when changing the output angle. It is clear from the plot that for larger lateral displacements there is less throughput of the light through to the optical fibre loom plane, however there is only an 8% difference between a beam on the axis and a beam offset by 8 mm which is acceptable. Again, the percentage throughput was calculated for each component and is shown in Figure 74, where the largest loss is at the scan lens, indicating a possibility of design improvement.

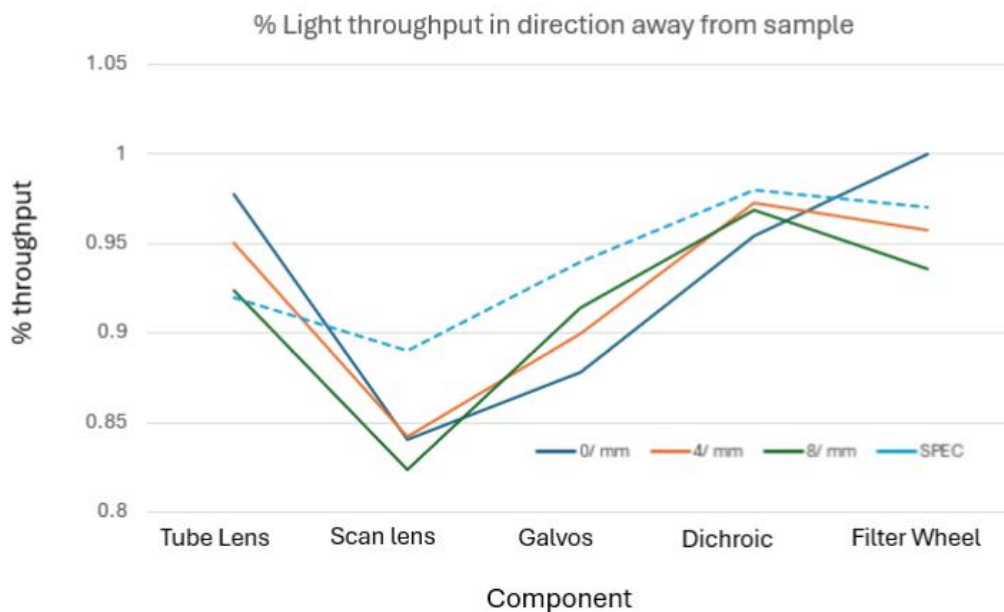


Figure 74 Percentage light throughput away from sample along fluorescence path, with specification throughput.

4.5.1.3 PMT lens relay

Using the same laser diode illumination simulating the fluorescence signal, light was coupled into each of the 1000 μm detection optical fibres in the loom using the galvos. The power was measured before and after the fibre to deduce the losses in the fibre. To mimic the two detection configurations of the PMT and fibre, namely with and without a relay lens on the fibre output, a 3 mm diameter aperture was used to act as the PMT active area. A power meter was placed after the aperture in both configurations to evaluate the benefit offered by the additional lens. The plots for each optical fibre are presented in Figure 75. The plot shows on average a 10% loss through the fibre and a 40-50% loss from the fibre to PMT when using no lens but recovered to a 5% loss when using the lens relay.

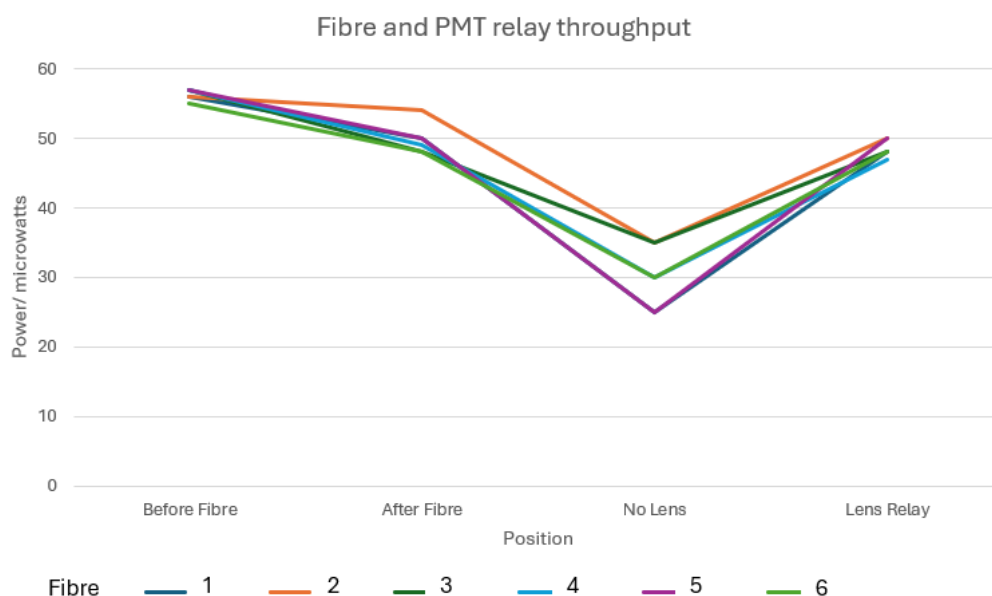


Figure 75 Throughput for each fibre, and throughput to PMT with lens relay and no lens.

4.5.1.4 NDD fluorescence throughput

The light throughput for fluorescence from the sample to the NDD was measured. The optical path for the NDD fluorescence consists of a long pass dichroic, a lens relay and a turning mirror. Due to difficulty in accessing the optical path, power measurements could only be made after the objective and after the turning mirror. The second lens of the lens relay was situated between the turning mirror and the PMT active area, so to provide the full power throughput the power measured after the turning mirror was multiplied by the specification throughput of the final lens to yield the data points in the final column in Figure 76. Figure 77 shows the ratio of the NDD to descanned fluorescence throughput as a function of field location. It shows that the NDD has a higher throughput, roughly averaging 1.5x greater over the FOV.

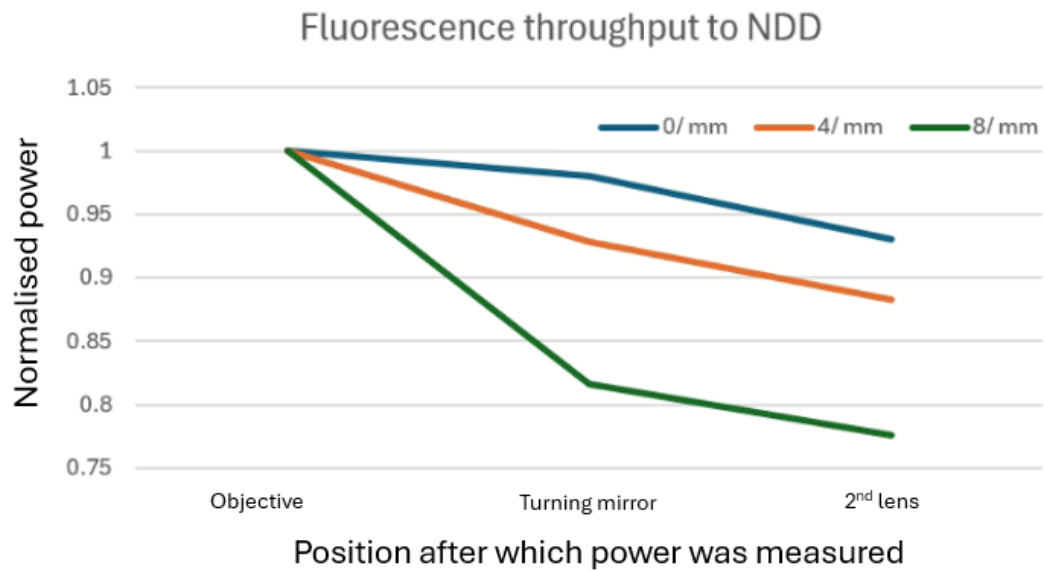


Figure 76 Fluorescence throughput in NDD path. Power measured was normalised to value after objective.

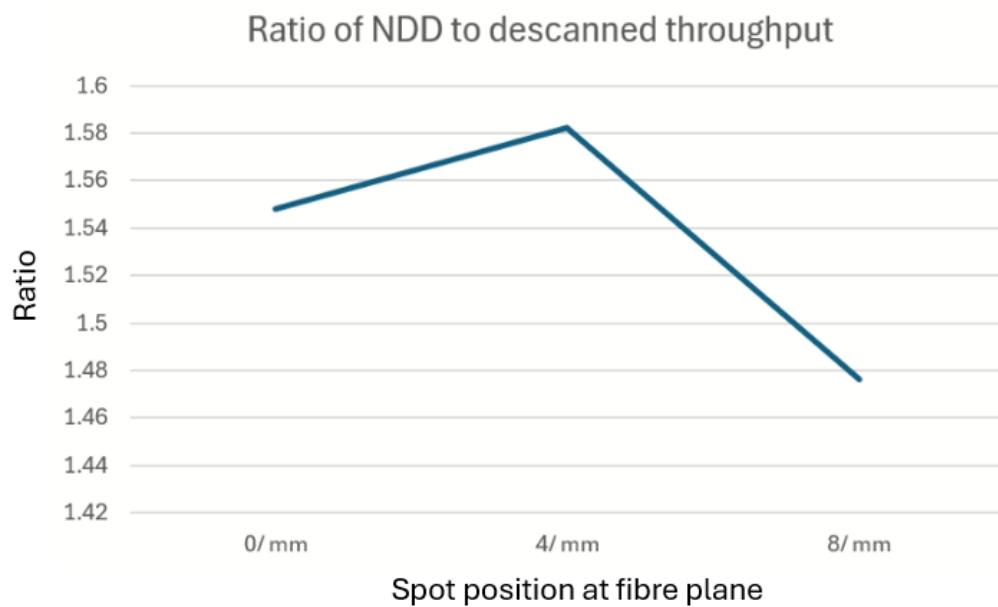


Figure 77 Ratio of NDD to descanned fluorescence throughput as function of spot position at fibre plane.

4.5.2 Temporal performance of fluorescence detection

To verify that the time-resolved fluorescence detection equipment was functioning as expected, one of the descanned photomultipliers was used to acquire TCSPC data of a (124 μM) solution of Rhodamine 6B, a reference dye with a known fluorescence lifetime, and gold nanorods, which have a sufficiently short emission lifetime to provide an instrument response function (IRF) of the system. The decay profiles of these reference samples are presented in Figure 78. The FWHM of the nanorod decay curve was 240 ps which is primarily attributed to the response time of the HPMA and enables useful lifetime measurements of most fluorophores encountered in fluorescence microscopy. The IRF profile also presented no after-pulsing, as expected for a HPMA. The lifetime measured for Rhodamine 6B (dissolved in water) was $\tau = 1.58$ ns which is in accordance with the literature [139].

Any difference in IRF width measured when using 200, 600 and 1000 micron core diameter optical detection fibres was found to be negligible, indicating that group velocity dispersion in the detection optical fibres is not a problem for this system. However, the centre point of the IRF was found to be shifted between measurements using different detectors. This offset could be corrected in the SymPhoTime programme or in the MultiHarp device settings. The range in time shift of the IRF measurements was 1 ns, which would be equivalent to ~ 20 cm difference in optical fibre lengths. As these optical fibres were of 2 m length, this seems unlikely to be the cause of the time shifts. It may be due to differences in the electronic circuitry.

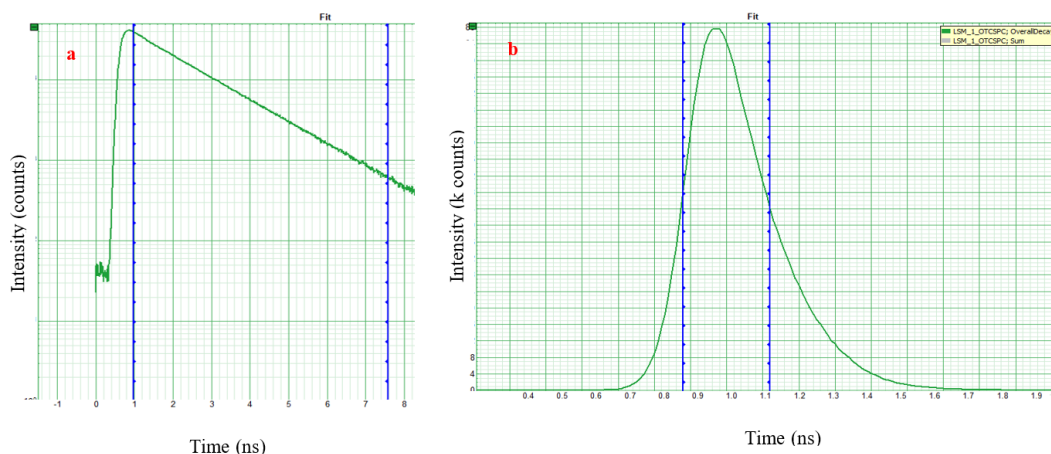


Figure 78 (a) Rhodamine 6g and (b) gold nanorods fluorescence decay profiles acquired using the PicoQuant Hybrid PMA40.

4.5.3 Comparison of multiple excitation beams with descanned detection and single beam excitation with non-descanned detection

Single-beam excited multiphoton imaging is most often carried out in a non-descanned configuration with a large detector, as this allows more efficient collection of scattered fluorescence. It is therefore interesting to compare the 6 excitation beam descanned configuration described here with single beam excitation and non-descanned detection implemented with the same excitation laser and objective lens.

A section of mouse tissue (described fully in the previous chapter) was imaged to 200 microns depth using both modalities, acquiring z-stacks with 20-micron steps between slices. In the non-descanned modality, a single 10 mW excitation beam (measured at the sample) was used, while in the descanned modality, six 10 mW beams were employed. This comparison is based on the assumption that the factor limiting the average excitation power per beam is phototoxicity in the sample. The total number of photons collected per slice as a function of depth was compared between the two modalities imaging the same FOV each time. To mitigate any photobleaching effects, the descanned z-stack was taken twice (before and after the non-descanned stack) and the ‘before’ and ‘after’ signal intensities per slice were averaged.

Figure 79 shows that the total signal for the 6-excitation beam descanned configuration is greater than for the single excitation beam non-descanned configuration until approximately 75 microns imaging depth. The fluorescence intensity then decays more quickly with depth for the descanned modality as the detection optical fibres collect less scattered fluorescence than the NDD.

The peak ratio in the signal acquired from the descanned to non-descanned modalities was ~ 1.2 . One can make a prediction of the ratio of signal acquired in descanned to non-descanned modalities using the models of spatial and temporal dispersion described in section 4.3.3 and Figure 50 to estimate the amount of fluorescence excited and the light throughput data described in section 4.5.1 to find the difference in fluorescence throughput between the channels. In the NDD channel, the SLM is effectively used as a mirror, so there are no losses with respect to spatial and temporal dispersion of the pulse. For the 6-beam channel with the beam spacings used, there is an average of 70% loss per beam due to spatial and temporal dispersion (looking at displacements d_0 of 5.4 mm and 7.2 mm divided by the magnification of 80x). For light throughput we saw an average of 1.5x more throughput for the NDD with respect to the descanned channel. This results in a predicted fluorescence signal for the 6-beam channel of $\frac{6 \cdot 0.3}{1.5} = 1.2$ matching the experimental data.

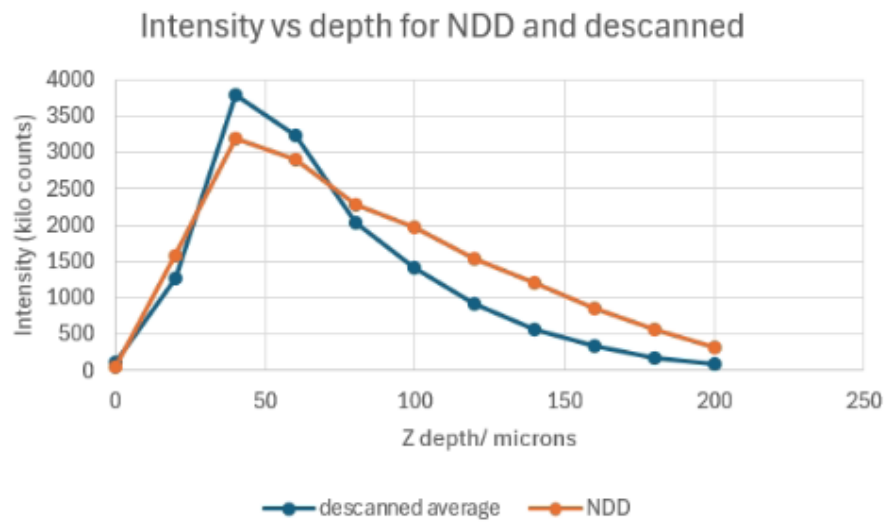


Figure 79 Intensity as a function of depth for 6-beam descanned and single-beam non-descanned configurations.

Although a signal acquisition ratio of 1.2 is far from the possible 6x advantage of the parallelised excitation, there are several issues to consider. Firstly, the fluorescence throughput for the descanned modality was expected to be lower due to the increased number of components, and the $1/1.5 = 65\%$ throughput is not far away from the cumulative effect of all the optics in place. Secondly, in the descanned multibeam system, the amount of phototoxicity caused to the sample will be decreased due to the spatially and temporally elongated pulses, as discussed earlier. If the goal is to optimise time-lapse multiphoton excited assays of live samples, a reasonable comparison would be to set the excitation peak intensities to be the same in the single excitation beam and each of the parallel excitation beams. Then the difference in detected signals between the descanned and non-descanned modalities would be determined by the difference in detection transmission efficiency – which is approximately 33%. Thus, the 6 parallel excitation beams would give a 4x advantage in imaging speed – and the phototoxicity

would be lower due to the impact of chromatic dispersion on the excitation pulses discussed above. However, this presupposes that sufficient excitation power is available to increase the power of the elongated SLM diffracted pulses, which is not the case in our current setup.

4.6 Biological imaging

4.6.1 Live FRET construct spheroid assay

Our laboratory has previously developed an approach to test FLIM systems applied to cell-based assays using genetically expressed FRET constructs [140]. The FRET constructs used here consisted of mTurquoise2FP and mVenusFP fluorescent proteins joined by amino acid linkers of 5, 17 and 32 amino acid lengths, as well as a construct containing only mTurquoise2FP with no mVenusFP acceptor. The 5 amino acid construct, referred to as mTq2-5-mV, has the shortest distance between the two fluorophores, meaning that it should present the highest FRET efficiency. The mTq2-17-mV and mTq2-32-mV constructs have consecutively larger distances between the fluorophores and therefore should exhibit lower FRET efficiencies. Finally, the mTq2FP with no acceptor fluorophore should not undergo FRET. As discussed in Chapter 2, increasing FRET efficiencies between the constructs causes a decrease in the donor lifetimes and so a multiwell plate array of cells expressing these constructs should present a predictable trend in fluorescence lifetime.

The experiment used HEK-293 cells transfected with plasmids encoding for the paired FRET constructs and DU-145 cells for the mTq2 only cells. These cells were cultured into spheroids using agarose moulds made from a (Sigma Aldrich Z764000) micro-mould template. The cells were allowed to grow in the agarose until they reached roughly 200 microns in diameter, after which they were transferred into a glass-bottomed imaging multiwell plate.

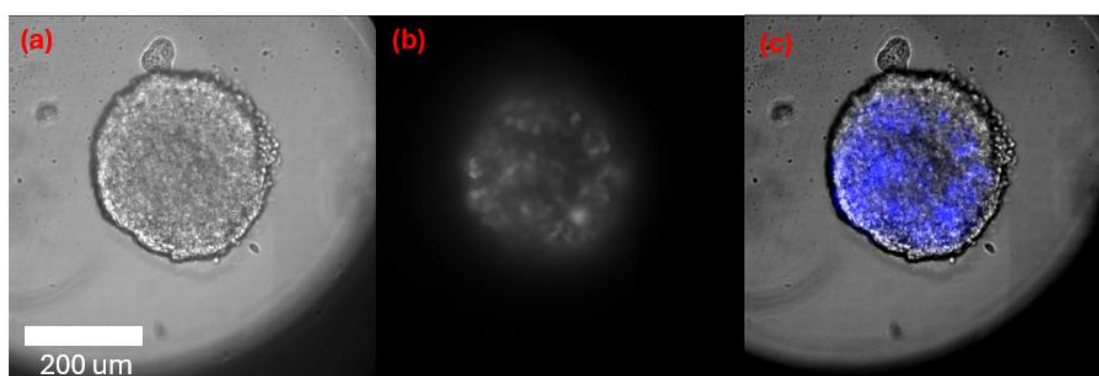


Figure 80 (a) Brightfield, (b) single photon widefield fluorescence image and (c) overlay of spheroid expressing the mTq2-5-mV construct.

Figure 80 shows brightfield and widefield single-photon excited fluorescence images of a spheroid expressing the mTq2-5-mV construct. These two wide-field imaging modalities are convenient for an

initial evaluation of a sample and can be used for sample pre-finding and to confirm that the sample is expressing the expected fluorophore(s). However, the 3D structure of the sample compromises the image quality, and so multiphoton imaging is desirable to assay such samples.

For this experiment 32 wells of a 96 well-plate were used in the layout shown in Figure 81 with increasing fluorescence lifetime per well expected from columns 3 to 6, and then repeated for columns 7 to 10, with the same condition in each well of each column. The brightfield modality was used to find a spheroid in each well and save the stage position. This position list was then loaded into the MicroManager acquisition engine where the MultiHarp acquisition script was executed at each location. The data was then processed in Python allowing it to be viewed and analysed using *FLIMfit* [141].

For each experiment presented in this thesis an IRF was taken on the day of the experiment which was subsequently used in for deconvolution in *FLIMfit*. Any intensities shown are the sum total of all photons collected in each pixel during the acquisition. Where multiple lifetime components were used, it will be indicated if a particular lifetime component, or a weighted mean lifetime is being presented. Sometimes due to a lack of photons, binning over multiple pixels was carried out, which will also be noted when such data is presented.

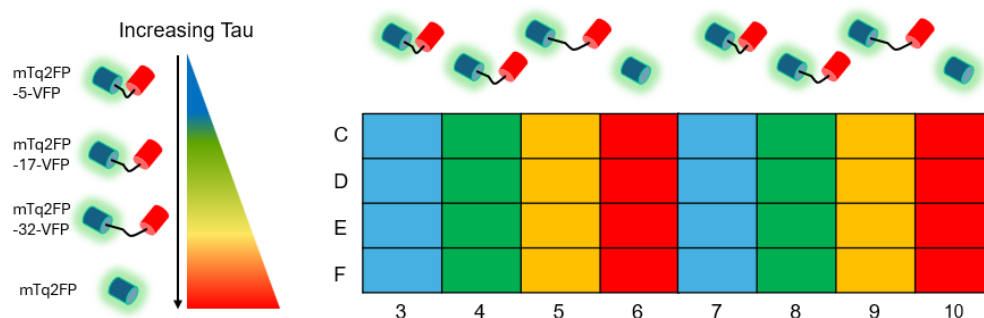


Figure 81 FRET construct assay plate layout with expected lifetime trend shown by colour.

A multiphoton excitation wavelength of 842 nm was used with a 482/35 nm emission filter and 15 mW per beam excitation power. This experiment was done before the M³M incubator was installed so a 37-degree plate warmer was used and there was no CO₂ atmosphere. The image data acquisition was undertaken 50 minutes after the cells were taken out of the incubator and the cells were kept in a CO₂ independent medium with no fetal bovine serum. Initial measurements showed that the spheroids were fairly uniform in diameter and in their axial distance from the coverslip, meaning that a fixed objective z-position was used for imaging each well, and no autofocus was required. One z-plane was imaged in each well at a distance of ~80 microns from the top of the coverslip. Using 15 second image data acquisitions per field of view, the overall acquisition time was approximately 9 minutes, with 8 minutes taken up with image data acquisition and the rest of the time taken up by stage movement and

software overhead time. The images were acquired using the original fibre loom and comprised 240 x 240 pixels across a 360 x 360 micron FOV.

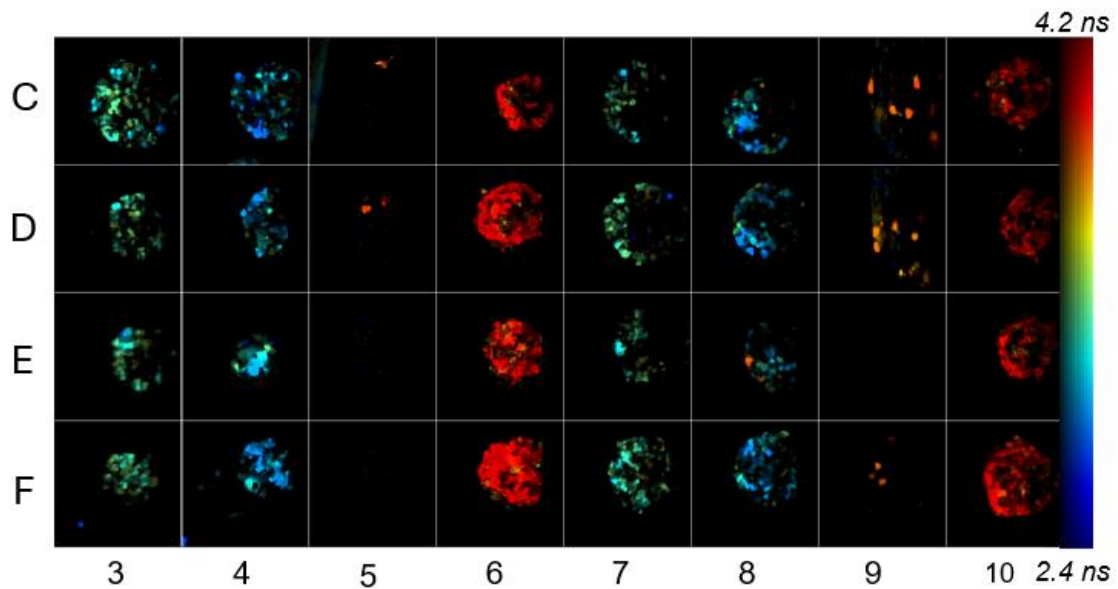


Figure 83 Multiwell-plate FLIM acquisition of spheroids expressing the FRET constructs, with one z-slice FOV acquired per well.

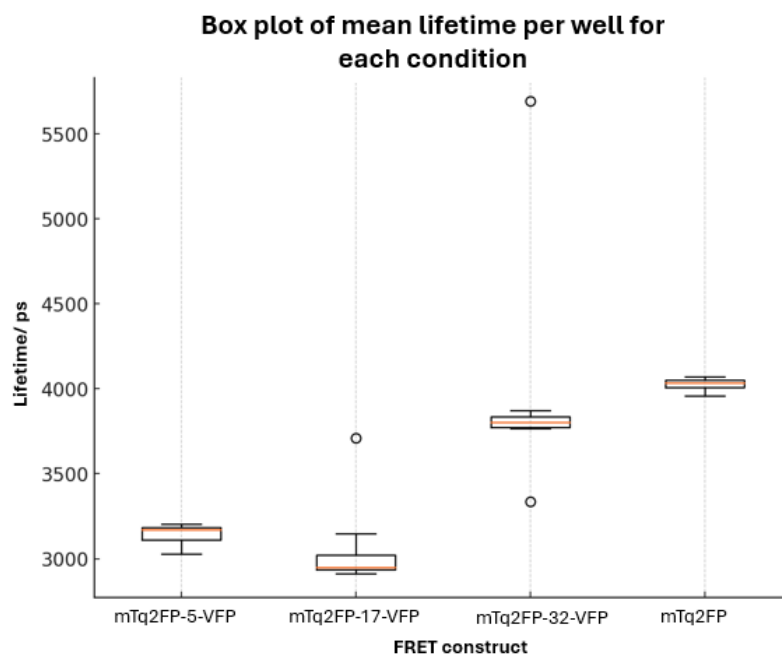


Figure 82 Box plot of mean lifetime per well for each FRET construct. (Outliers shown as circles)

The processed data taken is represented in Figure 82, showing a FLIM image of each spheroid in its respective well. The lifetime fitting was done with a pixel-wise single exponential fit with a 11 x 11 pixel spatial smoothing to provide more accurate fitting. From the data it was noted that the mTq2-32-mV spheroids showed less fluorescence than the others. It was also noted that the expected lifetime trend was not seen, with the mTq2-5-mV showed a longer lifetime than the mTq2-17-mV cells. As these observations were consistent over the 8 wells of each condition, this was attributed to an issue with the cell biology as opposed to the fluorescence lifetime imaging. This data is also presented in Figure 83 where the mean lifetime per well has been calculated and collected into a box plot for each construct.

Z-stacks for 3D imaging were also acquired of the same spheroid sample with 1 micron per z plane. Figure 84 presents z-stacks from separate image data acquisitions of spheroids in wells C3 and D10 with the false colour showing the lifetimes fitted across the whole z-stack. This approach to multiwell plate imaging utilising an initial single z-plane acquisition followed by z-stacks of spheroids of specific interest could be generally useful.

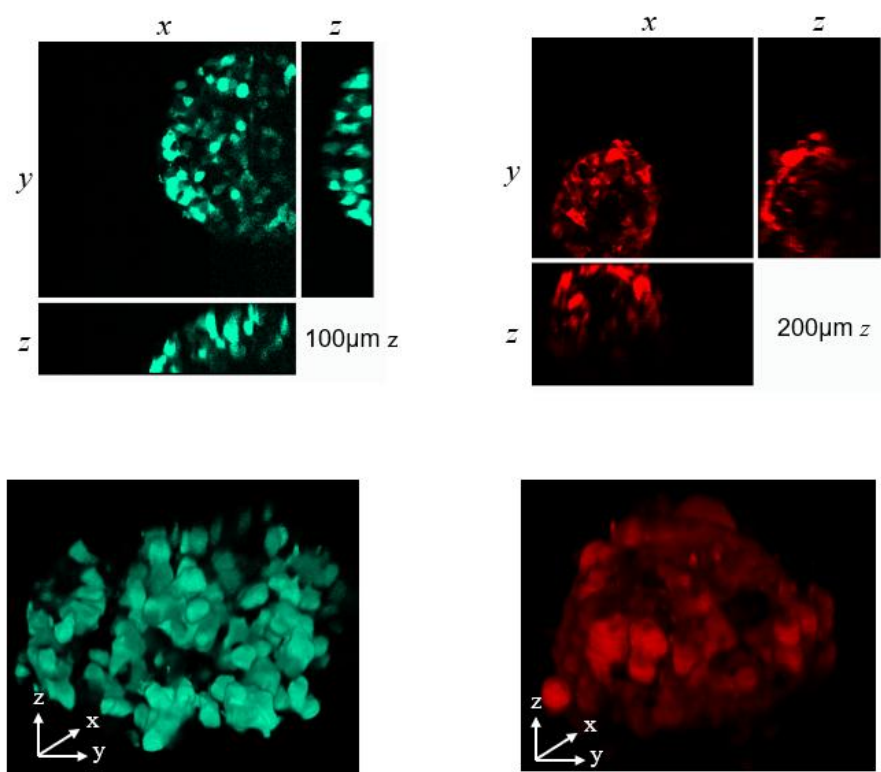


Figure 84 3D imaging of wells C3 and D10 with colour showing lifetime fit for total stack.

4.7 Summary

This chapter has described the development of the M³M after its initial construction. A description of the software written to allow communication with the MultiHarp TCSPC box via MicroManager was given, which ultimately allowed automated multiwell plate acquisitions to be executed using only MicroManager (with a Labview programme running in the background). A further piece of software was written to interpret the files saved by the MultiHarp in such a format that they could be loaded into *FLIMfit* allowing easier high content analysis on larger datasets. The improvements that were made to the components used in the system were discussed, including a new laser with a wider spectral range as well as an AOM which allowed faster power modulation of the excitation beam. The power throughput to the sample was increased by lowering the fill factor onto the objective lens which was done without any noticeable change in resolution. The effect of chromatic dispersion was investigated, with it being shown to cause a significant decrease in the fluorescence emitted for a given excitation power.

Analysis was carried out both experimentally and via simulation on the effect that scattering had on the imaging process. Signal gain and cross-talk were evaluated, indicating that we could increase the diameter of our fibres to collect more signal, without incurring image degrading levels of cross-talk until at least 100 microns imaging depth. Cross-talk was lowered by placing the fibres in a grid pattern so the average inter-fibre distance was increased compared to a line pattern. Further efficiency was gained by using a more tightly spaced beam pattern with a higher magnification onto the fibres due to the decrease in chromatic dispersion and increase in SLM diffraction efficiency.

The light throughput in the system was measured providing supporting data for a comparison of the overall imaging speed benefit when using 6 descanned beams compared to one non-descanned beam. The signal collection ratio of 1.2x for 6x descanned to 1x non-descanned at shallower imaging depths was discussed in the context of the lower damage the chromatically spread excitation pulses would be causing to the sample. An exemplar 3D FLIM FRET multiwell plate assay was carried out to show the efficacy of the system for its intended purpose.

5 Quantitative phase imaging implementation in the M³M

The modular nature of the M³M makes it straightforward to integrate further imaging modalities to complement the multiphoton fluorescence microscopy. Quantitative phase imaging (QPI) is of particular interest because it is a label-free imaging modality that can provide morphological information to complement multiphoton excited fluorescence image data, which can also be label-free if applied to autofluorescence. This combination of label-free imaging modalities can be useful to study live cells with no genetic or chemical manipulation, potentially better recapitulating “natural” biological processes in live cell assays. A key application of phase contrast imaging is to acquire image data with minimal phototoxicity that can be used to provide cell segmentation masks, which can be used for fluorescence binning, or cell tracking.

As discussed in Chapter 2, there are a range of approaches to QPI that usually require significant modifications to the microscope and often require the sequential acquisition of multiple images. For automated multiwell plate assays where imaging rate is a key consideration, it is preferable to use a “single-shot QPI” technique. Typically such techniques are based on digital (off-axis) holography, e.g., [142], or phase stepping holography, e.g., [143], requiring the implementation of an interferometer in the microscope imaging path, or shearing/differential interferometric measurements of phase gradients [144], [145], [146] that also typically require modification of the imaging path and face the challenge of integrating the acquired phase gradient data to obtain quantitative phase profiles. We chose to implement polarisation-resolved differential phase contrast (*pDPC*) microscopy, which is a semi-quantitative phase contrast technique that I previously helped develop and evaluate during my MRes [147]. *pDPC* is a 2D imaging technique that requires minor modification of an existing microscope to provide semiquantitative phase contrast imaging. This chapter will introduce *pDPC* and describe how it was implemented on the M³M. It will present exemplar *pDPC* assay and timelapse data and discuss how the data can be used for cell segmentation.

5.1 Introduction to *pDPC*

pDPC is an extension of differential phase contrast (*DPC*) [148] that typically involves modifying the microscope transillumination or detection path and recording a sequence of at least 4 images under conditions of oblique illumination or imaging. *pDPC* is a single-shot implementation of *DPC* that exploits a polarisation resolving camera to simultaneously record images of a sample illuminated from four different directions.

5.1.1 Differential phase contrast (DPC) microscopy

DPC [148] entails illuminating the sample with a set of source intensity distributions that provide oblique illumination at complementary angles and recording the transmitted light images such that pairs of images acquired with opposite illumination angles can be subtracted.

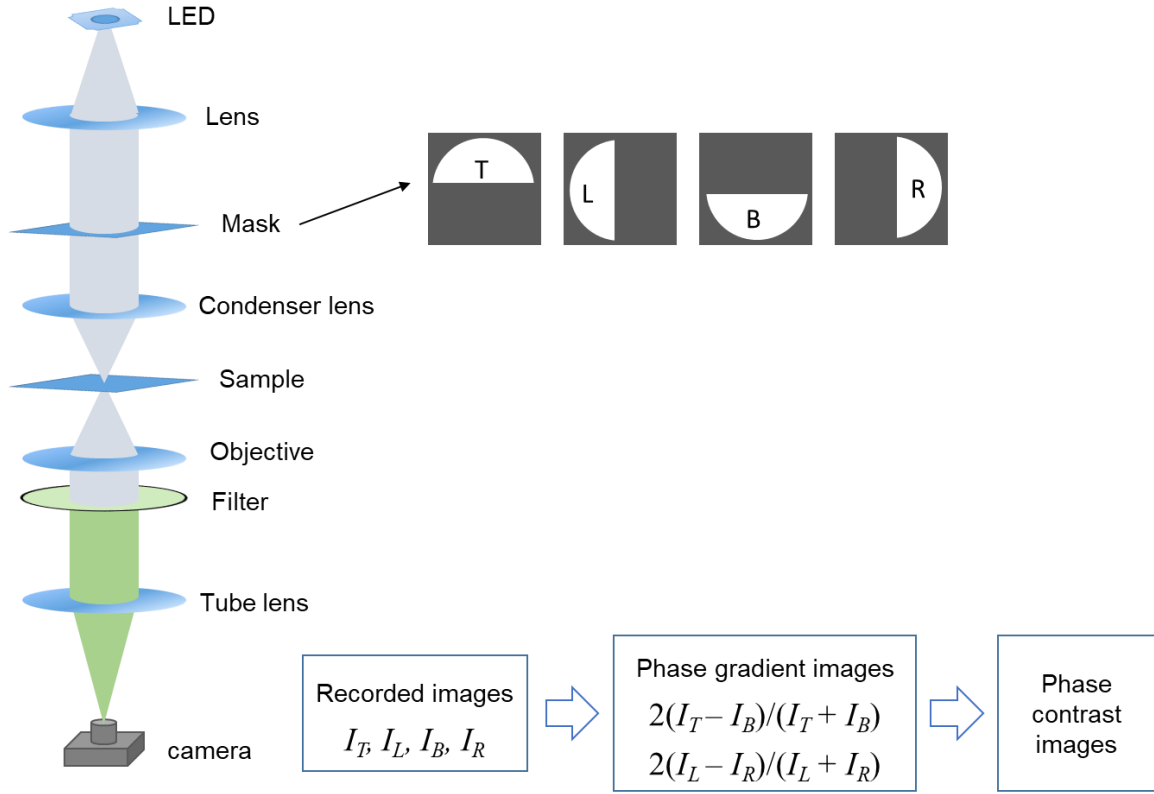


Figure 85 Schematic of differential phase contrast microscopy (DPC) implemented with sequential acquisition of images using masks to provide oblique illumination by blocking half pupil plane of condenser lens.

The mathematical reconstruction of phase images, i.e., maps of optical path length (OPL) across the sample, assumes that the sample is a weakly interacting object, with a linearised transmission function: $t = 1 - \mu + i\varphi$, where μ is the sample absorption and φ is the phase of the sample. For phase measurement along one direction an intensity image related to phase gradient, I^{DPC} , is constructed by calculating, e.g.,

$$\text{Equation 28} \quad I^{DPC} = \frac{I_T - I_B}{I_T + I_B}$$

where I_T is a source illumination allowing only the top semi-circle of light to pass through, and I_B the bottom. One can intuitively see that a phase gradient at the sample modulates the oblique angles of

illumination coming from a semi-circular aperture, therefore shifting the image of the condenser mask that is formed at the objective pupil plane. This shift affects the relative transmission efficiency, therefore coupling phase gradient to measured intensity [149]. A first approximation finds that the intensity at the camera is linearly proportional to the gradient of the phase, meaning integration of the intensity allows reconstruction of the sample phase profile, e.g [150]. A more rigorous analysis carried out by Waller et al [25] showed that the phase gradient approximation is inaccurate for high spatial frequencies and offered an alternative approach using the phase transfer function $H_{ph}(u)$, which can be calculated using the properties of the source illumination and objective pupil function. They showed that the spatial frequencies found in the I^{DPC} image for an in-focus and unaberrated system can be described by:

$$\text{Equation 29} \quad \tilde{I}^{DPC}(u) = H_{ph}(u) \tilde{\varphi}(u)$$

Where $\tilde{\varphi}(u)$ denotes the spatial frequency spectrum of the sample phase. Therefore, by measuring I^{DPC} , calculating $\tilde{I}^{DPC}$ and applying a deconvolution using $H_{ph}(u)$ one can calculate φ [10]. Due to excessive amplification of noise components along the zeros of H_{ph} , in practice an iterative method is used to calculate φ namely solving the least squares problem of

$$\text{Equation 30} \quad \min \sum_j |\tilde{I}^{DPC}_j - H_j \cdot \tilde{\varphi}|^2 + \alpha |\tilde{\varphi}|^2$$

where the j index indicates axes over which DPC measurements have been taken, of which there are usually at least two so that a greater range of spatial frequencies can be reconstructed. The first term in Equation 30 is in accordance with Equation 29, while the second term penalises high frequency content of the phase, therefore suppressing noise. Tuning of the α parameter dictates the level of noise suppression. Figure 85 shows a possible configuration of a microscope for DPC where a set of masks are introduced into the back focal plane of the condenser lens in the transillumination light path.

An inherent limitation of DPC is that the phase transfer function of all semi-circular illumination profiles contains a zero value at the origin, meaning that the DC component of the phase (OPL) map cannot be reconstructed, so phase images can only show relative phase changes across a FOV. A further consideration is that the numerical aperture (NA) of the objective lens must be less than that of the illumination NA; this is parameterised in DPC analysis by the coherence parameter, σ , which should be greater than 1:

$$\text{Equation 31} \quad \sigma = \frac{NA_{illum}}{NA_{obj}}$$

DPC has been elegantly realised using a programmable LED array [25] instead of a mask in the pupil plane of a condenser lens to provide the four necessary source illumination profiles for the two

pairs of complementary asymmetrically illuminated images that can then be used to calculate the phase (OPL) distribution across the sample. However, as the technique acquires four sequential image acquisitions, it is vulnerable to motion artefacts, and replacing the microscope transillumination path with the LED array is a significant modification that may not be practical on an established (commercial) instrument. The same laboratory went on to develop a single-shot implementation they described as colour differential phase contrast (*cDPC*) [151] microscopy, which uses a quadrant spectral mask in the back focal plane of the condenser lens in a Kohler illumination configuration, and a camera with an RGB Bayer filter sensor. Appropriate processing of the RGB sub-images enabled synthesis of the images that would have been recorded with 4 semi-circular masks in the pupil plane of the condenser lens. These images served as the input for their DPC phase reconstruction algorithm and hence they could acquire *DPC* phase images in a single shot. However, the use of a spectral mask to multiplex the illumination profiles resulted in limited spectral availability for other imaging modalities and the visible radiation could excite unwanted fluorescence or be phototoxic to live cells. Furthermore, *cDPC* could also be impacted by chromatic aberrations in the microscope and chromatic absorption in the sample. [12]

5.1.2 pDPC

In *pDPC*, a quadrant polarisation filter is used to multiplex four images acquired with different asymmetric illumination profiles on a single polarisation resolving camera [152]. This is conceptually similar to *cDPC* but replacing the quadrant spectral mask with a quadrant polarisation mask and replacing the RGB camera with the recently available polarisation resolving camera (“PolCam”)

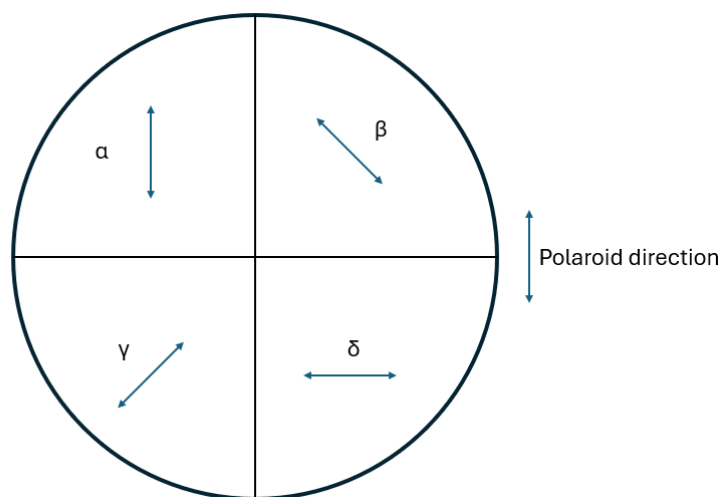


Figure 86 Polarisation mask placed in the back focal plane of the condenser lens to multiplex the illumination patterns needed for *pDPC* microscopy.

incorporating the Sony Polarsens™ sensor. The quadrant polarisation mask (QPM) is illustrated in Figure 86. The quadrants contain polaroid sheet with its transmission axis oriented at 0, 45, 90 and 135 degrees from vertical respectively going anti-clockwise.

The light passing through the QPM transilluminates the sample and is imaged into the PolCam, which incorporates a polarisation mask with sets of 2x2 polarisation filters across the image pixels of the sensor that are orientated at the same angles as the QPM, as depicted in Figure 87. This PolCam sensor had 2448 x 2048 pixels of size 3.45 microns, providing a total FOV under a 20x magnification of 422 x 353 microns. As each 2 x 2 super-pixel is used to construct one phase pixel, the resulting phase image has a sampling of 345 nm/ pixel.

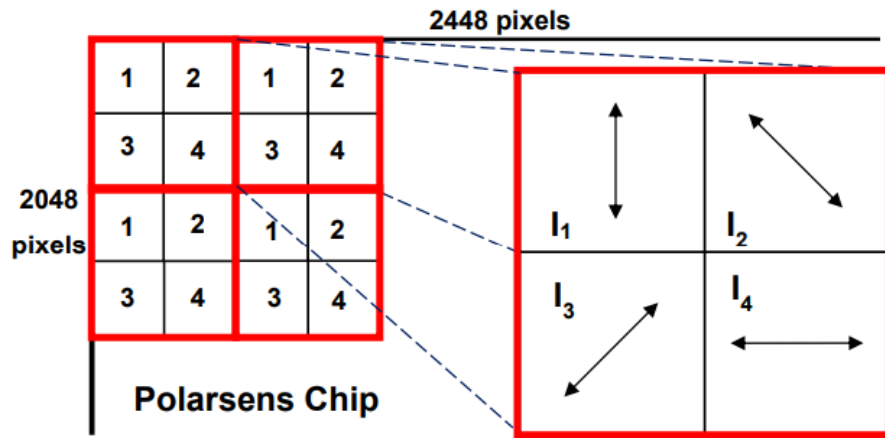


Figure 87 Polarisation masks placed over every 2 x 2 pixels in the Polcam sensor.

Sub-images can be extracted from each PolCam image containing pixels of only one polarisation filter orientation, e.g., all of the pixels with label 1 in Figure 87. From four such sub-images (labelled $I_{1,2,3,4}$), the contribution from each quadrant of the QPM, $I_{\alpha,\beta,\gamma,\delta}$, can be calculated using Malus's Law; for example, the I_1 PolCam sub-image would detect all light from resulting from I_α quadrant and half of that from the I_β and I_γ quadrants but and no light from the I_δ quadrant. From this reasoning we can construct a matrix equation:

$$\text{Equation 32} \quad \begin{bmatrix} I_1 \\ I_2 \\ I_3 \\ I_4 \end{bmatrix} = \frac{1}{2} \begin{bmatrix} 2 & 1 & 1 & 0 \\ 1 & 2 & 0 & 1 \\ 1 & 0 & 2 & 1 \\ 0 & 1 & 1 & 2 \end{bmatrix} \begin{bmatrix} I_\alpha \\ I_\beta \\ I_\gamma \\ I_\delta \end{bmatrix}$$

To relate these images to those that would have been generated using illumination with each of the four semi-circular apertures, we can use:

$$\text{Equation 33} \quad \begin{bmatrix} I_T \\ I_R \\ I_B \\ I_L \end{bmatrix} = \begin{bmatrix} 1 & 1 & 0 & 0 \\ 0 & 1 & 0 & 1 \\ 0 & 0 & 1 & 1 \\ 1 & 0 & 1 & 0 \end{bmatrix} \begin{bmatrix} I_\alpha \\ I_\beta \\ I_\gamma \\ I_\delta \end{bmatrix}$$

And rearrange to find the mapping of $I_{1,2,3,4}$ corresponding to the synthetic semi-circular apertures images $I_{T,R,B,L}$:

$$\text{Equation 34} \quad \begin{bmatrix} I_T \\ I_R \\ I_B \\ I_L \end{bmatrix} = \frac{1}{4} \begin{bmatrix} 3 & 3 & -1 & -1 \\ -1 & 3 & -1 & 3 \\ -1 & -1 & 3 & 3 \\ 3 & -1 & 3 & -1 \end{bmatrix} \begin{bmatrix} I_1 \\ I_2 \\ I_3 \\ I_4 \end{bmatrix}$$

From $I_{T,R,B,L}$, the phase images can be calculated using the DPC phase reconstruction algorithm. Thus, single-shot DPC imaging can be implemented using the QPM in the back focal plane of the condenser lens and a PolCam mounted for brightfield imaging. This *pDPC* technique can be implemented in most microscopes, requiring only the insertion of the QPM in an existing transillumination set-up and the mounting of a PolCam. It is not sensitive to chromatic aberration and can be implemented with near infrared radiation, e.g., from a low-cost LED, for minimal phototoxicity. This also leaves the entire visible spectrum free for other modalities including fluorescence microscopy. We have already demonstrated *pDPC* on an *openFrame*-based microscope [153] and in a commercial (Olympus IX-83; unpublished work) fluorescence microscope where it has been combined with fluorescence microscopy. It is important to note, however, that the *pDPC* analysis assumes the sample is not polarisation sensitive; birefringence or linear dichroism in the sample could compromise the calculation of phase images using *pDPC*. To date, this has not been a problem when imaging cells or small organisms. Figure 88 shows some exemplar *pDPC* phase images of HEK293 cells, illustrating the quality of the reconstructed phase images that can provide morphological readouts of cell state and can be used for cell segmentation.

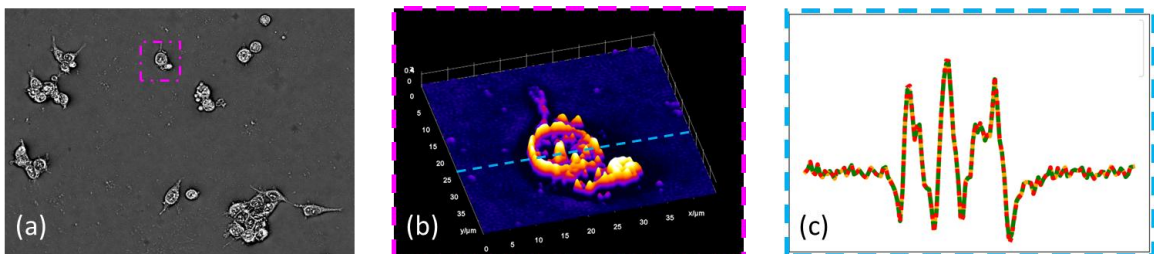


Figure 88 (a) Exemplar *pDPC* phase image acquired on an *openFrame*-based microscope with (b) surface plot and (c) line profile . (Figure adapted from [153])

5.2 Implementation of *pDPC* in the M³M

Implementing *pDPC* on the M³M was relatively straightforward because of the flexibility of the modular *openFrame*-based microscope stand. However, in order to enable simultaneous QPI and multiphoton fluorescence microscopy & FLIM, it was necessary to adapt the *pDPC* implementation so that we could work with the high NA microscope objective lens, noting that this NA exceeds that of any condenser lens we could reasonably use.

5.2.1 Experimental set-up

To implement *pDPC* on the M³M, a transillumination assembly was mounted into the SM2 circular aperture on the top surface of the incubator. By design, this is centred on the optical axis of the objective lens within the *openFrame*. This transillumination assembly comprised a broadband (‘MonoLED’, Cairn Research Ltd) white light source with a filter centred at 635 nm and a collimating lens. The red filter provided quasi-monochromatic illumination as assumed for the *pDPC* reconstruction algorithm and this wavelength presented lower phototoxicity than shorter wavelength light while remaining in the viable spectral detection window of the PolCam – for which the quantum efficiency and polarisation

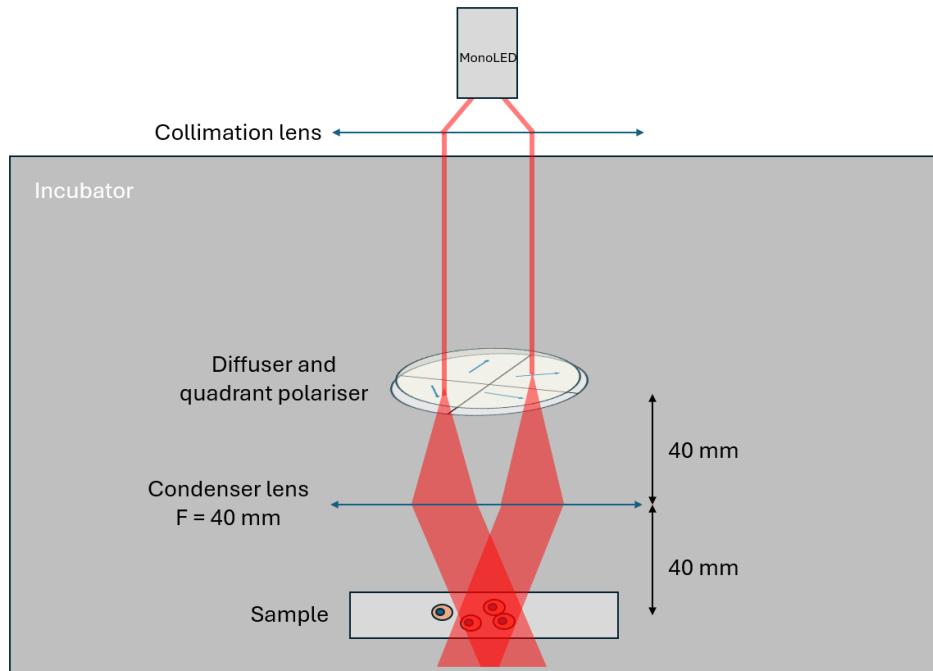


Figure 89 *pDPC* illumination arm following beamlets at two emission angles from the MonoLED.

discrimination both decrease with wavelength [154]. At ~ 635 nm, this red light would also be transmitted through the autofocus cube and short pass dichroic beamsplitter in the excitation layer of the M³M. The light illuminates a diffuser placed directly behind the QPM located in the back focal plane of the condenser lens ($f = 40$ mm) which effectively makes the QPM plane the source plane of the pDPC illumination. See reference [153] for details on the fabrication of the QPM. As illustrated in Figure 89, each ‘point source’ of light scattered from the diffuser is collimated by the condenser lens and illuminates the sample plane at an angle with the angle of illumination increasing for points further away from the optical axis on the diffuser.

As discussed, *pDPC* requires that the illumination $NA \geq$ imaging NA , but the multiphoton fluorescence imaging is implemented with a 0.95 NA objective lens. However, a condenser lens with $NA > 0.95$ is not readily available and it would be challenging to mount it above the sample; further, the

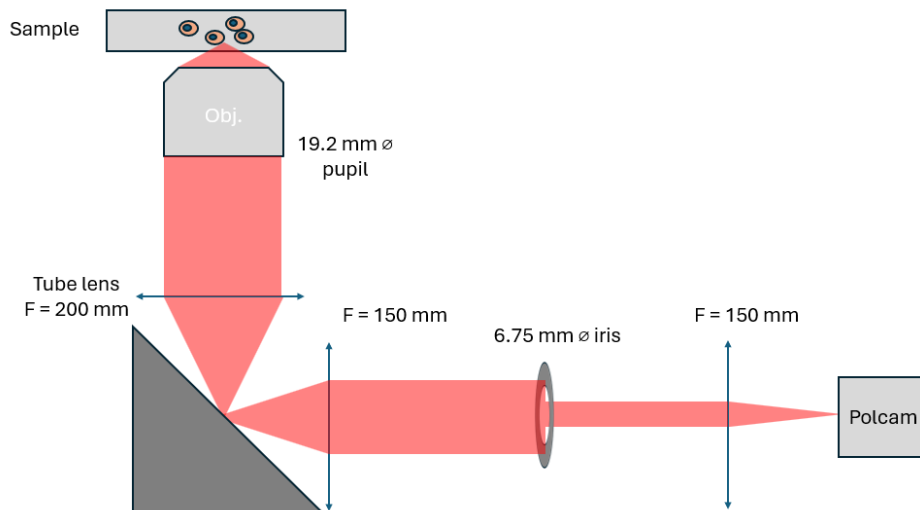


Figure 90 Imaging side of the pDPC setup showing the light collected from one point at the sample plane.

“chimney effect” encountered when imaging in a multiwell plate limits the illumination NA – particularly for sample ROI away from the centre of the well. The highest practical illumination NA we can achieve is 0.55 – realised using a condenser lens of $f = 40$ mm (Thorlabs ACL5040U-A). Therefore, in order to implement *pDPC* while retaining the 0.95 NA objective lens for multiphoton microscopy, it is necessary to lower the imaging NA only for the pDPC detection.. This was done by adding an additional $4-f$ optical relay using achromatic lenses of $f = 150$ mm in the brightfield imaging path to the PolCam that imaged the BFP of the tube lens through an adjustable iris placed at the Fourier plane of the first relay lens.

By considering the relationship $\tan(\theta) = \frac{D}{2f}$, where f and D are respectively the focal length (10 mm) and back aperture diameter (19.2 mm) of the objective lens, and noting $NA = n \sin(\theta)$, one can calculate that reducing the back aperture to 9 mm diameter would produce a 0.55 imaging NA. The $f = 200$ mm tube lens within the *openFrame* and the first relay lens demagnified the objective lens BFP, by a factor of 0.75x, and therefore it needed to be apertured to 6.75 mm diameter to realise the imaging NA < 0.55 required for *pDPC*. This setup is shown in Figure 90 with the light emanating from a point on the optical axis traced through the system.

To implement this new light path, a fixed turning mirror was placed in the *openFrame* at the bottom of the stack and steering mirrors were placed after each relay lens to enable the *pDPC* image light to be accurately directed through the centre of the 6.75 mm diameter iris and onto the PolCam. These mirrors also facilitated a more compact folded imaging system.

5.2.2 *pDPC* reconstruction software

A dedicated MicroManager plug-in with an improved *pDPC* phase image reconstruction algorithm was developed in the group by Huihui Liu [153] which provided live viewing of the *pDPC* phase image and allowed user setting of the phase reconstruction parameters. The plug-in could also be used to reconstruct acquired PolCam image data post-acquisition in a batch process. Figure 91 shows the settings needed to be entered by the user into the *pDPC* reconstruction algorithm. From left to right these are the *preset name*, *illumination NA*, *wavelength*, *imaging NA*, *magnification*, *sequence* (related to the orientation of QPM and camera), *binning* (binned images can be reconstructed faster for live viewing), *use pupil function* (unused), two *regularisation parameters*, and the *camera pixel size*.

Current settings										
preset name	NA_illu	lbd_um	NA_img	mag	seq	binning	use_pupil	reg_p	reg_u	px_cam_um
preset0	0.55	0.6	0.55	20.0	1	1	false	0.005	1.0E-5	3.45

Figure 91 Settings panel on the *pDPC* MicroManager plug-in.

5.2.3 Exemplar M³M pDPC phase images

Figure 92 presents a *pDPC* phase image of HEK293K cells acquired on the M³M. It is clear that the technique provides information on cell morphology with sufficient contrast for cell segmentation. Within the limits of this semi-quantitative phase imaging technique, the “dry mass” of each cell can be determined by integrating the OPL over the lateral cell profile. The dry mass of a cell quantifies the non-water content of the cell and can be used as a readout of cell size, e.g., during the cell cycle [155]. Figure 93 shows a *pDPC* phase image of OVCAR-4 cells, for which the phase values are much smaller, indicating that the cells exhibit a shorter optical path length compared to HEK293K cells.

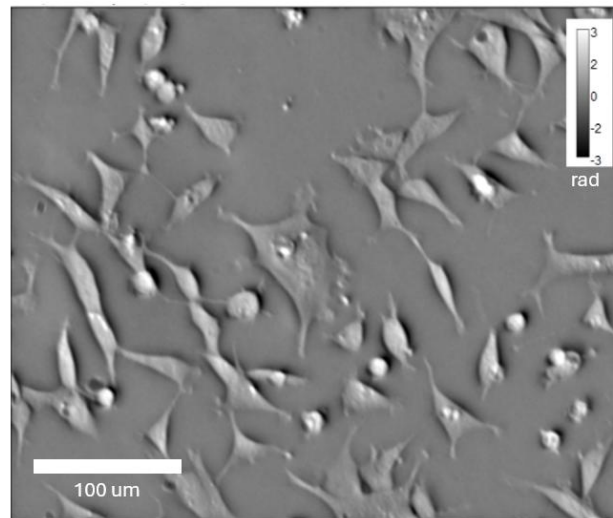


Figure 92 Exemplar pDPC image taken of HEK cells using the M3M.

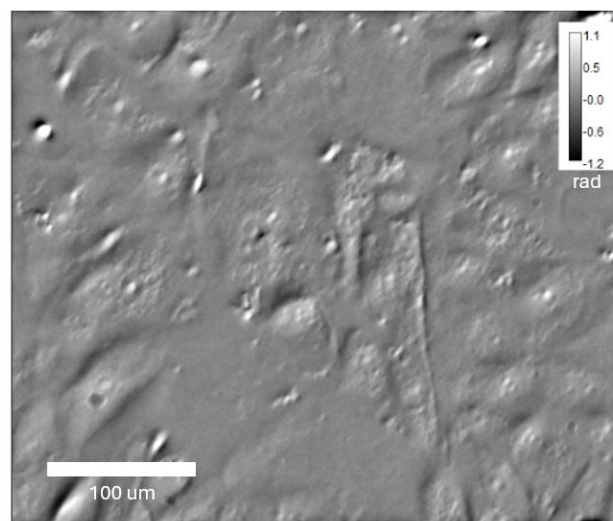


Figure 93 Exemplar pDPC image taken of OVCAR-4 cells using the M3M.

5.3 Exemplar applications of *pDPC* on the M³M

The *pDPC* imaging modality could be used for live cell HCA on the M³M. Using micromanager scripting *pDPC* acquisitions could also be interleaved with multibeam multiphoton imaging.

5.3.1 Multimodal M³M HCA with *pDPC* + NADH and FAD autofluorescence in OVCAR-4 cells

For an initial trial of this multimodal assay, 32 wells of a 96-well plate were seeded with OVCAR-4 cells. In an earlier study [156], both NADH and FAD had excited at 800 nm and autofluorescence signals were detected using 450/50 nm and 560/80 nm emission filters for NADH and FAD respectively. In our experiment, excitation at 810 nm was used to match the autofocus dichroic beamsplitter window and emission filters of 460/50 nm and 578/ 105 nm were used for the NADH and FAD channel respectively.

These two channels were imaged sequentially with the same excitation wavelength being used for both channels as a device adapter had not yet been written for the (Spectra Physics, Insight X3) excitation laser and so the excitation wavelength could not be changed within a MicroManager script. 20 mW average excitation per beam was for imaging in both the NADH and FAD channels, with 16 second acquisitions. 270 x 270 microns FOVs were imaged with 240 x 240 pixels. After acquiring fluorescence data in each channel, the configuration of the microscope was switched to acquire a *pDPC* phase image. The settings for each type of image acquisition are presented in Table 8. The cell were not confluent within the wells and so a manual pre-finding stage was necessary to identify suitable FOVs for imaging, which were added to the position list for automated imaging.

Settings	Image Type		
	NADH	FAD	pDPC
Laser + PMT shutter	Open	Open	Closed
Brightfield shutter	Closed	Closed	Open
Emission filter	460/50 nm	578/ 105 nm	None
Excitation layer cube	Mirror	Mirror	Short pass dichroic
Detector	HPMAs + MH	HPMAs + MH	Polcam

Table 8 M3M configuration settings for NADH, FAD and pDPC imaging.

After acquiring the images, they had to be co-registered, which required the *pDPC* images to first be rotated by -15.5 degrees to match the scan angle used in the multibeam multiphoton modality. The *pDPC* image then had to be rescaled, with the rescale factor roughly matching the ratio of multiphoton to *pDPC* pixel size of 3.3. A translation was then applied to precisely overlap the FOVs. After establishing the appropriate co-registration parameters with one FOV, these image manipulations were applied to the whole *pDPC* image stack. Figure 94 shows the NADH and FAD fluorescence intensity, *pDPC* and overlay of a region in well C7 of the multiwell plate

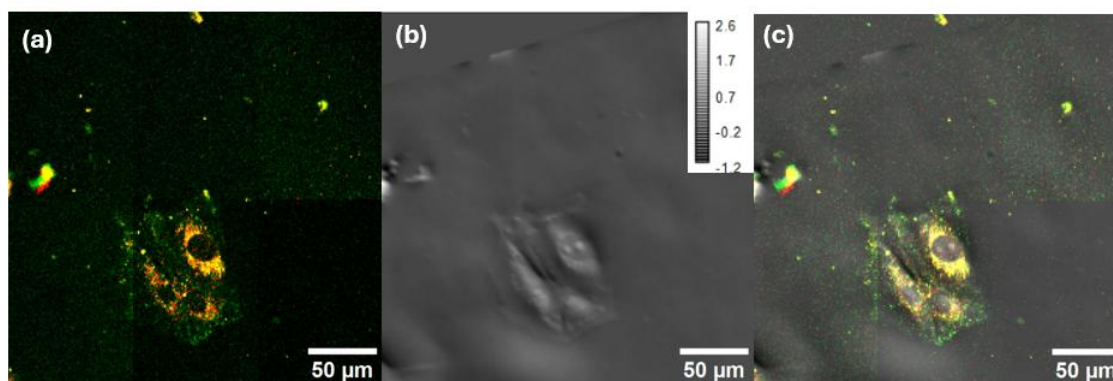


Figure 94. M3M autofluorescence and pDPC imaging of OVCAR4 cells. (a) NADH (green) and FAD (red) multibeam multiphoton fluorescence overlay image, (b) pDPC image and (c) NADH, FAD

Figure 95 shows the overlaid *pDPC*, FAD and NADH intensity image data for the 32 wells imaged. As the confluency of the wells was low and only 1 FOV was taken per well, there were relatively few cells per condition. The experiment demonstrated that interleaved *pDPC* and multibeam multiphoton imaging could be carried out in a HCA format. The interleaved nature of the image data acquisition also minimises the effect of sample movement between images, meaning they can be overlaid accurately.

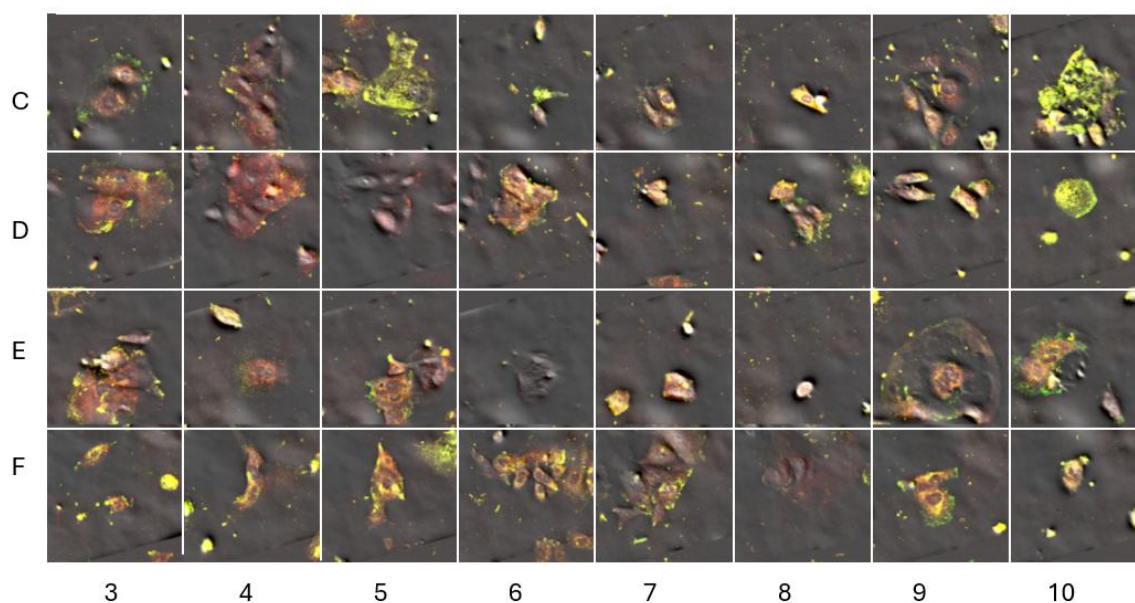


Figure 95 Multiwell plate acquisition of OVCAR4 cells. pDPC (grey scale), NADH (green) and FAD (red)

5.3.2 pDPC time lapse with NADH FLIM endpoint measurements

One application of pDPC is the tracking of cells throughout time. Due to the low intensity red illumination used, cells are less perturbed by pDPC imaging, meaning that long time-lapse imaging can be carried out to no detriment of the sample. An exemplar experiment was conducted to show how pDPC can provide cell tracking information between two NADH FLIM measurements, allowing one to correlate information on a per cell basis between FLIM images.

OVCAR-4 cells were mounted inside a multiwell plate in just PBS, rather than in a more complete culture medium, and placed inside the M³M incubator. The lack of nutrients in the PBS medium was intentional so that the cells would be stressed and show clear morphological changes over the timelapse. *pDPC* images were taken every 30 minutes for 15 hours, and FLIM images were taken at the 0 and 15 hour time points. FLIM images were taken in the 6 beam modality using the new fibre loom with 240 x 240 pixels, 270 x 270 micron FOV, 20 mW per beam, 20 second acquisitions per image. Excitation was at 750 nm and a 450/60 nm emission filter was used to view the NADH signal. The data from the experiment is shown in Figure 96, with *pDPC* images shown every 3 hours for ease of viewing. The experiment shows how *pDPC* imaging provides data that can be used to track cells between FLIM images, allowing confident identification of the same cells and observation of morphological dynamics between time points.

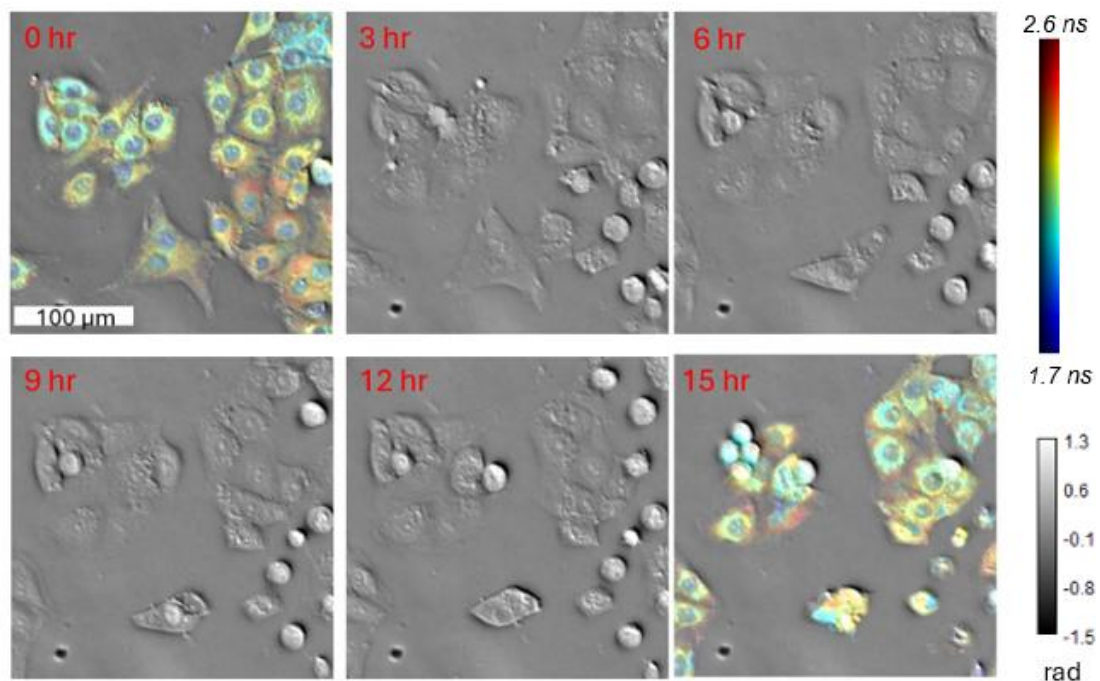


Figure 96 *pDPC* timelapse of OVCAR4 cells with NADH autofluorescence FLIM endpoint measurements at 0 and 15 hours.

5.3.3 Analysis on *pDPC* image data

Various image analysis tools can be applied to *pDPC* data, including to create segmentation masks for quantitative single-cell-resolved analysis and cell tracking, and to calculate morphological

parameters such as the dry mass of cells. To date, the following software tools have been explored with the M³M.

5.3.3.1 Labkit

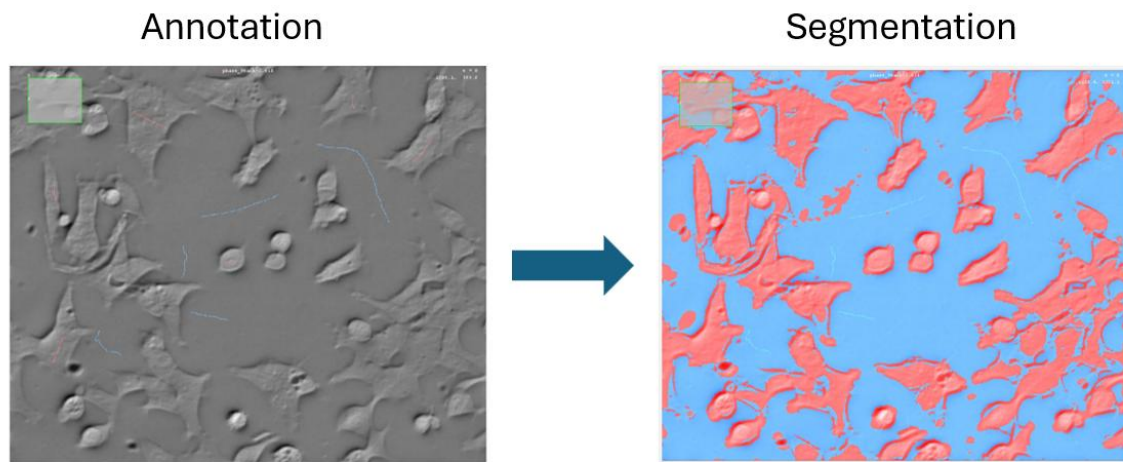


Figure 97 pDPC image of HEK293 cells with manually annotated foreground (red lines) and background (blue lines) in Labkit (left) and resulting segmented image (right).

Labkit is an open-source tool that is available as an *ImageJ* plug-in [157]. It requires a user to provide some basic initial labelling, after which a random forest classifier is applied to annotate the rest of the images in a dataset. Figure 97 shows the annotation process; only a few areas need to be annotated for the algorithm to be able to classify foreground and background. Once the foreground and background

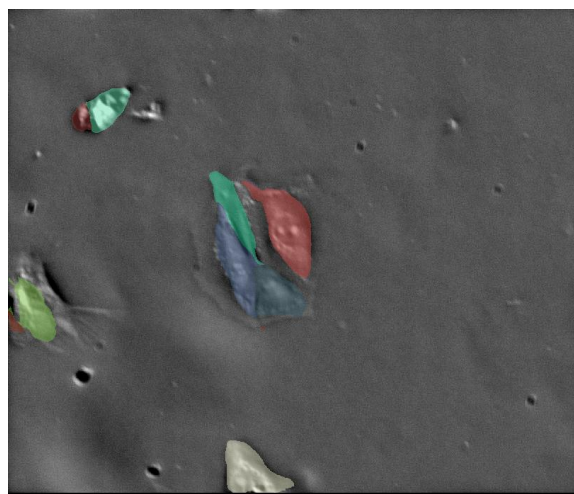


Figure 98 Cellpose segmented pDPC image of OVCAR-4 cells

have been successfully established, the total dry mass across a FOV can be calculated by summing all the pixels in the foreground. The average dry mass per pixel can also be calculated by dividing the dry mass by the total number of foreground pixels. More labels can be applied to images; for example, specific cell phenotypes present in a data set can be labelled to enable the algorithm to classify the foreground into distinct classes.

5.3.3.2 Cellpose

Cellpose is a commonly used open-source cell segmentation tool [129]. Segmentation entails dividing a FOV into discrete objects (typically cells), thereby enabling single-cel-resolved analysis - and tracking if each time frame can be segmented. Cellpose recognises cells within FOV using deep learning models that have been trained on many fluorescence images of cells. Users can also annotate their own datasets and then train their own models. Figure 98 shows a *pDPC* image of OVCAR-4 that has been segmented using Cellpose. Using this data, shape information for each cell may be found, such as cell area and eccentricity.

5.3.3.3 CellProfiler

CellProfiler is an open-source image analysis tool for HCA that allows a user to configure their own image processing and analysis pipelines [158]. Standard image processing tools can be implemented in a sequential manner, allowing complex processing pipelines to be built up step by step. Figure 99 shows some images from an exemplar CellProfiler processing pipeline using a HEK293 cell *pDPC* image as input, the steps for which are described below:

1. Objects are identified in a given input image.
2. Objects are filtered according to their size, rejecting those with an area < 500 pixels
3. The filtered objects are then filled using a convex hull algorithm.
4. Information on the shape and intensity distribution for each object is measured.
5. The data measured data is output to an excel file and segmented images saved.

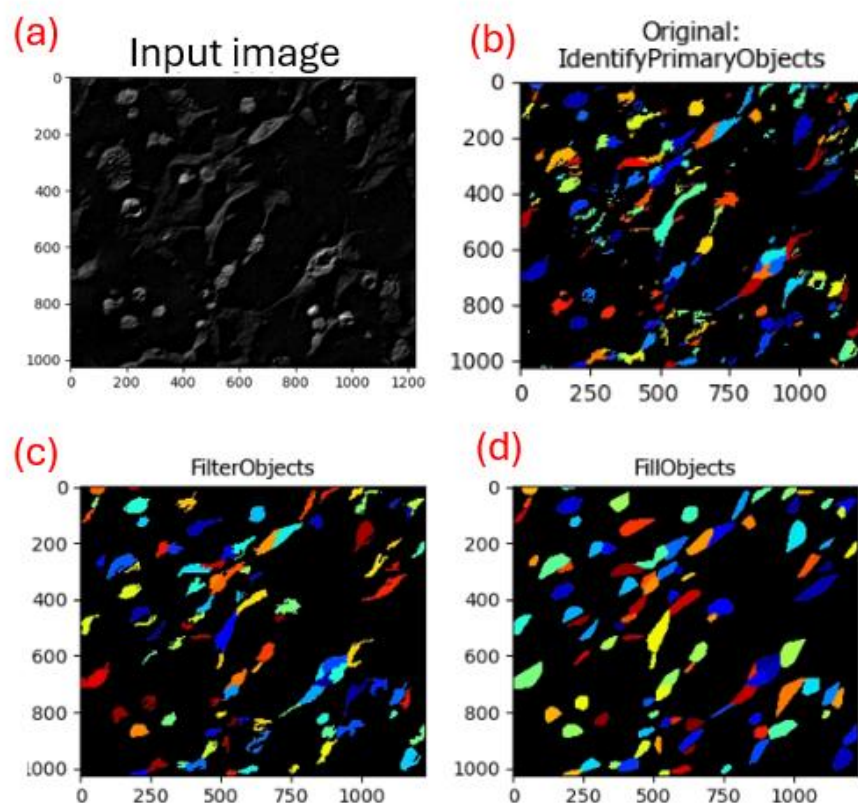


Figure 99 Exemplar CellProfiler processing pipeline illustrated with pDPC image of HEK293 cells. An (a) input image is used for (b) object identification, which are then (c) filtered based on their size, the remaining objects are (d) filled from which final shape properties such as area and eccentricity can be measured.

5.3.4 Summary

This chapter has discussed the design and implementation of a semi-quantitative phase imaging technique, namely polarisation-resolved differential phase contrast (pDPC), on the M³M. An introduction to pDPC was first given, with the specific implementation used with the high NA objective of the M³M shown afterwards. As the technique demands that the illumination NA be greater than the imaging NA, an aperture was employed at a conjugate plane of the objective BFP to reduce the imaging NA. Applications of pDPC were then shown in the form of a pDPC/ FLIM multiwell plate acquisition and a pDPC time-lapse acquisition with FLIM endpoints. Some open-source tools available to analyse pDPC data were then shown with applications in cell segmentation.

6 Application of the M³M to assays of metabolism

This chapter describes the application of the M³M to automated multiphoton fluorescence lifetime imaging readouts of changes in cellular metabolic state; FLIM of NADH provided label-free time-resolved readouts in 2D and 3D cell cultures treated with compounds known to affect metabolic function. These FLIM readouts obtained were compared to readouts using T2AMPKAR, a previously demonstrated FRET biosensor. Quantitative phase imaging using *pDPC* following drug treatment was also applied to provide complementary information.

6.1 Readouts of changes in cell metabolic state

This section introduces NAD(P)H, an endogenous metabolite that is a major contributor to cellular autofluorescence that is frequently interrogated using fluorescence lifetime measurements. I also introduce the genetically expressed T2AMPKAR FRET biosensor [75] that provides a readout of AMPK that in turn probes the intracellular ratio of ADP to ATP.

6.1.1 Intracellular NAD(P)H

NADH is a fluorescent molecule that is involved in the production cycle of adenosine triphosphate (ATP), the molecule responsible for providing the energy needed in many cellular reactions. NADH carries electrons from other parts of the cell to the mitochondrial electron transport chain (ETC), initially delivering electrons to complex 1 in the inner mitochondrial membrane [159]. Once an NADH molecule has donated an electron, it reverts to its oxidised form NAD⁺ which doesn't have an excitation peak centred at a wavelength of 350 nm. The electrons delivered to the mitochondria result in the transport of protons to produce a charge gradient, which is ultimately responsible for the production of ATP. This method of ATP production is known as oxidative phosphorylation and can only be carried out in aerobic conditions [160]. NADH can also be converted back to NAD⁺ through fermentation [161], which can proceed in anaerobic conditions where oxidative phosphorylation isn't possible. Glycolysis is the second main ATP production pathway in the cell, resulting in the reduction of NAD⁺ molecules into NADH through the breakdown of glucose molecules, directly producing ATP [162]. As glycolysis converts NAD⁺ to NADH, and oxidative phosphorylation converts NADH into NAD⁺, the dominant ATP production method will be indicated by the relative abundances of these two molecules, which will impact the cellular autofluorescence. To support rapid proliferation, cancer cells typically utilise glycolysis more than non-cancer cells, even in aerobic conditions. Described as the Warburg effect [163], this has led to NADH autofluorescence imaging being investigated as a means to detect cancer, including *in vivo* [164], [165], [166]. In its interactions with the ETC and in other interactions within the cell, NADH binds to a range of enzymes that can modify (typically increasing) its fluorescence

lifetime. It has been shown that the population of enzyme bound NADH is less variable with respect to respiratory changes [167].

Nicotinamide adenine dinucleotide phosphate (NADPH) is a molecule related to NADH by the addition of a phosphate group, which is also fluorescent in the same spectral window as NADH and cannot be separated spectrally. NADPH has different cellular functions to NADH, such as maintaining the cellular redox balance and supporting biosynthesis [168]. Since their fluorescence spectra cannot be distinguished, imaging intracellular autofluorescence is often described as imaging NAD(P)H. Care must be taken to consider whether the population of NADPH can impact cellular autofluorescence readouts, as discussed in [169].

6.1.2 FLIM of NAD(P)H

When imaging NADH, the free and (enzyme) bound forms emit fluorescence with different average fluorescence lifetimes. Their respective lifetimes quoted in the literature are often $\tau_{free} = 400$ ps and $\tau_{bound} = 2500$ ps [170], however as NAD(P)H binds at least 334 known proteins in cells [171], τ_{bound} may contain multiple decay components from different bound forms; indeed bound NAD(P)H has been found to present lifetimes varying in the range of 1-9 ns [172]. A key factor allowing useful metabolic FLIM analysis, however, is that the abundance of the bound form of NAD(P)H doesn't fluctuate greatly with respect to metabolic changes compared to the unbound form. This means that metabolic changes that increase or decrease the amount of free NADH will result in mean

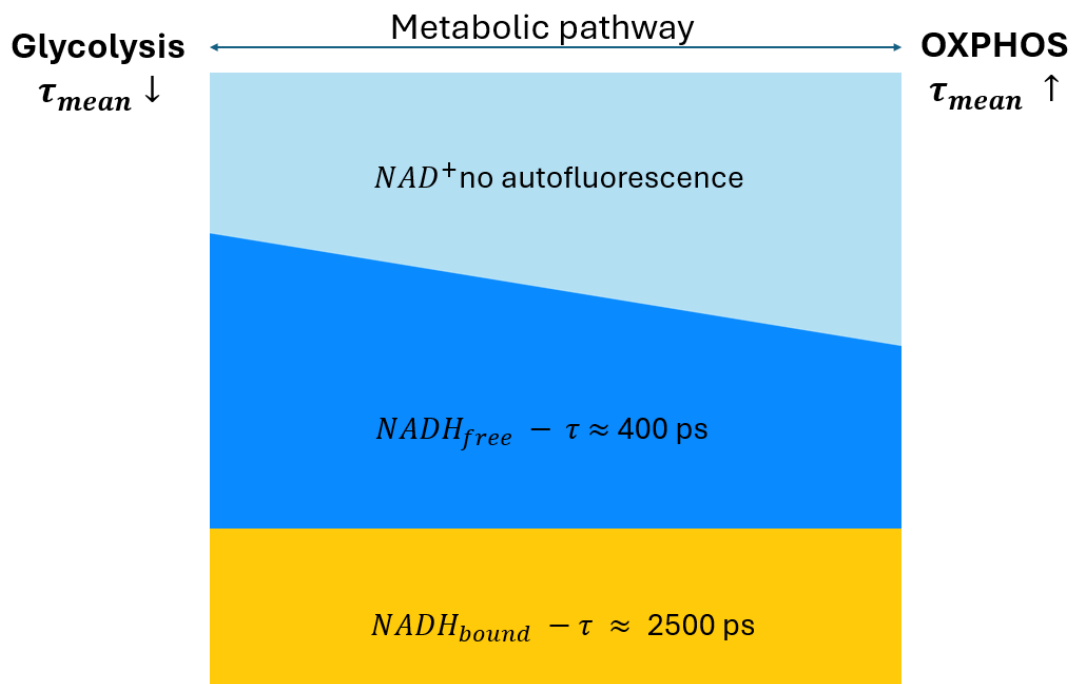


Figure 100 Relation between predominant metabolic pathway, abundances of NADH & NAD⁺, and overall lifetime measured.

autofluorescence lifetimes that decrease or increase respectively, as indicated in Figure 100 (adapted from [160]).

As the number of photons needed to accurately fit decay curves increases rapidly with the number of lifetime components (e.g. corresponding to NAD(P)H bound to a variety of enzymes), practical considerations result in a double exponential fit being most often applied with the short lifetime component attributed to free NAD(P)H and the longer lifetime component representing the ensemble of lifetime contributions from all bound NAD(P)H components. As FLIM readouts are independent of fluorescence intensity, they provide a more quantitative readout than fluorescence intensity measurements [173].

6.1.3 T2AMPKAR bio-sensor

mTurquoise2 AMPK activity reporter (T2AMPKAR) is a FRET biosensor [75] adapted from the previously published AMPKAR biosensor [174] at Imperial that can be used for metabolic readouts. AMPK is an enzyme that senses the energetic status of a cell by responding to the ratio of AMP + ADP to ATP. AMPK is activated at low energy levels, promoting ATP generating pathways, for example by increasing the amount of glucose uptake and glycolysis whilst inhibiting biosynthesis. The FRET biosensor takes advantage of a change in conformation of the enzyme upon activation: the FRET pair, mTurquoise2FP (donor) and cpVenus173FP (acceptor) are attached to different ends of the enzyme to realise the T2AMPKAR sensor. The conformational change decreases the distance between the two fluorophores upon enzyme activation and therefore the donor fluorescence lifetime decreases. Thus, the energetic status of a cell expressing T2AMPKAR can be analysed by observing the fluorescence lifetime of mTurquoise2FP [75]. A non-functional mutant T2AMPKAR sensor was also made to provide a negative control - showing no lifetime changes with metabolic changes in the cell.

6.1.4 Metabolism modulating drugs

In the experiments carried out in this chapter a set of compounds known to affect the cell metabolism were used: phenformin, rotenone and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). Phenformin inhibits complex 1 of the mitochondrial ETC, diminishing the cell's ability to carry out oxidative phosphorylation. With lower levels of oxidative phosphorylation, one expects to see higher levels of free NADH and activation of the AMPK enzyme in response to lower energy levels [175]. Rotenone also inhibits complex 1 of the mitochondrial ETC and therefore produces a similar effect [176]. FCCP is a mitochondrial uncoupler that reduces the proton gradient built up in the mitochondrial membrane used to produce ATP by allowing another method of proton crossing in the mitochondrial membrane [177]. This over-activates the ETC in response, meaning more NADH oxidation and less free NADH present in the cell, resulting in longer NADH fluorescence lifetimes [178]. Despite the over-activation of the ETC, still no ATP is produced due to

the uncoupling from FCCP, therefore AMPK is also activated. Phenformin, rotenone and FCCP have all been used in cancer treatment studies [179], [180], [181].

6.2 Multiwell plate assay comparing NADH and T2AMPKAR readouts in cell monolayers following treatment with metabolism modulating drugs

Multiwell plate experiments were carried out to compare responses between both NADH and T2AMPKAR FLIM readouts when exposed to drugs affecting metabolic pathways. These experiments were carried out in a “2D” cell monolayer before progressing to 3D cell cultures.

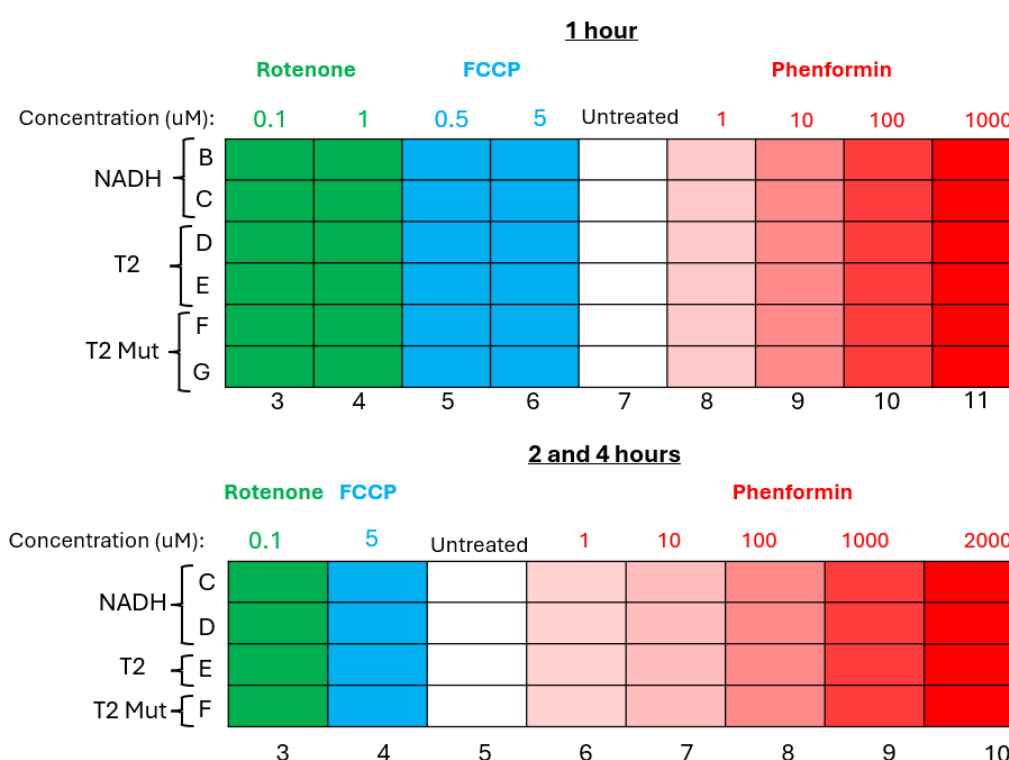


Figure 101 Plate maps for experiments carried out at 1 hour and 2 & 4 hours after drug application.

6.2.1 Acquisition details

<u>Experiment</u>	<u>FOVs</u> <u>per</u> <u>well</u>	<u>Dwell</u> <u>time</u> <u>NADH (s)</u>	<u>Dwell time</u> <u>T2AMPKAR (s)</u>	<u>T2AMPKAR</u> <u>filters</u>	<u>T2AMPKAR</u> <u>power (mW per</u> <u>beam)</u>
1 hour	2	16	8	850 exc 450/60 em	20
2 hours	4	16	4	900 exc No emission	10
4 hours	1	8	4	850 exc No emission	10

Table 9 Experimental details for three 2D metabolic imaging experiments

Three experiments were carried out on different days looking at the effect of rotenone, FCCP and phenformin on NADH and T2AMPKAR fluorescence lifetimes in HEK293 cells. The three experiments were undertaken at different times after drug application with the plate layouts shown in Figure 101. Due to unexpected issues during the imaging sessions, there were some differences between the experiments such as the number of repeats per well, or dwell time per FOV. The details for each experiment are shown in Table 9. 240 x 240 pixels were used to image a 270 x 270 micron FOV in each experiment and NADH imaging used 750 nm excitation and a 450/60 (centre / bandpass wavelength in nm) emission filter in each experiment. In the 1-hour and 4-hour post treatment experiment a *pDPC* image data acquisition was interleaved with the FLIM data acquisition.

The FLIM data was analysed using a global double exponential fit for the NADH autofluorescence to capture the short and long lifetime components attributed to free and enzyme bound NADH respectively. A pixel-wise single exponential fit was used for the T2AMPKAR data in the acquisitions taken with no emission filter. The reason for using no emission filter was to increase acquisition rate, reasoning that minimal mVenusFP would be excited using our excitation wavelength, and the FRET efficiency would be low enough that effectively only mTurquoise2FP fluorescence would be collected. A double exponential fit per image was used for the T2AMPKAR acquisition taken with the 450/60 emission filter in place as the decay showed another lifetime component in this configuration, possibly the short lifetime from NADH. This configuration and lifetime fitting is in line with the procedure carried out on the T2AMPKAR biosensor previously in the group [75].

6.2.2 NADH shows greater sensitivity to metabolic changes than T2AMPKAR at 1 hour after drug treatment

Figure 102 shows exemplar FLIM images taken for each cell type in the experiment carried out 1 hour after drug treatment. In the work done previously in the group [75] the values found for the lifetime of the T2AMPKAR mutant subjected to 50 micro molar of AMPK activator 991 (which shouldn't activate the mutant) were $\tau_1 = 3.3 \text{ ns}$ and $\tau_2 = 0.7 \text{ ns}$ and $\frac{a_1}{a_2} = 2.3$, whilst in our experiments we found $\tau_1 = 3.1 \text{ ns}$ and $\tau_2 = 0.4 \text{ ns}$ and $\frac{a_1}{a_2} = 1.9$. Though these parameters are not directly comparable due to the previous work using single photon excitation, and with a different drug (991 is an AMPK activator), the values are similar to those found in our experiment. The short and long lifetimes found for NADH were 370 ps and 3750 ps respectively.

The full dataset for the 1-hour experiment is shown in Figure 103 in the form of an average

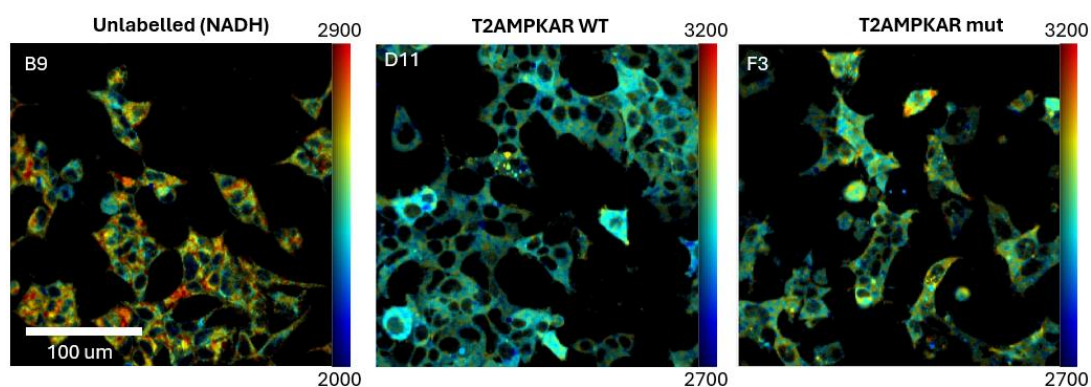


Figure 102 Images of unlabelled cells imaged in the NADH channel as well as active and mutant T2AMPKAR cells imaged in the mTurquoise2 channel. From the 1 hour post treatment experiment

weighted mean lifetime heatmap over the multiwell plate. The data is plotted per column (each column having the same condition) in Figure 104. Looking only at the NADH data we see that the lifetime exhibits the expected dose response trend for increasing concentrations of phenformin (decreasing mean lifetime due to a more glycolytic state). The increase in weighted mean lifetime seen for FCCP also follows the expended trend. However, while the lifetime decreases as expected for treatment with rotenone, the lifetime at the larger concentration is longer, whereas it was expected to be shorter.

In comparison, there is minimal change in mTq2FP lifetime of the T2AMPKAR FRET biosensor across all conditions, indicating that the NADH signal responds with more sensitivity at earlier time points. The mutant non-functional sensor also maintained a relatively constant lifetime as expected, apart from the 5 μM dose of FCCP seeming to increase the mTq2FP lifetime.

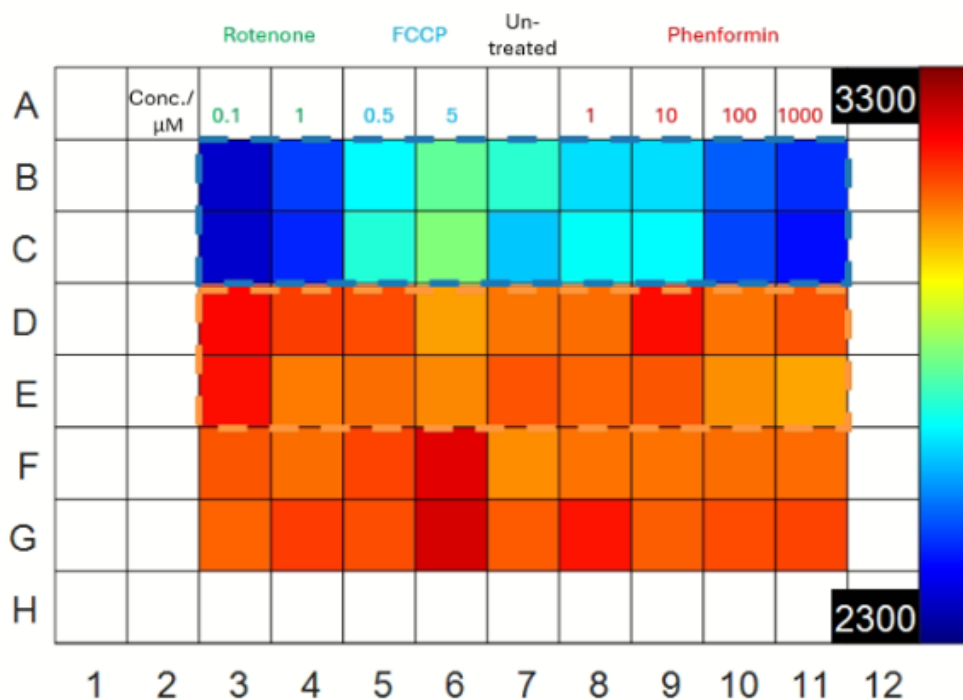


Figure 103 Average mean weighted lifetime of NADH (rows B to C) and mTurquoise2 of T2AMPKAR FRET sensor (rows D-G) per well for the 1-hour experiment.

Weighted mean tau of NADH and T2AMPKAR for varying drug conditions

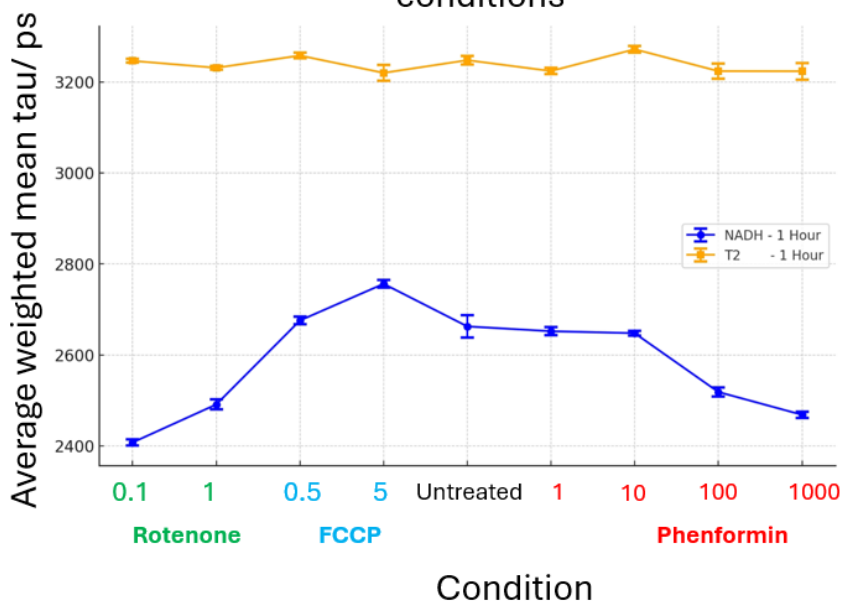


Figure 104 Average mean weighted lifetime per column across the plate for both NADH and T2AMPK.

6.2.3 Time response after drug application

The change in lifetime following drug application was analysed for the unlabelled cells in the NADH channel, and the T2AMPKAR biosensor/mutant biosensor expressing cells in the mTq2FP channel. Figure 105 shows how the weighted mean NADH lifetime varied with time across treatment conditions and Figure 106 and Figure 107 show the corresponding variation in Tq2FP lifetime for the cells expressing the T2AMPKAR biosensor and its non-functional mutation respectively. The temporal variation of mean NADH lifetime remains fairly consistent from 1 to 4 hours across the conditions. Figure 106 and 8 indicate that the T2AMPKAR biosensor only indicates a dose response curve for the 4-hour timepoint. It is also worth noting that the relative change in weighted mean lifetime is about 5 times greater for the NADH channel. Figure 107 shows that the cells expressing the non-functional T2AMPKAR mutation show no dose response at any time point, apart from small changes for the FCCP treatment at 1 hour. This may not be significant and requires further investigation.

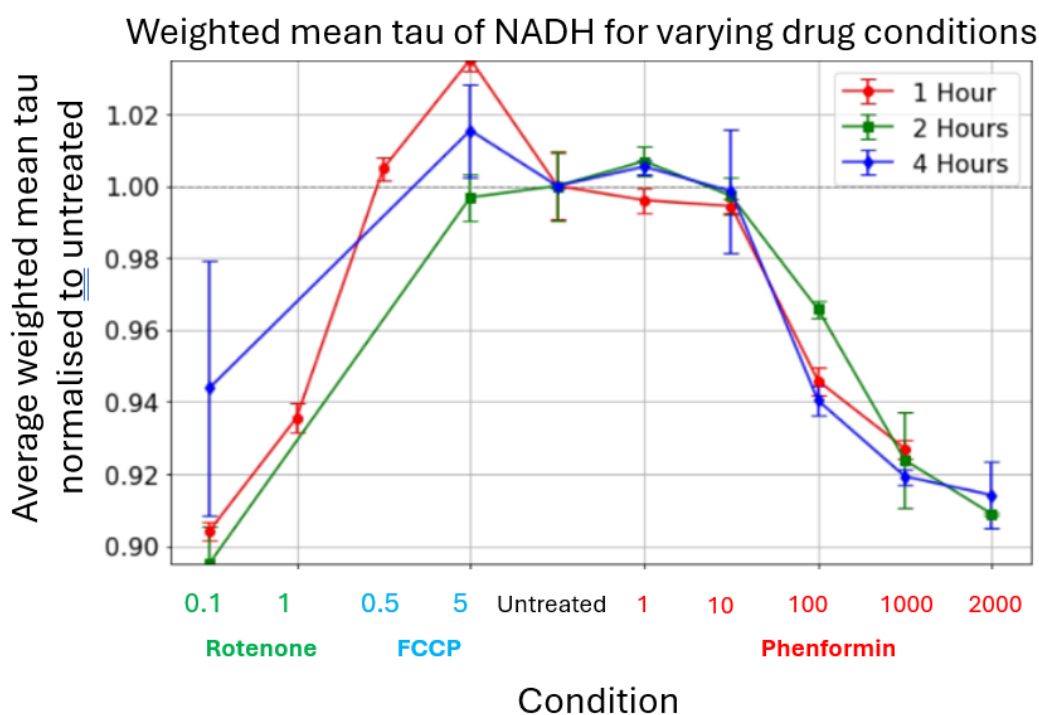


Figure 105 Average HEK293 NADH weighted mean lifetime per condition for each time point.

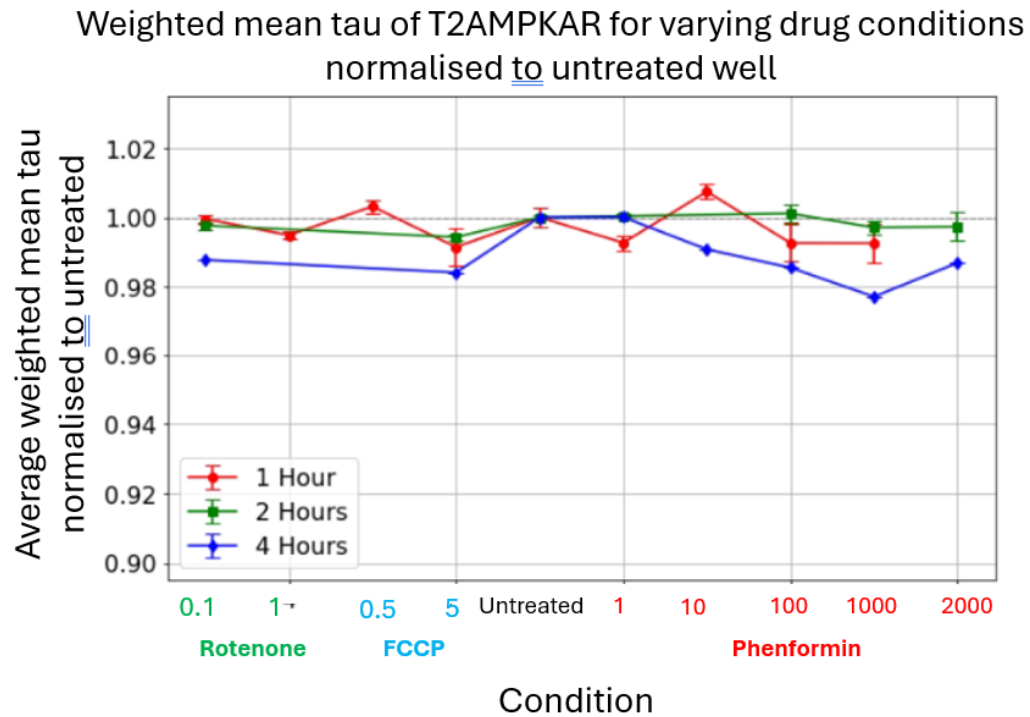


Figure 106 Average HEK293 T2AMPKAR weighted mean lifetime per condition for each time point. (Data point at 2 hours for 10 uM phenformin wasn't taken due to a brief autofocus malfunction during the acquisition)

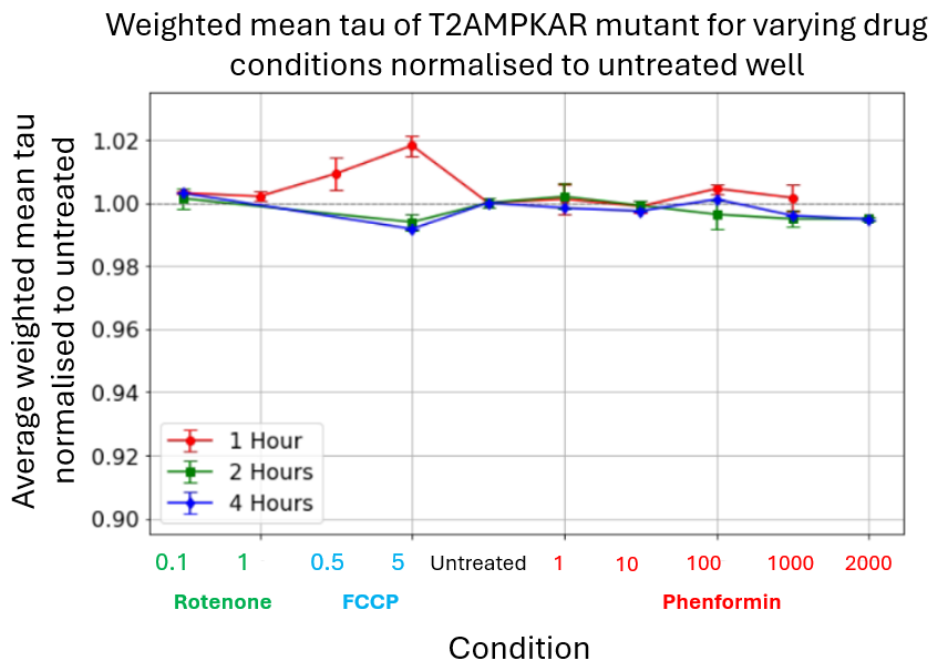


Figure 107 Average HEK293 T2AMPKAR mutant weighted mean lifetime per condition for each time point.

6.2.4 pDPC morphological analysis

pDPC acquisition was interleaved with the FLIM acquisition for the experiments at 1-hour and 4-hour post drug treatment. Exemplar phase and fluorescence lifetime images and overlay are shown in Figure 108. As an unexpected higher lifetime was seen for the higher concentration of rotenone in the FLIM data, the phase image data was investigated to see if there were any morphological changes.

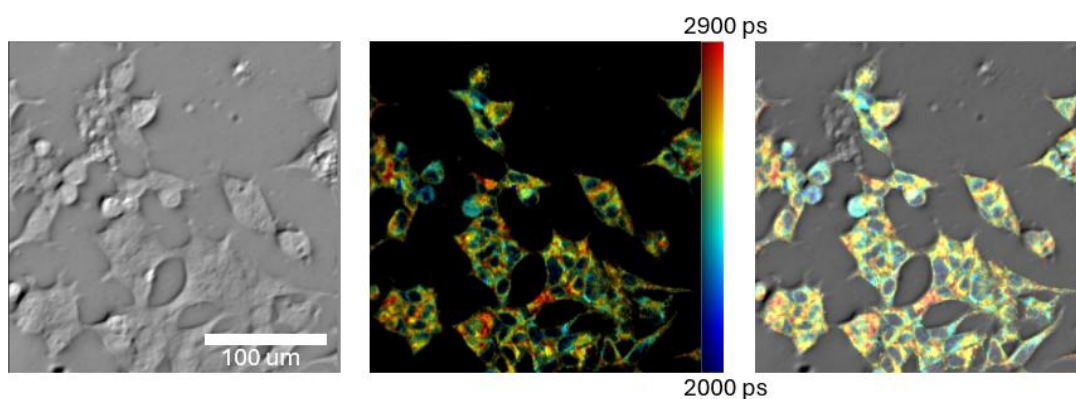


Figure 108 pDPC image, FLIM image and overlay of HEK293 cells in well B9 from the 1-hour experiment.

Figure 109 shows the *pDPC* and *pDPC*/FLIM overlay images for the whole multiwell plate. Figure 110 shows a comparison of *pDPC* FOVs corresponding to increasing concentrations of rotenone where one can see increasing phase values for increasing rotenone concentration. Increasing phase values may be explained by cells rounding up as they enter apoptosis due to stress from the drug treatment, increasing the dry mass per pixel as it is packed into a smaller space [182]. Phase contrast microscopy has been used previously to assess cell morphological dynamics including showing that apoptosis causes an increase in average cell phase value [183], [184].

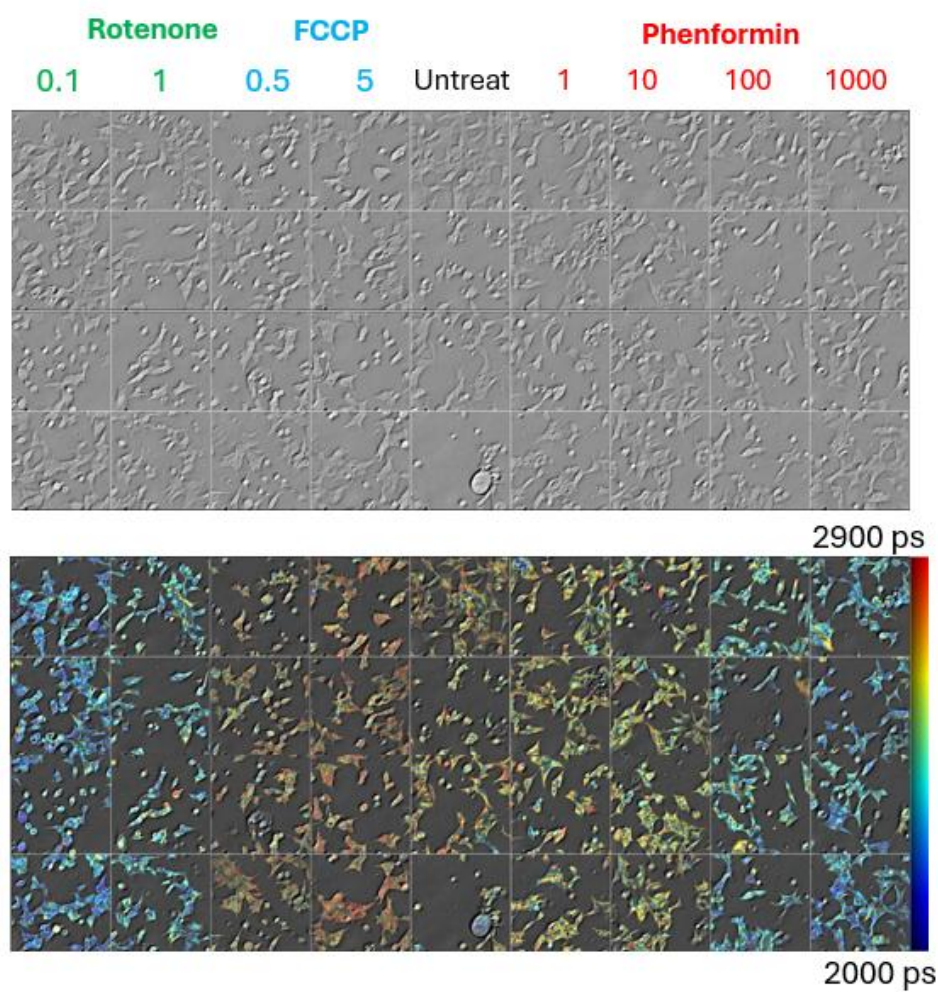


Figure 109 pDPC and pDPC/FLIM overlay of HEK293 cells across multiwell plate in the 1 hour experiment.

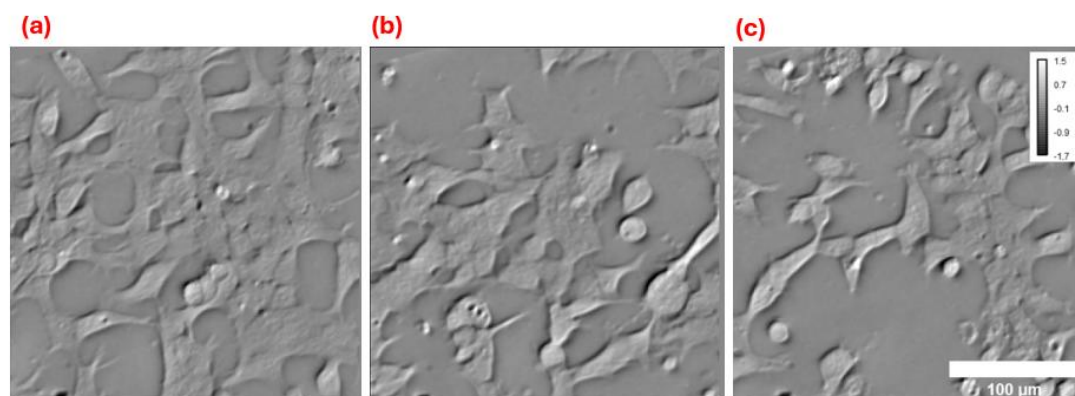


Figure 110 pDPC image for (a) untreated, (b) 0.1 μM rotenone and (c) 1 μM rotenone treated HEK293 cells (1-hour experiment).

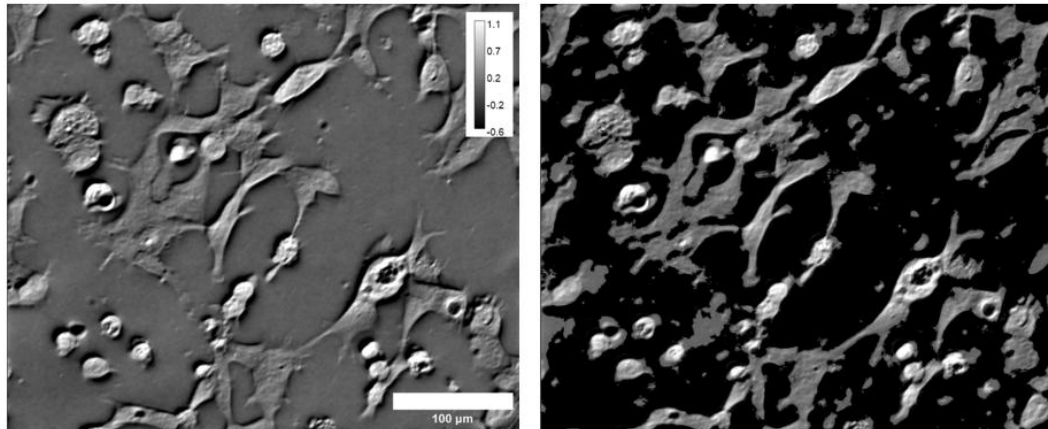


Figure 111 Original pDPC image and labkit foreground/ background segmented image.

In order to analyse the morphological changes across the plate more quantitatively, labkit [157] was used to segment cells from background. An original frame and its segmented counterpart are shown in Figure 111. Using the segmented image one can calculate the number of pixels attributed to cells found in each image, as well as the average phase value across the segmented region in each image. We reasoned that cell rounding could be identified by observing fewer cell pixels or higher average phase values per FOV. Plots of average phase per cell pixel are shown for each multiwell plate column in Figure 112 and Figure 113 for the 1-hour and 4-hour time points respectively.

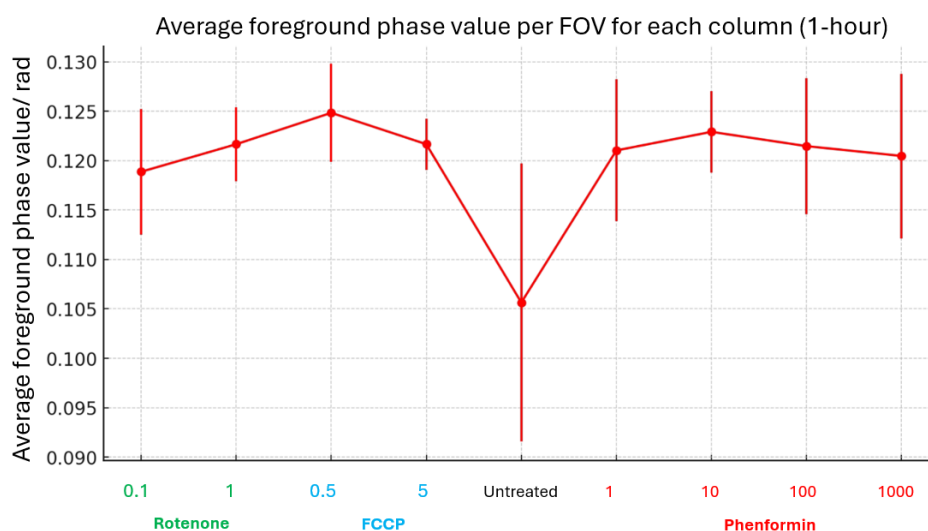


Figure 112 Average phase per segmented foreground pixel in HEK293 cells plotted for each column in the 1-hour experiment.

Figure 112 shows a clear lower average phase value per cell pixel in the untreated cells compared to treated cells 1 hour after treatment. If we attribute higher phase values to more stressed cells, we could expect the untreated cells to have lower average phase values. The average phase value does increase for the higher concentration of rotenone, in agreement with the hypothesis of average cell phase correlating with increased cell stress. Interestingly the average phase increases for the initial phenformin treatments and decreases for the highest doses. The data in Figure 113 at 4 hours after treatment again shows increasing phase values for drug treated cells. The average phase value seen for the plot in Figure 113 is greater than that of Figure 112 because the *pDPC* system was still being optimised during this time and some settings had been changed between the two experiments, however

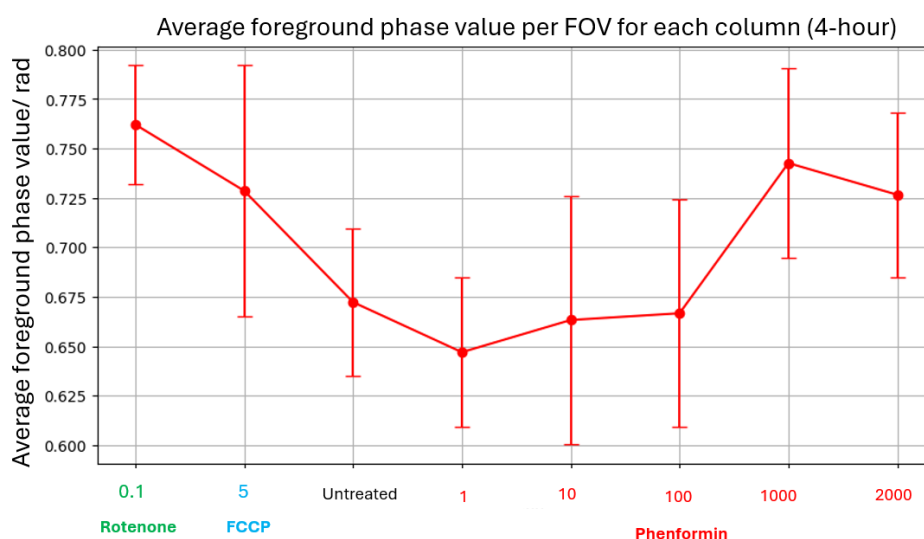


Figure 113 Average phase per segmented foreground pixel plotted for each column in the 4-hour experiment

these data still allow quantitative comparisons of relative phase change within each experiment.

This analysis shows that the average phase (optical path length) per cell pixel may serve as a morphological readout of cell stress that can complement FLIM data. It also demonstrates that labkit can be used to segment the cells in order to calculate the average phase per cell pixel.

6.3 Multiwell plate FLIM of NADH reporting dose response of cells to phenformin in OVCAR-4 spheroids

A multiwell plate experiment was carried out using the M³M to investigate the dose response of OVCAR-4 spheroids to phenformin. Having seen a dose response of decreasing lifetime with respect

to increasing concentration in cell monolayers, we were interested to compare this to the dose response of cells in 3D, using this to explore the capabilities of the M³M.

6.3.1 Acquisition details

To produce the OVCAR-4 spheroids a cell suspension was mixed with basement membrane extract (BME) (R&D Systems, #3533-005-02). The BME/cell mixture was placed into the wells of a 96-well plate which was incubated at 37 °C for 20 minutes to allow BME polymerisation. 3D medium was added to each well to cover the BME droplets. 3D media consisted of: DMEM (Gibco 11965092), supplemented with 10% heat-inactivated foetal bovine serum (Gibco 10270-106), 2 mM L-Glutamine (Thermo Fisher Scientific 25030-081) and 100 ug/mL penicillin/streptomycin (P/S) (Thermo Fisher Scientific 15070-063) 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).

Spheroids were cultured for 7–10 days and imaged in CO₂-dependent imaging medium. With this 3D cell culture method, cells can sometimes grow on the coverslip in a monolayer as well as in spheroidal structures. The plate layout is shown in Figure 114 and consisted of 3 columns of untreated wells (in columns 3, 9 and 10) with increasing doses of phenformin for columns 4–8 in concentrations of 1, 10, 100, 1000 and 2000 µM respectively.

The spheroids were initially located using the brightfield imaging modality in a pre-find procedure to create a position list with the XY locations of the 64 spheroids. The midplane of the spheroid was approximately located using the multiphoton imaging capability such that a Z value was added to each

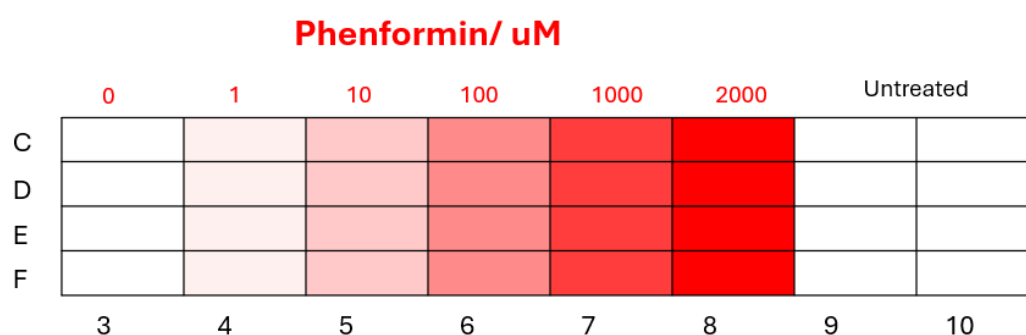


Figure 114 Plate layout for NADH FLIM phenformin dose response in OVCAR-4 spheroids.

of the XY locations in the position list. 2 FOVs of midplanes in separate spheroids were imaged per well over 32 wells resulting in a total of 64 images. A 60 second FLIM data acquisition was carried out for each FOV using 20 mW per excitation beam at the sample plane, resulting in a ~1 hour total FLIM

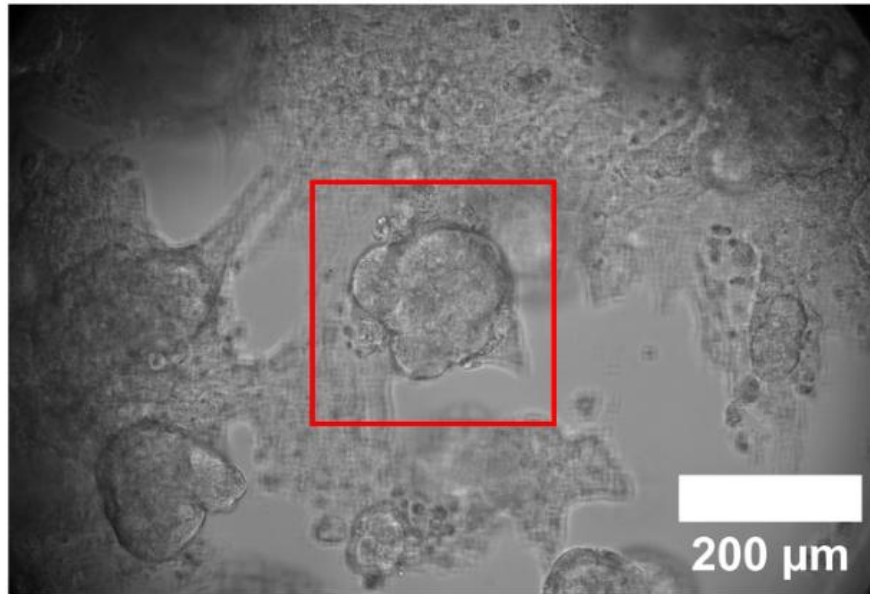


Figure 115 Brightfield image of OVCAR4 spheroid used in pre-finding step. Monolayer cell colonies are seen alongside the spheroid. Red square shows multiphoton FOV.

data acquisition time. Acquisitions were carried out at two time points: at 1 hour and 4 hours after drug treatment. Multiphoton excitation at 750 nm was used with a 450/60 nm emission filter. A representative brightfield image acquired during the pre-find procedure is shown in Figure 115, with the corresponding multibeam multiphoton fluorescence FOV also indicated.

6.3.2 Phenformin dose response of OVCAR-4 spheroids at two time points

Figure 117 shows intensity weighted mean lifetime images taken across the plate for the FLIM data acquisitions at both 1 and 4 hours. FLIM data were globally fitted to a double exponential model yielding component lifetimes of approximately 400 and 4100 ps. A minimum number of photons per

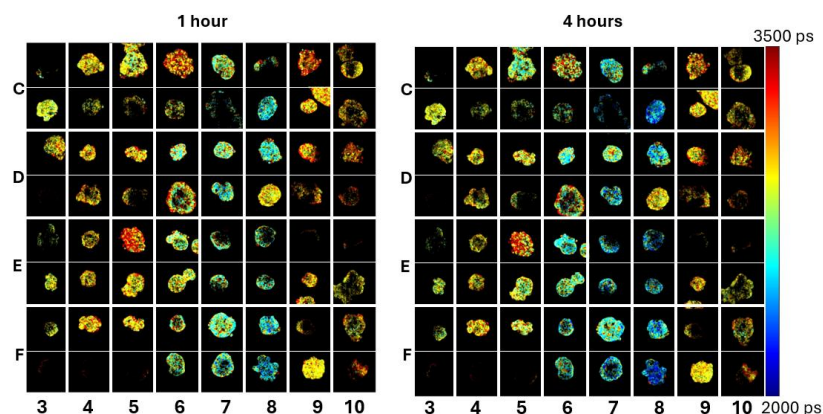


Figure 117 NADH FLIM multi-well plate acquisition of phenformin dose response in OVCAR-4 spheroids 1 (left) and 4 (right) hours after drug application.

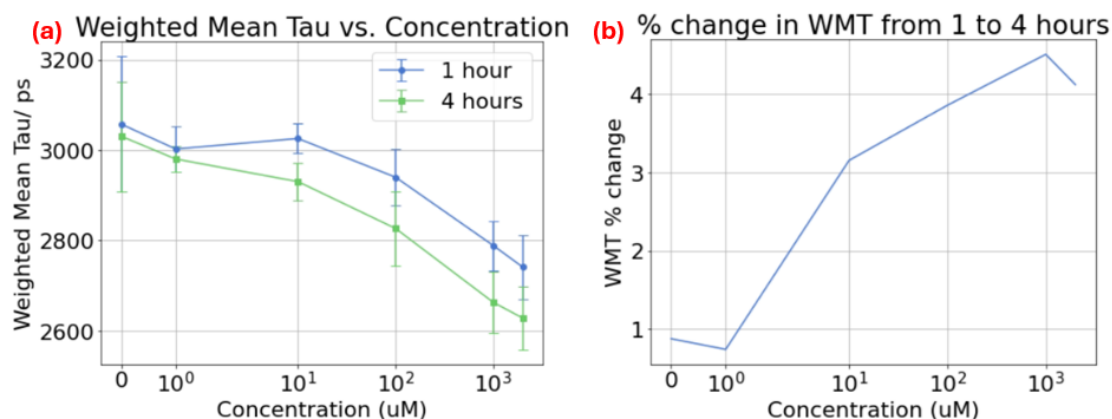


Figure 116 Quantification of OVCAR-4 spheroid response to phenformin. (a) Weighted mean lifetime for 1 hour and 4 hour measurements and (b) % change in weighted mean lifetime plotted as a function of concentration on a logarithmic scale. 8 spheroids were imaged per concentration when phenformin is applied and 24 for untreated spheroids.

pixel of 85 and 5 x 5 smoothing were used to produce accurate lifetime fitting. Manual segmentation was applied to each of the 64 FOVs in the 1-hour dataset. These segmentation masks were then applied to the 4-hour data set as the cells had not moved significantly.

Figure 116 presents the weighted mean lifetimes for each condition and the % change in lifetime between time points as a function of phenformin concentration. These plots show a decrease in the

weighted mean lifetime as a function of increasing phenformin concentration for both time points and that the % change between time points increases for greater concentrations. Similar plots for NADH fluorescence intensity are shown in Figure 118.

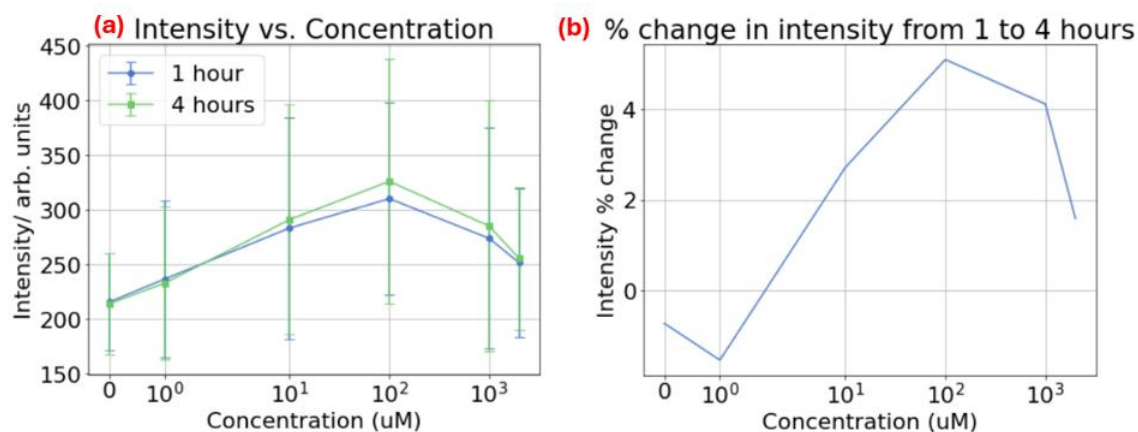


Figure 118 OVCAR-4 NADH fluorescence (a) intensity for 1 hour and 4 hour measurements and (b) % change in intensity plotted as a function of phenformin concentration on a logarithmic scale. 8 spheroids imaged per concentration for phenformin treated spheroids and 24 for untreated spheroids.

As expected, the application of phenformin to the spheroids resulted in a reduction of the weighted mean lifetime in a manner proportional to the dose applied. This can be attributed to greater levels of free NADH due to the reduction in oxidative phosphorylation. The decrease in lifetime from the 1-hour to 4-hour time point is most likely due to more engagement of phenformin in the cell.

The intensity plots show an increase in intensity with respect to phenformin concentration until the two largest doses. As treatment with phenformin increases the amount of NADH relative to the non-fluorescent NAD^+ in the cell, this increase in intensity seems reasonable. The decrease in intensity for concentrations greater than 100 micromolar may indicate the cells are now dying. Intensity measurements are less reliable readouts due to their dependence on various imaging factors, including depth of z-slice into the spheroid and the presence of any occluding objects in the sample.

Figure 119 shows heatmaps of the average weighted mean lifetime and intensity per well across the multiwell plate. There are some clear systematic trends in intensity across the plate, for example column 9 is consistently brighter than column 10 despite them sharing the same biological conditions. The same systematic trends are not seen in the average lifetime per well shown in the same figure.

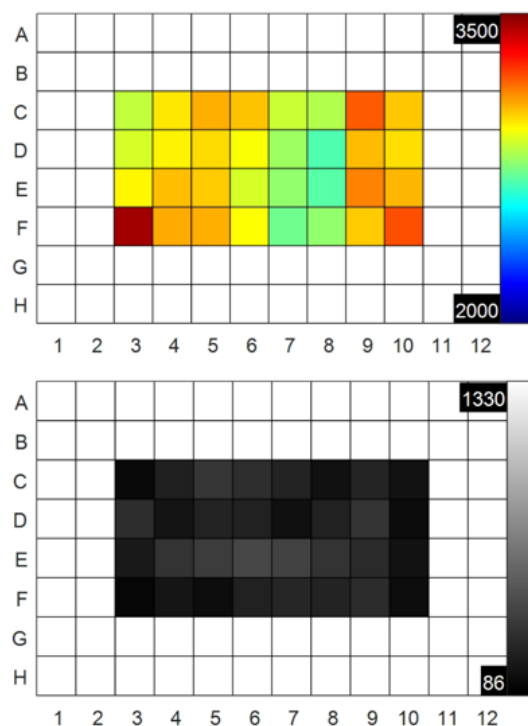


Figure 119 Average weighted mean lifetime (top) and intensity (bottom) per well across the multi-well plate for the measurement taken after 1 hour in phenformin treated OVCAR-4 spheroids.

6.3.3 Variation of phenformin dose response across OVCAR-4 spheroids

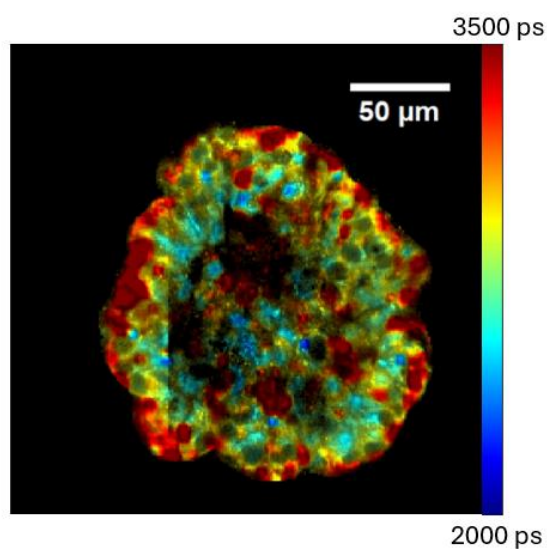


Figure 120 NADH autofluorescence from spheroid in well D6 suggesting difference in mean lifetime between inner and outer regions.

Previous work [75] had reported that the mTq2FP lifetime of the T2AMPKAR biosensor expressed in HEK293 spheroids showed a radial gradient decreasing from the inside to the outside when treated with phenformin – with the longer lifetime of the outer regions indicating less activation of the biosensor. Previous work has shown an increasing trend in NADH lifetime as a function of distance from spheroid centre [185]. It was noted that some of the OVCAR-4 spheroids imaged here showed similar characteristics; one such spheroid is shown in Figure 120. Analysis was carried out to determine if there was a significant difference between inner and outer regions in the spheroid across the plate. To undertake a plate-wide analysis, a python script was written to load the FLIM images and create a mask around the region of interest (spheroid) using intensity thresholding. A second mask was made by shrinking the first mask by 25% in order to select the inside of the spheroid. The original mask minus the inner mask then yielded the outer region. Images with spheroid regions below a certain area threshold were discarded as the level of fluorescence didn't allow accurate specifying of inner and outer regions using the method presented. The inner and outer segmented regions along with rejected FOVs are shown in Figure 121.

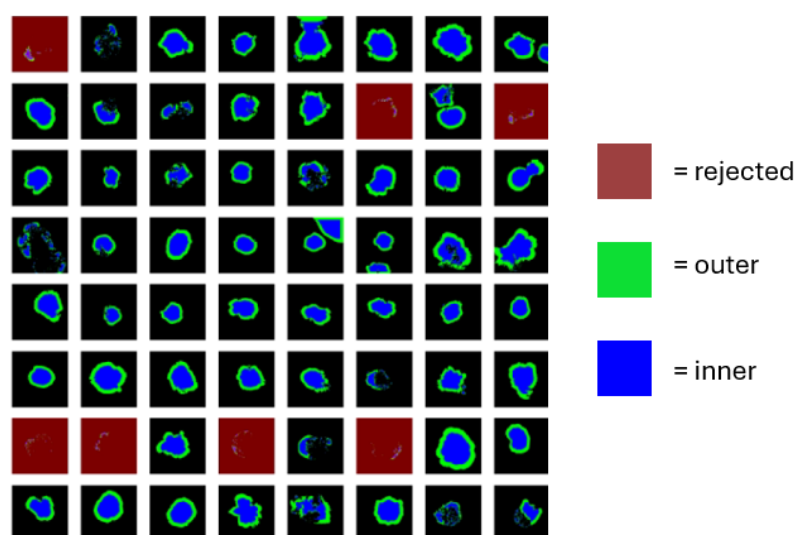


Figure 121 Segmented regions found for inside (blue) and outside (green) of spheroid with rejected FOVs shown in red

Once the segmented masks had been made, the average lifetime within the inner and outer masked regions were calculated for each condition and both time points. A scatter plot showing the difference between mean lifetimes found in the outer and inner regions for both time points is shown in Figure 122. No visually apparent trend can be seen in this data set, although there appears to be a larger variance in the lifetime difference at higher phenformin concentrations. However, this processing pipeline can be applied to future data to explore differences between fluorescence lifetime in outer and inner regions of spheroids.

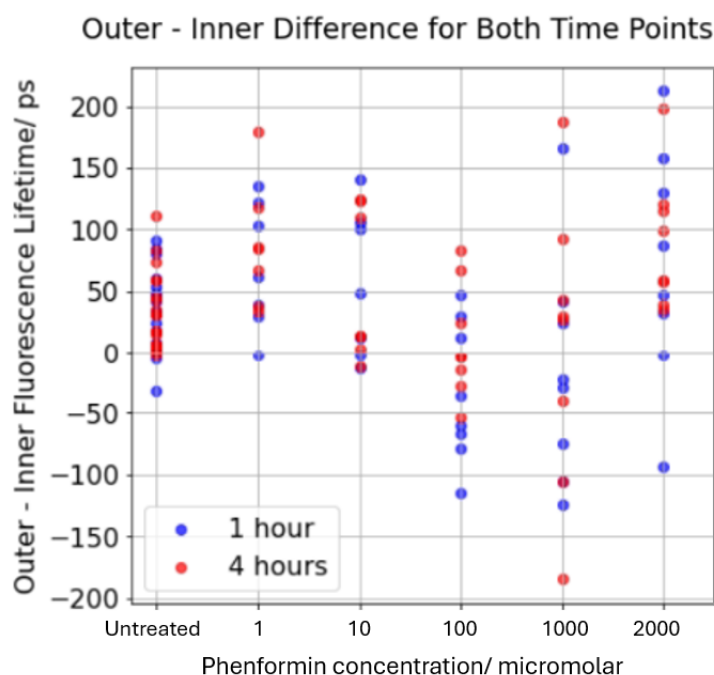


Figure 122 Difference (outer – inner) in fluorescence lifetime between segmented regions shown for both time points as a function of phenformin concentration.

6.3.4 Z-stack imaging

Z-stacks were taken of the OVCAR-4 spheroids in wells C10, F6 and D8 that had been treated with a range of phenformin concentrations. This data was acquired 5 hours after drug application and so could be expected to present larger lifetime deviations than the previously shown dataset. The imaging parameters for the multiwell plate acquisition were the same as for the previous experiment, with z-slices acquired at a z-spacing of 5 microns. FLIM z-stacks for the three treatment conditions are shown in Figure 123. This was done to approximately image to the equator of each spheroid, imaging was undertaken to a depth of 55 microns depth for the spheroid in C10 and to 85 microns depth for the spheroids in F6 and D8. The longer lifetimes seen in the outer region of the F6 spheroid are also seen in the lower z values of the z-stack, imaging the bottom of the spheroid. This suggests a radial trend in lifetime throughout that particular spheroid.

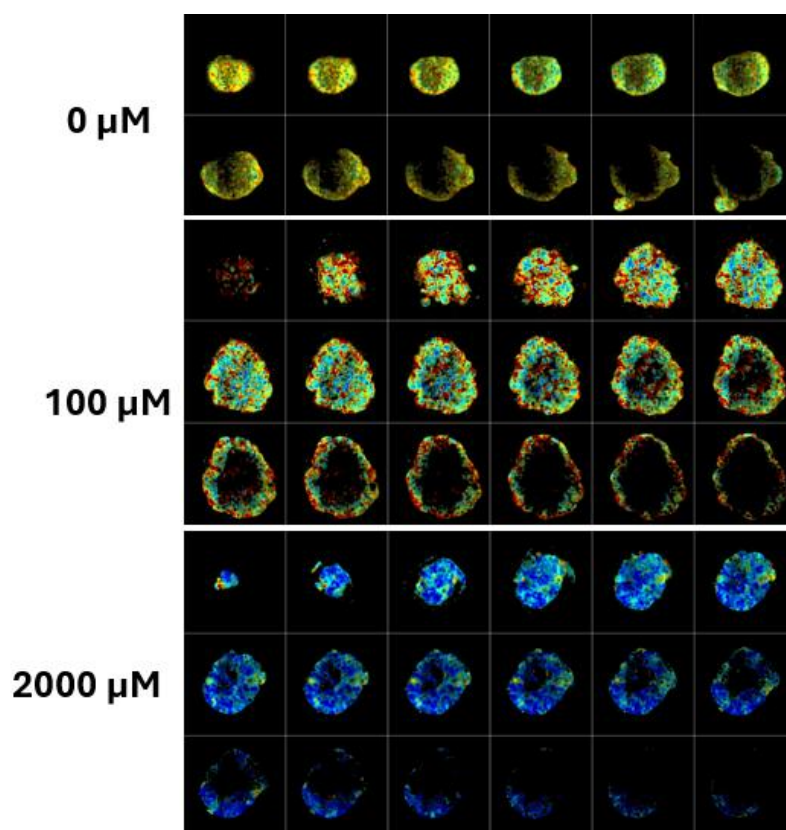


Figure 123 Z-stacks taken of spheroids in 0, 100 and 2000 micromolar phenformin conditions. Z-stacks are shown in a montage with 5 micron increments between images going from left to right and then top to bottom.

6.4 Summary

This chapter has presented 2D and 3D NAD(P)H FLIM assays on the response of cells under the treatment of drugs known to affect cell metabolism. The 2D assays were also combined with quantitative phase imaging to provide complementary readouts showing that the average phase per cell pixel showed higher values in drug treated cells. Work has previously been carried out correlating cell phase values with the progression to apoptosis [183], [184] showing that apoptosis leads to rounding of cells into a smaller volume and therefore higher average phase values per pixel. This is the first work to the author's knowledge using quantitative phase and FLIM readouts simultaneously in a multiwell plate assay, although interleaved phase contrast and fluorescent lifetime imaging has been carried out previously at Imperial [186].

The combination of phase contrast and NADH FLIM readouts offers multiple potential benefits due to the label-free nature of both modalities. The multibeam multiphoton and phase contrast imaging techniques both allow measurements with reduced phototoxicity levels compared to conventional multiphoton fluorescence microscopy, which may allow longer time lapse experiments to be carried out, revealing more information on cancer cell dynamics, for example, varying across multiple cell cycles. As no external fluorophore needs to be introduced for the imaging methods, e.g., using genetic engineering, cells remain close to their native state. Thus, assays may be carried out on primary cells from patients [187] which could ultimately lead to advances in personalised medicine [188].

There are a few previous studies have realised multiphoton 3D NADH FLIM assays in spheroids. The work done in [189] showed spatial metabolic changes in breast cancer spheroids, work in [185] investigated the sensitivity of quiescent cancer cells in bone marrow spheroids and the study in [190] investigated the metabolic response of breast tumour spheroids to novel compounds. Of these only [185] implemented multiphoton FLIM in a multiwell plate assay, albeit with a single excitation beam. It is hoped that the M³M can support further such studies at higher throughput and with reduced phototoxicity.

7 Application of the M³M to investigate cellular rucaparib dynamics

The previous chapter illustrated the capability of the M³M to facilitate label-free assays of primary cells with no requirement for genetic manipulation in order to express fluorescent proteins or other genetically expressed constructs. This chapter continues the theme of applicability to primary cells with no genetic manipulation and explores the ability to image intrinsic fluorescence of a cancer drug called rucaparib [191]. The drug is known to bind to the Poly(ADP-ribose) polymerase 1 (PARP1) DNA repair molecule, disabling one of the DNA repair pathways found in cells therefore causing a synthetic lethality in cancer cells. The work reported here extends an earlier study [191] where the fluorescence properties of rucaparib in cells were characterised.

7.1 Introduction

7.1.1 Biological background

DNA damage, including single-strand and double-strand breaks, can lead to cell death and so DNA damaging agents are used for cancer therapy. However, cells have multiple pathways to repair DNA strand breaks. PARP1 is a key molecule involved in the repair of DNA strand breaks, binds to DNA damage sites and recruits further molecules that aid in fixing the DNA damage. PARP inhibitors (PARPi) are a class of cancer drug that trap PARP at a DNA damage site, removing the ability for cells to repair further DNA breakages through the PARP pathway, and causing further toxicity to the PARP trapped DNA breakage site. A normal functioning cell has multiple DNA repair pathways and so a PARP inhibitor will not necessarily be lethal. However, some cancer cells contain mutations in certain genes, such as the BRCA gene, that limit the cells' ability to repair DNA damage through pathways other than PARP. This results in PARP inhibitors being specifically lethal to cancer cells, especially when used in conjunction with DNA damaging compounds [192]. PARP inhibitors have shown great efficacy in treating cancer in some patients, however usually cancer recurs as patients become resistant to the cancer drugs [193]. Understanding mechanisms of drug resistance is a vital area of cancer research, for which it is important to understand the interactions of cancer drugs within cells. Rucaparib is a PARP inhibitor that is fortuitously intrinsically fluorescent and so provides a means to study how PARPi interact with cells.

Previous single photon widefield fluorescence imaging of rucaparib [191] in cells showed that the drug presents a high fluorescence signal in the nucleus, where the majority of PARP is expected to be found. Treatment with rucaparib was found to result in puncta of high fluorescence intensity outside of the nucleus after ~1 hour. Furthermore, the fluorescence lifetime of rucaparib was measured in

solutions with a range of pH levels and found to decrease from ~7 ns at neutral levels to ~5 ns in more acidic environments.

7.1.2 Initial rucaparib imaging experiments

We first tested whether rucaparib could be imaged with the M³M using multiphoton excitation, for which OVCAR-4 cells were treated with 10 μ M rucaparib and left for 2 hours before imaging. Consistent with the single-photon excitation spectrum previously reported [191], a two-photon excitation wavelength of 750 nm in combination with a 460/50 nm emission filter was found to be suitable for rucaparib excitation – and is less damaging than single-photon excitation in the ultraviolet spectrum. Given that NADH is imaged in the same channel, some untreated cells were also imaged to see the extent to which NADH would bleed through into the rucaparib channel. The rucaparib to NADH signal ratio was approximately 60x. However, it is important to note that, due to the short lifetime of NADH compared to rucaparib, the NADH fluorescence still must be accounted for in FLIM data analysis. FLIM data was analysed in *FLIMfit* using a 5x5 pixel smoothing mask and fitting pixel wise to a double exponential decay model, with one exponential accounting for short lifetime autofluorescence components and the other for the longer rucaparib lifetime.

As shown in Figure 1, the fluorescence lifetime image recapitulated the bright puncta seen in the previous work [1], with the bright puncta presenting a much shorter lifetime than the fluorescence from the surrounding cytoplasm. The fluorescence lifetime of rucaparib in the nucleus was close to the previous value reported for a neutral pH, whereas the puncta in the cytoplasm (seen in blue in Figure 124) presented a shorter lifetime suggesting a more acidic pH. It was observed that the distribution of

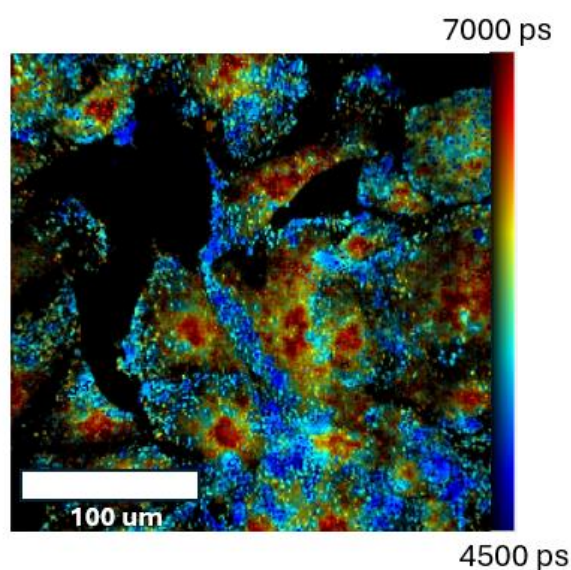


Figure 124 M3M FLIM image of rucaparib in OVCAR-4 cells.

bright puncta presenting a lower lifetime seemed similar to the expected distribution of lysosomes in the cell – noting that lysosomes have an acidic environment, which could be the possible cause of lower lifetimes found in those regions. Intermediate lifetime values between those of the bright puncta (blue) and nucleus (red) are also seen.

7.2 2D rucaparib multiwell plate assay

To explore the action and compartmentation of rucaparib, we imaged 2D OVCAR-4 cell monolayers treated with rucaparib (5 μ M) and with the addition of lysosome staining (LysoTracker Red 50 nM) or nuclear staining (SYTO Deep Red 1 μ M) dyes, and/or a different PARP inhibitor (olaparib 50 μ M [194]) and a lysosome inhibitor (bafilomycin 50 nM [195]) within a multiwell plate for which the layout of the plate is indicated in Figure 125. Two FOVs in each well of the plate were imaged in the rucaparib channel 2 hours after treatment with the multibeam multiphoton modality with excitation at 750 nm and a 460/50 nm emission filter. The average excitation power in each of the 6 beams was 12 mW per beam and the rucaparib FLIM data acquisition time was 4 seconds per FOV. The same FOVs were then imaged using the single-photon modality in the columns in which dyes were added. LysoTracker Red was imaged using an mCherryFP-like cube (excitation filter 540/30 nm and emission filter 700/75 nm) with excitation at 540 nm, and SYTO Deep Red was imaged with a Cy5 cube (excitation filter 624/40 nm and emission filter 692/40 nm) with excitation at 630 nm. The experiment was carried out on live cells held at 37 degrees Celsius and 5% CO₂ in the incubator. Standard cell culture procedures were carried out in line with the work done in [191].

	2	3	4	5	6	7	8	9	10	11
B										untreat
C										untreat
D										olap10x
E										olap10x
	ruc+syto+lyso+BF1A	ruc+syto+lyso	Olup10X-Ruc1X	Olup10X-SYTO-Ruc1X	Ruc		ruc+BF1A	ruc+syto	ruc + lyso	

Figure 125 Multiwell plate layout used for variety of rucaparib experiments using rucaparib (ruc) in OVCAR-4 cells, LysoTracker Red (lyso), SYTO Deep Red (syto), the olaparib PARP inhibitor (Olap) and a lysosome inhibitor (BF1A). 1x and 10x indicates 5 and 50 μ M concentrations

7.2.1 Variation of rucaparib lifetime in different cellular compartments

After observing the spatial variation of rucaparib lifetime in the OVCAR-4 cells, experiments were undertaken to see whether rucaparib reported different lifetimes in different cellular

compartments. By inspection, the longer rucaparib lifetime regions appeared in the cell nuclei, and the regions presenting shorter rucaparib lifetimes appeared to be associated with lysosomes- which was reasonable since lysosomes are expected to present a more acidic environment. To confirm this hypothesis, the longer and shorter lifetime regions were correlated with fluorescence images of SYTO Deep Red and LysoTracker Red stained cells.

To aid image registration, an image of the SYTO Deep Red channel was taken using both single photon and multiphoton modalities. Figure 126 shows an overlay of multiphoton excited and single-photon excited SYTO Deep Red intensity images of the same FOV from well B9. The multiphoton excitation was at 850 nm imaged using a 643/50 nm emission filter. Co-registering the images in Figure 126 required a rotation to account for the scan angle of the galvo scanners, a scaling of the wide-field camera image to account for the different pixel sizes compared to the multiphoton image, a translation to account for a small mismatch of the two FOVs, and a crop of the wide-field fluorescence image to match the FOV of the multiphoton image.

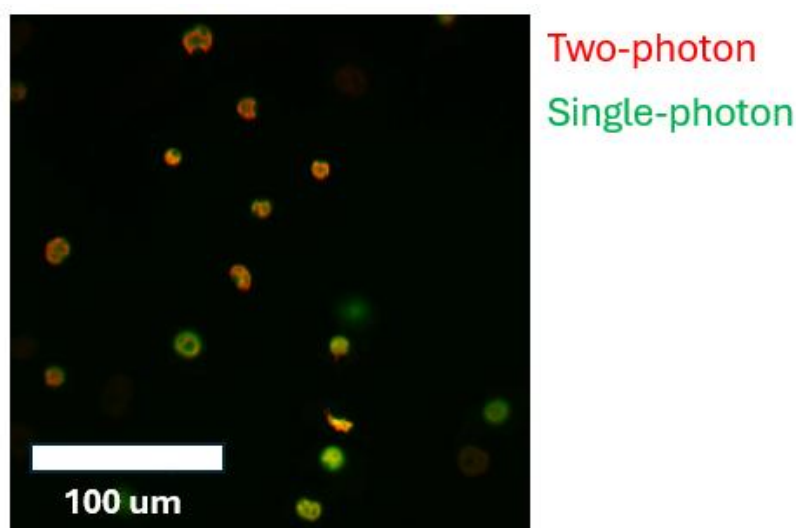


Figure 126 Co-registered image of SYTO Deep Red nuclear staining with two-photon imaging shown in red and single-photon imaging shown in green.

7.2.1.1 Rucaparib in lysosomes

Figure 127 shows the rucaparib FLIM and LysoTracker Red images of OVCAR-4 cells in the same field of view and their overlay with a binary mask applied to the lysosome channel. It is clear from inspection that the lysosome channel overlaps with the regions of lower rucaparib fluorescence lifetime in the cell. To show this more quantitatively, the LysoTracker Red image was converted into a binary mask, converting pixels above a certain intensity threshold to the value of 1, and below the threshold to 0. It is worth noting there were regions set to 0 with some portion of LysoTracker signal,

indicating that there may be high density aggregations of lysosomes as well as more homogenously distributed regions, this is supported by work done in [196] with more information on the distribution

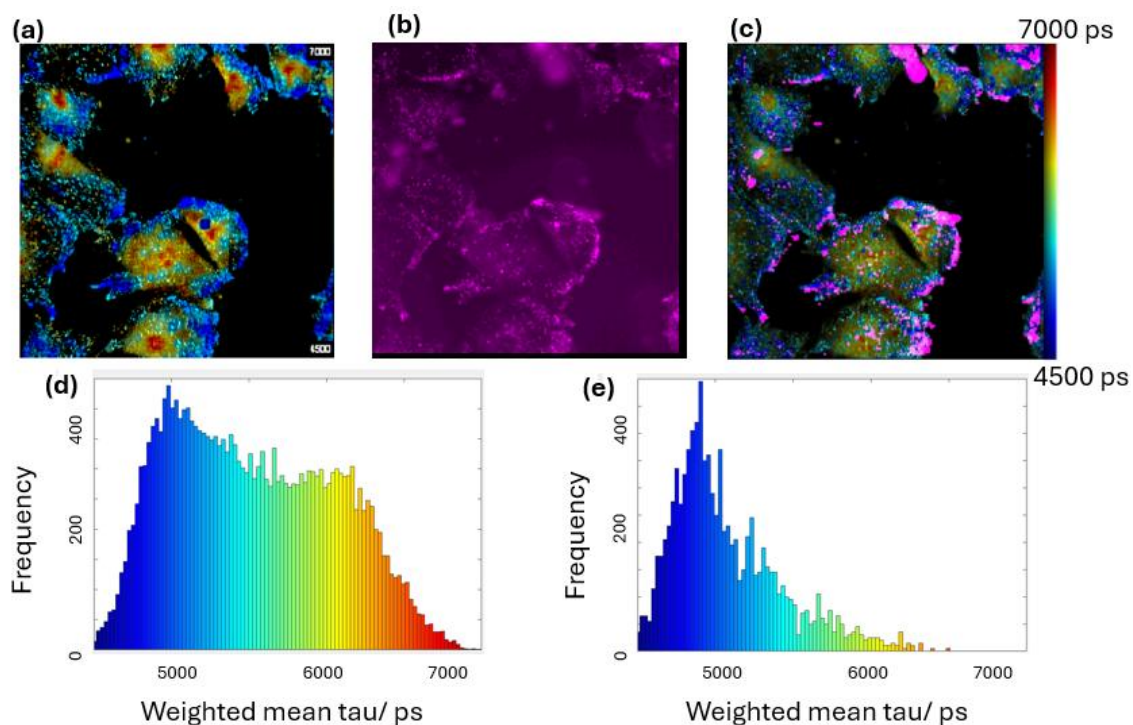


Figure 127 OVCAR-4 cells and (a) rucaparib M3M FLIM image (b) wide-field single-photon excited LysoTracker Red image, and (c) overlay image using binarised mask of LysoTracker Red image, as well as lifetime histograms of (d) unsegmented and (e) LysoTracker mask segmented images of OVCAR-4 cells from well D10 of multiwell plate.

of lysosomes in the cell found in [197]. The binary image was then used to segment the rucaparib fluorescence lifetime image. The rucaparib lifetime histogram of lifetimes for the unsegmented and segmented rucaparib lifetime images are shown in the bottom row of Figure 127, confirming the correlation of with the lower lifetime regions. Similar correlation was observed for other FOV imaged in rows B-E of column 10.

7.2.1.2 Rucaparib in nuclei

The same approach was applied to the nuclear stained cells in well D9 of the multiwell plate. Figure 128 presents a FLIM image of the rucaparib channel, the corresponding SYTO Deep Red fluorescence image of the nuclear channel and an overlay that indicates the longer rucaparib lifetime regions coincide with the nuclear stain. A binary mask was made using the SYTO Deep Red image to segment the fluorescence lifetime image and lifetime histograms were again produced of the complete FOV and nuclear masked lifetime images, as shown in the bottom row of Figure 128. Applying the nuclear segmented mask caused the histogram to be weighted towards longer lifetimes. Further measurements of FOVs in all of the wells in column 9 similarly presented the regions of longer lifetimes co-localised with the nuclear SYTO Deep Red stain.

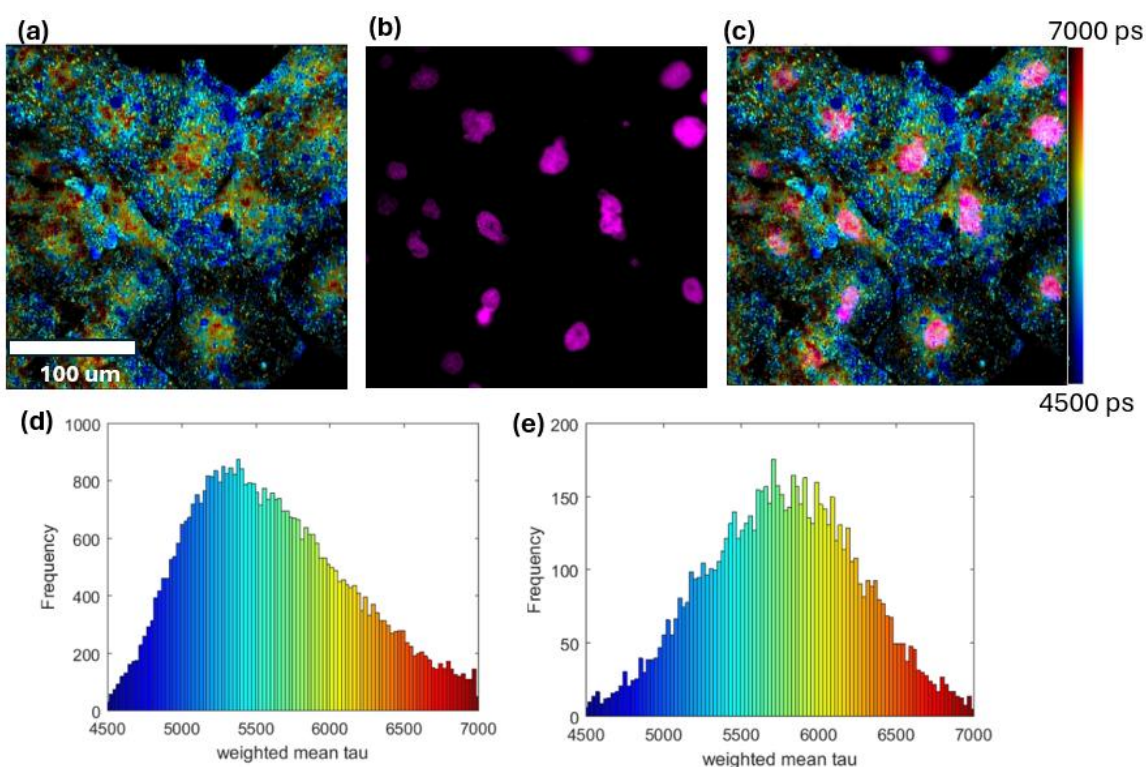


Figure 128 OVCAR-4 cells. (a) Rucaparib M3M FLIM image (b) wide-field single-photon excited SYTO Deep Red image, (c) overlay image using binarised mask of SYTO Deep Red image, as well as lifetime histogram of (d) unsegmented and (e) SYTO Deep Red mask segmented images of OVCAR-4 cells from D9 of multiwell plate.

It is apparent in the rucaparib fluorescence lifetime images that there are regions of fluorescence that are neither in the nucleus or co-localised with the lysosomes. These contributions of intermediate rucaparib lifetime could be from the drug in transit from the nucleus to the lysosome. One can see that

the longer fluorescence lifetimes (found in the nucleus) are made shorter in the presence of SYTO Deep Red when comparing Figure 127 (d) and Figure 128 (d), this will be elaborated on later.

7.2.2 Verifying lysosomal aggregation of rucaparib using bafilomycin

To further probe the dynamics of rucaparib within the cell, a lysosome inhibitor named bafilomycin 1A was applied to the wells in column 8 to see the effect on the shorter rucaparib lifetime puncta observed, e.g., in Figure 124. Bafilomycin functions by blocking a pump that draws protons into the lysosome, making the lysosomal environment more acidic. The application of bafilomycin therefore reduces the acidity of the lysosome, increasing the pH closer to neutral levels, and also resulting in less affinity of the rucaparib molecule to the lysosome. The rucaparib lifetime data from columns 7 and 8 are presented in Figure 129, comparing rucaparib lifetime with and without bafilomycin treatment.

Figure 129 shows that the application of bafilomycin removes the shorter lifetime pixels seen in the FLIM images of cells in column 7. This was seen consistently over all of the wells in column 8. The

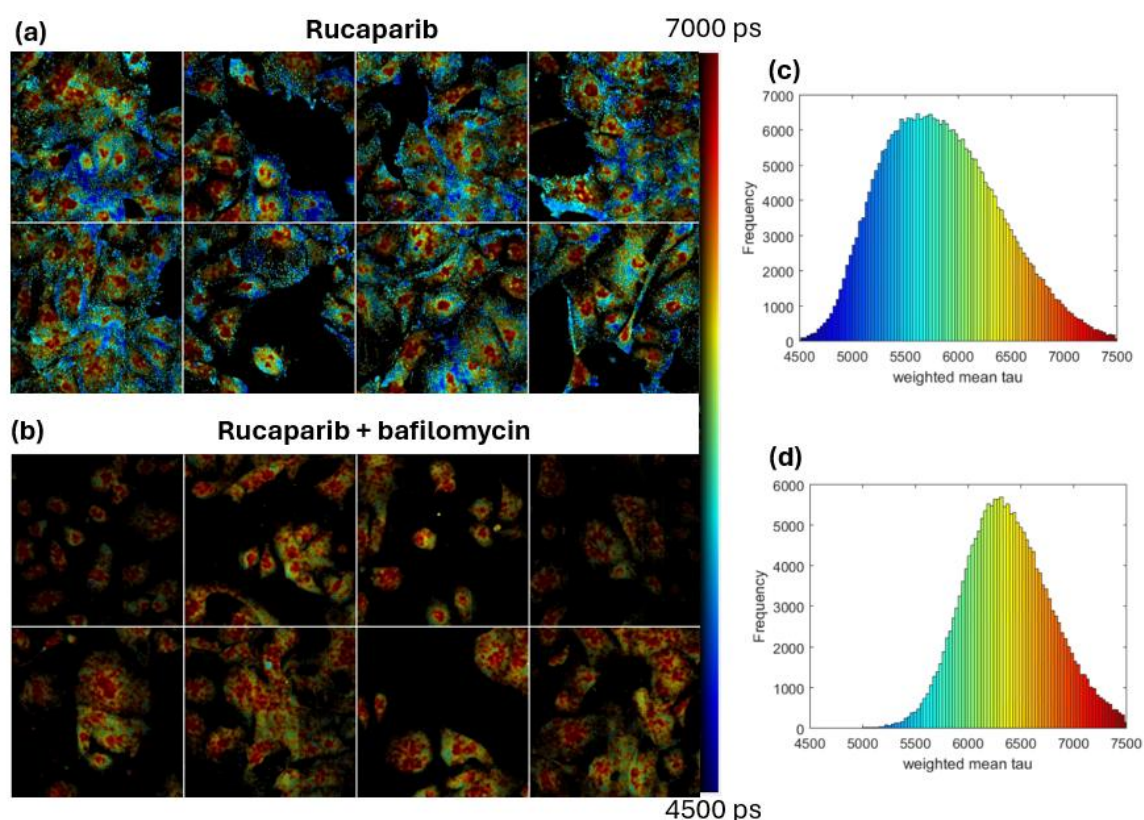


Figure 129 M3M FLIM of rucaparib treated OVCAR-4 cells (a) with and (b) without bafilomycin with their respective lifetime histograms across all 8 images shown in (c) and (d). The addition of bafilomycin removes the low lifetime regions seen in the rucaparib only images.

bright puncta are also removed upon the addition of bafilomycin. This was verified quantitatively by observing that when applying the ‘find maxima’ function in ImageJ to the FLIM images in Figure 129 with the same maxima criteria, 0 maxima were found in the images of cells treated with bafilomycin whereas 1600 were found for the images without bafilomycin. The difference in intensity distributions is further highlighted when looking at the Fourier transform of images from B7 and B8, as shown in

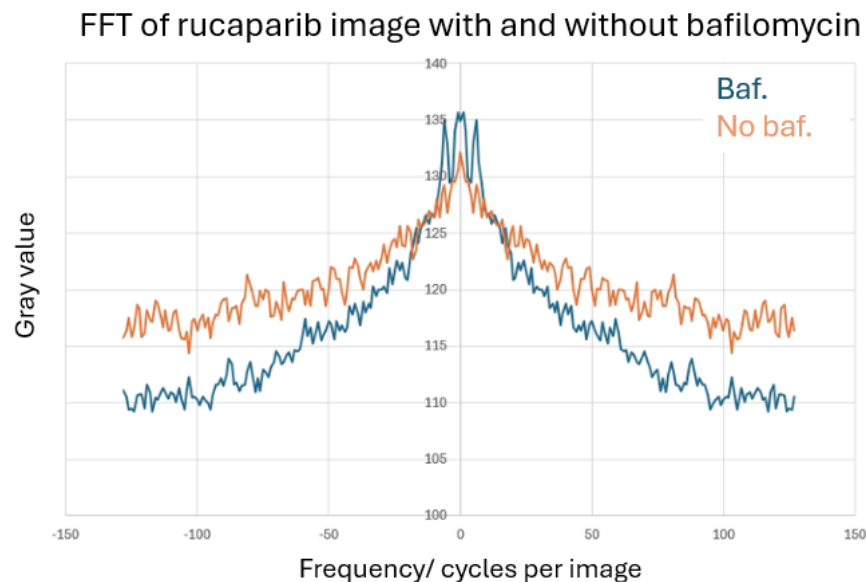


Figure 130 FFT of rucaparib imaged in cells treated with and without bafilomycin.

Figure 130. The FFT of the bafilomycin treated image shows a decrease in high frequency content when compared to the non-bafilomycin treated images. This can be attributed to the fewer puncta within the image.

The addition of bafilomycin removed the bright, short-lifetime puncta indicating that less lysosomal accumulation was occurring due to the neutralised lysosomal pH levels. The images in Figure 129(b) still show that the nuclear regions display a longer lifetime than cytoplasmic regions. This may be due to different fluorescence properties of rucaparib in the nucleus compared to the cytoplasm, due to its DNA binding or the local viscosity, as it is known viscosity can affect lifetime. Another reason could be that the bafilomycin had not inhibited all the lysosomes, therefore meaning some population remain active and reduce the overall lifetime within the cytoplasm.

A box plot of the total intensity per FOV in both conditions is shown in Figure 131, showing that the bafilomycin treated cells also present a lower overall rucaparib fluorescence intensity, possibly due to less drug accumulation in the lysosomes.

7.2.3 Observing the effect of olaparib in rucaparib treated cells

Olaparib is another PARP inhibitor that is used in cancer treatments, however, it does not exhibit significant fluorescence. The effect of adding olaparib and rucaparib simultaneously to the cells was investigated, an experiment that had been carried out in the work done in [191]. Their work showed that by incubating cells with rucaparib (5 μ M) and Olaparib (50 μ M) simultaneously, the rucaparib signal was diminished. This was attributed to the olaparib outcompeting the rucaparib for binding sites in the nucleus, meaning less rucaparib binds and therefore less drug is accumulated in the cell. In our

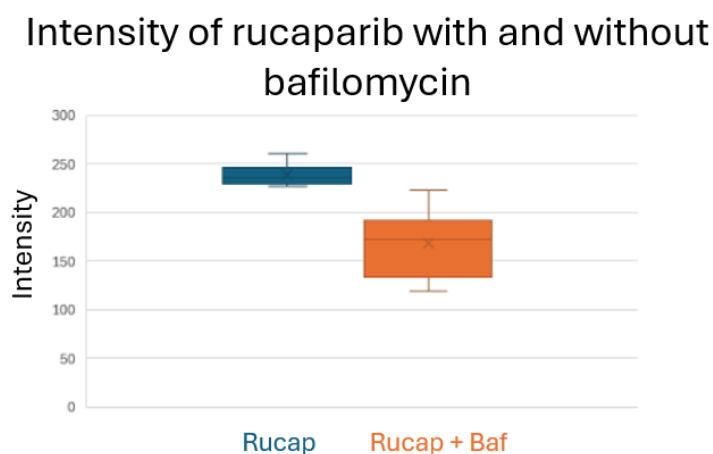


Figure 131 Rucaparib intensity with and without the presence of lysosome inhibitor bafilomycin.

experiment olaparib was added at a 50 μ M concentration, then 90 minutes later a rucaparib and olaparib treatment was applied yielding a 10x final concentration ratio between Olaparib (50 μ M) and rucaparib (5 μ M). The images taken of both conditions show no obvious differences upon observation. However, subtle differences can be seen in the box plots for average intensity and mean weighted lifetime per

well (both calculated across all pixels with counts above a threshold of 75 to reject background pixels) with and without olaparib treatment seen in Figure 132.

The olaparib treated cells show a slightly higher intensity and a slightly lower weighted mean lifetime compared to the non-treated cells. This is in contrast to the previous work showing a decrease in rucaparib signal. However, our experiment used OVCAR-4 cells rather than HT29 cells, which may cause some differences, and also they had taken images ~1 hour after treatment, compared to our images that were ~2 hours after treatment. Further experiments would have to be carried out to determine the exact cause for the data seen in Figure 132. One possibility is that lack of binding sites in the nucleus due to the presence of olaparib means more rucaparib ends up accumulated in the lower lifetime lysosomal regions.

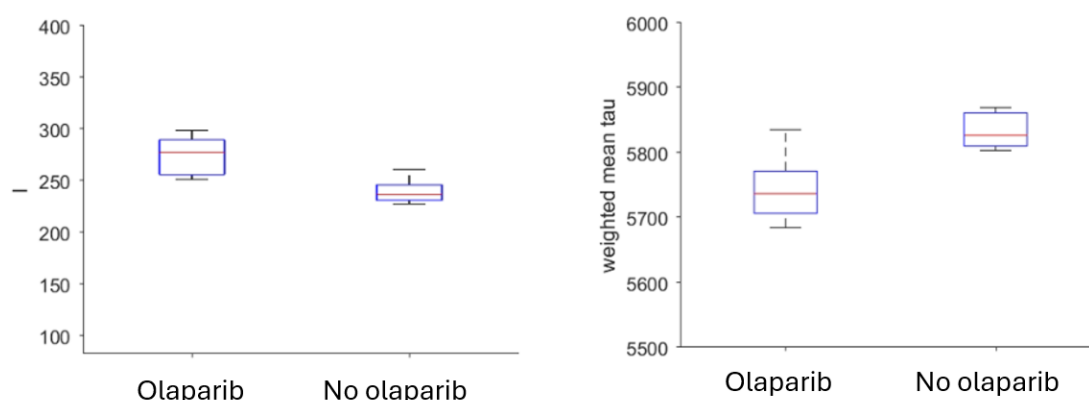


Figure 132 Intensity and weighted mean tau box plots for 5 microM rucaparib treated OVCAR-4 cells with and without prior 50 microM olaparib treatment.

7.2.4 FRET of rucaparib to nucleic dye

It was shown in Figure 127 and Figure 128 that the long rucaparib lifetimes tended towards lower values in the presence of the nuclear dye than in the presence of the lysosome dye. This effect is further shown in Figure 133 which shows the FLIM histograms for rucaparib (with no LysoTracker) and rucaparib with SYTO Deep Red. The figure shows that the addition of SYTO shifts the rucaparib lifetime to shorter values.

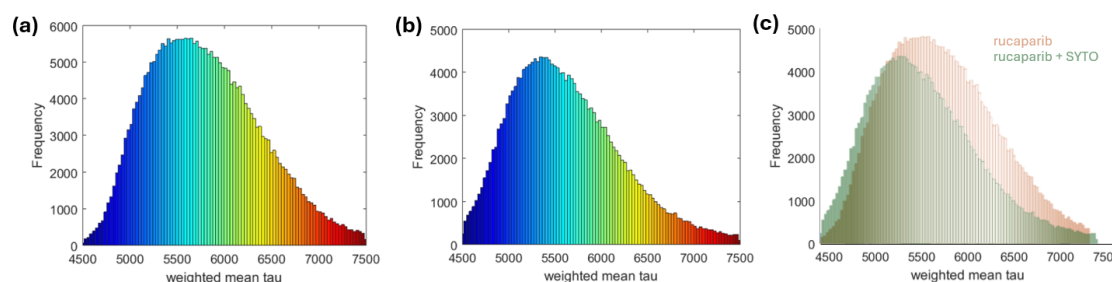


Figure 133 Weighted mean tau histograms OVCAR-4 cells treated with (a) rucaparib and (b) rucaparib with SYTO Deep red nuclear dye, with (c) overlay of the histograms showing rucaparib in orange and rucaparib + SYTO in green. Histograms generated from 8 FOVs per condition.

The possibility of this lifetime shift being caused by bleed-through of the SYTO deep red dye into the rucaparib channel seems small given the negligible overlap of the SYTO emission spectra with the emission filter used. This is shown in Figure 134. Actual imaging of cells with SYTO deep red and no rucaparib in the rucaparib channel would further verify this point, however unfortunately this data was not taken at the time.

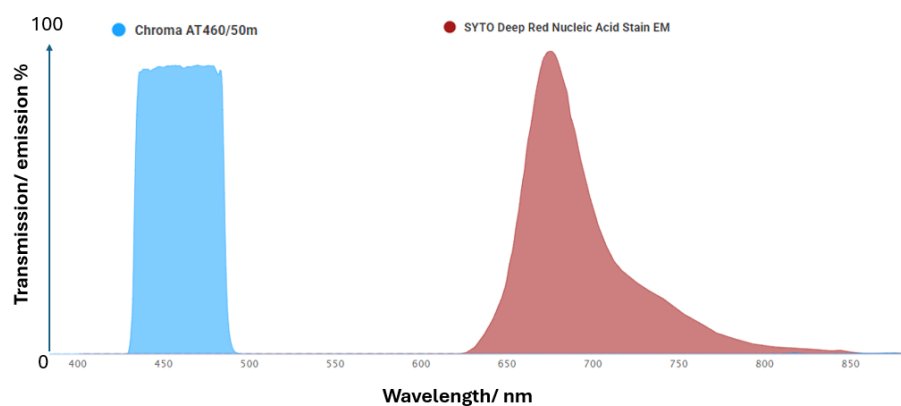


Figure 134 Emission spectra of SYTO deep red dye plotted with the transmission spectra of the emission filter used for rucaparib imaging.

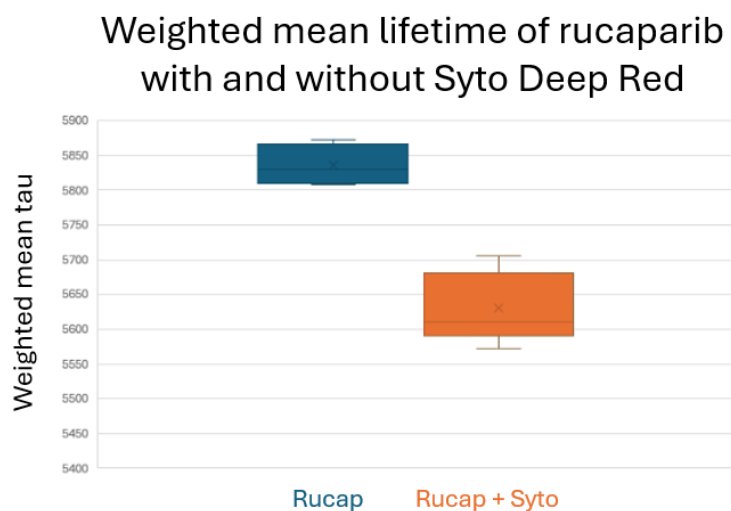


Figure 135 Box plots of weighted mean lifetime of rucaparib per well for cells treated with and without SYTO deep red.

The box plots in Figure 135 show how the average weighted mean lifetime per well is ~200 ps lower for rucaparib when in the presence of the nuclear dye. This could be due to FRET of rucaparib with SYTO deep red.

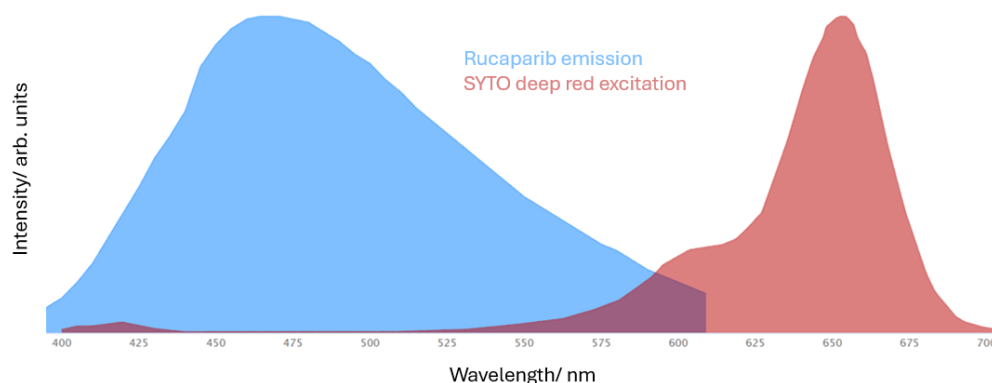


Figure 136 Spectral overlap of the emission spectra of rucaparib and excitation spectra of SYTO Deep dye.

The spectra shown in Figure 136 show that the emission spectra of rucaparib and excitation spectra of SYTO deep red provide a sufficient overlap for FRET. As the SYTO Deep Red dye and the rucaparib both bind to DNA it is reasonable to expect the two molecules to be co-localised to within ~10 nm.

The emission spectra of rucaparib also overlaps with the excitation spectra of the lysosome dye, however due to the already decreased rucaparib lifetime in the lysosome from the low pH levels it may be that the FRET effect is less significant and therefore it wasn't observed.

7.3 3D rucaparib penetrance timelapse in spheroid

The 3D imaging capability of the M³M was applied to study the penetrance of rucaparib into a live OVCAR-4 cell spheroid over time using both intensity and fluorescence lifetime readouts. The experiment was carried out on live cells held at 37 degrees Celsius and 5% CO₂ in the incubator. Drug penetrance follows different dynamics in 3D and can be a very useful readout in evaluating drug efficacy [198].

7.3.1 Experimental description

To produce the OVCAR-4 spheroids a cell suspension was mixed with basement membrane extract (BME) (R&D Systems, #3533-005-02). The BME/cell mixture was placed into the wells of a glass imaging 96-well plate (SENSOPLATE MICROPLATE, 96 WELL, PS, F-BOTTOM) which was

incubated at 37 °C for 20 minutes to allow BME polymerisation. Spheroids were cultured for 6–11 days and imaged in CO₂-dependent imaging medium. This method produces spheroids evenly distributed throughout the BME droplet. Given that the droplet is in contact with the coverslip, some of the spheroids will be closer to the coverslip and within the 1 mm working distance of the objective.

A spheroid of 150 microns diameter that was sufficiently near to the coverslip to be imaged was found and the multiphoton excitation was set to image the midplane of the spheroid. Rucaparib was added to the well and the spheroid was imaged (with excitation at 750 nm using a 450/60 nm emission filter) every 10 seconds for 6 minutes using the multibeam multiphoton excitation modality. Imaging started 15 seconds after the initial drug application. A segmentation mask was created for the inner and outer regions of the spheroid and applied to each frame in the time series, as shown in Figure 137.

7.3.2 Results

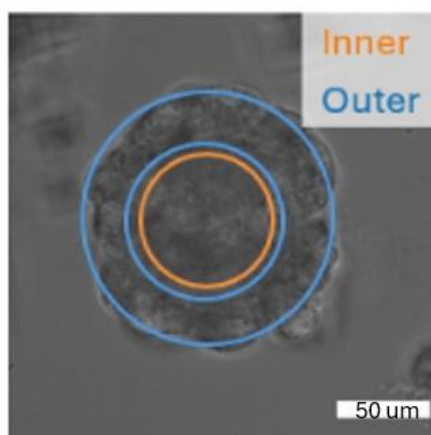


Figure 137 OVCAR-4 spheroid with inner and outer segmentation regions shown.

Figure 138 shows the intensity and weighted mean lifetime images taken during the first 3 minutes of the acquisition. The lifetime fitting was carried out with a global double exponential fit per segmented region per frame.

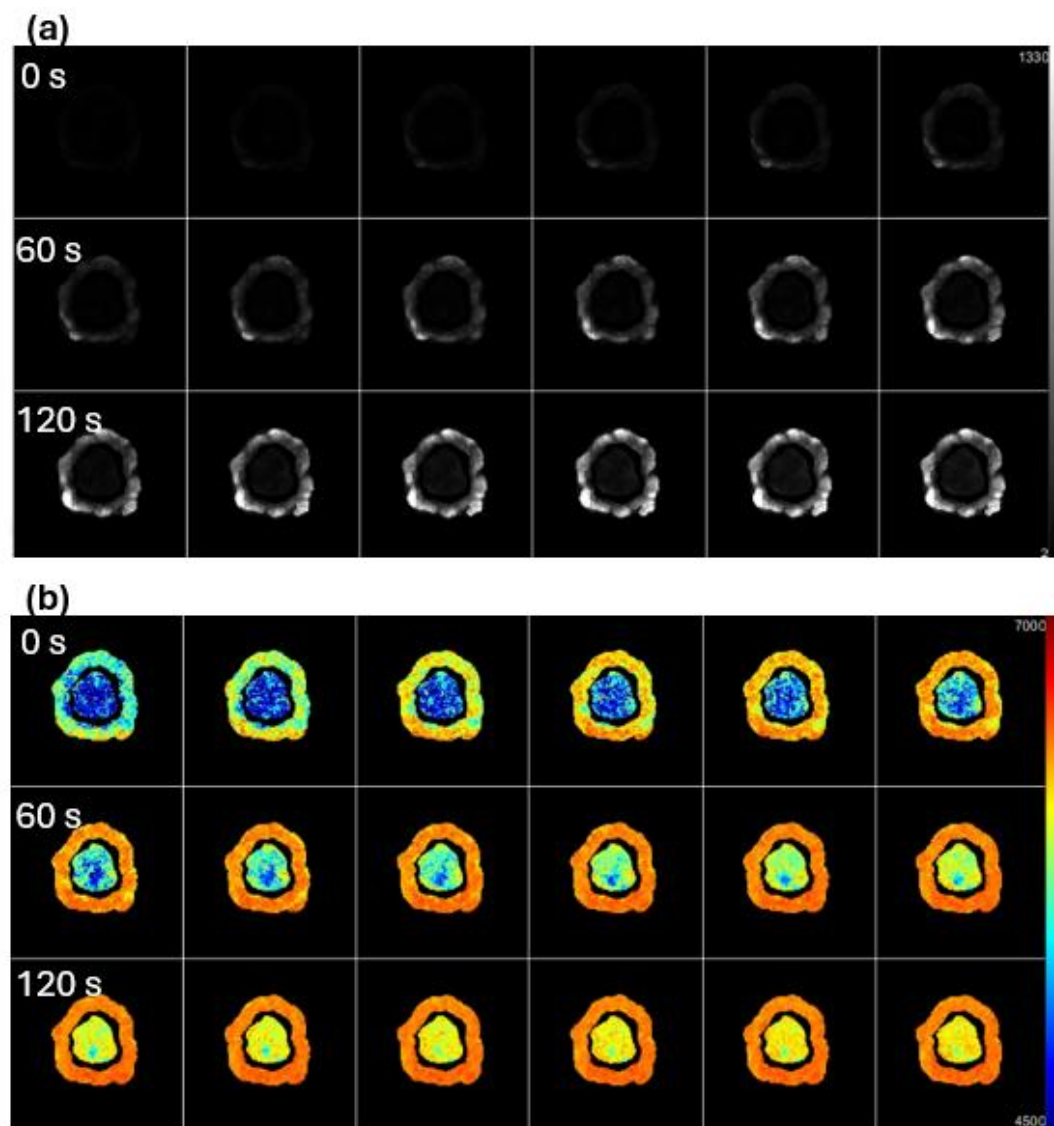


Figure 138 (a) Intensity and (b) weighted mean lifetime in rucaparib treated spheroid shown at 10 second intervals for inner and outer segmented regions.

Figure 139 shows that over time the average fluorescence intensity per segmented region in both the inner and outer regions increased for 3 minutes into the acquisition, with the inner region exhibiting a lower rucaparib fluorescence intensity throughout the time-series. After 3 minutes the intensity decreased, possibly due to a different plane being imaged in the spheroid due to movement of the spheroid. This was supported by the images taken showing a different distribution of cells from the 3-minute time point.

Figure 140 shows the intensity of both inner and outer regions normalised to their maximum values, which shows that the inner region exhibited a delayed increase in intensity compared to the outer region. These results illustrate that the rate of drug uptake varies between inner and outer regions as expected for a diffusion process. It is challenging to quantify intensity differences between the inner and outer regions as attenuation of excitation light due to scattering will have diminished the signal in the centre of the spheroid more than the outside.

The rucaparib imaging channel also allowed the collection of NADH fluorescence, so NADH autofluorescence would be the dominant signal in the absence of rucaparib. NADH exhibits a lower weighted mean lifetime (~ 3 ns) than rucaparib (~ 7 ns), resulting in an increase in the mean fluorescence lifetime as more rucaparib is present. This is seen in Figure 141 which shows the variation of weighted mean lifetime as a function of time for the inner and outer regions. The higher initial lifetime value at the outside of the spheroid can be attributed to some amount of drug penetrance already having occurred, as imaging started 15 seconds after drug application.

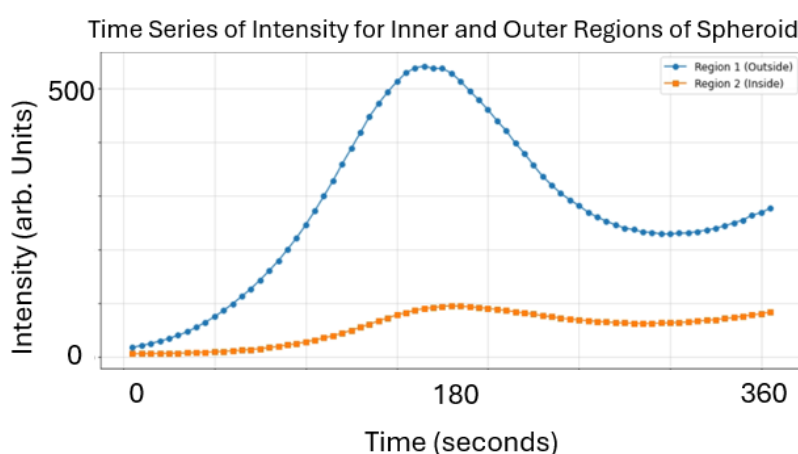


Figure 139 Average rucaparib intensity in OVCAR-4 spheroids as a function of time for the inner and outer segmented regions over 6 minutes.

Figure 141 shows that the weighted mean lifetime converges to a value of 6250 ps for both inner and outer regions by the end of the acquisition, with the outer region having converged to this value at ~ 75 seconds and the outer region at ~ 200 seconds. The variations in intensity seen after 180 seconds in Figure 139 don't affect the weighted mean lifetime significantly indicating that lifetime is a more robust readout of drug penetrance in the presence of small movements of the spheroid.

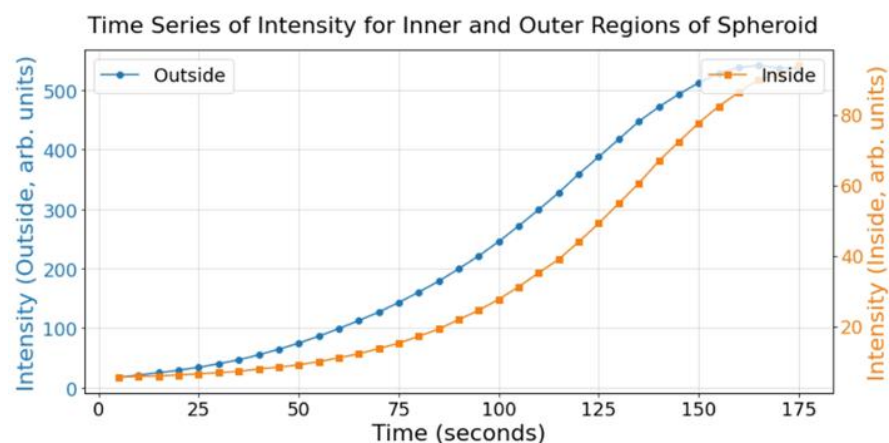


Figure 140 OVCAR-4 spheroids treated with rucaparib. Intensity as a function of time shown until 3 minutes with intensity of inner and outer regions shown normalised to their maximum value.

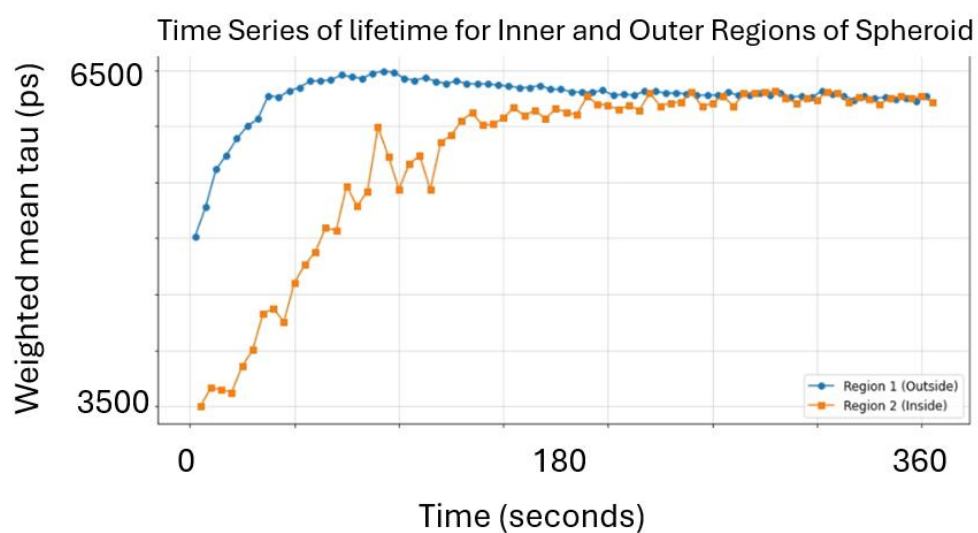


Figure 141 OVCAR-4 spheroids treated with rucaparib. Weighted mean lifetime as a function of time for inner and outer regions

7.4 Summary

This chapter has shown the investigation of cellular dynamics of the intrinsically fluorescent PARP inhibitor drug rucaparib. Heterogeneity in the fluorescence lifetime of the drug was probed using nuclear and lysosome dyes, indicating that regions with longer and shorter rucaparib lifetime coincided with the nucleus and lysosome respectively. The lysosomal regions also showed bright highly localised puncta. The addition of a lysosome inhibitor, bafilomycin, removed the short lifetime puncta, further indicating that the bright short lifetime pixels seen previously were due to lysosomal rucaparib. The nucleus still exhibited a longer lifetime than the cytoplasm in bafilomycin treated cells. Possible explanations for this are that the rucaparib has a different local environment in the nucleus due to different viscosity, or the fact it is bound to DNA, or possibly that not all lysosomes had been inhibited meaning there were still some present in the cytoplasm acting to reduce the cytoplasmic lifetime compared to the nucleus. Cells treated with olaparib and rucaparib were imaged, however the reduction in rucaparib intensity seen in previous work was not observed. This may have been due to the cell type being used, or that we had imaged the cells 1 hour later than in their experiment; further experiments are needed to confirm the results. We also observed a possible FRET signal in the cells treated with rucaparib and the SYTO nuclear dye.

This section has shown the utility of rucaparib as a tool to explore cellular PARP inhibitor dynamics as well as the utility of FLIM to sense changes in a fluorophores environment (pH/ presence of other fluorophores). Cells with a genetically expressed PARP biosensor containing mCherry / GFP/ both fluorescent proteins at ends of the PARP molecule have been developed in the group. The treatment of these cells with rucaparib could show further application in understanding rucaparib binding dynamics due to FRET between rucaparib and GFP. Such experiments could be revealing in studies using cells that show resistance to PARP inhibitors, possibly elucidating resistance mechanisms. The use of multiphoton FLIM measurements to quantify drug uptake in spheroids was also shown, which could also be applied to organoids and tissue.

8 Conclusions and future work

This thesis has presented the implementation and development of a multibeam, multiphoton FLIM microscope for usage in 2D/3D multiwell plate assays. This chapter will discuss the outcomes of the project as well as possible improvements and directions for the future.

8.1 Conclusions

8.1.1 Imaging speed comparison

A significant aim for the project was to increase the imaging speed of a single-beam point-scanning multiphoton microscope by using 6 spatially multiplexed beams. Out of the various components on offer to multiplex the beams an SLM was chosen, as it could provide fine tuning adjustments of the 6-beam pattern to couple the fluorescence directly into the constructed fibre loom. Furthermore, producing new beam patterns (e.g line, grid, more/ less than 6 beams) could easily be done with the SLM, allowing flexibility in the investigation of new configurations. The SLM posed some disadvantages with regards to the amount of fluorescence signal excited. The first issue came from the diffraction efficiency of the 6-beam pattern, which was shown to be approximately 80% for the 6-beam line pattern, however in terms of power throughput this figure wasn't too detrimental. The second issue came from the chromatic dispersion of the outgoing diffracted light from the SLM, which in turn caused elongation of the laser pulses at the sample and ~70% loss in fluorescence signal for the same average illumination power. For the same average power a spatially and temporally expanded pulse will produce less fluorescence, however it will also produce less photodamage. Previous work has shown that maintaining the same photodamage rate by increasing both pulse duration and average illumination power, one can excite more fluorescence. The same is true for increasing the beams spatial width. This means that with a large laser power reserve, the pulse spreading can be counteracted by increasing the power without increasing photodamage. However, we found that for deeper imaging we did not have this excess laser power necessary, meaning that the pulse spreading was detrimental to signal acquisition rate. It is worth noting that increasing pulse width spatially and temporally also results in corresponding losses in spatial and temporal resolution

The experiment carried out at the end of chapter 4 showed that the 6 beam (10 mw per beam) configuration yielded a maximum ~1.2x speed advantage at shallow imaging depths over the single-beam (10mW per beam) non-descanned configuration. However as mentioned, it is reasonable to say that each of the beams in the 6-beam configuration was causing less photodamage due to the pulse spreading caused by the SLM. Therefore, it would be more appropriate to either determine the power for the SLM diffracted beams which cause the same rate of photodamage as a non-diffracted beam and compare the fluorescence signal using this power per beam, or another method of fair comparison would

be to increase the power of the 6-beam configuration such that each beam excites the same amount of fluorescence excited in the single-beam configuration multiplied by the ratio of fluorescence throughput for both paths (~70%). This would result in 4.2x signal gained for less photodamage. The comparison in chapter 4 also showed that the ratio of the amount of signal collected in the non-descanned detector compared to the descanned detectors increased as a function of imaging depth, as expected as it collects more scattered light. This indicates that the 4.2x speed benefit would only be for shallow imaging and would decrease for increasing imaging depths.

A new beam multiplexing unit is currently being constructed at Imperial based on reflective rather than diffractive components, making use of beam splitters and mirrors to produce 4 equal intensity beams. The unit will direct the four beams at the appropriate angles towards the galvo-mirrors, mimicking the optical setup currently in place. The L1 and L2 lens relay will no longer be needed, however to achieve the appropriate field size on the galvos, a reflective beam expander (3x) will be used before the beam multiplexing unit. As the unit relies on reflection to multiplex the beams, the effects of chromatic dispersion will be removed, therefore meaning multibeam imaging can be carried out at maximum signal acquisition rate up to greater depths within the sample.

8.1.2 Scattering investigation

One of the main difficulties in 3D biological imaging is overcoming the effect of photon scattering. As emission photons scatter they may be left undetected by their intended detector, and in multifocal imaging can end up being detected by neighbouring detectors. This results in both the loss of signal and image-degrading cross-talk when imaging at greater depths. Experiments and a simulation were carried out to quantify the effect photon scattering had on fluorescence collection levels and cross-talk in our fibre loom detection scheme. One experiment evaluated the signal detected by various sized fibres at a range of imaging depths, and another experiment quantified the amount of signal entering the fibres at different imaging depths as a function of the fibre-beam distance. The former experiment quantifies the gain in signal when using larger detection fibres, and the second experiment quantifies the amount of cross-talk one expects to see at different imaging depths for different spacings and fibre sizes. As expected, we found that greater signal collection and cross-talk was seen at larger imaging depths for larger fibres. The experiment revealed that we could afford to make the fibres larger by moving from a line to grid pattern, increasing the inter-fibre distances and decreasing the increased cross-talk incurred by the larger fibres.

To verify the simulation, the same two experiments described previously were recreated. As the simulation required the input of two sample parameters, namely the scattering anisotropy and scattering mean free path length, these two parameters were first tuned so that the output of the simulation matched

the data from the first experiment. These tuned parameters were then used for the simulation of the second experiment, where it was again found that the data and simulation were in good agreement, providing confidence in the output of the simulation. This simulation tool could be used when designing new detection configurations and considering different sample types. Given the effort and time taken to gather the experimental scattering data, the simulator provides a simple and easy alternative of determining the expected cross-talk and signal gain one can expect for different detection configurations.

8.1.3 FLIM of rucaparib to analyse drug dynamics

The investigation of cellular rucaparib dynamics carried out using the M³M showed the efficacy of the microscope for real biological investigations. The single-photon fluorescence modality complemented the multiphoton FLIM modality well, shown by the acquisition of the lysosomal and nuclei masks using the single-photon modality, taking considerably less time than if the acquisition was done with multibeam, multiphoton imaging. These masks were then used to determine the regions in the cell displaying distinct lifetimes, ultimately identifying the nucleus and the lysosome to be two specific regions of interest with regards to rucaparib lifetime within the cell. This made sense as the molecule that rucaparib binds to (PARP) exists mostly in the nucleus and the lysosome is often used for degradation of molecules within the cell. Experiments adding bafilomycin (a lysosomal inhibitor) to the cells further verified the lysosomal degradation of the drug. These experiments were all carried out in a high content analysis fashion with multiple FOVs from separate wells automatically acquired, providing more data and more robust conclusions.

A PARP biosensor has been engineered at Imperial in which the PARP molecule is fluorescently tagged on both ends of the molecule. As one of the tags is GFP (a possible FRET acceptor to rucaparib due to their overlapping spectra) this biosensor could be used in FLIM FRET experiments to determine rucaparib binding dynamics. This could be particularly interesting in cell lines showing resistance to PARP inhibitors that have also been developed in the group.

8.1.4 pDPC and NADH FLIM readouts for analysis of cellular metabolic changes

Further relevant biological experiments were carried out in the form of investigating NADH FLIM readouts in 2D and 3D in cells treated with drugs known to affect metabolic functioning. The autofluorescent NADH molecule offers a convenient way of analysing changes in cellular metabolism using FLIM given its free and bound forms (the abundancies of which change depending on the metabolic state of the cell) exhibit different fluorescent lifetimes. The multiwell plate assays showed

that the NADH weighted mean lifetime decreased as expected under the application of phenformin and rotenone and increased with FCCP treatment. A dose response curve was observed for increasing concentrations of phenformin, however after an initial decrease in lifetime due to rotenone treatment, larger doses caused the lifetime to increase again. This increase in lifetime was thought to result from the high rotenone dose inducing cell death, therefore changing the cell phenotype and affecting the lifetime distribution observed. pDPC images were then used to complement the FLIM data acquired in the 2D assays, with a quantitative readout of average phase per cell pixel offering a convenient metric for insights into cellular morphological changes induced by the drugs. Further phase analysis using shape metrics such as cell eccentricity could offer more insight into the morphological changes induced. This type of analysis could be carried out in high throughput batch processing with the CellProfiler software described in chapter 5.

The gold standard in metabolic imaging is taking the FLIRR metric, calculated as the ratio of enzyme bound NADH to enzyme bound FAD. Therefore, further 3D assays imaging FAD as well as NADH in drug treated spheroids would be an interesting prospect.

8.2 Future work

8.2.1 Software

Various pieces of software were written for this project. The main piece was a device adapter that could communicate with the MultiHarp TCSPC unit from MicroManager, allowing us to execute acquisitions and view images in real time all inside the MicroManager GUI. A socket connection was made between MicroManager and Labview allowing initiation of the Labview galvo scanning code from MicroManager, further simplifying image acquisitions. However, full integration between MicroManager and Labview has not yet been completed. Further work would completely synchronise the settings set in MicroManager to the parameters sent to Labview for scanning, meaning a user would never have to open the Labview window. As the aim of the project was to make the microscope run on as much open-source software as possible, ideally the use of Labview would be removed given its proprietary nature.

8.2.2 Automated multibeam to single beam switching

Given the increased scattered light collection of the non-descanned detector and increased cross-talk of the multibeam descanned detection configuration at increased imaging depths, there will often be a particular imaging plane at which it would be beneficial to switch from multibeam to single beam

imaging. It would be beneficial for this switch to be done automatically, meaning some metric must be used to determine when it is beneficial to switch modalities. One possible method based on detector cross-talk could use cross-correlation analysis between sub-images formed by the different beams. When no cross-talk is present the cross-correlations should be low, unless a particularly repetitious sample is used. With greater cross-talk, stronger “ghost images” will be formed between neighbouring detectors, which would result in a greater cross-correlation signal. One could then set a certain cross-correlation threshold at which the imaging modality would automatically change from multibeam to single beam illumination. Another method could be to analyse the rate at which the signal is decreasing as a function of imaging depth and use this to infer the scattering properties of the sample. Knowing these properties, one could then determine the optimal plane to switch configurations at using the scattering simulation described in this thesis.

8.2.3 Adaptive optics for deeper imaging

Another common function of an SLM is to apply aberration correction to an excitation beam such that the aberrations induced by the sample are pre-compensated and a more tightly focussed excitation volume is produced within the sample. This can improve signal at increased imaging depths and has been applied to multiphoton multibeam microscopy previously to correct for spherical aberration [199]. They used a precalculated wavefront distortion due to the mismatch in refractive indices of their air objective and water-based sample. This may be the easiest first step in applying aberration correction as it doesn't require image-based optimisations of the correction hologram, and the same correction is applied for each beam. To apply unique corrections to each individual beam would require more thought and could be possible future work.

8.2.4 Second harmonic generation imaging

The current M³M setup could easily be expanded to incorporate some further imaging modalities. One such modality is second harmonic generation imaging (SHG). SHG uses the doubling in frequency of light transmitted through a sample containing some non centro-symmetric repeating structure, such as collagen. SHG signal can be detected either in transmission or from back scattered light in a epi-illumination configuration. Either of these methods could be implemented on the M³M, with the epi configuration simply using an appropriate emission filter for the excitation wavelength used placed in front of the fibre loom. The transmitted configuration would require further optics above the sample (opposite side to objective) to collect the SHG signal, as well as the appropriate emission filter. A PMT used to detect the signal could then be mounted to the top of the incubator where the transillumination column is currently positioned. Collagen imaging can provide useful complementary information to the existing modalities as there is significant research currently on the interaction of cancer spheroids with

the extra-cellular matrix, of which collagen is a large component [200]. The SHG signal would have the same temporal profile as the illuminating laser pulse, meaning that multiple beams each undergoing SHG could be detected on the same PMT if the beams are temporally shifted by an amount similar to the IRF width. Given the IRF width measured of ~ 300 ps this would require a ~ 10 cm propagation length difference in respective beam paths, which is easily possible with beam delay lines. Given the temporal profile and shifts of the beams can be measured exactly beforehand, temporal shifts much less than ~ 300 ps would probably suffice.

References

- [1] Hopt, A., & Neher, E. (2001). *Highly nonlinear photodamage in two-photon fluorescence microscopy*. *Biophysical Journal*, 80(4), 2029–2036. [https://doi.org/10.1016/S0006-3495\(01\)76174-4](https://doi.org/10.1016/S0006-3495(01)76174-4).
- [2] J. R. Lakowicz, ‘Principles of fluorescence spectroscopy’, *Principles of Fluorescence Spectroscopy*, pp. 1–954, 2006, doi: 10.1007/978-0-387-46312-4/COVER.
- [3] M. Göppert-Mayer, ‘Über Elementarakte mit zwei Quantensprüngen’, *Ann Phys*, vol. 401, no. 3, pp. 273–294, Jan. 1931, doi: 10.1002/ANDP.19314010303.
- [4] W. Kaiser and C. G. B. Garrett, ‘Two-Photon Excitation in $\text{CaF}_2 : \text{Eu}^{2+}$ ’, *PhRvL*, vol. 7, no. 6, pp. 229–231, 1961, doi: 10.1103/PHYSREVLETT.7.229.
- [5] R. Datta, T. M. Heaster, J. T. Sharick, A. A. Gillette, M. C. Skala, and "J Biomed, ‘Fluorescence lifetime imaging microscopy: fundamentals and advances in instrumentation, analysis, and applications’, *J Biomed Opt*, vol. 25, no. 7, p. 071203, May 2020, doi: 10.1117/1.JBO.25.7.071203.
- [6] S. Kalies, K. Kuertemeyer, and A. Heisterkamp, ‘Mechanisms of high-order photobleaching and its relationship to intracellular ablation’, *Biomed Opt Express*, vol. 2, no. 4, p. 805, Apr. 2011, doi: 10.1364/BOE.2.000816.
- [7] P. Fischer, K. König, I. Riemann, K.-J. Halbhauer, and T. W. Becker, ‘Pulse-length dependence of cellular response to intense near-infrared laser pulses in multiphoton microscopes’, *Optics Letters*, Vol. 24, Issue 2, pp. 113–115, vol. 24, no. 2, pp. 113–115, Jan. 1999, doi: 10.1364/OL.24.000113.
- [8] V. Magidson and A. Khodjakov, ‘Circumventing photodamage in live-cell microscopy’, *Methods Cell Biol*, vol. 114, pp. 10.1016/B978-0-12-407761-4.00023–3, 2013, doi: 10.1016/B978-0-12-407761-4.00023-3.
- [9] O. I. Kolenc and K. P. Quinn, ‘Evaluating Cell Metabolism Through Autofluorescence Imaging of NAD(P)H and FAD’, *Antioxid Redox Signal*, vol. 30, no. 6, p. 875, 2019, doi: 10.1089/ARS.2017.7451.
- [10] ‘The bh TCSPC Handbook 10th edition, 2023 - Becker & Hickl GmbH’. Accessed: Dec. 22, 2024. [Online]. Available: <https://www.becker-hickl.com/literature/documents/flim/the-bh-tcspc-handbook/>

- [11] M. Papanicolaou *et al.*, ‘Temporal profiling of the breast tumour microenvironment reveals collagen XII as a driver of metastasis’, *Nature Communications* 2022 13:1, vol. 13, no. 1, pp. 1–21, Aug. 2022, doi: 10.1038/s41467-022-32255-7.
- [12] L. Loew *et al.*, ‘Quantification of collagen fiber organization using three-dimensional Fourier transform-second-harmonic generation imaging’, *Optics Express*, Vol. 20, Issue 19, pp. 21821–21832, vol. 20, no. 19, pp. 21821–21832, Sep. 2012, doi: 10.1364/OE.20.021821.
- [13] B. Banerjee *et al.*, ‘Tryptophan autofluorescence imaging of neoplasms of the human colon’, *J Biomed Opt*, vol. 17, no. 1, p. 016003, 2012, doi: 10.1117/1.JBO.17.1.016003.
- [14] N. Bagheri, H. Chen, M. Rabasovic, and J. Widengren, ‘Non-fluorescent transient states of tyrosine as a basis for label-free protein conformation and interaction studies’, *Scientific Reports* 2024 14:1, vol. 14, no. 1, pp. 1–8, Mar. 2024, doi: 10.1038/s41598-024-57054-6.
- [15] J. Kapuscinski, ‘DAPI: a DNA-specific fluorescent probe’, *Biotech Histochem*, vol. 70, no. 5, pp. 220–233, 1995, doi: 10.3109/10520299509108199.
- [16] J. Marshall, R. Molloy, G. W. J. Moss, J. R. Howe, and T. E. Hughes, ‘The jellyfish green fluorescent protein: a new tool for studying ion channel expression and function’, *Neuron*, vol. 14, no. 2, pp. 211–215, 1995, doi: 10.1016/0896-6273(95)90279-1.
- [17] M. Hirano *et al.*, ‘A highly photostable and bright green fluorescent protein’, *Nature Biotechnology* 2022 40:7, vol. 40, no. 7, pp. 1132–1142, Apr. 2022, doi: 10.1038/s41587-022-01278-2.
- [18] R. Dreyer, R. Pfukwa, S. Barth, R. Hunter, and B. Klumperman, ‘The Evolution of SNAP-Tag Labels’, *Biomacromolecules*, vol. 24, no. 2, pp. 517–530, Feb. 2023, doi: 10.1021/ACS.BIOMAC.2C01238/ASSET/IMAGES/MEDIUM/BM2C01238_0022.GIF.
- [19] A. Van Leeuwenhoek, ‘III. A letter from Mr Antony van Leeuwenhoek, F. R. S. concerning the flesh of whales, crystalline humour of the eye of whales, fish, and other creatures, and of the use of the eye-lids.’, *Philos Trans R Soc Lond*, vol. 24, no. 293, pp. 1723–1730, Oct. 1704, doi: 10.1098/RSTL.1704.0035.
- [20] ‘Micrographia - National Library of Wales’. Accessed: Dec. 22, 2024. [Online]. Available: <https://www.library.wales/discover-learn/digital-exhibitions/printed-material/micrographia>
- [21] Rayleigh, ‘XXXI. Investigations in optics, with special reference to the spectroscope’, *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science*, vol. 8, no. 49, pp. 261–274, Oct. 1879, doi: 10.1080/14786447908639684.
- [22] T. Wilson, ‘Resolution and optical sectioning in the confocal microscope’, *J Microsc*, vol. 244, no. 2, pp. 113–121, 2011, doi: 10.1111/J.1365-2818.2011.03549.X.

- [23] F. Zernike, 'How I discovered phase contrast', *Science* (1979), vol. 121, no. 3141, pp. 345–349, Mar. 1955, doi: 10.1126/SCIENCE.121.3141.345/ASSET/05CA7055-602B-4878-B0CE-78C472BB8494/ASSETS/SCIENCE.121.3141.345.FP.PNG.
- [24] R. Allen, G. B. David, and G. Nomarski, 'The zeiss-Nomarski differential interference equipment for transmitted-light microscopy.', *Z Wiss Mikrosk*, 1969.
- [25] L. Waller and L. Tian, 'Quantitative differential phase contrast imaging in an LED array microscope', *Optics Express*, Vol. 23, Issue 9, pp. 11394-11403, vol. 23, no. 9, pp. 11394–11403, May 2015, doi: 10.1364/OE.23.011394.
- [26] G. Zheng, R. Horstmeyer, X. Ou, and C. Yang, 'Quantitative phase imaging via Fourier ptychographic microscopy', *Optics Letters*, Vol. 38, Issue 22, pp. 4845-4848, vol. 38, no. 22, pp. 4845–4848, Nov. 2013, doi: 10.1364/OL.38.004845.
- [27] S. Lagies *et al.*, 'Cells grown in three-dimensional spheroids mirror in vivo metabolic response of epithelial cells', *Communications Biology* 2020 3:1, vol. 3, no. 1, pp. 1–10, May 2020, doi: 10.1038/s42003-020-0973-6.
- [28] M. Minsky, 'Memoir on inventing the confocal scanning microscope', *Scanning*, vol. 10, no. 4, pp. 128–138, Jan. 1988, doi: 10.1002/SCA.4950100403.
- [29] A. R. Carlini and T. Wilson, 'Size of the detector in confocal imaging systems', *Optics Letters*, Vol. 12, Issue 4, pp. 227-229, vol. 12, no. 4, pp. 227–229, Apr. 1987, doi: 10.1364/OL.12.000227.
- [30] A. Nakano, 'Spinning-disk confocal microscopy -- a cutting-edge tool for imaging of membrane traffic', *Cell Struct Funct*, vol. 27, no. 5, pp. 349–355, Oct. 2002, doi: 10.1247/CSF.27.349.
- [31] H. Ishida, T. Tanaami, M. Shimizu, N. Tomosada, S. Otsuki, and Y. Kosugi, 'High-speed 1-frame/ms scanning confocal microscope with a microlens and Nipkow disks', *Applied Optics*, Vol. 41, Issue 22, pp. 4704-4708, vol. 41, no. 22, pp. 4704–4708, Aug. 2002, doi: 10.1364/AO.41.004704.
- [32] K.-B. Im, B.-M. Kim, D. Kim, H. Park, and S. Han, 'Simple high-speed confocal line-scanning microscope', *Optics Express*, Vol. 13, Issue 13, pp. 5151-5156, vol. 13, no. 13, pp. 5151–5156, Jun. 2005, doi: 10.1364/OPEX.13.005151.
- [33] E. Mei, P. A. Fomitchov, R. Graves, and M. Champion, 'A line scanning confocal fluorescent microscope using a CMOS rolling shutter as an adjustable aperture', *J Microsc*, vol. 247, no. 3, pp. 269–276, 2012, doi: 10.1111/J.1365-2818.2012.03642.X.
- [34] W. Denk, J. H. Strickler, and W. W. Webb, 'Two-photon laser scanning fluorescence microscopy', *Science*, vol. 248, no. 4951, pp. 73–76, 1990, doi: 10.1126/SCIENCE.2321027.

- [35] C. Xu, W. Zipfel, J. B. Shear, R. M. Williams, and W. W. Webb, 'Multiphoton fluorescence excitation: new spectral windows for biological nonlinear microscopy.', *Proceedings of the National Academy of Sciences*, vol. 93, no. 20, pp. 10763–10768, Oct. 1996, doi: 10.1073/PNAS.93.20.10763.
- [36] N. G. Horton *et al.*, 'In vivo three-photon microscopy of subcortical structures within an intact mouse brain', *Nature Photonics* 2013 7:3, vol. 7, no. 3, pp. 205–209, Jan. 2013, doi: 10.1038/nphoton.2012.336.
- [37] J. Bewersdorf and S. W. Hell, 'Picosecond pulsed two-photon imaging with repetition rates of 200 and 400 MHz', *J Microsc*, vol. 191, no. 1, pp. 28–38, Jul. 1998, doi: 10.1046/J.1365-2818.1998.00379.X.
- [38] M. Eibl, B. Sauer, S. Karpf, G. Hüttmann, F. Reinholz, and R. Huber, 'Two-photon microscopy using fiber-based nanosecond excitation', *Biomedical Optics Express*, Vol. 7, Issue 7, pp. 2432-2440, vol. 7, no. 7, pp. 2432–2440, Jul. 2016, doi: 10.1364/BOE.7.002432.
- [39] E. Tal, D. Oron, and Y. Silberberg, 'Scanningless depth-resolved microscopy', *Optics Express*, Vol. 13, Issue 5, pp. 1468-1476, vol. 13, no. 5, pp. 1468–1476, Mar. 2005, doi: 10.1364/OPEX.13.001468.
- [40] E. Tal, D. Oron, and Y. Silberberg, 'Improved depth resolution in video-rate line-scanning multiphoton microscopy using temporal focusing', *Optics Letters*, Vol. 30, Issue 13, pp. 1686-1688, vol. 30, no. 13, pp. 1686–1688, Jul. 2005, doi: 10.1364/OL.30.001686.
- [41] J. Bewersdorf, R. Pick, and S. W. Hell, 'Multifocal multiphoton microscopy', *Optics Letters*, Vol. 23, Issue 9, pp. 655-657, vol. 23, no. 9, pp. 655–657, May 1998, doi: 10.1364/OL.23.000655.
- [42] T. Nielsen, M. Fricke, D. Hellweg, and P. Andresen, 'High efficiency beam splitter for multifocal multiphoton microscopy', *J Microsc*, vol. 201, no. Pt 3, pp. 368–376, 2001, doi: 10.1046/J.1365-2818.2001.00852.X.
- [43] M. R. Taghizadeh, F. S. Pavone, A. Choudhury, L. Sacconi, E. Froner, and R. Antolini, 'Multiphoton multifocal microscopy exploiting a diffractive optical element', *Optics Letters*, Vol. 28, Issue 20, pp. 1918-1920, vol. 28, no. 20, pp. 1918–1920, Oct. 2003, doi: 10.1364/OL.28.001918.
- [44] P. Barber *et al.*, 'A high speed multifocal multiphoton fluorescence lifetime imaging microscope for live-cell FRET imaging', *Biomedical Optics Express*, Vol. 6, Issue 2, pp. 277-296, vol. 6, no. 2, pp. 277–296, Feb. 2015, doi: 10.1364/BOE.6.000277.

- [45] J. Huiskens, J. Swoger, F. Del Bene, J. Wittbrodt, and E. H. K. Stelzer, 'Optical sectioning deep inside live embryos by selective plane illumination microscopy', *Science (1979)*, vol. 305, no. 5686, pp. 1007–1009, Aug. 2004, doi: 10.1126/SCIENCE.1100035/SUPPL_FILE/HUISKEN.SOM.PDF.
- [46] D. J. X. Chow *et al.*, 'Quantifying DNA damage following light sheet and confocal imaging of the mammalian embryo', *Scientific Reports 2024 14:1*, vol. 14, no. 1, pp. 1–12, Sep. 2024, doi: 10.1038/s41598-024-71443-x.
- [47] Y. Wu *et al.*, 'Inverted selective plane illumination microscopy (iSPIM) enables coupled cell identity lineaging and neurodevelopmental imaging in *Caenorhabditis elegans*', *Proc Natl Acad Sci U S A*, vol. 108, no. 43, pp. 17708–17713, Oct. 2011, doi: 10.1073/PNAS.1108494108/SUPPL_FILE/SM06.MOV.
- [48] C. Dunsby, 'Optically sectioned imaging by oblique plane microscopy', *Optics Express, Vol. 16, Issue 25, pp. 20306-20316*, vol. 16, no. 25, pp. 20306–20316, Dec. 2008, doi: 10.1364/OE.16.020306.
- [49] V. Voleti *et al.*, 'Real-time volumetric microscopy of in vivo dynamics and large-scale samples with SCAPE 2.0', *Nature Methods 2019 16:10*, vol. 16, no. 10, pp. 1054–1062, Sep. 2019, doi: 10.1038/s41592-019-0579-4.
- [50] V. Maioli *et al.*, 'Time-lapse 3-D measurements of a glucose biosensor in multicellular spheroids by light sheet fluorescence microscopy in commercial 96-well plates', *Scientific Reports 2016 6:1*, vol. 6, no. 1, pp. 1–13, Nov. 2016, doi: 10.1038/srep37777.
- [51] J. Sharpe, 'Optical projection tomography as a new tool for studying embryo anatomy', *J Anat*, vol. 202, no. 2, p. 175, Feb. 2003, doi: 10.1046/J.1469-7580.2003.00155.X.
- [52] M. A. Bray *et al.*, 'Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes', *Nature Protocols 2016 11:9*, vol. 11, no. 9, pp. 1757–1774, Aug. 2016, doi: 10.1038/nprot.2016.105.
- [53] T. Thestrup *et al.*, 'Optimized ratiometric calcium sensors for functional in vivo imaging of neurons and T lymphocytes', *Nature Methods 2013 11:2*, vol. 11, no. 2, pp. 175–182, Jan. 2014, doi: 10.1038/nmeth.2773.
- [54] J. T. Lock, I. Parker, and I. F. Smith, 'A comparison of fluorescent Ca²⁺ indicators for imaging local Ca²⁺ signals in cultured cells', *Cell Calcium*, vol. 58, no. 6, pp. 638–648, Dec. 2015, doi: 10.1016/J.CECA.2015.10.003.
- [55] H. Y. Wen, Y. Hu, Y. Y. Duo, L. Zhao, Z. G. Wang, and S. L. Liu, 'Ratiometric Fluorescence Probes for In Situ Imaging of Membrane Tension in Live Cells', *Bioconjug Chem*, vol. 35, no.

- 7, pp. 934–943, Jul. 2024, doi: 10.1021/ACS.BIOCONJCHEM.4C00101/SUPPL_FILE/BC4C00101_SI_001.PDF.
- [56] W. Becker, ‘Advanced Time-Correlated Single Photon Counting Techniques’, vol. 81, 2005, doi: 10.1007/3-540-28882-1.
- [57] D. M. Owen *et al.*, ‘High speed optically sectioned fluorescence lifetime imaging permits study of live cell signaling events’, *Optics Express*, Vol. 15, Issue 24, pp. 15656–15673, vol. 15, no. 24, pp. 15656–15673, Nov. 2007, doi: 10.1364/OE.15.015656.
- [58] K. C. Benny Lee *et al.*, ‘Application of the stretched exponential function to fluorescence lifetime imaging’, *Biophys J*, vol. 81, no. 3, pp. 1265–1274, Sep. 2001, doi: 10.1016/s0006-3495(01)75784-0.
- [59] M. Köllner and J. Wolfrum, ‘How many photons are necessary for fluorescence-lifetime measurements?’, *Chem Phys Lett*, vol. 200, no. 1–2, pp. 199–204, Nov. 1992, doi: 10.1016/0009-2614(92)87068-Z.
- [60] S. C. Warren *et al.*, ‘Rapid Global Fitting of Large Fluorescence Lifetime Imaging Microscopy Datasets’, *PLoS One*, vol. 8, no. 8, p. e70687, Aug. 2013, doi: 10.1371/JOURNAL.PONE.0070687.
- [61] M. A. Digman, V. R. Caiolfa, M. Zamai, and E. Gratton, ‘The Phasor Approach to Fluorescence Lifetime Imaging Analysis’, *Biophys J*, vol. 94, no. 2, p. L14, Jan. 2007, doi: 10.1529/BIOPHYSJ.107.120154.
- [62] A. A. Heikal, ‘Intracellular coenzymes as natural biomarkers for metabolic activities and mitochondrial anomalies’, *Biomark Med*, vol. 4, no. 2, p. 241, Apr. 2010, doi: 10.2217/BMM.10.1.
- [63] A. J. Walsh, L. Hu, N. Wang, and E. Cardona, ‘Fluorescence intensity and lifetime redox ratios detect metabolic perturbations in T cells’, *Biomedical Optics Express*, Vol. 11, Issue 10, pp. 5674–5688, vol. 11, no. 10, pp. 5674–5688, Oct. 2020, doi: 10.1364/BOE.401935.
- [64] A. Gijsbers, T. Nishigaki, and N. Sánchez-Puig, ‘Fluorescence Anisotropy as a Tool to Study Protein-protein Interactions’, *J Vis Exp*, vol. 2016, no. 116, p. 54640, Oct. 2016, doi: 10.3791/54640.
- [65] W. W. Webb, ‘Fluorescence correlation spectroscopy: inception, biophysical experimentations, and prospectus’, *Applied Optics*, Vol. 40, Issue 24, pp. 3969–3983, vol. 40, no. 24, pp. 3969–3983, Aug. 2001, doi: 10.1364/AO.40.003969.

- [66] F. Becker, ‘Using Fluorescence Cross Correlation Spectroscopy to Monitor and Quantify Drug Target Occupancy in Cells, Tissues and Patient Biopsies’, *Eur J Cancer*, vol. 174, p. S102, Oct. 2022, doi: 10.1016/S0959-8049(22)01069-3.
- [67] R. M. Dörlich *et al.*, ‘Dual-color dual-focus line-scanning FCS for quantitative analysis of receptor-ligand interactions in living specimens’, *Scientific Reports 2015 5:1*, vol. 5, no. 1, pp. 1–11, May 2015, doi: 10.1038/srep10149.
- [68] M. E. Van Royen, P. Farla, K. A. Mattern, B. Geverts, J. Trapman, and A. B. Houtsmuller, ‘Fluorescence recovery after photobleaching (FRAP) to study nuclear protein dynamics in living cells’, *Methods in Molecular Biology*, vol. 464, pp. 363–385, 2008, doi: 10.1007/978-1-60327-461-6_20/TABLES/1.
- [69] T. Foerster, ‘DELOCALIZED EXCITATION AND EXCITATION TRANSFER. Bulletin No. 18’, Jan. 1964, doi: 10.2172/4626886.
- [70] P. Tsou, B. Zheng, C. H. Hsu, A. T. Sasaki, and L. C. Cantley, ‘A fluorescent reporter of AMPK activity and cellular energy stress’, *Cell Metab*, vol. 13, no. 4, pp. 476–486, Apr. 2011, doi: 10.1016/j.cmet.2011.03.006.
- [71] A. Pietraszewska-Bogiel and T. W. J. Gadella, ‘FRET microscopy: from principle to routine technology in cell biology’, *J Microsc*, vol. 241, no. 2, pp. 111–118, Feb. 2011, doi: 10.1111/J.1365-2818.2010.03437.X.
- [72] P. T. Arsenovic, C. R. Mayer, and D. E. Conway, ‘SensorFRET: A Standardless Approach to Measuring Pixel-based Spectral Bleed-through and FRET Efficiency using Spectral Imaging’, *Scientific Reports 2017 7:1*, vol. 7, no. 1, pp. 1–15, Nov. 2017, doi: 10.1038/s41598-017-15411-8.
- [73] F. S. Wouters, P. J. Verveer, and P. I. H. Bastiaens, ‘Imaging biochemistry inside cells’, *Trends Cell Biol*, vol. 11, no. 5, pp. 203–211, May 2001, doi: 10.1016/S0962-8924(01)01982-1/ASSET/BB591626-1BB2-4765-BEE1-C00C258F276E/MAIN.ASSETS/GR3.SML.
- [74] A. Esposito, ‘How many photons are needed for FRET imaging?’, *Biomed Opt Express*, vol. 11, no. 2, p. 1186, Feb. 2020, doi: 10.1364/BOE.379305.
- [75] G. Chennell *et al.*, ‘Imaging of Metabolic Status in 3D Cultures with an Improved AMPK FRET Biosensor for FLIM’, *Sensors (Basel)*, vol. 16, no. 8, Aug. 2016, doi: 10.3390/S16081312.
- [76] D. J. Kelly *et al.*, ‘An automated multiwell plate reading flim microscope for live cell autofluorescence lifetime assays’, *J Innov Opt Health Sci*, vol. 7, no. 5, p. 1450025, 2014, doi: 10.1142/S1793545814500254.

- [77] S. You *et al.*, ‘Intravital imaging by simultaneous label-free autofluorescence-multiharmonic microscopy’, *Nature Communications* 2018 9:1, vol. 9, no. 1, pp. 1–9, May 2018, doi: 10.1038/s41467-018-04470-8.
- [78] J. Ryu *et al.*, ‘Multimodal microscopy for the simultaneous visualization of five different imaging modalities using a single light source’, *Biomedical Optics Express*, Vol. 12, Issue 9, pp. 5452–5469, vol. 12, no. 9, pp. 5452–5469, Sep. 2021, doi: 10.1364/BOE.430677.
- [79] ‘The Nobel Prize in Chemistry 2014 - NobelPrize.org’. Accessed: Dec. 23, 2024. [Online]. Available: <https://www.nobelprize.org/prizes/chemistry/2014/summary/>
- [80] J. Wichmann and S. W. Hell, ‘Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy’, *Optics Letters*, Vol. 19, Issue 11, pp. 780–782, vol. 19, no. 11, pp. 780–782, Jun. 1994, doi: 10.1364/OL.19.000780.
- [81] D. Wildanger *et al.*, ‘STED microscopy with a supercontinuum laser source’, *Optics Express*, Vol. 16, Issue 13, pp. 9614–9621, vol. 16, no. 13, pp. 9614–9621, Jun. 2008, doi: 10.1364/OE.16.009614.
- [82] M. Heilemann *et al.*, ‘Subdiffraction-Resolution Fluorescence Imaging with Conventional Fluorescent Probes’, *Angewandte Chemie International Edition*, vol. 47, no. 33, pp. 6172–6176, Aug. 2008, doi: 10.1002/ANIE.200802376.
- [83] M. J. Rust, M. Bates, and X. Zhuang, ‘Stochastic optical reconstruction microscopy (STORM) provides sub-diffraction-limit image resolution’, *Nat Methods*, vol. 3, no. 10, p. 793, Oct. 2006, doi: 10.1038/NMETH929.
- [84] D. A. Helmerich *et al.*, ‘Photoswitching fingerprint analysis bypasses the 10-nm resolution barrier’, *Nature Methods* 2022 19:8, vol. 19, no. 8, pp. 986–994, Aug. 2022, doi: 10.1038/s41592-022-01548-6.
- [85] F. Ströhl and C. F. Kaminski, ‘Frontiers in structured illumination microscopy’, *Optica*, Vol. 3, Issue 6, pp. 667–677, vol. 3, no. 6, pp. 667–677, Jun. 2016, doi: 10.1364/OPTICA.3.000667.
- [86] X. Li *et al.*, ‘Three-dimensional structured illumination microscopy with enhanced axial resolution’, *Nature Biotechnology* 2023 41:9, vol. 41, no. 9, pp. 1307–1319, Jan. 2023, doi: 10.1038/s41587-022-01651-1.
- [87] M. J. Booth, ‘Adaptive optical microscopy: the ongoing quest for a perfect image’, *Light: Science & Applications* 2014 3:4, vol. 3, no. 4, pp. e165–e165, Apr. 2014, doi: 10.1038/lsa.2014.46.

- [88] P. N. Marsh, D. Burns, J. M. Girkin, D. S. Wan, M. Rajadhyaksha, and R. H. Webb, ‘Practical implementation of adaptive optics in multiphoton microscopy’, *Optics Express*, Vol. 11, Issue 10, pp. 1123–1130, vol. 11, no. 10, pp. 1123–1130, May 2003, doi: 10.1364/OE.11.001123.
- [89] J. Won Cha and P. T. C. So, ‘Shack-Hartmann wavefront-sensor-based adaptive optics system for multiphoton microscopy’, *J Biomed Opt*, vol. 15, no. 4, p. 046022, 2010, doi: 10.1117/1.3475954.
- [90] E. Durech, E. Durech, E. Durech, W. Newberry, J. Franke, and M. V. Sarunic, ‘Wavefront sensor-less adaptive optics using deep reinforcement learning’, *Biomedical Optics Express*, Vol. 12, Issue 9, pp. 5423–5438, vol. 12, no. 9, pp. 5423–5438, Sep. 2021, doi: 10.1364/BOE.427970.
- [91] S. Kumar *et al.*, ‘FLIM FRET Technology for Drug Discovery: Automated Multiwell-Plate High-Content Analysis, Multiplexed Readouts and Application in Situ’, *ChemPhysChem*, vol. 12, no. 3, pp. 609–626, Feb. 2011, doi: 10.1002/CPHC.201000874.
- [92] M. A. Bray *et al.*, ‘Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes’, *Nat Protoc*, vol. 11, no. 9, p. 1757, Sep. 2016, doi: 10.1038/NPROT.2016.105.
- [93] Y. Song, V. Madahar, and J. Liao, ‘Development of FRET Assay into Quantitative and High-throughput Screening Technology Platforms for Protein–Protein Interactions’, *Ann Biomed Eng*, vol. 39, no. 4, p. 1224, Apr. 2010, doi: 10.1007/S10439-010-0225-X.
- [94] A. Boes, S. Olatunji, T. Mohammadi, E. Breukink, and M. Terrak, ‘Fluorescence anisotropy assays for high throughput screening of compounds binding to lipid II, PBP1b, FtsW and MurJ’, *Scientific Reports 2020 10:1*, vol. 10, no. 1, pp. 1–8, Apr. 2020, doi: 10.1038/s41598-020-63380-2.
- [95] S. Kumar *et al.*, ‘FLIM FRET Technology for Drug Discovery: Automated Multiwell-Plate High-Content Analysis, Multiplexed Readouts and Application in Situ’, *ChemPhysChem*, vol. 12, no. 3, pp. 609–626, Feb. 2011, doi: 10.1002/CPHC.201000874.
- [96] J. Lightley *et al.*, ‘ROBUST OPTICAL AUTOFOCUS SYSTEM UTILIZING NEURAL NETWORKS APPLIED TO AUTOMATED MULTIWELL PLATE STORM MICROSCOPY’, *European Conferences on Biomedical Optics 2021 (ECBO) (2021)*, paper ES1A.1, p. ES1A.1, Jun. 2021, doi: 10.1364/ECBO.2021.ES1A.1.
- [97] S. Aknoun *et al.*, ‘Quantitative phase microscopy for non-invasive live cell population monitoring’, *Scientific Reports 2021 11:1*, vol. 11, no. 1, pp. 1–11, Feb. 2021, doi: 10.1038/s41598-021-83537-x.

- [98] V. Maioli *et al.*, ‘Time-lapse 3-D measurements of a glucose biosensor in multicellular spheroids by light sheet fluorescence microscopy in commercial 96-well plates’, *Scientific Reports* 2016 6:1, vol. 6, no. 1, pp. 1–13, Nov. 2016, doi: 10.1038/srep37777.
- [99] G. H. Patterson and D. W. Piston, ‘Photobleaching in two-photon excitation microscopy’, *Biophys J*, vol. 78, no. 4, pp. 2159–2162, 2000, doi: 10.1016/S0006-3495(00)76762-2.
- [100] C. Gu, Q. Geng, J. Cheng, and S. Chen, ‘Digital micromirror device-based two-photon microscopy for three-dimensional and random-access imaging’, *Optica*, Vol. 4, Issue 6, pp. 674–677, vol. 4, no. 6, pp. 674–677, Jun. 2017, doi: 10.1364/OPTICA.4.000674.
- [101] E. P. Perillo *et al.*, ‘Deep in vivo two-photon microscopy with a low cost custom built mode-locked 1060 nm fiber laser’, *Biomedical Optics Express*, Vol. 7, Issue 2, pp. 324–334, vol. 7, no. 2, pp. 324–334, Feb. 2016, doi: 10.1364/BOE.7.000324.
- [102] K. Hean Kim *et al.*, ‘Multifocal multiphoton microscopy based on multianode photomultiplier tubes’, *Optics Express*, Vol. 15, Issue 18, pp. 11658–11678, vol. 15, no. 18, pp. 11658–11678, Sep. 2007, doi: 10.1364/OE.15.011658.
- [103] A. H. Buist, M. Müller, J. Squier, and G. J. Brakenhoff, ‘Real time two-photon absorption microscopy using multi point excitation’, *J Microsc*, vol. 192, no. 2, pp. 217–226, 1998, doi: 10.1046/J.1365-2818.1998.00431.X.
- [104] M. Straub and S. W. Hell, ‘Fluorescence lifetime three-dimensional microscopy with picosecond precision using a multifocal multiphoton microscope’, *Appl Phys Lett*, vol. 73, no. 13, pp. 1769–1771, Sep. 1998, doi: 10.1063/1.122276.
- [105] P. Georges, G. Roger, M. P. Fontaine-Aupart, and S. Lévêque-Fort, ‘Fluorescence-lifetime imaging with a multifocal two-photon microscope’, *Optics Letters*, Vol. 29, Issue 24, pp. 2884–2886, vol. 29, no. 24, pp. 2884–2886, Dec. 2004, doi: 10.1364/OL.29.002884.
- [106] R. K. P. Benninger *et al.*, ‘3D high-speed time-resolved fluorescence imaging of solvent interactions in microfluidic devices’, *Micro Total Analysis Systems - Proceedings of MicroTAS 2005 Conference: 9th International Conference on Miniaturized Systems for Chemistry and Life Sciences*, vol. 1, no. 16, pp. 16–18, 2005.
- [107] S. Padilla-Parra, N. Audugé, M. Coppey-Moisán, and M. Tramier, ‘Quantitative FRET Analysis by Fast Acquisition Time Domain FLIM at High Spatial Resolution in Living Cells’, *Biophys J*, vol. 95, no. 6, pp. 2976–2988, Sep. 2008, doi: 10.1529/BIOPHYSJ.108.131276.
- [108] K. H. Kim *et al.*, ‘Multifocal multiphoton microscopy based on multianode photomultiplier tubes’, *Optics Express*, Vol. 15, Issue 18, pp. 11658–11678, vol. 15, no. 18, pp. 11658–11678, Sep. 2007, doi: 10.1364/OE.15.011658.

- [109] C. Buehler, K. H. Kim, U. Greuter, N. Schlumpf, and P. T. C. So, ‘Single-photon counting multicolor multiphoton fluorescence microscope’, *J Fluoresc*, vol. 15, no. 1, pp. 41–51, Jan. 2005, doi: 10.1007/S10895-005-0212-Z/METRICS.
- [110] D. M. Owen *et al.*, ‘Multifocal multiphoton excitation and time correlated single photon counting detection for 3-D fluorescence lifetime imaging’, *Optics Express*, Vol. 15, Issue 20, pp. 12548–12561, vol. 15, no. 20, pp. 12548–12561, Oct. 2007, doi: 10.1364/OE.15.012548.
- [111] F. Görlitz *et al.*, ‘easySLM-STED: Stimulated emission depletion microscopy with aberration correction, extended field of view and multiple beam scanning’, *J Biophotonics*, vol. 11, no. 11, p. e201800087, Nov. 2018, doi: 10.1002/JBIO.201800087.
- [112] J. Lightley *et al.*, ‘openFrame: A modular, sustainable, open microscopy platform with single-shot, dual-axis optical autofocus module providing high precision and long range of operation’, *J Microsc*, vol. 292, no. 2, pp. 64–77, Nov. 2023, doi: 10.1111/JMI.13219.
- [113] ‘Mai Tai® Ti:Sapphire Ultrafast Laser’. Accessed: Dec. 24, 2024. [Online]. Available: <https://www.spectra-physics.com/en/f/mai-tai-ultrafast-laser>
- [114] ‘SLM-200 | Santec’. Accessed: Dec. 24, 2024. [Online]. Available: <https://www.santec.com/en/products/components/slm/slm-200/>
- [115] I. Moreno, B. K. Gutierrez, M. M. Sánchez-López, J. A. Davis, H. P. Khanal, and D. M. Cottrell, ‘Diffraction efficiency of stepped gratings using high phase-modulation spatial light modulators’, *Opt Lasers Eng*, vol. 126, p. 105910, Mar. 2020, doi: 10.1016/J.OPTLASENG.2019.105910.
- [116] ‘Thorlabs - QS7XY-AG Dual-Axis, Ø7 mm Beam Galvo System, Protected Silver Mirrors (Power Supply & Cables Sold Separately)’. Accessed: Dec. 24, 2024. [Online]. Available: <https://www.thorlabs.com/thorproduct.cfm?partnumber=QS7XY-AG>
- [117] J. B. Pawley, ‘Handbook of biological confocal microscopy: Third edition’, *Handbook of Biological Confocal Microscopy: Third Edition*, pp. 1–985, 2006, doi: 10.1007/978-0-387-45524-2/COVER.
- [118] ‘USB-6363 - NI’. Accessed: Dec. 24, 2024. [Online]. Available: https://www.ni.com/en-gb/shop/model/usb-6363.html?srsId=AfmBOor2tqBlnye7mxwc9I9dHFfp6qkBjiSuiKgT-42Im-ekZ7QfIz_N
- [119] ‘Objective Comparison | Objective Selector | Nikon Instruments Inc.’ Accessed: Dec. 24, 2024. [Online]. Available: <https://www.microscope.healthcare.nikon.com/products/optics/selector/comparison/-1918>

- [120] M. Wahl, H. J. Rahn, I. Gregor, R. Erdmann, and J. Enderlein, ‘Dead-time optimized time-correlated photon counting instrument with synchronized, independent timing channels’, *Review of Scientific Instruments*, vol. 78, no. 3, Mar. 2007, doi: 10.1063/1.2715948/687902.
- [121] ‘MultiHarp 150 - High-Throughput Multichannel Event Timer & TCSPC Unit | PicoQuant’. Accessed: Dec. 24, 2024. [Online]. Available: <https://www.picoquant.com/products/category/tcspc-and-time-tagging-modules/multiharp-150-high-throughput-multichannel-event-timer-tcspc-unit>
- [122] R. Gerchberg, ‘A practical algorithm for the determination of phase from image and diffraction plane pictures’, *Optik (Stuttg)*, 1972.
- [123] ‘CuPy: NumPy & SciPy for GPU’. Accessed: Dec. 24, 2024. [Online]. Available: <https://cupy.dev/>
- [124] Q. Li, Z. Wu, M. Wang, H. Song, Y. Pu, and Y. Luo, ‘Design of Uniform Intensity Field Forming System Based on Improved G-S Algorithm’, *2022 23rd International Vacuum Electronics Conference, IVEC 2022*, pp. 442–443, 2022, doi: 10.1109/IVEC53421.2022.10292345.
- [125] N. Krstajić, R. D. Knight, S. M. Ameer-Beg, S. P. Poland, and R. K. Henderson, ‘Development of a doubly weighted Gerchberg-Saxton algorithm for use in multibeam imaging applications’, *Optics Letters*, Vol. 39, Issue 8, pp. 2431–2434, vol. 39, no. 8, pp. 2431–2434, Apr. 2014, doi: 10.1364/OL.39.002431.
- [126] ‘Cairn Research Ltd – Precision Optics for Breakthrough Discoveries’. Accessed: Jan. 15, 2025. [Online]. Available: <https://cairn-research.co.uk/>
- [127] J. Lightley *et al.*, ‘Robust deep learning optical autofocus system applied to automated multiwell plate single molecule localization microscopy’, *J Microsc*, vol. 288, no. 2, pp. 130–141, Nov. 2022, doi: 10.1111/JMI.13020.
- [128] ‘SumeetRohilla (SumeetRohilla) / Repositories · GitHub’. Accessed: Jan. 23, 2025. [Online]. Available: <https://github.com/SumeetRohilla?tab=repositories>
- [129] C. Stringer, T. Wang, M. Michaelos, and M. Pachitariu, ‘Cellpose: a generalist algorithm for cellular segmentation’, *Nature Methods* 2020 18:1, vol. 18, no. 1, pp. 100–106, Dec. 2020, doi: 10.1038/s41592-020-01018-x.
- [130] O. Mendoza-Yero, J. Pérez-Vizcaíno, R. Martínez-Cuenca, P. Andrés, G. Mínguez-Vega, and J. Lancis, ‘Dispersion management in two-photon microscopy by using diffractive optical elements’, *Optics Letters*, Vol. 38, Issue 4, pp. 440–442, vol. 38, no. 4, pp. 440–442, Feb. 2013, doi: 10.1364/OL.38.000440.

- [131] J. A. Galbraith and M. Terasaki, ‘Controlled Damage in Thick Specimens by Multiphoton Excitation’, *Mol Biol Cell*, vol. 14, no. 5, p. 1808, May 2003, doi: 10.1091/MBC.E02-03-0163.
- [132] E. B. Berens, J. M. Holy, A. T. Riegel, and A. Wellstein, ‘A Cancer Cell Spheroid Assay to Assess Invasion in a 3D Setting’, *J Vis Exp*, vol. 2015, no. 105, p. 53409, Nov. 2015, doi: 10.3791/53409.
- [133] ‘ECE 532, 3. Optical Properties’. Accessed: Mar. 03, 2025. [Online]. Available: <https://omlc.org/classroom/ece532/class3/gdefinition.html>
- [134] Q. Liu and F. Weng, ‘Combined Henyey-Greenstein and Rayleigh phase function’, *Applied Optics*, Vol. 45, Issue 28, pp. 7475-7479, vol. 45, no. 28, pp. 7475–7479, Oct. 2006, doi: 10.1364/AO.45.007475.
- [135] S. L. Jacques, ‘Tutorial on Monte Carlo simulation of photon transport in biological tissues [Invited]’, *Biomedical Optics Express*, Vol. 14, Issue 2, pp. 559-576, vol. 14, no. 2, pp. 559–576, Feb. 2023, doi: 10.1364/BOE.477237.
- [136] P. D. Cook, P. D. Cook, J. N. Bixler, R. J. Thomas, and E. A. Early, ‘Prediction of tissue optical properties using the Monte Carlo modeling of photon transport in turbid media and integrating spheres’, *OSA Continuum*, Vol. 3, Issue 6, pp. 1456-1476, vol. 3, no. 6, pp. 1456–1476, Jun. 2020, doi: 10.1364/OSAC.377805.
- [137] S. M. Ross, ‘Generating discrete random variables’, *Simulation*, pp. 47–67, Jan. 2023, doi: 10.1016/B978-0-32-385738-3.00009-2.
- [138] S. Yongrong Deng, W. Yan, and C. Zhang, ‘Optical properties of biological tissues: a review’, *Phys Med Biol*, vol. 58, no. 11, p. R37, May 2013, doi: 10.1088/0031-9155/58/11/R37.
- [139] A. S. Kristoffersen, S. R. Erga, B. Hamre, and Ø. Frette, ‘Testing Fluorescence Lifetime Standards using Two-Photon Excitation and Time-Domain Instrumentation: Rhodamine B, Coumarin 6 and Lucifer Yellow’, *J Fluoresc*, vol. 24, no. 4, p. 1015, 2014, doi: 10.1007/S10895-014-1368-1.
- [140] H. Sparks *et al.*, ‘Characterisation of new gated optical image intensifiers for fluorescence lifetime imaging’, *Review of Scientific Instruments*, vol. 88, no. 1, p. 13707, Jan. 2017, doi: 10.1063/1.4973917/367723.
- [141] S. C. Warren *et al.*, ‘Rapid Global Fitting of Large Fluorescence Lifetime Imaging Microscopy Datasets’, *PLoS One*, vol. 8, no. 8, p. e70687, Aug. 2013, doi: 10.1371/JOURNAL.PONE.0070687.
- [142] B. Javidi *et al.*, ‘Roadmap on digital holography [Invited]’, *Optics Express*, Vol. 29, Issue 22, pp. 35078-35118, vol. 29, no. 22, pp. 35078–35118, Oct. 2021, doi: 10.1364/OE.435915.

- [143] S. Cui, S. Gao, C. Li, W. Zhang, X. S. Yao, and X. S. Yao, ‘Quantitative phase imaging based on polarization encoding’, *Optics Express*, Vol. 30, Issue 24, pp. 43622–43632, vol. 30, no. 24, pp. 43622–43632, Nov. 2022, doi: 10.1364/OE.472373.
- [144] B. Wattellier, S. Monneret, P. Bon, and G. Maucort, ‘Quadriwave lateral shearing interferometry for quantitative phase microscopy of living cells’, *Optics Express*, Vol. 17, Issue 15, pp. 13080–13094, vol. 17, no. 15, pp. 13080–13094, Jul. 2009, doi: 10.1364/OE.17.013080.
- [145] Y. Park and K. Lee, ‘Quantitative phase imaging unit’, *Optics Letters*, Vol. 39, Issue 12, pp. 3630–3633, vol. 39, no. 12, pp. 3630–3633, Jun. 2014, doi: 10.1364/OL.39.003630.
- [146] X. Wang *et al.*, ‘Single-shot isotropic differential interference contrast microscopy’, *Nature Communications* 2023 14:1, vol. 14, no. 1, pp. 1–9, Apr. 2023, doi: 10.1038/s41467-023-37606-6.
- [147] R. Kalita *et al.*, ‘Single-shot phase contrast microscopy using polarisation-resolved differential phase contrast’, *J Biophotonics*, vol. 14, no. 12, Dec. 2021, doi: 10.1002/JBIO.202100144.
- [148] D. K. Hamilton and C. J. R. Sheppard, ‘Differential phase contrast in scanning optical microscopy’, *J Microsc*, vol. 133, no. 1, pp. 27–39, Jan. 1984, doi: 10.1111/J.1365-2818.1984.TB00460.X.
- [149] C. J. R. Sheppard and S. B. Mehta, ‘Quantitative phase-gradient imaging at high resolution with asymmetric illumination-based differential phase contrast’, *Optics Letters*, Vol. 34, Issue 13, pp. 1924–1926, vol. 34, no. 13, pp. 1924–1926, Jul. 2009, doi: 10.1364/OL.34.001924.
- [150] T. N. Ford, K. K. Chu, A. B. Parthasarathy, and J. Mertz, ‘Quantitative phase imaging using a partitioned detection aperture’, *Optics Letters*, Vol. 37, Issue 19, pp. 4062–4064, vol. 37, no. 19, pp. 4062–4064, Oct. 2012, doi: 10.1364/OL.37.004062.
- [151] Z. F. Phillips, M. Chen, and L. Waller, ‘Single-shot quantitative phase microscopy with color-multiplexed differential phase contrast (cDPC)’, *PLoS One*, vol. 12, no. 2, p. e0171228, Feb. 2017, doi: 10.1371/JOURNAL.PONE.0171228.
- [152] R. Kalita *et al.*, ‘Single-shot phase contrast microscopy using polarisation-resolved differential phase contrast’, *J Biophotonics*, vol. 14, no. 12, Dec. 2021, doi: 10.1002/JBIO.202100144.
- [153] H. Liu *et al.*, ‘Open-source implementation of polarisation-resolved single-shot differential phase contrast microscopy (pDPC) on a modular openFrame-based microscope’, *HardwareX*, vol. 21, p. e00622, Mar. 2025, doi: 10.1016/j.ohx.2024.e00622.
- [154] ‘Polarization Image Sensor | Image Sensor for Industrial Use [Overview] | Image Sensor | Products & Solutions | Sony Semiconductor Solutions Group’. Accessed: Feb. 27, 2025. [Online]. Available: <https://www.sony-semicon.com/en/products/is/industry/polarization.html>

- [155] Y. R. He *et al.*, ‘Cell Cycle Stage Classification Using Phase Imaging with Computational Specificity’, *ACS Photonics*, vol. 9, no. 4, pp. 1264–1273, Apr. 2022, doi: 10.1021/ACSPHOTONICS.1C01779/ASSET/IMAGES/MEDIUM/PH1C01779_M003.GIF.
- [156] R. Cao, H. K. Wallrabe, and A. Periasamy, ‘Multiphoton FLIM imaging of NAD(P)H and FAD with one excitation wavelength’, *J Biomed Opt*, vol. 25, no. 1, p. 1, Jan. 2020, doi: 10.1117/1.JBO.25.1.014510.
- [157] M. Arzt *et al.*, ‘LABKIT: Labeling and Segmentation Toolkit for Big Image Data’, *Front Comput Sci*, vol. 4, p. 777728, Feb. 2022, doi: 10.3389/FCOMP.2022.777728/BIBTEX.
- [158] A. E. Carpenter *et al.*, ‘CellProfiler: Image analysis software for identifying and quantifying cell phenotypes’, *Genome Biol*, vol. 7, no. 10, pp. 1–11, Oct. 2006, doi: 10.1186/GB-2006-7-10-R100/FIGURES/4.
- [159] M. Ahmad, A. Wolberg, and C. I. Kahwaji, ‘Biochemistry, Electron Transport Chain’, *StatPearls*, Sep. 2023, Accessed: Mar. 06, 2025. [Online]. Available: <https://www.ncbi.nlm.nih.gov/books/NBK526105/>
- [160] P. M. Schaefer, S. Kalinina, A. Rueck, C. A. F. von Arnim, and B. von Einem, ‘NADH Autofluorescence—A Marker on its Way to Boost Bioenergetic Research’, *Cytometry Part A*, vol. 95, no. 1, pp. 34–46, Jan. 2019, doi: 10.1002/CYTO.A.23597.
- [161] S. J. Berrios-Rivera, G. N. Bennett, and K. Y. San, ‘The effect of increasing NADH availability on the redistribution of metabolic fluxes in Escherichia coli chemostat cultures’, *Metab Eng*, vol. 4, no. 3, pp. 230–237, 2002, doi: 10.1006/mben.2002.0228.
- [162] W. Ying, ‘NAD⁺ and NADH in cellular functions and cell death’, *Frontiers in Bioscience*, vol. 11, no. SUPPL. 3, pp. 3129–3148, Sep. 2006, doi: 10.2741/2038/PDF.
- [163] M. V. Liberti and J. W. Locasale, ‘The Warburg Effect: How Does it Benefit Cancer Cells?’, *Trends Biochem Sci*, vol. 41, no. 3, p. 211, Mar. 2016, doi: 10.1016/J.TIBS.2015.12.001.
- [164] E. V. Potapova *et al.*, ‘Detection of NADH and NADPH levels in vivo identifies shift of glucose metabolism in cancer to energy production’, *FEBS J*, vol. 291, no. 12, pp. 2674–2682, Jun. 2024, doi: 10.1111/FEBS.17067.
- [165] M. T. Erkkilä *et al.*, ‘Macroscopic fluorescence-lifetime imaging of NADH and protoporphyrin IX improves the detection and grading of 5-aminolevulinic acid-stained brain tumors’, *Scientific Reports 2020 10:1*, vol. 10, no. 1, pp. 1–15, Nov. 2020, doi: 10.1038/s41598-020-77268-8.
- [166] J. McGinty *et al.*, ‘Wide-field fluorescence lifetime imaging of cancer’, *Biomed Opt Express*, vol. 1, no. 2, p. 627, Sep. 2010, doi: 10.1364/BOE.1.000627.

- [167] P. M. Schaefer Diana Hilpert Moritz Niederschweiberer Larissa Neuhauser Sviatlana Kalinina Enrico Calzia Angelika Rueck Bjoern von Einem Christine A F von Arnim Patrick M Schaefer *et al.*, ‘Mitochondrial matrix pH as a decisive factor in neurometabolic imaging’, <https://doi.org/10.1117/1.NPh.4.4.045004>, vol. 4, no. 4, p. 045004, Nov. 2017, doi: 10.1117/1.NPH.4.4.045004.
- [168] W. Xiao, R.-S. Wang, D. E. Handy, and J. Loscalzo, ‘NAD(H) and NADP(H) Redox Couples and Cellular Energy Metabolism’, doi: 10.1089/ars.2017.7216.
- [169] T. S. Blacker *et al.*, ‘Separating NADH and NADPH fluorescence in live cells and tissues using FLIM’, *Nature Communications* 2014 5:1, vol. 5, no. 1, pp. 1–9, May 2014, doi: 10.1038/ncomms4936.
- [170] ‘The bh TCSPC Handbook 10th edition, 2023 - Becker & Hickl GmbH’. Accessed: Feb. 18, 2025. [Online]. Available: <https://www.becker-hickl.com/literature/documents/flim/the-bh-tcspc-handbook/?token=5dd34d251b10b5d8fc1b1b7367b8c28a>
- [171] J. T. Sharick, P. F. Favreau, A. A. Gillette, S. M. Sdao, M. J. Merrins, and M. C. Skala, ‘Protein-bound NAD(P)H Lifetime is Sensitive to Multiple Fates of Glucose Carbon’, *Scientific Reports* 2018 8:1, vol. 8, no. 1, pp. 1–13, Apr. 2018, doi: 10.1038/s41598-018-23691-x.
- [172] S. Ranjit, L. Malacrida, M. Stakic, and E. Gratton, ‘Determination of the metabolic index using the fluorescence lifetime of free and bound NADH in the phasor approach’, *J Biophotonics*, vol. 12, no. 11, p. e201900156, Nov. 2019, doi: 10.1002/JBIO.201900156.
- [173] J. Vergen, C. Hecht, L. V. Zholudeva, M. M. Marquardt, R. Hallworth, and M. G. Nichols, ‘Metabolic imaging using two-photon excited NADH intensity and fluorescence lifetime imaging’, *Microsc Microanal*, vol. 18, no. 4, p. 10.1017/S1431927612000529, Aug. 2012, doi: 10.1017/S1431927612000529.
- [174] P. Tsou, B. Zheng, C. H. Hsu, A. T. Sasaki, and L. C. Cantley, ‘A fluorescent reporter of AMPK activity and cellular energy stress’, *Cell Metab*, vol. 13, no. 4, pp. 476–486, Apr. 2011, doi: 10.1016/J.CMET.2011.03.006.
- [175] M. E. G. Rubiño, E. Carrillo, G. R. Alcalá, A. Domínguez-Martín, J. A. Marchal, and H. Boulaiz, ‘Phenformin as an Anticancer Agent: Challenges and Prospects’, *Int J Mol Sci*, vol. 20, no. 13, p. 3316, Jul. 2019, doi: 10.3390/IJMS20133316.
- [176] S. Heinz, A. Freyberger, B. Lawrenz, L. Schladt, G. Schmuck, and H. Ellinger-Ziegelbauer, ‘Mechanistic Investigations of the Mitochondrial Complex I Inhibitor Rotenone in the Context of Pharmacological and Safety Evaluation’, *Scientific Reports* 2017 7:1, vol. 7, no. 1, pp. 1–13, Apr. 2017, doi: 10.1038/srep45465.

- [177] Y. H. HAN, Y. M. YANG, and W. H. PARK, ‘Carbonyl Cyanide P-(Trifluoromethoxy) Phenylhydroazone Induces Caspase-independent Apoptosis in As4.1 Juxtaglomerular Cells’, *Anticancer Res*, vol. 30, no. 7, 2010.
- [178] J. Sutin *et al.*, ‘Fluorescence lifetime microscopy of NADH distinguishes alterations in cerebral metabolism in vivo’, *Biomedical Optics Express*, Vol. 8, Issue 5, pp. 2368–2385, vol. 8, no. 5, pp. 2368–2385, May 2017, doi: 10.1364/BOE.8.002368.
- [179] H. Zhao, K. D. Swanson, and B. Zheng, ‘Therapeutic repurposing of biguanides in cancer’, *Trends Cancer*, vol. 7, no. 8, p. 714, Aug. 2021, doi: 10.1016/J.TRECAN.2021.03.001.
- [180] Y. L. Shi, S. Feng, W. Chen, Z. C. Hua, J. J. Bian, and W. Yin, ‘Mitochondrial inhibitor sensitizes non-small-cell lung carcinoma cells to TRAIL-induced apoptosis by reactive oxygen species and Bcl-XL/p53-mediated amplification mechanisms’, *Cell Death & Disease* 2014 5:12, vol. 5, no. 12, pp. e1579–e1579, Dec. 2014, doi: 10.1038/cddis.2014.547.
- [181] R. Shrestha, E. Johnson, and F. L. Byrne, ‘Exploring the therapeutic potential of mitochondrial uncouplers in cancer’, *Mol Metab*, vol. 51, p. 101222, Sep. 2021, doi: 10.1016/J.MOLMET.2021.101222.
- [182] I. Vorobjev and N. S. Barteneva, ‘Temporal Heterogeneity Metrics in Apoptosis Induced by Anticancer Drugs’, *Journal of Histochemistry and Cytochemistry*, vol. 63, no. 7, pp. 494–510, Jul. 2015, doi: 10.1369/0022155415583534.
- [183] T. Vicar, M. Raudenska, J. Gumulec, and J. Balvan, ‘The Quantitative-Phase Dynamics of Apoptosis and Lytic Cell Death’, *Sci Rep*, vol. 10, no. 1, p. 1566, Dec. 2020, doi: 10.1038/S41598-020-58474-W.
- [184] B. Janicke, A. Kårnsnäs, P. Egelberg, and K. Alm, ‘Label-free high temporal resolution assessment of cell proliferation using digital holographic microscopy’, *Cytometry Part A*, vol. 91, no. 5, pp. 460–469, May 2017, doi: 10.1002/CYTO.A.23108.
- [185] S. P. Cavnar *et al.*, ‘Imaging Sensitivity of Quiescent Cancer Cells to Metabolic Perturbations in Bone Marrow Spheroids’, *Tomography*, vol. 2, no. 2, p. 146, Jun. 2016, doi: 10.18383/J.TOM.2016.00157.
- [186] D. J. Kelly *et al.*, ‘An automated multiwell plate reading flim microscope for live cell autofluorescence lifetime assays’, <https://doi.org/10.1142/S1793545814500254>, vol. 7, no. 5, p. 15, Sep. 2014, doi: 10.1142/S1793545814500254.
- [187] N. Yu *et al.*, ‘Patient-derived cell-based pharmacogenomic assessment to unveil underlying resistance mechanisms and novel therapeutics for advanced lung cancer’, *Journal of*

- Experimental and Clinical Cancer Research*, vol. 42, no. 1, pp. 1–16, Dec. 2023, doi: 10.1186/S13046-023-02606-3/FIGURES/5.
- [188] V. V. Pipiya, Z. E. Gilazieva, S. S. Issa, A. A. Rizvanov, and V. V. Solovyeva, ‘Comparison of primary and passaged tumor cell cultures and their application in personalized medicine’, *Open Exploration* 2019 5:3, vol. 5, no. 3, pp. 581–599, Jun. 2024, doi: 10.37349/ETAT.2024.00237.
- [189] K. Karrobi *et al.*, ‘Fluorescence Lifetime Imaging Microscopy (FLIM) reveals spatial-metabolic changes in 3D breast cancer spheroids’, *Scientific Reports* 2023 13:1, vol. 13, no. 1, pp. 1–12, Mar. 2023, doi: 10.1038/s41598-023-30403-7.
- [190] A. Cong, R. M. L. Pimenta, H. B. Lee, V. Mereddy, J. Holy, and A. A. Heikal, ‘Two-photon fluorescence lifetime imaging of intrinsic NADH in three-dimensional tumor models’, *Cytometry Part A*, vol. 95, no. 1, pp. 80–92, Jan. 2019, doi: 10.1002/CYTO.A.23632.
- [191] S. Kossatz, B. Carney, C. Farley, W. A. Weber, C. M. Drain, and T. Reiner, ‘Direct Imaging of Drug Distribution and Target Engagement of the PARP Inhibitor Rucaparib’, *Journal of Nuclear Medicine*, vol. 59, no. 8, p. 1316, Aug. 2018, doi: 10.2967/JNUMED.117.205765.
- [192] H. J. Park *et al.*, ‘The PARP inhibitor olaparib potentiates the effect of the DNA damaging agent doxorubicin in osteosarcoma’, *Journal of Experimental and Clinical Cancer Research*, vol. 37, no. 1, pp. 1–15, May 2018, doi: 10.1186/S13046-018-0772-9/FIGURES/8.
- [193] H. Li, Z. Y. Liu, N. Wu, Y. C. Chen, Q. Cheng, and J. Wang, ‘PARP inhibitor resistance: the underlying mechanisms and clinical implications’, *Molecular Cancer* 2020 19:1, vol. 19, no. 1, pp. 1–16, Jun. 2020, doi: 10.1186/S12943-020-01227-0.
- [194] R. E. Norris, P. C. Adamson, V. T. Nguyen, and E. Fox, ‘Preclinical Evaluation of the PARP Inhibitor, Olaparib, in Combination with Cytotoxic Chemotherapy in Pediatric Solid Tumors’, *Pediatr Blood Cancer*, vol. 61, no. 1, p. 10.1002/pbc.24697, 2013, doi: 10.1002/PBC.24697.
- [195] C. Mauvezin and T. P. Neufeld, ‘Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-lysosome fusion’, *Autophagy*, vol. 11, no. 8, p. 1437, Jan. 2015, doi: 10.1080/15548627.2015.1066957.
- [196] Q. Ba, G. Raghavan, K. Kiselyov, and G. Yang, ‘Whole-Cell Scale Dynamic Organization of Lysosomes Revealed by Spatial Statistical Analysis’, *Cell Rep*, vol. 23, no. 12, p. 3591, Jun. 2018, doi: 10.1016/J.CELREP.2018.05.079.
- [197] B. Cabukusta and J. Neefjes, ‘Mechanisms of lysosomal positioning and movement’, *Traffic*, vol. 19, no. 10, pp. 761–769, Oct. 2018, doi: 10.1111/TRA.12587.
- [198] G. J. LaBonia, S. Y. Lockwood, A. A. Heller, D. M. Spence, and A. B. Hummon, ‘Drug penetration and metabolism in 3-dimensional cell cultures treated in a 3D printed fluidic device:

- Assessment of irinotecan via MALDI imaging mass spectrometry', *Proteomics*, vol. 16, no. 11–12, p. 1814, Jun. 2016, doi: 10.1002/PMIC.201500524.
- [199] N. Matsumoto *et al.*, 'Correction of spherical aberration in multi-focal multiphoton microscopy with spatial light modulator', *Optics Express, Vol. 25, Issue 6, pp. 7055-7068*, vol. 25, no. 6, pp. 7055–7068, Mar. 2017, doi: 10.1364/OE.25.007055.
- [200] K. Karrobi *et al.*, 'Fluorescence Lifetime Imaging Microscopy (FLIM) reveals spatial-metabolic changes in 3D breast cancer spheroids', *Sci Rep*, vol. 13, no. 1, p. 3624, Dec. 2023, doi: 10.1038/S41598-023-30403-7.