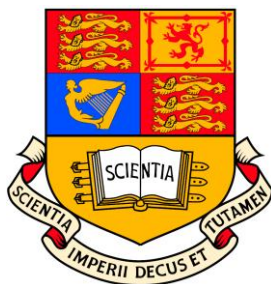


TOOLS FOR SINGLE CELL PROTEOMICS

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2011

Submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

Abstract

Despite recent advances that offer control of single cells, in terms of manipulation and sorting and the ability to measure gene expression, the need to measure protein copy number remains unmet. Measuring protein copy number in single cells and related quantities such as levels of phosphorylation and protein-protein interaction is the basis of single cell proteomics.

A technology platform to undertake the analysis of protein copy number from single cells has been developed. The approach described is 'all-optical' whereby single cells are manipulated into separate analysis chambers using an optical trap; single cells are lysed by mechanical shearing caused by laser-induced microcavitation; and the protein released from a single cell is measured by total internal reflection microscopy as it is bound to micro-printed antibody spots within the device. The platform was tested using GFP transfected cells and the relative precision of the measurement method was determined to be 88%. Single cell measurements were also made on a breast cancer cell line to measure the relative levels of unlabelled human tumour suppressor protein p53 using a chip incorporating an antibody sandwich assay format. This demonstrates the ability count protein copy number from single cells in a manner which could be applied in principle to any set of proteins and for any cell type without the need for genetic engineering.

Metabolism can undergo alteration in diseases such as cancer and heart failure and also as cells differentiate during development. In order to assess how it may inform a proteomic measurement, multidimensional two-photon fluorescence metabolic imaging is conducted on a cultured cancer cell line, primary adult rat cardiomyocytes and human embryonic stem cells. By measuring the parameters of fluorescence such as intensity and lifetime of the autofluorescent metabolic co-factors NADH and FAD, it was found to be possible to contrast cells under various conditions and metabolic stimuli. In particular, human embryonic stem cells were able to be contrasted at 3 stages of development as they underwent differentiation into embryonic stem cell derived cardiomyocytes.

Metabolic imaging provides a non-destructive method to monitor cellular metabolic activity with high resolution. This is complimentary to the single cell proteomic platform and the convergence of both techniques holds promise in future investigations into how metabolism influences cell function and the proteome in development and disease.

Author declaration

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

- p53 pulldowns were conducted with Joseph Kaplinsky and Edward Burgin
- Antibody validation by Western blotting was performed by Heather Rada
- Work carried out or assisted by additional parties is attributed in the text as appropriate

Acknowledgements

“If it bleeds, we can kill it.”

This piece of insight comes from the 1987 sci-fi action film *Predator*. A film which follows an elite special forces team on a mission to rescue hostages from guerrilla territory in Central America. Unbeknownst to the group, they are being hunted by a technologically advanced form of extra-terrestrial life, the Predator. The climax of the film depicts Dutch, played by Arnold Schwarzenegger, barely defeating the Predator who activates a self-destruct mechanism resulting in a massive explosion from which Dutch manages to narrowly escape. To me the film represents how my PhD has unfolded and how ultimately one must ‘go it alone’ but you are only able to do so because of the help, support and strength of those around you. I’d like to take this opportunity to thank those who have helped me along the way.

It is difficult to overstate my appreciation and gratitude to my supervisors, David Klug, Paul French and Mark Neil, for giving me the amazing opportunity to work on this project and have found their ideas, insight and patience invaluable. I would also like to thank Keith Willison, Andrew de Mello and especially Oscar Ces. I wish to thank Sian Harding and Nadire Ali for their wonderful collaboration. I would also like to thank Anca Margineanu, Chris Dunsby, Peter Lanigan and Tanja Ninkovic for their scientific guidance, without which the project would not be where it is today. I also wish to express thanks to my fellow SCPers and Photonics group who have been a great help over the years. Particularly, Ed Burgin and Joe Kaplinsky who I’ve worked with very closely and always enjoy brainstorming with.

Special thanks go to my friends and to my remarkable family, especially my beautiful sisters, Zahra and Ameneh, and my protégé, little Afshin. Special thanks also my beloved Monks, aka Hannah, who has always kept me smiling. Lastly, it is a pleasure and an honour to thank my parents, Berys and Morteza. To you I dedicate this thesis.

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List of abbreviations

1PE	single photon excitation
2PE	two photon excitation
a_x	fluorescence lifetime contribution
AB	antibody
ARCMs	adult rat cardiomyocytes
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAD	computer-aided design
CFD	computational fluid dynamics
CIN	α -cyano-4-hydroxy cinnamate
Da	Dalton
DNA	deoxyribonucleic acid
DPSS	diode-pumped solid state
eGFP	enhanced green fluorescent protein
EM-CCD	electron multiplying charge coupled device
ETC	electron transport chain
FAD	flavin adenine dinucleotide
FIFO	first in, first out (TCSPC data acquisition)
FLIM	fluorescence lifetime imaging microscopy
G	glucose
hESC	human embryonic stem cells
hESCM	human embryonic stem cell-derived cardiomyocytes
IR	infra-red
LDH	lactate dehydrogenase
LIM	laser induced microcavitation
MCT	monocarboxylate transporter
MT	mitochondria
NA	numerical aperture
NADH	nicotinamide adenine dinucleotide
OT	optical trap
OXA	oxamate
p53	tumour protein 53
PDMS	poly(dimethylsiloxane)
PYR	pyruvate
Re	Reynolds number
RNA	ribonucleic acid
RR	redox ratio
S/N	signal to noise ratio
TCA	tricarboxylic acid cycle
TCSPC	time-correlated single photon counting
TE	trapping efficiency
TIRF	total internal reflection fluorescence
λ	radiation wavelength
τ	fluorescence lifetime

1 Single Cell Analysis

1.1 The single cell advantage

Why study proteomics and why single cells?

There are a number of reasons to analyse proteins, their copy number and protein post-translational modifications at the level of single cells. There may be simple practical reasons as in the case of rare or precious cells where the limited quantities available give the researcher no choice. A good example of this is the analysis of circulating tumour cells where there can be as few as one cell in an entire clinical blood sample [1]. There is the situation where cell to cell variation masks the properties of interest or where the variation from cell to cell actually is the property of interest, typified by the studies of genetically identical bacterial populations [2]. There is also the interest in sub-cellular properties such as location and/or co-location of cellular components.

Proteins are of *primary importance* and carry out the actions necessary for cellular function. Unlike the genome, the proteome is not static. Instead, levels of protein expression are constantly changing and their concentrations have direct phenotypic consequences. A major goal in biology is to provide a complete quantitative description of cellular behaviour. This task, however, has been hampered by the difficulty in measuring protein abundances, their variation and exactly which proteins are expressed in a particular cell to begin with.

The typical protein content of a cell is approximately 10 % (w/w) [3]. A typical mammalian cell has a diameter of 10 μm and a volume of 0.5 pL, which, assuming an average molecular mass of 30 kDa [4], suggests a total protein content in the region of 1×10^9 copies. There are upwards of 20×10^3 protein coding genes in human DNA [5] which are estimated to produce, including splice variants and post-translational modifications, approximately 1×10^6 proteins [6]. It is not known how many different proteins are expressed in a typical mammalian cell. Although somewhat arbitrary, even if 1 % of the total coded proteins are present then a typical cell expresses 1×10^4 different proteins. Therefore, on average, a typical protein is expressed at 1×10^5 copies per cell. However, this is not an appropriate concept since proteins may be present at single copy number or be highly abundant.

Conventional cellular analyses work at the level of 10^5 - 10^6 cells and their responses are pooled together as an ensemble measurement. They assume the most relevant measure of a stimulus response is one which is averaged over large populations. Taking data from many cells provides information on major patterns and features such as high abundance proteins or common signalling networks. Averaging completely ignores effects from cell cycle dependent states, different and

inhomogeneous cellular responses or genotypic and phenotypic variability. Bulk experiments may also be misleading. Population heterogeneity may be governed by non-normal distributions. Such cells may exhibit a bimodal response, where a population may contain high or low responders or fast or slow responders. For example, figure 1.1a portrays the case whereby 50% of cells are in 1 state, say off, and 50% are in another, say on. By measuring these cells with a population averaging technique, one would be unable to determine whether the underlying biochemistry permits a bimodal, where 50 % of cells respond with full intensity, or graded, all cells responding with 50 % intensity, response. Another example of misleading bulk experiments is illustrated in figure 1.1b. A cellular response may be fast in individual cells but its onset may vary in time in the population and as such the kinetics may be misinterpreted.

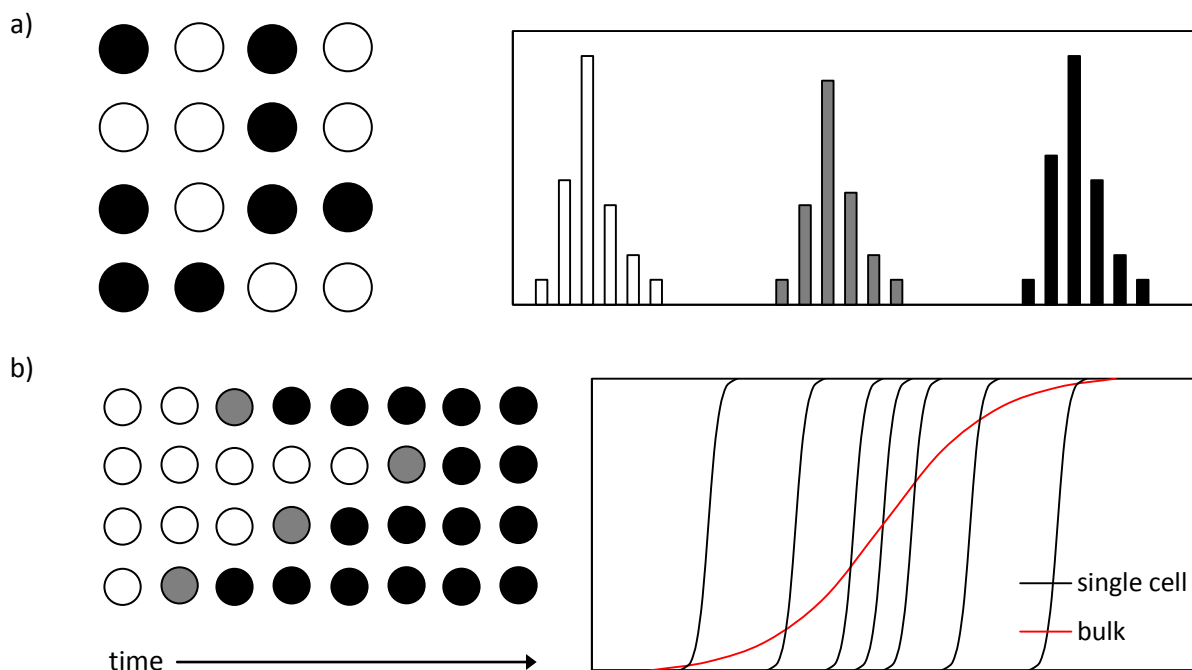


Figure 1.1: Two classic examples of how averaging can mislead results. a) A population of cells contains 50 % of cells in state 1 (white circles and bars) and 50 % in state 2 (black circles and bars). Averaging may result in a non-physiological average (grey bars). b) A population of cells may contain early or late responders to a stimulus but with similar kinetics (black lines). Again, averaging may be misleading toward the functional model of cell behaviour (red line).

The case for single cell proteomics is perhaps best made for disease. The enormous efforts of genomic sequencing, especially in the 1990s, have advanced our understanding of molecular alterations in human disease tremendously. However, genomic approaches have limitations. A genomic sequence is essentially a list of proteins that can be produced in a cell but it does not specify post translational modifications. Nor does it specify which proteins interact, how they

interact or where they interact. In addition, transcript copy number does not always correlate with protein abundances. However, proteomics is not so much about creating another master list but categorising these interactions and how these pathways and interaction networks play a role in pathology. Indeed, the vast majority of pharmacological agents are directed at proteins as opposed to genes and so one promise of proteomics is to discover biomarkers for medicine and improve drug development.

Cancer is often considered a genetic disease but in a functional sense is a disease of the proteome [7]. Tumours are invariably heterogeneous and investigating cancer at the single cell level will help identify subpopulations based on their proteome and altered signalling networks. Of importance is finding what drives the tumour and to determine what conditions lead to metastasis, a process which is responsible for over 90 % of cancer related deaths [8]. Another leading cause of morbidity and death is heart failure [9]. The causes of cardiac dysfunction in most heart diseases is still largely unknown but are likely able to be elucidated by proteomic studies.

1.2 Project aims

A simple shift from population assays to single cells will not be sufficient in order to achieve these goals. The fact that protein copy numbers may be as low as 1 or 2 per cell [10] calls for the development of new technologies and the improvement of existing systems [11]. Since there exists no amplification technique for protein analogous to polymerase chain reaction for DNA then this requires single cell proteomic methods to have appropriately wide dynamic ranges of detection. The comprehensive analysis of the proteome of a cell will comprise information on the protein content, relative or absolute levels of expression, protein-protein interactions and post-translational modification.

Despite recent advances that offer control of single cells [12], in terms of manipulation and sorting and the ability to measure gene expression, the need to measure protein copy number remains unmet. Measuring protein copy number in single cells and related quantities such as levels of phosphorylation and protein-protein interaction is the basis of single cell proteomics.

The combination of fluorescent labelling of proteins, particularly using genetically engineered constructs, such as fluorescent fusion proteins, and modern microscopy has proven to be a particularly powerful combination for single cell analysis. This approach has in recent years started to uncover the connectivity of protein networks including some fascinating reports of pulsatile

behaviour such as in the case of the creation of pulses of p53 in response to a DNA-damaging stimulus [13]. The combination of microscopy and fusion protein labelling is extremely powerful and will no doubt continue to provide important new insights. It does, however, have some significant limitations. Firstly, there is always the concern that the inclusion of fusion proteins can alter the biology observed [14, 15]. Perhaps more limiting, however, are the restrictions caused by the need to use genetically engineerable cells. This is particularly problematic for human samples and in particular for human clinical samples. Moreover, although immortal cell lines are easily engineered and studied, many of these cell lines have biology which is greatly altered from the biology of the human tissue from which it originates. This is due to a combination of their origins as cancerous cells and their genetic instability over many years of culture in many different laboratories around the world under many different conditions [16]. Furthermore, there is no practicable method for engineering human clinical samples either from healthy volunteers or from sick patients such that they can be studied using fusion protein labels. Finally, there is the restriction on multiplexing signals from multiple labels for multiple proteins. The current practical limit for live cells is three or four different labels, and even then, if stable cell lines are required, the practicalities mean that such experiments are rarely undertaken.

The aim of this project is to develop an integrated technology platform capable of manipulating, lysing and analysing the protein content of a single cell in a precise and reproducible manner. Considerable interest lies in metabolism and how this is altered in disease and development. The study of rare cells such as stem cells does not lend itself to high-throughput population-based methods. Therefore, the development of techniques that allow multiple measurements to be conducted on the same cell to maximise information on cell state is vital. This project also aims to develop multidimensional fluorescence imaging technology for the monitoring and analysis of the metabolic phenotype of single cells, which will inform a proteomic measurement of a cell.

1.3 Thesis outline

This thesis is structured into two main areas:

Part I Microfluidic antibody capture with TIRF detection

Part II Autofluorescence determination of metabolic phenotype

The focus of part I is the development of an integrated technology platform that is capable of performing a proteomic analysis at the single cell level. In order to do so, the platform must be capable of first sorting and trapping cells into defined locations, secondly lysing them and, thirdly, qualitatively or quantitatively measuring the protein content of the lysed cell. Chapter 2 outlines the multitude of different approaches that may be taken in order to achieve each individual capability and discusses the merits of each toward single cell analysis with justification of why a particular method was chosen over the alternatives. Chapter 3 follows the design and development of the microfluidic system and how it is exploited to effect cell trapping. Simulations of the fluid dynamics of different designs are explored and a full characterisation of the device in terms of trapping efficiency is made. Chapter 4 demonstrates the capability of laser induced microcavitation as a method to achieve single cell lysis and describes the experimental microscope-based platform upon which measurements will be conducted. Chapter 5 describes the antibodies used to capture protein originating from a single cell and the total internal fluorescence microscopy method used to detect protein that is bound to surface immobilised antibodies in a microarray format. Validation of the antibodies used in this work is described along with the extensive optimisation of microarray production parameters. In addition, a simple reaction-diffusion model is employed to explore the transport limited kinetics of protein binding within the reduced volume of the microfluidic analysis chambers. Chapter 6 pulls together the systems and methods developed in the preceding chapters to demonstrate a platform capable of single cell proteomic analysis. It is shown that the precision and fidelity of the combined workflow is sufficient to produce data that is revealing of cell to cell variation in protein copy number and demonstrates the first measurement of 'native' cell to cell protein copy number variation using the tumour suppressor protein p53 as an example.

The focus of part II shifts to development of a non-destructive multidimensional fluorescence imaging method of determining the metabolic phenotype of a cell. It is based on two-photon excitation of endogenous fluorophores that are cofactors in redox metabolism. Chapter 7 introduces these fluorophores and their role in cellular metabolism and how metabolism may be altered in disease and development. Chapter 8 describes the basic properties of fluorescence and the modes of microscopy which will be employed. Chapter 9 presents the results of the investigation into the metabolism of a cancer cell line, the differentiation of embryonic stem cells and of cells sourced from failing and non-failing adult rat hearts.

The thesis is concluded with chapter 10 which reviews the achievements of this work and briefly discusses future directions.

PART I

MICROFLUIDIC ANTIBODY CAPTURE CHIP WITH TIRF DETECTION

2 Methods in Single Cell Analysis

2.1 Concept workflow

In order to perform quantitative single cell proteomics, 3 basic steps are required – cells must first be isolated, then lysed to expose cell contents to the fluidic environment and measurements must be made upon these contents. This chapter will describe the possible solutions to each of the 3 steps and the justification for each chosen method that will be integrated into the technology platform.

- Cell handling, isolation and trapping

Hydrodynamic, optical, chemical, dielectrophoretic, magnetic and acoustic methods.

- Cell Lysis

Optical, electrical, chemical and mechanical methods.

- Proteomic Analysis

Microarray affinity capture, capillary electrophoresis and mass-spectrometry.

- Chosen workflow

Hydrodynamic trapping of cells into defined locations which are then lysed optically using laser-induced microcavitation followed by TIRF-based detection of protein binding to affinity capture microarrays.

2.2 Cell handling, isolation and trapping

Cell cultures of 10^3 - 10^6 cells/mL can be easily manipulated using bench-top assays and conventional pipettes in volumes typically on the order of microlitres to millilitres. Single cell analysis is difficult since in order to study a single cell you must first isolate it and handling single cells is inherently difficult due to their small size, typically 10 μ m in diameter.

Flow cytometry is able to analyse and separate single cells. It has become an established method since its introduction by Moldovan in 1934 [17] who demonstrated the principle of counting and the examination of cells by suspending them in a stream of fluid and passing them by a detection

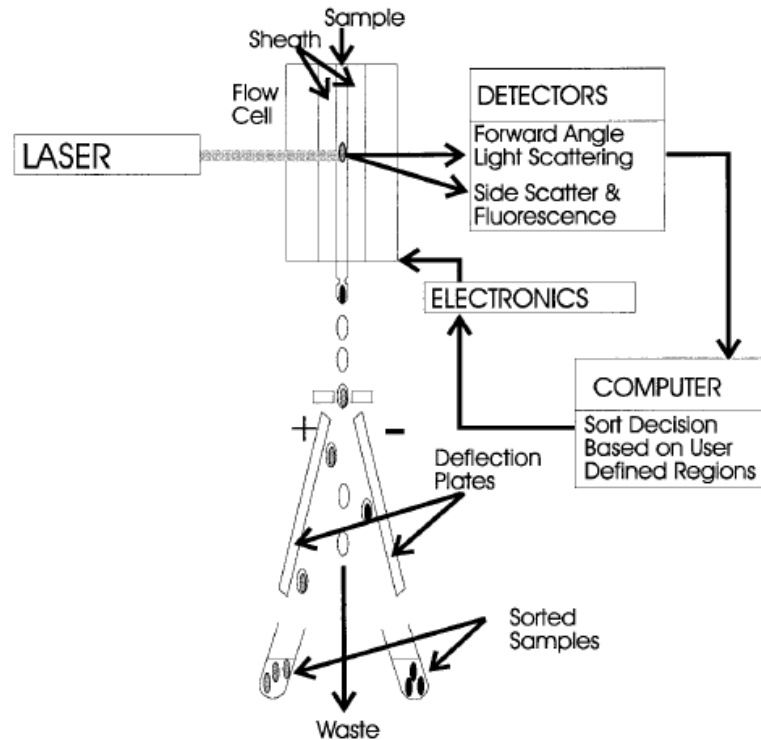
apparatus. Development by Gucker et al. [18] and Wallace Coulter [19] allowed electrical measurement of the cell volume to be made. Optical flow cytometry quickly replaced electrical techniques due to the increased dynamic range of particle size and the independence of cell orientation on scattering signals (side scatter determines granularity and forward scatter determines cell volume). Advancements in ink jet printing made possible the first flow sorter, described by Fulwyler [20]. In flow sorters, cells are flowed at high speed, $1 - 10 \text{ ms}^{-1}$, and pass an interrogation zone where light scatter and fluorescence signals are detected. Based on these parameters, as flow is broken up into a uniform stream of droplets, each droplet is electrostatically charged and deflected causing the cell to be sorted (figure 2.1a). Fulwyler's system measured 3 parameters – one wavelength and the two from scatter measurements. The most advanced fluorescence activated cell sorting instruments today are equipped with 4 light sources and 8 detectors and can achieve 19 parameter measurements [21]. They are capable of sorting up to 100,000 cells/s [22] and serve as high throughput platforms.

Flow cytometry allows multiparameter data acquisition and multivariate data analysis, high speed analysis and the ability to effect cell sorting [23]. However, flow cytometers require of the order $10^5 - 10^6$ cells to be injected and are not suited to study rare cell types such as circulating tumour cells. A fundamental drawback to flow cytometry is that it does not address time dependent measurements of the same individual cell, or spatial localisation within a cell. Although, conventional flow cytometry will not be a part of this work, it still plays an irreplaceable role in single cell proteomics as demonstrated by Newman et al. [24].

Laser scanning cytometry (LSC) is an imaging based technique which involves scanning one or several lasers over fluorescently labelled or stained cells on a microscope slide. Early LSC sacrificed the throughput of flow cytometry for the ability to obtain time resolved measurements of cellular processes from the same cell. Rapid advances in optoelectronics have all but eliminated the disparity of the two main modes of cytometry as far as throughput is concerned. Krivacic et al. [25] developed a cytometer incorporating a fibre-optic bundle to process 300,000 cells/s for the detection of rare cells (figure 2.1b).

High throughput is not necessarily a primary goal of the work presented in this thesis. Of distinct importance is the ability to manipulate and isolate single cells to predetermined locations or compartments to perform a proteomic measurement. The microfluidic format is ideally suited to single cell studies.

a)



b)

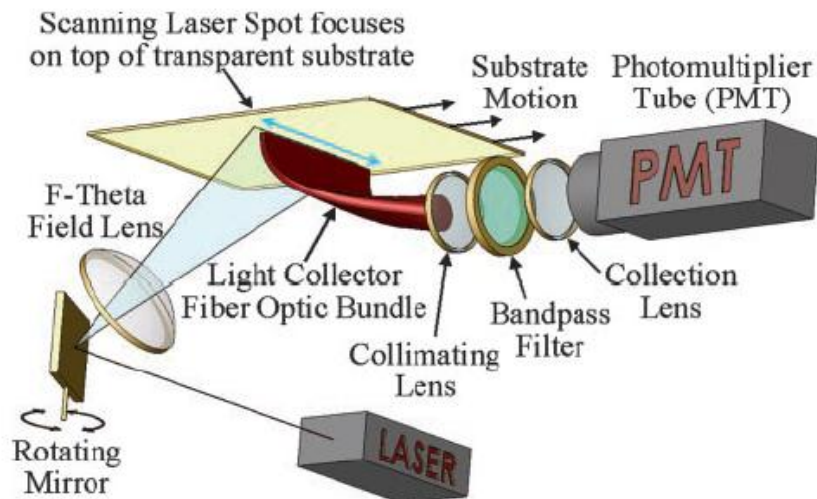


Figure 2.1: a) In fluorescence activated cell sorting, a cell passes an interrogation zone where its light scattering and fluorescence properties are measured and a decision is made to sort it based on the measurement. b) Optical detection strategy of laser scanning cytometry which is capable of scanning 300,000 cells/sec. Reproduced from [23] and [25].

Microfluidic devices process and manipulate, typically, nanolitre volumes of fluid using channels and features on the order of 10 – 100 μm . It is as much a distinct field of study as it is a technology. Some cite post Cold War chemical and biological threats as the main stimulus for the rapid growth of microfluidic technology [26] but microfluidics emerged primarily from molecular biology, where it arose from the need for greater throughput and higher sensitivity. Capitalising on the success of the microelectronics industry to build and fabricate on micron length scales, the possibility of

automating chemistry and biology was, and still is, extremely attractive. Indeed, fabrication of early devices was made using silicon but this has been largely replaced by bio-compatible elastic polymers such as poly(dimethylsiloxane) (PDMS).

The microfluidic length scale is on the same order as that of typical cell dimensions making cell handling straightforward. The microfluidic advantage is derived as much from characteristic fluid flow in microchannels as it does from characteristic length scale and offers capabilities of control impossible at larger scales, such as laminar flow and hydrodynamic focussing. Due to reduced volumes, the ability to precisely control the local environment is afforded, such as pH, temperature and the rapid exchange of buffer or introduction of a chemical stimulus. In addition, microfluidics offers pragmatic advantages such as low cost, low quantity sample consumption and small footprints for analytical devices.

Much of microfluidics early success can be attributed to DNA amplification via the polymerase chain reaction (PCR) and chemical microreactors. PCR is not complicated but conventional thermal cyclers are often attributed with slow and inefficient reaction times. Y. Schaerli et al. [27] reported a device which completed 34 cycles of PCR in 17 min compared to conventional bench-top solutions which typically require 1 - 2 hours.

The application of microfluidics is highly diverse and has been reviewed extensively. Particular insight can be gained from historical perspectives and a review by Whitesides is especially noteworthy [26]. This section will focus on microfluidic techniques to trap or isolate single cells.

Cell trapping methods may require surface contact or are contactless. Methods requiring surface contact are hydrodynamic trapping and chemical immobilisation. Contactless trapping methods involve applying external field gradients to induce forces such as magnetic, acoustic, optical and electrical fields. These may be used in isolation but are often used in a complementary fashion.

2.2.1 Hydrodynamic trapping

Hydrodynamic trapping within a microfluidic channel is a term applied when using barriers, wells or obstacles to physically sequester cells or particles from flow and position them at predefined sites. It is the most common and straightforward method to effect trapping.

Microwell arrays are a highly-parallelised format whereby cells are allowed to settle or are filtered in microwells on a chip surface (figure 2.2a). By exploiting the size difference between circulating

tumour cells (CTCs) and erythrocytes, Zheng et al [28] demonstrated a 90 % recovery rate of CTCs in a microarray format. An array of 10 μm diameter holes was etched in a parylene film to capture the CTCs, which were electrically lysed after capture and demonstrated downstream analysis by β -actin gene PCR.

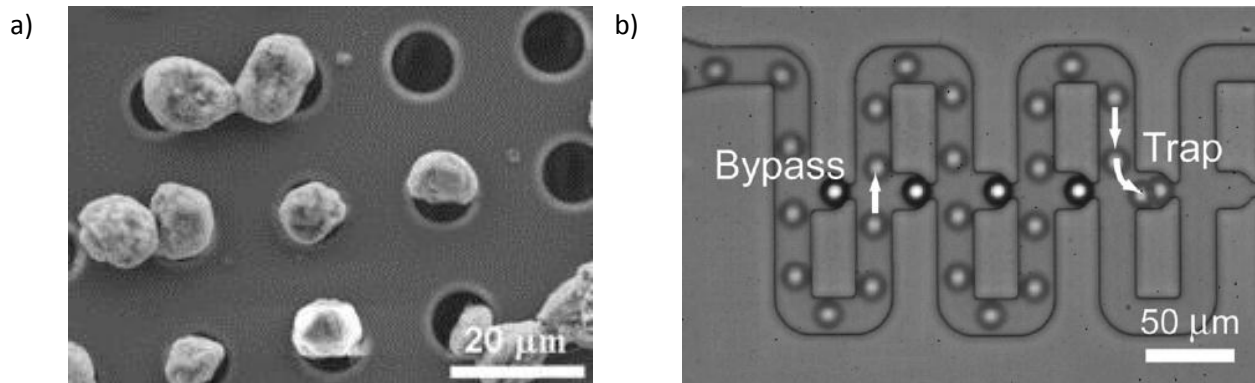


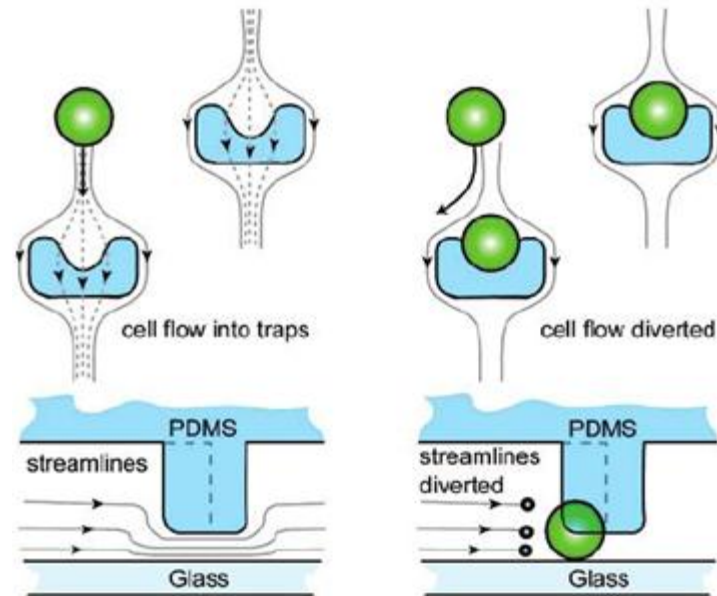
Figure 2.2: a) Cells captured by a parylene membrane filter as imaged by scanning electron microscopy. b) Trapping mechanism of arresting objects from flow. When the trap is empty resistance through the trap is lower than that of the loop channel and the stream flows through the trap, otherwise, flow is carried along the loop to the first available trap. Reproduced from [28] and [29].

Weirs, pillars or topologically similar features are popular in effecting a hydrodynamic trap. Wheeler et al. [30] employed several fluid streams to load a cell in a dock placed at a point of stagnation at a T-junction. By exploiting fluid dynamics cells were loaded and subsequently perfused with reagents to perform cell viability and Ca^{2+} assays. Although not demonstrated for single cells, Li et al. [31] used U-shaped weirs placed in the channel and perpendicular to flow to retain cells as fluid is flowed. Cell loading was opportunistic as flow was diverted down specific channels.

A more directed approach based on the principle of fluidic resistance was shown by Tan & Takeuchi [32]. They designed a device comprising a meandering square-wave channel superimposed onto a narrow straight channel and traps were formed where the two intersected (figure 2.2b). The channels are designed such that the straight channel has a lower fluidic resistance than the meandering channel and so particles in flow become trapped at the narrowed intersections as they try to pass. But the trapped particle acts as a plug increasing resistance along the straight channel so flow is subsequently redirected via the meandering channel and on to the next intersection where successive traps are filled. Coupled with laser-based bubble generation, the system allowed selective retrieval of trapped particles along the channel. Following on from this Tan & Takeuchi [29]

encapsulated cells in alginate beads to demonstrate the suitability of the device as a dynamic cell microarray for drug screening.

a)



b)

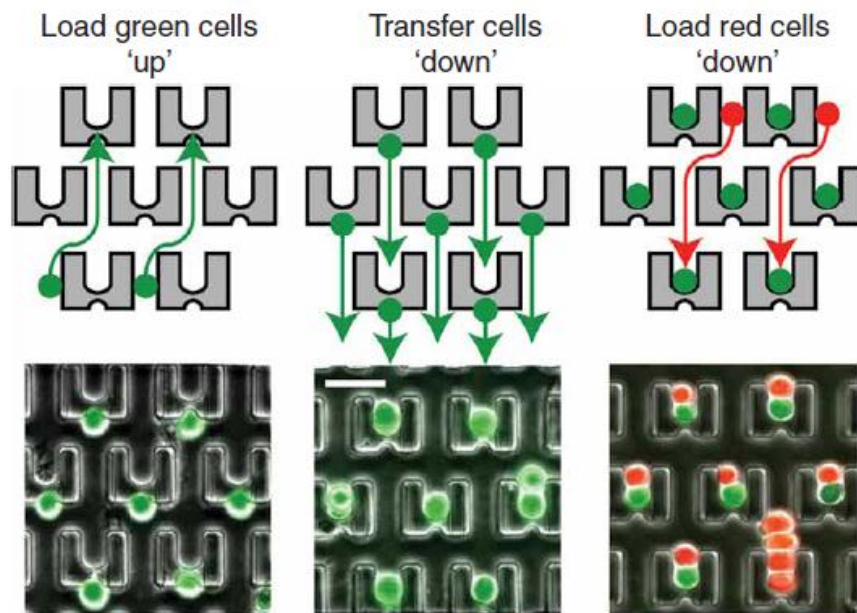


Figure 2.3: a) Mechanism of self-sealing cell trapping using PDMS features suspended from the chamber ceiling. b) The mechanism was modified to facilitate cell pairing and cell fusion in a multiplexed fashion in a single chip. Reproduced from [33] and [34].

U-shaped geometries designed to retain only single cells have been demonstrated and feature often in array-format for single cell analysis. The array of traps is similar to that of a Pachinko game whereby cells cascade through the device eventually becoming trapped. In the implementation by

Di Carlo et al. [35], the structures do not extend the full channel depth but hang a few microns short of the glass coverslip bottom surface (figure 2.3a). Free trapping sites allow a fraction of fluid streamlines to enter the traps and any cell flowing along one of these streams becomes trapped. Once trapped, the cell partially occludes the gap and streamlines are diverted. Hydrodynamic traps that self-seal enhance the number of single cells that are trapped above that of a Poissonian distribution for a random process [33].

In-flow traps, such as that demonstrated by Di Carlo et al. [35] allow rapid perfusion of media and solution switching rates are only limited by diffusion of the solution front. Such devices have been used to demonstrate culturing of HeLa cells and in enzyme profiling to estimate kinetics and concentration of carboxylesterases in cancer cells. Wldokowic et al. [36] quantified anti-cancer drug induced apoptosis and reported similar statistical variation with 300 cells to fluorescence activated cell sorting (FACS) using 15000 - 30000 cells. These devices will prove useful for rare-cell populations and, unlike FACS, can dynamically monitor the cell population.

Kim et al. [37] developed a more accurate cell trapping model by considering inertial, in addition to viscous, components in the Navier-Stokes equation. Viscous terms are sufficient to describe motion through the device but only inertial terms can describe the cell's deceleration as it becomes confined in the traps. Three trap geometries were tested within chambers – the flat-type, U- and C-sieve. The flat type consisted of rectangular features with single apertures which dictated the location of the trapping site. The U-sieve contained either 1 or 2 apertures and the C-sieve, which was a semi-circular feature consisting of 13 apertures. It was shown that each was suited for trapping single, tens or hundreds of cells with good agreement between simulation and experiment. The simulation consisted first of determining the fluid field and then modelling cell trajectories through the device based on this field. The presence of trapped cells had no consequence on the fluid field. Behaviour in self-healing traps would be better reproduced if there existed coupling between cell trajectory and fluid flow field through the device. This would be relatively straightforward to implement given the work by Kim et al. but is computationally demanding.

Skelley et al. [34] modified Di Carlo's cell trapping array to facilitate cell pairing and induce fusion in several cell types including mouse embryonic stem cells (figure 2.3b). The trap structures were modified to include a deeply-recessed back-side "cup." Cells are flowed and trapped but when flow is subsequently reversed cells are transferred to the back-side cup of the opposite trapping post. Once this is completed for a first cell type, it is repeated for a second cell type.

2.2.2 Optical trapping

The transfer of momentum from light to transparent particles, such as a cell, enables non-invasive trapping and manipulation. In 1970, Ashkin showed that photon pressure from a focused laser beam produced forces which could significantly affect the dynamics of small transparent micrometre sized particles and used two opposing beams to confine particles at their coincident foci [38]. As originally intended, the method was applied to trapping and cooling atoms by Chu et al [39] but found a completely different application in biology that started with the single-beam gradient force optical trap – more commonly known as the optical tweezer – which was able to stably confine dielectric particles in the range of 25 nm – 10 μ m [40]. The same apparatus was capable of trapping individual tobacco virus mosaic and *Escherichia coli* bacteria and individual mitochondria within eukaryotic cells as well as the cells themselves [41, 42]. Important applications of the optical tweezer in biology have involved the measurement of molecular motors such as bacterial flagella [43] and nucleic motor enzymes such as RNA polymerase [44]. The understanding of detailed motion of kinesin motors owes much to studies employing optical tweezers [45-47].

Trapping may be described under two broad regimes. For particles whose size is much less than the wavelength of the trapping beam, i.e. $r \ll \lambda$, then the Rayleigh approximation is applied and the particle is treated as an electric dipole in an electromagnetic field. For $r \gg \lambda$ the geometric optics approximation is applied and the forces are derived from the reflection and refraction of light by the particle. Eukaryotic cells are on the order of 10 – 20 μ m and trapping wavelengths are typically in the near infra-red (800 – 1100 nm) to minimise photodamage and minimise heating effects. While the two are comparable, and therefore neither regime is strictly valid, the geometric approach generally suffices. The associated forces are the scattering force, which operates in the direction of the incident beam and arises from radiation pressure, and a gradient force, which operates along the intensity gradient of the beam. Figure 2.4 illustrates the refraction of two rays, *a* and *b*, from a focussed Gaussian beam, which gives rise to the forces F_a and F_b in the direction of the momentum change. The vector sum of these forces is always restoring for axial (figure 2.4a) and transverse (figure 2.4b) displacements of the particle from the trap focus *f* ensuring the trap is stable.

An optical trap is made simply by constructing a three-dimensional electromagnetic field gradient with high intensities in the centre. Such gradients occur near any focus. The sharper the focus, the steeper the gradient. Sufficiently high gradients can be achieved simply by focusing laser light with a high numerical aperture microscope objective and provide the most stable traps since they provide the largest fraction of rays giving rise to the gradient force. The same objective is then able to image the trapped particle.

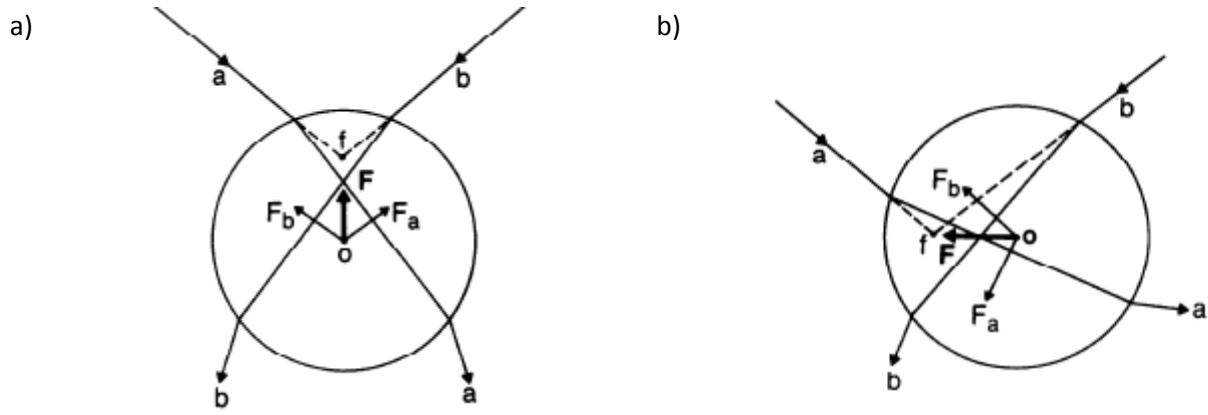


Figure 2.4: Geometric view of optically trapping dielectric particles illustrating the restoring force F toward the trap focus f for a) axial and b) transverse displacements. Reproduced from [48], where the refraction of rays a and b produce the forces F_a and F_b .

Optical traps have been used to manipulate cells in a microfluidic environment and enable particle flow to be independent from that of fluid flow. Hellmich et al. [3] used an optical trap to transfer cells from a holding reservoir to a rectangular channel crossing in the device and deposited them in vertical posts, which would physically maintain the cells in place. Each cell was subsequently chemically lysed and electrophoretic measurements were made on intracellular proteins. The optical trap is a directed method that provides the ability to examine, select and analyse single cells from a population. This device consisted of only one channel over which electrophoresis was performed and any serial separations would require extensive wash steps. Munce et al. [49] reported a device whereby an optical trap was used to transport selected cells to multiple parallel channels, which increased throughput and eliminated cross-contamination between separations.

Rapid solution change is usually afforded by the switching of solutions flowed through the microfluidic device. To achieve a stepwise change, fluid is flowed at high rates to minimise diffusion of the solution front. However, the trapping force is not sufficient to maintain confinement under such conditions. The utility of moving cells independently of fluid flow was demonstrated by Enger et al. [50] in displacing cells from one media containing reservoir to another to investigate environmental stimuli. The reservoirs were separated by relatively long channels, which limited the temporal resolution to minutes. Eriksson et al. [51] showed that by exploiting laminar flow in a Y-shaped microfluidic system, the trapped cell could be quickly switched between adjacent streams of media within 0.2s. The flow rate was slow enough such that cells were not lost from the trap. This results in only regions close to the Y-junction will have a stepwise concentration gradient due to mutual diffusion of the two streams.

Optical trapping's ability to fully manipulate or alter particle trajectories has been utilised in microfluidic sorting applications. Buican et al. [52] demonstrated a system in which cells were propelled by a laser along a default trajectory and upon entering a measurement chamber would be manipulated into a separate channel by an orthogonal deflection beam. Grover et al. [53] sorted peripheral blood by morphological discrimination between biconcave erythrocytes and spherical leukocytes and platelets – the erythrocytes would align into an upright position in the trap and be recognised and sorted out. Wang et al. [54] demonstrated comparatively high-throughput using all-optical switching. The device comprised an input channel and two output channels, one, waste, and the other, collection. Cells are aligned by flow focussing to the centre of the channel and enter an analysis region. Upon a positive fluorescence measurement the cell is displaced laterally by action of the trap and is diverted to the collection channel. By using only optical forces to effect sorting, and not transiently displacing fluid flow, throughputs of 20 – 100 cells/s were achieved with nearly 280,000 cells sorted in 44 min. Transcription factors for heat shock and shear stress were examined and sorted cells were determined to be viable and unstressed. The throughput is not comparable to FACS; however, the recoverability and ability to sort small starting populations of ~1000 cells is unmatched.

2.2.3 Chemical trapping

Functionalising surfaces with species such as cell adhesion molecules or antibodies which recognise surface proteins allow cells to be chemically anchored at specific locations. This is the general approach to cell patterning but may be used as a method to trap cells. Cell patterning is required for studies involving cell adhesion, cell growth on novel substrates, stem cell fate dependence and cell-cell interactions, among others.

Before cells may attach to surfaces, those surfaces must first be functionalised. This can be achieved through methods such as micro-contact printing, chemical vapour deposition or laminar-flow deposition. Micro-contact printing uses an elastomeric stamp which is inked with extracellular matrix proteins and then stamped onto the substrate where proteins adsorb to the substrate surface. Substrates are typically treated with poly-L-lysine-polyethylene glycol, which renders unstamped regions resistant to cell adhesion. Stamps may be fabricated to any desired pattern to facilitate single or multiple cell adhesion to predefined regions.

Exploiting laminar flow in a microfluidic to impregnate channel surfaces with a desired chemistry has been demonstrated by Juncker et al. [55]. Surfaces patterned with linear or graded shapes of functionalised surface were fabricated.

In flow, delivery of cells to patterned sites so that attachment can occur is not trivial. Rosenthal et al. [56] demonstrated a delivery system whereby cells first filled a microwell chip which was subsequently inverted over a functionalised substrate. The system is able to deliver cells into locations defined by the microwell grid upon a substrate which may or may not be chemically patterned itself.

A drawback of patterning substrates with cell adhesion proteins, such as collagen or fibronectin, is the inability to immobilise non-adherent cells. This may be remedied by the use of affinity capture agents, such as antibodies, aptamers or peptides, which recognise cell surface proteins. Affinity capture has also been particularly beneficial to the enrichment and capture of rare cancer cells. Murthy et al. [57] used the antibodies anti-CD5 and anti-CD19 to separate T and B lymphocytes, respectively, from model mixtures and were able to obtain high purity (>90 %) subpopulations even where when concentration of the target cell type was low (<10 %).

Aptamers are another example of affinity capture agents and are gaining popularity for selective cell adhesion. Using aptamers, Phillips et al. [58] captured rare cancer cells with >97% purity and >80% efficiency while Dharmasiri et al. [59] developed a device to capture low abundance prostate cancer cells from whole blood. In the latter study, cells were released by tryptic digestion and their electrical properties measured – the over-expression of membrane glycoproteins associated with many tumour or cancer cells result in an increase in the number of negatively charged sialic acid molecules.

Cells are irreversibly trapped at capture surfaces unless adhesion proteins are cleaved to permit release. Chemical surfaces whose adhesive properties may be modified or reversibly switched upon a certain stimulus are therefore highly sought after. Stimuli may include solvent change, temperature, pH or an electrical trigger. Lahann et al. [60] designed a surface which changed its wetting behaviour between a hydrophilic and moderately hydrophobic state in response to an electrical potential. Surface linkers which are photo-activatable have also been used to augment a surface's adhesion properties and rely on the ability of UV light to remove anti-adhesive coatings or to activate photosensitive linkers [61].

2.2.4 Dielectrophoretic trapping

A force is exerted on a dielectric particle when it is subject to a non-uniform electric field. This is dielectrophoresis, which is similar to electrophoresis which concerns the migration of charged particles in an electric field. Dielectrophoresis (DEP) is the translational motion of polarisable particles, such as cells, in a non-uniform field. The force is proportional to the radius of the object cubed, the relative permittivity (K) between the object and the medium and the square of the electric field gradient. If the permittivity of the object is greater than that of the surrounding medium then objects will experience a positive dielectrophoretic force (p-DEP) and will be attracted towards higher electric field densities. Conversely, objects will experience a negative dielectrophoretic force (n-DEP) and be repelled from higher electric field densities when the permittivity of the object is less than that of the surrounding medium. Permittivity is frequency dependent which permits particle behaviour to be governed by p- or n-DEP at given frequencies.

The DEP force is on the order of pN for mammalian cells with field strengths on the order of tens of kV cm^{-1} required. Miniaturisation to the microfluidic format with the use of microelectrodes is advantageous where only several volts are required across typical microchannel length scales, which reduces Joule heating.

DEP is primarily used for separation and can be used to differentiate particles which experience p-DEP at a particular frequency to particles which experience n-DEP at the same or different frequency [62]. Braschler et al. [63] demonstrated sorting by opposing two DEP force fields from an array of electrodes that was placed either side of a channel. By driving each array with AC signals at different frequencies, particles are focussed laterally towards different positions based on their dielectric response. They were able to perform such subtle separations as sorting yeast cells into viable and nonviable fractions and to enrich pathogen infected red blood cells.

Particles may also be separated based on the magnitude of the DEP force. A popular implementation of this is to apply a perpendicular DEP force which moves cells to varying heights in a channel which become separated out when a velocity profile is applied to fluid flow. Separating cells this way removes the cubic radius dependence since cell height is an equilibrium between the DEP force and gravity. Wang et al. [64] employed this fractionation technique to separate breast cancer cells from normal T-lymphocytes and from CD34^+ haematopoietic stem cells and the enrichment of leukocytes from human blood. Unlike, the continuous separation afforded by field frequency response, separations relying on variations in DEP force magnitude are batch techniques.

DEP devices capable of trapping cells and arraying them at specific locations have been demonstrated. Fuhr et al. [65] demonstrated n-DEP cell trapping with quadrupole electrodes (four electrodes with alternating voltage polarities). It was possible to trap single or multiple cells by varying the spacing of the electrodes. n-DEP traps enable positive in-plane confinement but act to push dielectric particles away from the electrodes. This limits the strength of n-DEP traps as voltage cannot be simply increased without taking into account the balance between the upward component of the n-DEP force and gravity. Alternatively, p-DEP traps act to confine cells at the electrodes but require artificial low-conductivity media. Therefore, stronger n-DEP traps requiring more elaborate geometries have been designed due to n-DEP compatibility with normal cell media.

Reichle et al. [66] mirrored the quadrupole onto the chamber ceiling, creating an opposed octopole trap. By increasing electrode voltage, the cell is driven to the midpoint between the two sets of quadrupoles. This design requires precise alignment of the electrodes and limits the chamber height since the fields from each quadrupole must be in sufficient proximity in order to interact. Voldman et al. [67] designed an extruded quadrupole geometry which confined cells 100 times more strongly than a planar quadrupole geometry while removing the restriction on chamber geometry set by planar octopoles. Each trap consisted of four cylindrical gold electrodes that are 50 μm high and 20 μm in diameter. Despite improvements to trap strength, extruded quadrupoles are significantly more difficult to fabricate.

A novel planar geometry n-DEP trap was introduced by Rosenthal and Voldman [68] consisting of a square electrode and a line electrode. Beads were pushed toward the substrate within the square electrode below a stability transition line whereas above it particles would experience an upward DEP force. Under no flow all beads were stably trapped but under increasing flow rates smaller and larger particles are lost when their centres cross the transition line. This allowed for size selectivity which could be tuned by altering the dimensions of the square electrode. The electrode pattern is simple and easily arrayed for multiplexed single cell studies.

2.2.5 Magnetic trapping

As with electromagnetic (optical) and electrical fields, magnetic fields can manipulate objects within channels at a distance thus removing the reliance on fluid flow.

The magnetic force upon a particle inside a magnetic field is proportional to the strength and gradient (∇B) of the applied magnetic field and the difference in magnetic susceptibilities ($\Delta\chi$)

between the particle and the surrounding medium. Depending on a material's magnetic susceptibility, it is classed as diamagnetic ($\chi < 0$), paramagnetic ($\chi > 0$) or ferromagnetic ($\chi \gg 0$). Most materials, including cells, proteins and water are weakly diamagnetic and for cells in water-based buffers, with $\Delta\chi \approx 0$, the force upon the particle is extremely small [69]. Since the force is dependent on the gradient of the magnetic field, only inhomogeneous fields, where the gradient is non-zero, are suitable to trap or transport cells.

Magnets may be microfabricated and incorporated within the microchannels or placed externally. Placing the magnet externally negates complex and expensive fabrication whereas placing it within the microchannel allows for more precise control of the field strength and spatial pattern. Depending on the application, a permanent or electromagnet may be used. Typically, permanent magnets exert much larger forces than electromagnets with forces on the order of pico-Newtons [70].

Magnetic methods were first applied to cell sorting of blood since erythrocytes (red blood cells) are naturally magnetic owing to a cytoplasm rich in paramagnetic haemoglobin. Sorting of other cell types soon followed by their attachment to super-paramagnetic beads. Particles range in the size from a few nm to microns and are surface functionalised with a multitude of chemistries, which permit the attachment of such biomolecules as antibodies, proteins, DNA or aptamers. Positive or negative selection allows the sorting and enrichment of the target cells. Magnetic activated cell sorting is similar to FACS in principle but uses magnetism as the parameter for sorting.

Kimura et al. [71] showed that diamagnetic (non-magnetic) cells were able to be trapped by the use of paramagnetic compounds and micropatterned mouse osteoblast cells upon a substrate consistent with a periodically modulated magnetic field. Doping media with manganese (II) compounds made the surrounding medium paramagnetic relative to the diamagnetic cells thus increasing trapping efficiency by increasing $\Delta\chi$. It is desirable to avoid the use of high concentrations of paramagnetic compounds with unknown biological consequences. The use of such compounds may not be required if higher strength fields are employed. However, field strength is extremely limited and so trapping cells by proxy with the use of magnetic particles in moderate fields remains the popular solution.

Deng et al. [72] demonstrated a system that uses an external magnet to magnetise an array of on-chip nickel posts to filter magnetic from non-magnetic beads. Smistrup et al. [73] designed a device whereby an external magnet was used to magnetise long, thin, periodic, strips of permalloy (nickel-iron magnetic alloy) fabricated outside the channel on the device substrate. The device was capable

of capturing 1 μm fluorescent magnetic beads from flow and subsequently releasing them. Recently, single cell arrays have been demonstrated using a similar system. Liu et al. [74] electroplated a set of parallel saw-tooth featured permalloy strips onto a substrate of glass or silicon which was coated in a protective polystyrene film. The strips were magnetised by an external magnet and magnetic flux density peaks in the valleys between the teeth, either side of the permalloy strips. The device was able to selectively capture Jurkat cells which were tagged with magnetic nanoparticles but the number of cells per trap in the array followed a Poisson distribution. A novel approach to ordered cell arrangement using magnetic nanowires was made by Tanase et al. [75] (figure 2.5). NIH-3T3 mouse fibroblast cells were first co-cultured with nickel nanowires, which adsorb extra-cellular matrix proteins present in the media and bind to cells. When cells are detached and resuspended they align along magnetic field lines and dipole-dipole interactions between nanowires allow cells to form chains tens of microns long (figure 2.5a). Nanowired cells were also arrayed when flowed through a flow chamber patterned with high aspect ratio ellipsoidal micromagnets, which were externally magnetised. Nanowired cells are then attracted to the ends of the micromagnets where the local field is most intense. The nickel wires have been shown to outperform paramagnetic beads and are very responsive to small fields.

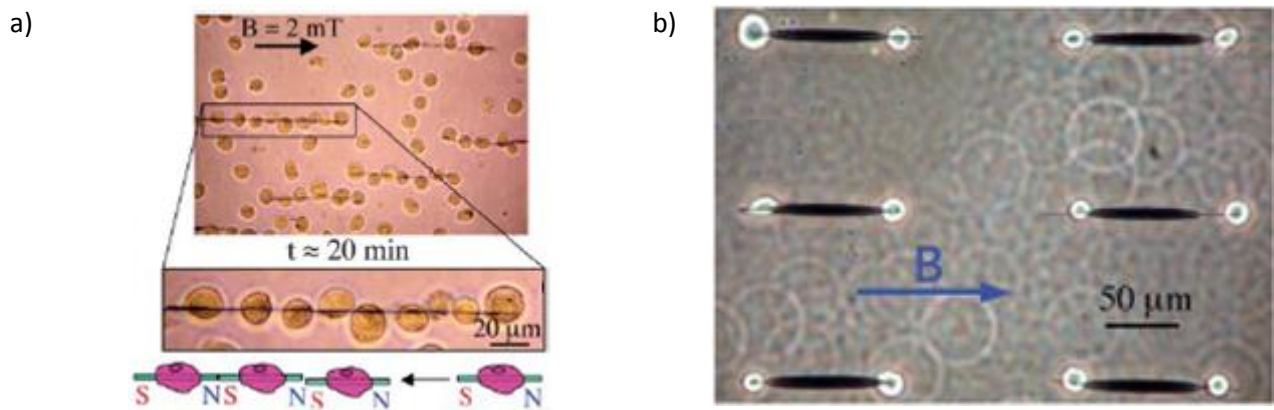


Figure 2.5: a) Chaining by cells attached to magnetic nanowires in a magnetic field. b) Trapping of single nanowired cells by ellipsoidal micromagnets in an ordered array. Reproduced from [75].

Complexity in fabrication is reduced in devices that require only a magnetisable film or feature on-chip in comparison to those that are designed with fully microfabricated electromagnets. Ohmic heating is also avoided. These devices are suitable for passive trapping but due to their fixed design cannot dynamically translate particles akin to their optical counterparts. Winkleman et al. [76] demonstrated a magnetic tweezer capable of 3D translation by independently manipulating two

cone-shaped magnets. However, this was performed on a coverslip and employed the use of paramagnetic compounds. Mirowski et al. [77] used a magnetised cantilever of a magnetic force microscope as a magnetorobotic arm to capture and translate magnetic beads while Lee et al. [78] demonstrated 2D translation and rotation of single yeast cells using a microelectromagnet matrix. The matrix consisted of two perpendicular layers of straight gold wires, capable of creating complex field patterns when particular current profiles were applied. Directed switching of these profiles allowed the magnetic field gradients and, in turn, bead-attached cells to be manipulated above the matrix. To avoid local heating, a thermoelectric cooler was used to maintain the device at 25°C.

Gosse and Croquette [79] demonstrated a more advanced system employing an array of six electromagnets in a feedback loop with a video-positioning system capable of real-time translation, rotation and simultaneous calibrated force measurement of magnetic beads. The drawback of the system is that it was incapable of applying a downward force and its reliance on gravity imposed a lower limitation on the weight of the trapped particles.

2.2.6 Acoustic trapping

In fluids, acoustic waves propagate as disturbances in the ambient pressure level. An object subject to an acoustic standing wave moves toward pressure nodes or anti-nodes depending on the acoustic contrast factor (figure 2.6). This is dubbed the primary radiation force (PRF) and acts parallel to the direction of the standing wave. A secondary radiation force (SRF), or Bjerknes force, arises due to the scattering of the incident acoustic wave and acts laterally contributing to particle agglomeration. The SRF is much weaker than the PRF and only becomes important as inter-particle distances decrease under the action of the PRF.

Acoustic devices consist of a transducer attached to a coupling layer beneath a fluidic layer which is topped by a reflecting layer. A transducer is most commonly made from a piezoelectric element capable of an operating frequency on the order of MHz, corresponding to microfluidic channel dimensions of around 10 – 100 μm . The coupling layer is chosen for good acoustic transmission or may be omitted. The material which defines the fluidic layer must be a good acoustic reflector and have good thermal conductivity to dissipate mechanically generated heat. Silicon, steel and glass have very low compressibility and high Young's modulus which make them suitable materials. The reflecting layer is often glass which also serves as an observation window into the device.

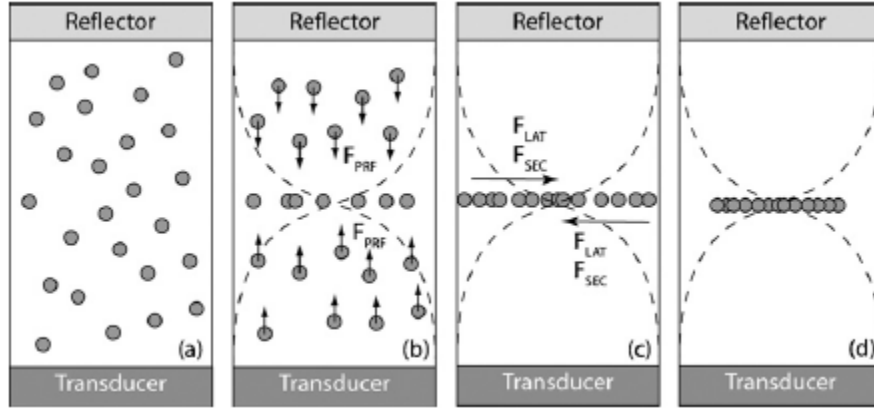


Figure 2.6: Schematic of acoustic trapping showing the forces acting to confine particles in a single node standing wave. Particles suspended in the medium (a) move toward the node under the primary radiation force. As the inter-particle distance is reduced the secondary radiation force (c), arising from acoustic scatter, acts laterally to agglomerate particles (d). Reproduced from [80].

Recently, Guo et al. [81] reported a device whose microchannels in the fluidic layer were defined by PDMS constructed upon a printed circuit board containing the piezoelectric element and capped with a glass reflector. Fabrication of PDMS based on standard soft-lithography represents a significant reduction in cost and complexity compared to microfabrication in silicon.

The acoustic contrast factor is analogous to the relative permittivity in dielectrophoresis. Based on this, some particles will therefore collect at nodes and others at antinodes and provide binary separations. For instance, Petersson et al. [82] were able to separate erythrocytes, which were forced to pressure nodes, from lipid microemboli, which were forced to antinodes. Particles were displaced along laminar flow streams in a channel by the PRF and were then separate by flowing down separate outlet channels based on their lateral position.

Using four outlet channels, Petersson et al. [83] have also demonstrated differential separation of 2, 5, 8 and 10 μm beads in a microfluidic based on the size of the particles and the magnitude of the acoustic contrast factor. It was also showed that, normally inseparable mixtures of, erythrocytes, platelets and leukocytes could be separated by suspending caesium chloride in the medium. The acoustic contrast factor is a function of the particle and carrier fluid compressibility and so the PRF may be altered by changing fluid density.

Using the idea of acoustic valving, Laurell et al. [84] proposed a rare event particle sorter which would divert cells or particles from a common flow based upon optical inspection, e.g. fluorescent label, as they flowed passed a detection zone. Once particles were sorted they could enter

subsequent sorters and be subject to n -levels of sorting into 2^n categories. This would require significant improvements in fabrication and has yet to be practically demonstrated.

Evander et al. [85] used a system, incorporating a miniature ultrasonic transducer integrated directly into the microchannels, to carry out viability assays on acoustically trapped clusters of yeast cells and neural stem cells. Neural stem cells were shown to be viable after 15 min as determined by uptake of perfused acridine orange while yeast cells were trapped and cultured for 6 hours while perfused with cell medium.

Due to their construction, acoustic-based devices are very restricted in the modes of microscopy that are possible. Wiklund et al. [86] developed a miniaturised acoustic particle trap called the ultrasonic particle concentrator (UPC). The small form factor UPC was able to be dipped into 96-well glass-bottomed micropates. Although only demonstrated with a 0.5 NA objective, the inverted mode of operation makes sensitive fluorescence assays upon inverted microscopes, such as confocal microscopy, possible.

2.2.7 Conclusions

In order to perform assays on single cells they must be first isolated and the methods outlined above are all capable of arraying single cells, with the exception of acoustic trapping. While arraying has been demonstrated with acoustic trapping, the low trapping resolution, on the order of 100 μm , permits only clusters of cells to be manipulated. Acoustic manipulation can result in significant rises in temperature due to dissipation of acoustic into thermal energy and induce a heat-shock response in cells.

Chemical trapping, with the use of adhesion proteins or affinity based chemistries, is arguably the easiest technique to produce arrays of defined shape and size and is easily scaled to trap one or multiple cells. Micro-contact printing allows arrays to be stamped upon a substrate. This substrate can be integrated into a microfluidic device but popular strong-bonding techniques such as plasma treatment are not permitted, since they would destroy printing proteins or chemical linkers. Surfaces may be patterned by laminar flow patterning [55], although these require complex valving and channel networks for ordered arrays, or by photoactivation of suitable linkers. Photoactivation is more versatile since cell arrays may be produced by simply imaging a patterned mask upon a surface. These arrays may also be dynamic, as demonstrated by Nakanishi et al. [87], who first micropatterned cells upon a substrate then investigated migration by photoactivating a region

alongside each cell. This may be adapted for studies of cell interactions whereby a second cell type is introduced alongside each cell, for example in cell-fusion studies. The main drawbacks of arraying cells chemically is that they must first be delivered to array locations and then undergo long residence times, perhaps hours, until they are strongly attached. Cells can only be detached by cleaving the anchorage proteins, using a protease such as trypsin.

DEP, magnetic and optical based devices are able to create dynamic non-contact microarrays. The trapping force of magnetic based devices is typically much lower than for DEP or optical traps. Consequently, magnetic trapping is enhanced by the use of paramagnetic beads that are attached to cells of interest and is preferable to the alternative of using paramagnetic compounds, with undesirable biological action, to dope the medium.

Electric and magnetic fields are set up in a microfluidic channel by microfabricated electrodes. The electrodes themselves are arrayed and can scale from trapping single to many cells. Fabrication techniques are complicated and expensive and drastically increase the time for rapid prototyping. Electrodes may be fouled and corrode limiting the lifetime of the device and electrolysis of water may occur when producing stronger fields. Electrolysis can lead to the formation of hydrogen peroxide, which can alter cell dynamics, and the evolution of gas bubbles, which can disrupt device performance. Heating of the media and cells via Joule heating is also disadvantageous although the large surface to volume ratio of microfluidics and on-chip thermoelectric cooling allows fast dissipation of heat. The electric field in DEP is also known to affect transmembrane voltages and influence membrane-bound components of the cell. The physiological effect on cells by magnetic field is generally considered to be minimal at short timescales but on the order of hours can start to affect critical cell functions, such as Ca^{2+} signalling [88].

Optical trapping and manipulation has no on-chip requirements and represents a significantly simpler system to fabricate and also to operate. It does require the integration of optical components into the wider experimental setup but this is not a problem as the microfluidic will form part of a microscopic apparatus, anyway. Whereas DEP and magnetic trapping devices are able to passively array cells across the entire device simultaneously, optical trapping is limited, however, to the field of view of the objective. Despite this, optical trapping is the most precise of the non-contact measurements as demonstrated by the classic work of Arai et al. [89], whereby an optical trap was used to tie knots in individual actin filaments and strands of DNA.

The power density at the focus of optical traps is very high and is known to affect cell viability. Modern systems employ lasers in the near IR range in order to minimise cellular photodamage.

Liang et al. [90] observed a wavelength dependence by the clonal growth of Chinese hamster ovary (CHO) cells after exposure to trapping wavelengths from 700 - 1064 nm. Maximum clonability was observed at 950 - 990nm, minimum clonability at 740 - 760 nm and 900 nm while the commonly used trapping wavelength of 1064 nm resulted in strongly reduced clonability. Liu et al. [91] further investigated the effects of trapping at 1064 nm on CHO cells by using fluorescent probes to assess temperature, DNA denaturation, cell viability and intracellular pH. Trapping powers up to 400 mW did not alter DNA structure or cellular pH. However, it was observed that for extended exposures greater than 2 - 3 min cell viability was compromised.

Absorption by water may lead to heating over extended periods of time. However, temperature rises of only 1-2°C have been measured due to an optical trap at 1064 nm and so different mechanisms of damage have been proposed. König et al. [92] reported that multiphoton mediated damage was responsible, especially at wavelengths below 800 nm where absorption by metabolic co-enzymes can lead to singlet oxygen generation. It was also found that cell damage is significantly reduced by the use of single-frequency, instead of multimode, traps. Neuman et al. [93] reported a reduction in photodamage to *Escherichia coli* under anaerobic conditions which further indicated singlet oxygen. This makes optical trapping more suited to the manipulation of cells from one position to another rather than to continuously array them. Although, Ramser et al [94] have reported trapping cells up to 20 min with minimal damage. Despite being more readily damaging to cells, optical trapping is the most versatile and simple to implement of the non-contact methods.

Hydrodynamic trapping is a passive method to array cells in locations defined by dam or sieve structures across an entire microfluidic device. By careful design, individual traps may be tuned to trap single or multiple cells and offer very strong confinement. Consequently, media may be rapidly exchanged throughout the device without the loss of cells from the array, which is a limitation to non-contact methods. The method is compatible with adherent as well as non-adherent cell lines without the requirement of immobilisation chemistry or any imposition upon the fluid medium – cells are able to remain in physiological buffer. Cells may also be trapped indefinitely as they are not subject to factors which compromise viability, such as shear stress or cytotoxic electromagnetic radiation.

Trapping structures may be fabricated from PDMS and so may be rapidly prototyped without complex fabrication steps required for the rendering of electrodes. There is also no need for function generators or other control boxes. The only requirements are off-chip tubing and pumps to deliver cells to the device, which are then trapped in defined locations without any user intervention.

To increase the number of traps in DEP or magnetic trapping employing electromagnets the number of electrodes must also be increased and scales as $2\sqrt{n}$ for row-column formats [95] and rapidly becomes complex. There is no such restriction with hydrodynamic traps and microarrays have been scaled to hundreds of traps without complicating device fabrication or operation [33-36].

A drawback of hydrodynamic traps is the lack of discrimination since all cells in flow will become trapped. For large arrays this may not be a problem as only certain cells at particular addresses in the array may be interrogated based on, say, an initial screening measurement. Hydrodynamic traps designed to increase fluidic resistance upon trapping a cell such that no further trapping is possible, or is reduced, are called self-healing traps. Self-healing traps are able to enhance the probability that only single cells are trapped in the device, rather than zero, two, or three, etc. In order to trap a particular cell type or subpopulation in an inhomogeneous starting population then a separation step must be performed before introduction to the hydrodynamic traps.

Furthermore, the choice of PDMS trapping structures to affect a hydrodynamic trap does not impose any downstream limitations regarding the choice of lysis method nor analysis. For this reason and the reasons outlined above (i.e. cheap, uncomplicated fabricated, no buffer restriction) then the method for isolating and trapping cells will be based on hydrodynamically trapping cells by the use of dam or sieve-like microstructures.

2.3 Lysis Methods

The trapping and isolation method has delivered cells to defined locations but in order to measure cellular processes which require high temporal resolution, sampling must be performed on sub-second to second timescales.

Cell lysis is the next step in the proteomic analysis of intracellular compounds. The lysis process must preserve the integrity of the relevant species at their pre-lysis concentrations while releasing them from the cell.

It takes on average 10s for a protein to traverse a HeLa cell, protein translation takes on average 10minutes whereas by far the most rapid intracellular process is signal transduction via protein kinases which can alter metabolite concentrations by 10-fold within a few seconds [96, 97]. The method of cell lysis must be performed on a timescale which does not allow the cell to respond, for instance, by activating stress pathways.

There are broadly 5 different approaches used to lyse cells: optical, electrical, chemical, mechanical and acoustical. Each approach is reviewed, with particular focus on how these methods have been applied to the microfluidic format. We define here that rapid lysis is that which completes on a sub-second timescale.

2.3.1 Optical lysis

Pulsed lasers have been employed in non-invasive ophthalmic surgery for several decades [98] but their application to lyse single cells has come about only recently [99, 100].

Pulsed laser-induced cell lysis involves directing a laser pulse through a high numerical aperture objective lens, which brings the pulse to a focus. At sufficiently high irradiance, non-linear light absorption in the media causes it to break down. The process of optical breakdown in liquids (figure 2.7) first involves multiphoton ionisation of an electron from a bound molecular orbital to a quasi-free state where it possesses sufficient kinetic energy to move without being captured by local molecular energy potentials. Sacchi [101] treated water as an amorphous semiconductor with an excitation energy of $\Delta E = 6.5$ eV. By $E = hc/\lambda$, excitation requires simultaneous absorption of 3 or 6 photons for pulses of typical wavelength 532 or 1064 nm, respectively.

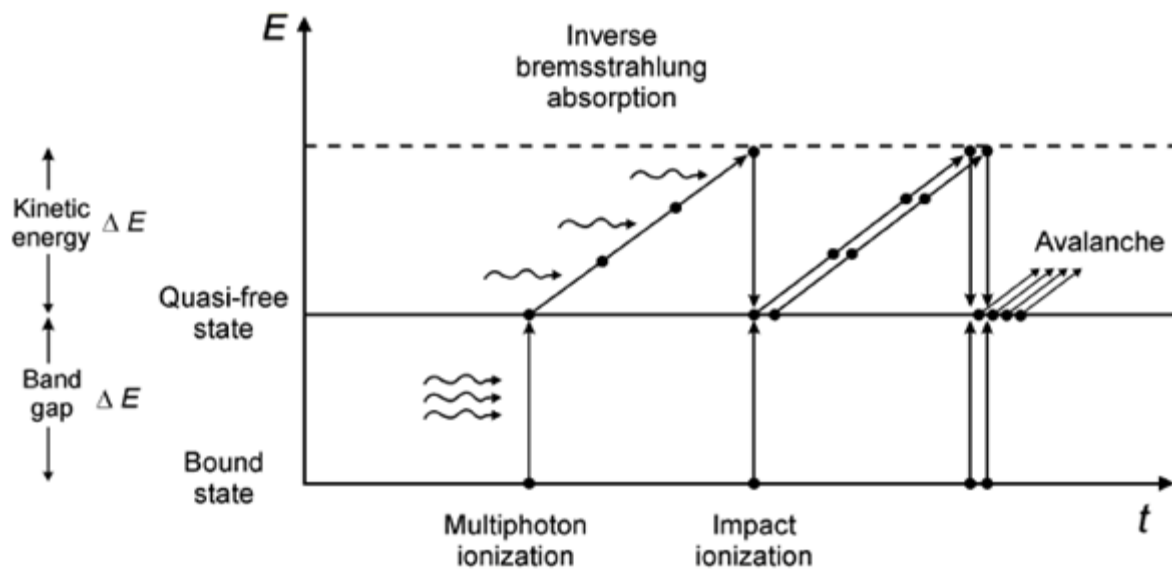


Figure 2.7: Schematic depicting the role of several electronic processes toward plasma formation in optical breakdown. Reproduced from [102].

The quasi-free electrons may absorb photons by undergoing inverse Bremsstrahlung absorption (IBA), which increases the electron's kinetic energy. After sufficient IBA events, the electron possesses kinetic energy exceeding ΔE and can produce another free electron via impact ionisation. Both electrons may gain energy through further IBA leading to a second impact ionisation event and a third quasi-free electron. This avalanche ionisation leads to a rapid growth in the number of free electrons provided the irradiance is sufficient to overcome losses, such as recombination or diffusion of the electron out of the focal volume, and results in the generation of a localised plasma.

During optical breakdown there is a rapid adiabatic temperature rise which causes a rapid rise in pressure. The plasma initially expands at hypersonic velocity and as this reduces to subsonic velocity it emits a shockwave. As the plasma expands further, a cavitation bubble is produced which expands to a maximum radius and then collapses.

The physical processes underlying plasma formation and the roles of the shockwave and expanding cavitation bubble are well established [102-106]. The absorbed energy from the pulse by the breakdown medium is spent evaporating the focal volume, radiated by the plasma and converted to mechanical energy in the shock wave and cavitation bubble [107]. The energy balance depends on pulse duration and wavelength.

With particular application to cell lysis, Rau et al. [103, 104] used time resolved imaging (figure 2.8) to determine that the primary agent of lysis to a monolayer of cells is cavitation bubble expansion when the pulse was focused at a distance of 10 μm above the monolayer. The maximum radius of the cavitation bubble was found to be much greater than that of lysed cells indicating that cells are lysed by dynamic shear stress produced by the fluid displaced by the expanding bubble. When the pulse was focussed much further above the cell monolayer at 400 μm distance cell lysis occurs during bubble collapse when a liquid jet is directed downward. As can be seen by the figure, optical lysis is extremely rapid and reaches completion well before 1 μs .

Hellman et al. [108] further characterised the injury to a cell monolayer and showed that, for a confluent layer of cells, there are four distinct zones of cellular injury. They are, in order of radial distance from the focus, the zone of lysis, a region denuded of cells; the zone of necrosis, where cells are necrotic and do not necessarily remain attached; the zone of permeabilisation, where cells remain viable, however, their membranes are compromised and are able to uptake small molecules; and beyond which cells appear unaffected.

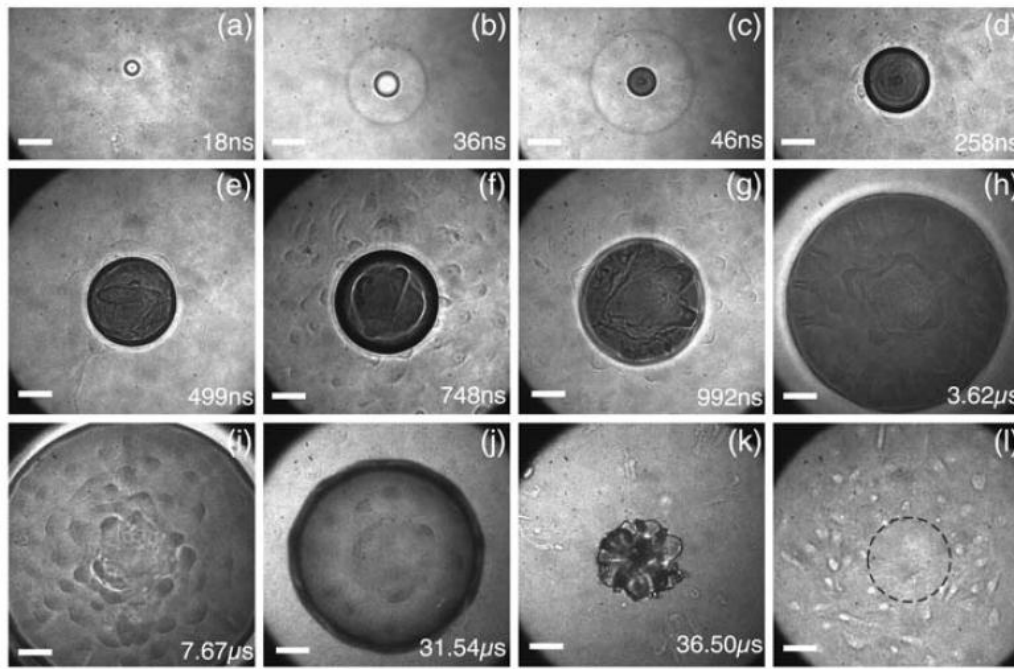


Figure 2.8: Time-resolved series of images of revealing the processes involved in lysis of a cell monolayer: plasma formation (a), shock wave propagation (b-c), cavitation bubble expansion (c-i) and collapse (i-k). Reproduced from [104].

Laser-induced microcavitation (LIM) may potentially disperse cellular material over a large area, however, Quinto-Su et al. [109] showed in a microfluidic channel that, although initial dispersion and dilution of the cytosolic CellTracker Red dye is significant, the collapse of the cavitation bubble reconcentrates material with minimal dilution. CellTracker Red is subsequently diluted by rapid diffusion and, like ATP, glucose, and metal ions, has a large mass diffusivity that suggests cellular reactions requiring small molecules are quickly terminated.

There have been several studies demonstrating how LIM can be successfully used as a single cell sampling strategy for electrophoretic separation. The first was made by Sims et al. [100] whereby the inlet of a standard capillary was positioned directly above a cell and, upon lysis, cellular constituents were loaded into the capillary and electrophoresis initiated. Cells were loaded with fluorescein and Oregon Green and a 100% loading efficiency was reported. In a follow-on study, Li et al. [110] improved control over sample loading. By varying the laser pulse energy, multiple cells down to desired fractions of a single cell could be sampled. Crucially, it was confirmed that lysis by LIM is sufficiently rapid to terminate cellular enzymatic reactions on the microsecond timescale. Protein kinase C (PKC) and calcium/calmodulin activated kinase II (CamKII) are two enzymes activated by phosphorylation in response to cellular damage. No phosphorylation was observed with LIM whereas when cells were lysed with a hypotonic solution almost complete phosphorylation

of PKC and CamKII was observed. The system has been ported to microfluidic format with electrophoresis on-chip by Lai et al. [111].

2.3.2 Electrical lysis

Electroporation is a widely used technique and has the advantage of being a non-contact method for permeabilisation or lysis of cells.

Depending on the magnitude of the field strength, a pulsed electric field can cause the lipid bilayer of a cell to form small pores or to rupture entirely [112]. Electrical pulses of defined width and voltage induce a potential difference across the cell leading to electroporation. Upon removal of the electric field, pores rapidly seal but beyond a critical field strength (typically $>1 \text{ kV cm}^{-1}$) and time of exposure, pore formation becomes irreversible and leads to cell lysis.

Traditional bulk experiments have required the application of hundreds to thousands of volts to large electrodes due to the high critical field strength required for electroporation. Lysis by this method on large populations of cells is impractical and unnecessarily dangerous when compared to more classic methods (see below). Using microelectrodes to lyse or electroporate a single cell avoids the use of high-voltage pulse generators due to the short length scales e.g. an electric field of 10 kV cm^{-1} represents a 1V drop over the length of a $10 \mu\text{m}$ cell.

The first integration within a microfluidic platform was made by Lee & Tai [113] using a saw-tooth electrode structure to lyse yeast, Chinese cabbage, radish and *Escherichia coli* and found field strengths on the order $1 - 10 \text{ kV cm}^{-1}$ were required. Lu et al. [114] demonstrated electro-lysis of human carcinoma cells in flow and identified how dielectrophoresis (DEP) as a consequence of the electrodes could be used immediately downstream to identify cytosolic proteins. DEP helps to prevent samples from being diluted by the carrier fluid and can be important for low-abundance proteins.

Capillary electrophoresis, employed to provide total analysis on chip, downstream of electro-lysis has been demonstrated [115, 116]. McClain et al. [116] reported a device capable of analysing Jurkat cells loaded with 3 fluorogenic dyes at a rate of 7 – 12 cells/min. Interestingly, they found a side population of cells, $\sim 9 \%$, which enzymatically hydrolysed the loaded dyes significantly differently from the majority of the cells. Such an observation would have been difficult to obtain using bulk methods. Gao et al. [115] used a similar approach to determine to cellular concentration

of glutathione in human erythrocytes. More recently, Sedgwick et al. [117] described a device which trapped (by DEP) and lysed A431 human epithelial carcinoma cells, expressing green fluorescent protein-labelled actin, and downstream analysis involved confocal microscopy of anti-B-actin antibody-coated microbeads. This demonstrates the compatibility of electro-lysis with affinity capture by antibodies, which can be specific to single proteins or to sub-proteomes such as post-translational modifications.

The high field strengths required in electro-lysis may lead to joule heating and electrolysis of the aqueous solution which can lead to local bubble formation at the electrodes. To lower overall power dissipation and Joule heating but still provide sufficient field strength for lysis, AC electric fields with DC offsets are employed.

At the cathode, electrolysis results in the generation of OH^- and is capable of cleaving fatty acid groups of membrane phospholipids. This process only requires modest field strengths ($10\text{-}40\text{Vcm}^{-1}$) and has been used to lyse cells [118, 119] in attempts to alleviate the problems associated with high field strength. pH significantly increases in the electrode's vicinity and, although unfolded proteins may refold as buffer pH returns to physiological level at the anode (OH^- quenched by H^+), this might affect analysis of protein complexes and proteins incapable of refolding. Another significant drawback is that the time to lyse is a function of the cell's distance from the electrode since it is dependent on the time required for OH^- to diffuse and reach lytic concentration. This time can be extremely long and of the order of 10s to 100s of seconds [119] and precludes this particular method for rapid lysis.

Lysis times of 40ms [115] and less than 33ms have been reported [120] for direct electro-lysis of cells. Such short lysis times are achieved by concurrent exposure of the cell to separation buffers which contain hypotonic surfactants, which osmotically swell cells and aid lysis.

2.3.3 Chemical lysis

The most common method used in biotechnology laboratories is chemical lysis by detergents because the protocols are well-established.

Detergents are amphiphatic and their behaviour is dictated by the properties of their polar hydrophilic head group and nonpolar hydrophobic tail. The cell membrane is made miscible as the

detergent inserts and bind to hydrophobic surfaces, solubilising membrane lipids and proteins. Lipid and protein interactions are disrupted causing pores to form which continues to full cell lysis.

They are either ionic, non-ionic or zwitterionic. Ionic detergents, such as sodium dodecyl sulphate (SDS), are generally strong and tend to denature proteins whereas non-ionic, such as Triton X-100, and zwitterionic detergents, such as CHAPS, are milder and are non-denaturing. Therefore, the choice of detergent will influence the range of applicable downstream analyses. SDS is capable of lysis within seconds but is not suitable in enzyme activity or immunoassays, unlike Triton-X, which is, but takes minutes. Some non-ionic detergents, such as NP-40 or Igepal CA-630, are not strong enough to break the nuclear membrane and so can be used to liberate only the cytoplasmic contents of the cell.

It is common practise to spike cell lysis buffers with a cocktail of protease inhibitor compounds to inhibit cellular proteases which, normally are regulated and compartmentalised, may breakdown proteins in the lysate.

Detergent lysis has been used on the single cell level in the microfluidic format. To quantify $\beta 2$ adrenergic receptors expressed in SF9 insect cells, Huang et al. [11] used a system of on-chip valves to trap a single cell then fill the chamber with a lysis buffer containing fluorescently labelled antibodies to tag target proteins. The non-ionic detergent, *n*-dodecyl- β -D-maltoside, was used to preserve antibody activity. Lysate was subsequently electrophoretically separated and target proteins quantified by fluorescence microscopy. The time for cell lysis was not reported, however, it is expected to be on the order of tens of seconds to minutes.

Ocvirk et al. [121] mixed lysis buffer and cells in a Y-shaped mixing junction in order to facilitate faster lysis. They compared denaturing 0.5 % SDS, lysis within 2 s, and non-denaturing 0.1 % Triton-X, lysis within 30 s. The study focussed on an enzymatic assay of β -Galactoside and, therefore, had no choice but to choose the slower method.

2.3.4 Mechanical and acoustical lysis

Mechanical lysis can simply involve crushing cells with a pestle and mortar, homogenisation by shearing forces as a suspension is forced through a narrow conduit or by rotating blades, which grind and disperse cells. Homogenisers can handle minimum sample volumes of 0.5 mL and are not well suited for lysis of a low number of cells.

Mechanical lysis was demonstrated in a microfluidic with nanostructured barbs by Di Carlo et al. [122]. Cells were driven under extremely high flow rate through a filter region composed of a 3µm grating with patterned with sharp ridges by a deep reactive ion etching process. Above a critical flow rate of 140 µL min⁻¹ 99 % cell lysis was achieved. However, only 4.8% of total protein was measured to be released. Kim et al. [123], while investigating mechanical stress to mammary gland epithelial (MCF7) cells, demonstrated cells could be lysed under compression by a deflected membrane of PDMS within 135ms.

Sonication is often used to lyse cells and shears cell membranes by the application of ultrasonic waves to generate cavitation. Sonication is slow, taking minutes to complete, and results in significant generation of heat, which can denature proteins. Sonication in a microfluidic device has been reported by Marentis et al. [124] whereby 80% of eukaryotic HL-60 cells were lysed within 3s. The piezoelectric transducers were integrated into the device. Localised sub-second cell lysis using a self-focused acoustic transducer has been reported by Zhu et al. [125] but poorly characterised.

2.3.5 Conclusions

There is a varied array of methods available for cell lysis. Each has their own particular limitations and applicability to the microfluidic experimental platform (summarised in table 2.1).

It is difficult to see how mechanical or acoustic lysis satisfies the requirements outlined in §2.3. Temporal resolution is far too low to confidently interrogate post translational modifications. The low total protein measured liberated from cells by Di Carlo et al. [122] suggests the method is not fully effective. The management of cell lysate would also be very difficult in such a high flow rate system.

lysis method	temporal resolution	single cell selectivity	protein denaturation	microfluidic applicability
optical	ns-µs	++	+	++
electrical	ms-s	+	+	+
chemical	s	-	+/-	+
mechanical	s-min	--	-	-
acoustic	s-min	-	--	--

Table 2.1: Distilled comparison of the lysis methods for single cell lysis reviewed above.

Chemical lysis is ubiquitous in biological laboratories and although it has been successfully demonstrated on chip it is not straightforward owing to the need for complex valving [11]. The main drawback of chemical lysis is the trade-off between temporal resolution and protein denaturation in the choice of ionic or non-ionic detergents [121]. Despite the cell being suspended in non-physiological buffer, this can restrict the choice of downstream analysis. Regardless of the disadvantages, chemical lysis represents the most economical method and may prove useful as a comparator in this work.

Electric lysis imposes less restriction on buffer conditions. Many studies, however, use hypotonic buffers to swell cells which aid lysis. Often, typical physiological buffers result in significant Joule heating as a result of high applied voltages. Electrolysis of the buffer causes bubble formation and can be very problematic in microfluidic systems. McClain et al. [116] encountered this problem due to the high conductivity of the buffer. To remedy this they employed an AC field with a DC offset for lysis. Consideration of the buffer is not where the main disadvantages of electrical lysis lie. The need for on-chip microelectrodes and microcircuitry to be fabricated for each and every device significantly increases complexity of rapid-prototyping and operation with the electrodes themselves having a limited lifespan. Lysis may only occur in spatially distinct locations in close proximity to the electrodes. One advantage of using electric lysis is the ease at which it can be coupled with electrokinetic injection and separation by electrophoresis.

As with electrical lysis, optical lysis is attractive since there is no need to introduce lytic agents. Cells may be maintained in physiological buffer right up to the point of lysis and beyond. The main point of concern with the method is denaturation or destruction of cell contents due to heating by the induced plasma or photochemistry by the high irradiance ultrashort pulse. Most demonstrations of LIM involve small molecule dyes, which are, perhaps, more resilient than proteins. Li et al. [110, 126] assayed the phosphorylation of the enzymes PKC and CamKII and Brown & Audet [127] used the sensitive autofluorescent green fluorescent protein (GFP) to measure the sample efficiency of a single-cell capillary electrophoresis system. A sampling efficiency of 100% was made under best conditions but typically was of the order of 60% and not statistically different from a control method that mechanically sheared cells with a capillary tip. The study suggested that GFP loss directly due to the laser pulse or plasma was minimal. Furthermore, Han et al. [120] concluded that there was no significant difference between optical and electrical lysis when sampling by a capillary for electrophoresis.

There are no on-chip requirements imposed by laser lysis, which makes fabrication simple as no addition features beyond channel and trapping geometries are required. Authors often cite the cost

of the laser itself as an additional disadvantage. The availability of ultrafast laser equipment and expertise is not an issue for the Single Cell Proteomics group (with which this project was undertaken); however, it may be a legitimate concern for some biological laboratories. Small form factor laser systems are becoming more widespread and represent a modest initial outlay when reconciled with the advantages of laser based lysis.

The optical lysis method is rapid offering the highest temporal resolution of any method, amenable to single, as well as multiple, cells, compatible with any downstream technique and easily integrated into the microfluidic platform with no spatial restrictions on where lysis can occur. For these reasons, it is the lysis method best suited for this work.

2.4 Analysis

A single cell has been delivered and lysed in a microfluidic chamber. Measuring the protein content originating from the cell is the final step in performing single cell proteomics. Proteins usually exist in higher quantities than DNA and mRNA, and are more difficult targets because they cannot be amplified and their identification is not straightforward. Proteins may be present at single copy number or be highly abundant in a cell and the chosen analysis method must have an appropriately wide dynamic range of detection.

2.4.1 Microarray analysis

Microarray technology is a powerful tool for highly parallel analysis of biological markers. A microarray is formed from a planar substrate, typically a glass slide, upon which thousands of small spots, each for a different biomolecule, are immobilised. The first microarrays were generated for DNA and quickly found success in genomics.

Fodor et al. [128] lay the foundation for microarrays in 1991 by demonstrating a process to build an array of synthesised peptides by sequentially depositing amino acid groups on a glass surface. The substrate was functionalised by a photolabile protecting chemical group, which when illuminated through a mask led to deprotection and the addition of the first chemical building block, which also contained a photolabile protecting group. By the use of different masks, an array of 1024 peptides was synthesised. The technology was subsequently used to construct DNA microarrays as nucleic acid synthesis was well known and as a direct result of efforts on genome sequencing and the high

throughput, which microarrays could offer. Schena et al. [129] used a robot to mechanically print complementary DNA in spots onto glass using a quill-type metal pin. Indeed, Schena's technique has become the most widespread technique today in the preparation of protein microarrays.

Upon completion of sequencing the human genome, attention focussed on the proteome and methods to study it. The success of the microarray to DNA studies was not immediately replicated in protein microarrays owing to the chemical complexity and diversity of the proteins themselves – proteins have a broad diversity of solubility, tertiary structures and amino acid content. All aspects of protein microarrays, such as printing, surface chemistry and capture probes, have undergone significant development, and continue to do so.

There are broadly two types of protein microarray - analytical and reverse-phase microarrays.

Analytical microarrays are used to profile and quantify a set of proteins from a mixture in order to measure protein expression in response to a stimulus or disease and are essentially a parallel series of micro-scaled immunoassays. A library of affinity capture agents, such as aptamers, affibodies and, most commonly, antibodies, are used to capture proteins of interest so that an appropriate measurement can be made. Arrays are commonly incubated with cell lysate but Belov et al. [130] showed that intact cells could be pulled down by antibodies against cluster of differentiation antigens, present on the surface of leukocytes.

Analytical protein microarrays are sometimes referred to as forward-phase arrays. Reverse-phase protein microarrays are the converse of this where antibodies are, instead, free in solution and protein antigens are immobilised on the surface. A subset of reverse-phase arrays, i.e. functional proteins microarrays, are composed of arrays of full-length functional proteins and are used to study protein interactions with other proteins, binding to DNA, RNA, lipid small molecules such as drugs. Using a functional protein microarray, Zhu et al. [131] tested 5800 yeast proteins, representing 80% of the yeast proteome, for their ability to interact with a set of proteins and phospholipids while Hall et al. [132] used the same library to identify previously unrecognised DNA binding activity.

Reverse-phase arrays are popular in readily assessing comparative proteomic changes during development or disease. This was first described by Paweletz et al. [133] who printed the lysate of histological samples taken from 3 progressive stages of prostate cancer, arrayed upon the same substrate. Progression was associated with increased phosphorylation of Akt, reduced phosphorylation of ERK and the suppression of apoptosis pathways.

The most common forward-phase arrays are the antibody protein microarrays. They are crucial in measuring abundance whereby captured proteins are detected and quantified.

Monitoring the binding of proteins over time or to measure the amount of bound protein can be conducted with a label-based strategy or label-free. Label-based techniques are, relatively, straightforward and involve labelling the antigen with fluorescent dyes, radioisotopes or epitope tags. Fluorescent labels are the most common in microarray analysis and allow for a highly sensitive platform. Fluorescent dyes may be functionalised with reactive succinimidyl-esters or maleimide for labelling proteins through lysine or cystine residues, respectively. Early, protein microarray employed the carbocyanine dyes Cy3 and Cy5 in order to be compatible with existing DNA microarray scanners. Nowadays, more flexible systems, incorporating different light sources and detection strategies, are able to use brighter, more photostable dyes such as the Alexa, ATTO and DyLight dyes.

However, direct labelling of proteins can alter surface characteristics can alter their natural activity and allosterically prevent antibody binding. Moreover, the labelling process may fail to label some proteins while excessively labelling others in complex samples, such as cell lysate. Binding may also be measured by using a sandwich assay or label-free detection methods. In a sandwich assay a second antibody is used to probe the presence of a protein on the array. This obviates labelling the protein since the secondary antibody may be labelled itself. Sandwich assays can have higher specificity than label-based strategies since two antibodies target each protein instead of one. Secondary antibodies can be conjugated to enzymes, such as horseradish peroxidase, which permit signal amplification. Horseradish peroxidase catalyses the oxidation of luminol to 3-aminophthalate with the concomitant emission of light in a process known as chemiluminescence. Amplification may also be afforded by rolling circle amplification (RCA) as demonstrated by Schweitzer et al. [134]. Their adaption of RCA for protein signal amplification involved attaching the 5' end of an oligonucleotide primer to an antibody. The antibody-DNA conjugate binds to its target and a circular DNA molecule hybridises to its complementary primer on the antibody. By adding DNA polymerase and nucleotides rolling circle replication occurs. The advantage of RCA is the amplified signal remains localised at the microarray spot. The sandwich assay format, however, does require two non-competing antibodies for each protein that may not be readily available and care must be taken to ensure there is little, or at least accounted for, cross reactivity or interference between antibodies. Label-free detection methods do not require a secondary antibody or labelling of the protein sample but instead rely on inherent property changes upon binding such as mass and dielectric properties [135]. Upon binding to an antibody coated surface, the refractive index at the

surface changes. This can be measured by surface plasmon resonance or ellipsometry-based techniques. However, label-free techniques often lack sensitivity and involve expensive fabrication techniques such as coating surfaces with silver or gold as in surface plasmon resonance.

For fluorescence based detection strategies, improvements have been employed to increase the sensitivity of the microarray. One way to do this is to use methods that discriminate unbound background protein fluorescence from that of antibody bound. Microarray scanners now incorporate pinholes in a confocal-based setup to achieve sectioning strength. A more powerful, and increasingly popular, technique is total internal reflection fluorescence (TIRF) microscopy. Light entering a medium of differing refracting index at an angle undergoes refraction in the new medium and partial reflection back into the existing medium. Beyond a critical angle all light is reflected from the boundary i.e. total internal reflection (TIR). An important effect of TIR is the evanescent field which is generated in the second medium with a frequency identical to that of the incident light. The field exponentially decays away from the boundary and so extends, at most, a few hundred nanometres into the second medium permitting only microarray surface-bound fluorophores to be excited. This negates the need for wash steps and will simplify any operation within a microfluidic as a result. Pawlack et al. [136] used thin waveguiding layers of high refractive index to produce an evanescent field in a system whose detection limit was found to be as low as 0.8 zmol of Cy5-labelled antibody per spot. Conducting TIRF by using only plain glass, Tessler et al. [137] demonstrated single molecule quantification by counting the binding of single labelled antibodies to surface immobilised proteins. The application of single molecule detection methods to protein microarrays can improve accuracy over bulk methods, potentially allowing protein quantification without calibration and can enable the detection of low-abundance proteins.

Other fluorescence-based methods include fluorescence anisotropy and fluorescence lifetime microscopy. For instance, Giraud et al. [138] used a change in fluorescence lifetime of quantum dots upon binding in a DNA microarray. The utility of such approaches, in light of single molecule detection using TIRF illumination, is demonstrative at best.

2.4.2 Capillary electrophoresis

Capillary electrophoresis, also known as capillary zone electrophoresis, is used to separate species based on their mass to charge ratio under the influence of a homogenous electric field.

Single cell lysate must be injected into the capillary in order to effect a separation. Conventionally, sample is electrokinetically injected or siphoned in once the cell is lysed. An increasingly popular method is to lyse the cell within the capillary. Krylov et al. [139] used surfactant in the run buffer to chemically lyse a single cell within 30s using SDS. Surfactant in the run buffer precludes certain modes of electrophoresis. Alternatively, the surfactant may be introduced as a plug, before or after the cell, but this relies on slow diffusion of surfactant out of the plug [140]. More rapid lysis methods such as optical lysis have been employed as a sampling method. Li et al. [110] aligned the capillary bore above and optically lysed a cell before electrokinetic injection.

Amperometry and chemiluminescence find niche application but the most often used methods to measure protein separation by CE are absorbance and fluorescence.

Absorbance measurements rely on the native fluorescence from proteins in the UV. Absorbance follows the Beer-Lambert law which prescribes a linear dependence on the path length available for absorption. Capillary inner diameters are typically on the order of 100 μm and is far less than that of a tradition 1 cm UV cell used for absorption measurements. Therefore, a highly sensitive detector is required. However, for proteins with low molar extinction coefficients sensitivity is still limited. Cavity techniques, such as cavity ring-down spectroscopy (CRDS), are becoming a serious alternative, spurred on by their success in high pressure liquid chromatography. Essentially, in this case, the cavity is formed by two mirrors, of reflectivity R , with the capillary position as the “gain” medium. A small fraction of light, $1 - R$, enters the cavity by the first mirror and proceeds to bounce back and forth, losing a fraction of intensity on each bounce, which corresponds to mirror reflectivity and the presence of absorbing species in the capillary bore. A detector placed behind the second mirror records the decay in intensity and is known as the ring-down profile. Using a variant of CRDS, Li et al. [141] achieved a detection limit of 5.3 fmol for human serum albumin.

Sensitivity may be increased by employing fluorescent markers and laser induced fluorescence (LIF) has become the method allowing ultra-sensitive detection of low abundance species in single cells. Low divergence, high intensity light excites fluorophores and emission is detected by a photomultiplier tube (PMT). Turner et al. [142] used CE with LIF to quantify the expression of GFP in a single bacterium. The limit of detection was 100 ymol, corresponding to only 60 copies of GFP in the detection volume. CE was used to separate GFP from native cellular autofluorescent components in order to reduce background and is useful for low-abundance proteins that may that would otherwise be masked by autofluorescence. The method was applied to determine expression at $\sim 2 \times 10^4$ GFP molecules/cell in *Deinococcus radiodurans* bacteria under control of the *recA* promoter.

CE with LIF is also able to temporally monitor secretion events from single cells. Monitoring of serotonin secretion from a single rat mast cell has been performed by Ho et al. [143] with a detection limit of approximately 1 amol.

Subcellular heterogeneity has also been studied by Johnson et al. [144] who used CE with LIF to detect the mitochondrial dye mito-DsRed2 in individual mitochondria released from single cells. Cells were gently lysed in the capillary and upon release intact organelles are electrophoretically separated and detected. They found that mitochondria were heterogeneous in their electrophoretic mobility and followed a distribution.

CE has been demonstrated in a microfluidic format. It was first demonstrated in a glass chip for the electrophoretic separation of amino acids by Harrison et al. [145]. Benefits of on-chip electrophoresis include reduction of sample volume and importantly the reduction of Joule heating owing to lower voltages required at shorter length scales. Several systems have been demonstrated in the capture, lysis and separation on chip and have been mentioned in the previous sections.

CE is a separation technique and has little ability to uniquely identify proteins. Proteins are separated based on their charge to mass ratio and it is easy to appreciate that many different proteins will co-migrate and be detected as a single peak. Complexity in electropherograms may be reduced by performing multidimensional separations but the problem of identification still remains. Hu et al. [146] suggested identification of proteins in electropherograms based on co-migration with standard proteins, however, co-migration is not sufficient for positive identification. One such solution was demonstrated by Zhang & Jin [147] who combined the separation efficiency and sensitivity of CE with the selectivity of an antibody immunoassay. Human natural killer cells were perforated with digitonin and then electrokinetically injected into the capillary followed by hydrodynamic injection of a fluorescein isothiocyanate labelled monoclonal antibody for the protein human interferon- γ (IFN- γ). The cell was completely lysed by ultra-sonication and left to incubate with the antibody. The sample was then separated and detected by CE with LIF. The system was capable of resolving the different forms of IFN- γ with a limit of detection of zmol.

Alternatively, Mellors et al. [148] coupled on-chip CE with mass spectrometry for protein identification. Haemoglobin from single erythrocytes was separated and introduced by electrospray ionisation to the mass spectrometer. The mass spectrometer employed was not sensitive enough to identify the second most abundant protein in erythrocytes, carbonic anhydrase I, whose abundance has been measured at ~ 7 amol/cell, compared to haemoglobin at 450 amol/cell.

2.4.3 Mass spectrometry

Mass spectrometers are used for a wide variety of applications and the nature of the mass spectrometer depends on the nature of the work, but the basic processes remain the same. A sample must be prepared and introduced, ionised and separated according to mass to charge ratios and recorded. Mass spectrometry (MS) can be used to study the behaviours of molecules under electron impact, help determine molecular structure and qualitatively and quantitatively determine the components of a mixture. For proteomic studies, the latter is very important.

However, the introduction of such small amounts of material as is required with single cell studies poses a technological barrier that needs to be overcome before MS can become competitive in single cell proteomics.

MS studies are only very recently beginning to detect analytes whose abundance is on the order of single cell quantities. Amantonico et al. [149] detected endogenous primary metabolites in a cell extract. Cell suspension was spotted onto a matrix of 9-aminoacridine and laser-ionised and examined by MALDI-TOF MS. Adenosine triphosphate was able to be detected down to tens of amol. Urban et al. [150] used an enzymatic amplification method to detect metabolites, such as ATP and AMP, providing up to 3 orders of magnitude greater sensitivity. ATP was detected down to ~50 amol.

Single cell studies using MS that have emerged so far focus on extremely large cell types such as plant cells [151] or larger neuron cells [152]. These cells contain large quantities of analyte. The quantities in most mammalian cells preclude MS from anything but studies in which highly abundant species are present. For example, Mellors et al. [148] used MS to identify haemoglobin in erythrocytes subsequent to an on-chip CE separation step.

Brown et al. [153] examined the proteolysis of β -APP peptide in single TF-1 cells by marrying the sensitivity of CE, which does not allow for definitive identification, with MS, which, although lacks the required sensitivity, does allow for protein identification. CE was used to profile the relative amounts of peptide fragments in single cells and MS was used to identify them in bulk cell lysate.

2.4.4 Conclusions

Unfortunately, there is no biochemical target amplification scheme, like PCR for nucleic acid, available for proteins. The analysis method must possess a read-out sensitivity that enables the

detection of low levels or transient states of proteins within single cells. It must also be compatible with the microfluidic format and be technologically straightforward to implement, allowing inexpensive and uncomplicated rapid prototyping. Proteins must be able to be separated, identified and quantified. The method must also be scalable to allow multiple proteins from varying phenotypes to be analysed simultaneously.

An advantage of MS, relative to other methods for protein analysis, is that it does not require a priori information on the proteins of interest. Microarrays, no matter how large, are still a directed approach whereas MS is able to detect unexpected changes in the protein sample. MS is also well suited to study PTMs as most result in characteristic increases in mass. However, the sensitivity of MS is still a fundamental barrier and significant technological improvements to sample introduction and detection must be made before the application of MS to single cell studies is a serious one.

Protein microarrays have the ability to sense and quantify a single protein species in a highly complex or congested sample requiring no pre-separation stage. It is compatible with the microfluidic format and, indeed, benefits from the reduced volume environment in respect of antibody binding kinetics.

There are several problems to deal with regarding protein microarrays and perhaps the most crucial is the antibodies themselves. Antibodies are currently the only class of molecules that serve as high affinity, high specificity ligands for almost any given protein. However, the majority of antibodies target relatively few, but common, proteins. For high throughput microarrays containing thousands of antibody spots, the isolation of such a large number of high affinity antibodies will prove very difficult if they are produced by traditional means in animals. For more conservative microarrays comprising a few to tens of antibodies, proper and thorough validation is necessary to quantify a particular antibody's behaviour. There is an increased need in microscale immunoassays for better sensitivity, especially in the hunt for low abundance proteins. Monoclonal antibodies are highly specific but lack the high affinities achievable with polyclonal antibodies. Moreover, validation must extend to the function of the antibody when immobilised on a solid surface as performance may not reflect that of the validating 2D gel since immobilisation can significantly alter their structure [154]. Suitable solid support and surface functionality are required and has been investigated in such works by Kusnezow et al. [155].

For a highly sensitive platform, the detection strategy should be based on TIRF microscopy using a sandwich assay. This approach increases sensitivity and overall specificity but, in terms, of antibody validation more than doubles the amount of work required – two, rather than one, antibodies are

needed and must be non-competitive. Although, the specificity of the secondary antibody need not be as high as the primary, immobilised, antibody, the scope of validation must cover cross reactivity and activity to other proteins and antibodies in the experiment.

Again, the greatest barrier is the heterogeneity of proteins and antibodies making it difficult to define strategies that do not discriminate between species. The need for thorough validation, accounting for affinity, specificity, cross-reactivity amongst others, is crucial to the performance and reliability of any protein microarray based assay.

CE has been used in single cell studies to investigate cellular and intracellular heterogeneity and to quantify intracellular expression levels of certain proteins. It has unparalleled sensitivity in combination with LIF detection, able to routinely demonstrate limits of detection on the order of zmol and below. CE has no inherent way of identifying proteins without any additional strategy. Fluorescently labelled antibodies may be introduced into the capillary and incubated with single cell lysate before any separation occurs in order to enable identification in the electropherogram. This has been performed for the interrogation of single species but nothing fundamental precludes the addition of more antibodies to simultaneously detect and quantify several protein species. Consideration must be taken if several antibody-protein complexes co-migrate then they may be distinguished in the orthogonal dimension of fluorescence wavelength, i.e. label different antibodies with different fluorophores, without the need for multidimensional separation.

One drawback of using this approach is, again, the antibodies themselves and also the inability to dynamically probe proteins. In the case of protein/antibody microarrays, secondary, tertiary, and further antibodies or species may be used to interrogate the captured protein. This is not possible with CE. The restriction for further experiments on the captured proteins is a significant limitation of CE.

Despite the need for time-consuming, and often laborious, validation protein microarray technology is well suited to the detection and quantification of low abundance proteins. It represents limited additional complexity in terms of microfluidic device fabrication and, apart from the antibodies themselves, is significantly less complicated than the other two methods to implement - the system will only require an additional laser source to effect TIRF illumination and be incorporated into the same optical setup as the microfluidic and lysis strategies. For these reasons it has been chosen as the method for analysis.

2.5 Summary

The technology to enable proteomic measurements at the single cell level will bring all 3 methods together on the same platform. To handle, isolate and trap cells, a microfluidic device containing sieve-type features will be employed to isolate and trap cells from flow. The locations of the cells will be within chambers containing one or several microarrayed antibody spots to detect protein originating from the cells under investigation. Lysis will be afforded by the delivery of a single laser pulse that will break down the media resulting in a cavitation bubble that in turn will mechanically disrupt the cell.

3 Microfluidic Single Cell Arrays

Microfluidics is the study of systems that process or manipulate nano- or even picolitre volumes using channels and features with dimensions of the order of a few to hundreds of micrometres. Upon shrinking to the micron-scale, the dominant forces of physical phenomena in a fluid change. Three characteristics that dominate are laminar flow, diffusion and surface area to volume ratio. Microfluidics may be used for a variety of applications. Here, microfluidics has been used as a tool to afford the isolation of single cells. This chapter introduces the concepts relevant to designing a microfluidic system that is capable of trapping cells in distinct analysis chambers and then characterises the efficiency of the resulting device.

- Design

The framework required to understand the microfluidic environment in terms of non-inertial flow and mass-transport by diffusion is introduced. Consideration of cross-talk between analysis chambers due to mass-transport by diffusion is also considered. Computational fluid dynamics simulations are made in order expedite design testing.

- Fabrication

Rapid-prototyping work-flow employed to construct chips is described.

- Results

The microfluidic design for hydrodynamic focussing and trapping of cells is tested and the cell trapping efficiency is measured.

3.1 Microfluidic theory

Fluids are continuum materials and so continuous fields such as density and force density, defined per unit volume, are better descriptors than discrete quantities such as mass. The velocity field, $\mathbf{u}(\mathbf{r}, t)$, for a Newtonian fluid (viscosity is not a function of velocity) obeys the Navier-Stokes equation. In an inertial frame of reference, the most general form is

$$\rho \left(\frac{\partial \mathbf{u}}{\partial t} + \mathbf{u} \cdot \nabla \mathbf{u} \right) = \nabla \cdot \vec{\sigma} + \mathbf{f} = -\nabla P + \mu \nabla^2 \mathbf{u} + \mathbf{f}, \quad [3.1]$$

where ρ is fluid density, $\vec{\sigma}$ is the tensor stress term consisting of normal and tangential components, ∇P is the pressure gradient arising from normal stresses, $\mu \nabla^2 \mathbf{u}$ describes viscosity and $\mathbf{f}$ represents other body forces such as gravity. The equation describes the conservation of momentum in a fluid and is an application of Newton's second law. It includes a non-linear inertial term $(\mathbf{u} \cdot \nabla) \mathbf{u}$, which describes turbulent effects predominant at the macroscale, and a viscosity term $(\mu \nabla^2 \mathbf{u})$, which damps inertial effects at the microscale.

Fluid flow may be characterised the Reynolds number. It is the ratio of inertial to viscous forces and consequently it quantifies the relative contribution of each for given flow conditions. Thus it is used to identify different flow regimes such as laminar or turbulent flow. It is given by

$$Re = \frac{\rho u L}{\mu}, \quad [3.2]$$

where u is the mean fluid velocity and L is the characteristic length, taken usually as the channel diameter. Unpredictable and chaotic fluid flow is characteristic of turbulent flow and occurs at high Reynolds number (typically $Re > 10^3$). Conversely, laminar flow is predictable and non-turbulent, occurring at low Reynolds number (typically $Re < 1$). The typical dimensions of microchannels in this work are $100 \mu\text{m} \times 50 \mu\text{m}$ ($w \times h$) and the carrier fluid is water, with density 1000kg/m^3 and viscosity 0.001 Pa s at 20°C . Since the channel has rectangular cross section, the hydraulic diameter, defined as $D_h = 4A/P$, where A is the cross-sectional area and P is the wetted perimeter of the cross section, is commonly used to obtain the Reynolds number for such non-circular channels. Thus the Reynolds number is approximately 0.22 and therefore in the limit of laminar flow.

Consequently, the inertial term in the Navier-Stokes equation is negligible and may be rewritten as

$$\rho \frac{\partial \mathbf{u}}{\partial t} = -\nabla P + \mu \nabla^2 \mathbf{u}, \quad [3.3]$$

while also neglecting body forces. Regardless of flow assumptions, mass must be conserved and is described by the continuity equation

$$\frac{\partial \rho}{\partial t} = \nabla \cdot (\rho \mathbf{u}), \quad [3.4]$$

which states that at all times the mass entering a volume is equal to mass exiting that volume. The continuity equation is valid for incompressible liquids therefore density is a constant and it follows that

$$\nabla \cdot \mathbf{u} = 0. \quad [3.5]$$

If flow is steady then the time derivative in eq. 3.3 is zero and we arrive at Stokes equation

$$\nabla P = \mu \nabla^2 \mathbf{u} . \quad [3.6]$$

Stokes flow has no time dependence, other than through time dependent boundary conditions, and so has the properties of instantaneity and time-reversibility. These properties mean that if time is reversed then the flow reverses along exactly the same path. This makes it difficult in the laminar regime to mix two fluids. Inertial forces that dominate turbulent flow produce random eddies, vortices and other chaotic fluctuations. As a result of turbulent mixing, concentration gradients are significantly enhanced and time scales for mixing are low. However, mixing in microfluidic systems is dominated by diffusion.

The diffusion time is

$$t_D = \frac{l^2}{2D} , \quad [3.7]$$

where l is the diffusion length, and D is the diffusion coefficient of the diffuser. The diffusion coefficient is given by

$$D = \frac{k_B T}{6\pi\mu a} , \quad [3.8]$$

where k_B is Boltzmann's constant, T is temperature in Kelvin and a is the diffusing particle's effective radius. Green fluorescent protein (GFP) is a 26.9 kDa protein that has a typical beta barrel structure 4 nm in length and 3 nm in diameter. According to eq. 3.8, in water at 295 K it has a diffusion coefficient of $54 \mu\text{m}^2/\text{s}$ and is very close to the measured value of $87 \mu\text{m}^2/\text{s}$ by Elowitz et al. [156]. The time it takes GFP to diffuse 10 μm is only 0.9 s whereas to diffuse 1 mm it would take 2.6 hours. This will have implications for the design of the device; specifically it imposes a limit on how close single cell chambers may be placed before they start suffer interference from adjacent chambers.

3.2 Fabrication

Early microfluidic fabrication relied on techniques developed by the microelectronics industry. Photolithography and etching in silicon (Si) are highly developed but are still expensive. Si is not transparent making integration into optical setups not so straightforward whereas glass is amorphous and vertical side walls are difficult to fabricate. In addition, batch processing requires a

cleanroom environment. These factors meant that the technology was open to only a few laboratories worldwide.

A particularly important contribution to device fabrication has been the development of soft lithography. Soft lithography involves cast moulding and a key element of soft lithography's success has been the elastomer poly(dimethylsiloxane) (PDMS). PDMS is a silicon-based viscoelastic polymer consisting of repeating $[\text{SiO}(\text{CH}_3)_2]$ units, which can be cross-linked by mixing with a curing agent to form a solid elastomer. Cured PDMS is optically transparent down to $\sim 300\text{nm}$; is highly inert allowing molecules to reversibly adhere to or react with the PDMS surface; is compatible with a range of solvents and most organic solvents do not swell PDMS to any significant degree; is gas-permeable and biocompatible allowing cell culture on-chip; and is also highly flexible, allowing it to be easily manipulated during construction of a PDMS-based device.

Components such as valves [157], mixers [158] and pumps [159] have all been demonstrated in PDMS devices. Etching in Si and glass is expensive and time consuming yet as the field has developed these materials have found use in specialised systems that require chemical and thermal stability and in nanofluidic systems [160, 161].

The process of rapid prototyping using soft lithography, summarised in figure 3.1, begins by designing the channel system and microstructures using a computer aided design program. The designs are then commercially printed at high resolution on a transparency, which will serve as a photomask (figure 3.1a). The epoxy SU-8 [162] is used as a negative photoresist since when exposed to near UV radiation (350 – 400 nm) it becomes insoluble in developing solutions due to the formation of cross linkages. SU-8 is spin-coated over a Si wafer, the thickness of which is dictated by the spin speed. After the resist has been applied it must be soft baked to densify the film and is normally carried out upon a hot plate. The photomask is placed upon the baked film and exposed briefly to near UV radiation. A post-exposure bake is performed to complete the cross-linking process. Unexposed SU-8 is removed by solvent washing in the development process leaving a positive relief of cured photoresist (figure 3.1b). After development, the wafer is washed with isopropyl alcohol and dried using nitrogen. This is the master and is subsequently used for the casting of PDMS devices. To enable easy lift-off of cured PDMS, the Si-wafer surface is passivated by vapour-deposition of (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane (Sigma-Aldrich, UK).

PDMS is prepared by mixing a prepolymer and curing agent, at a ratio which controls elastomer stiffness and is typically 10:1, degassing the mix in a desiccator and then pouring over the master. It may be left to set overnight but owing to its high thermal stability may be set within a few hours on

a hotplate at 60 – 80°C. Once the PDMS is set and has been peeled off the master is ready to be reused. So long as appropriate care is taken during pouring, peel-off and handling, the wafer may be reused indefinitely.

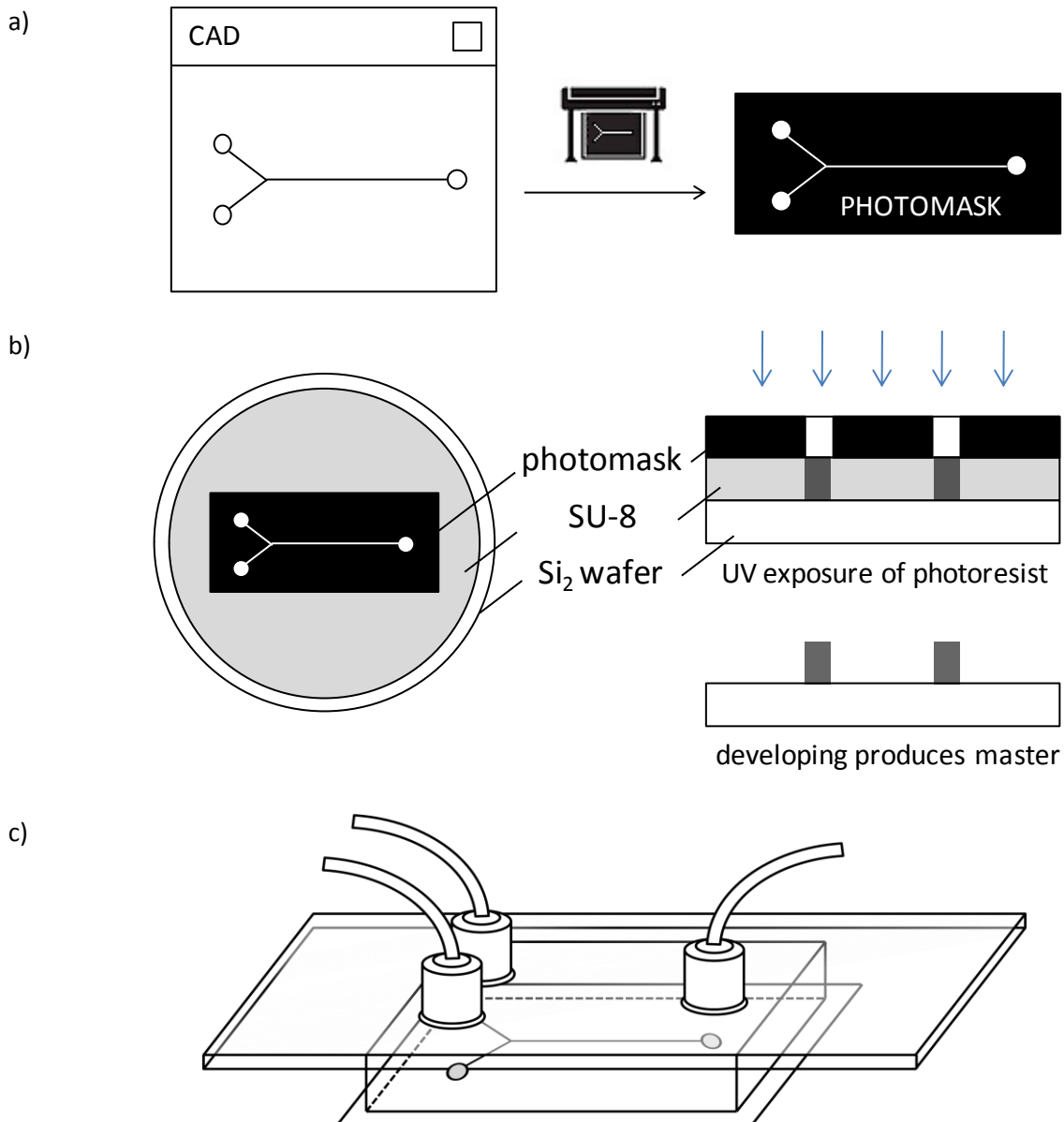


Figure 3.1: Major steps in producing a PDMS based microfluidic device using soft lithographic techniques [163]. a) Channel designs are drawn up in a CAD program from which a photomask is commercially produced. b) A photoresist SU-8 is spin coated onto a silicon wafer. The resist is exposed through the photomask and crosslinks which forms the channel patterns after development. c) PDMS is poured onto the master, cured and cut out. The top surface is bonded to a coverslide to provide structural support and contains access holes to interface chip-to-world tubing. The channel side is bonded to a coverslip which facilitates high NA imaging.

There are various approaches to PDMS bonding and what to bond it to in order to provide device rigidity and fluidic access to the microchannels. All devices in this work are built in the following order.

PDMS is cut into pieces measuring 24 mm x 50 mm appropriate for bonding the channel-side to a microscope coverslip. Access holes are punched using a filed down 25-gauge biopsy needle, which cored a vertical channel of approximate 300 μm diameter into the PDMS piece. Alignment was made by eye and resulting holes checked using a brightfield microscope that they overlapped the microchannels. Corresponding holes were drilled through a 25 mm x 50 mm x 1 mm microscope slide, which, along with the piece of PDMS, was plasma treated before bonding to form the 'top half' of the device. PDMS and glass may bond, or seal, reversibly by simply pushing the two surfaces into contact. Contact bonding is facilitated by weak van der Waals forces as the PDMS conforms to the flat surface of the glass slide and is unable to withstand high pressures from fluid pumping. In order to withstand higher pressures, PDMS and glass may be irreversibly bonded by short treatment in a plasma oven. Surface Si-OH groups are created on each surface by an air plasma, which, when in contact with the opposing surface replete with Si-OH groups, undergo a condensation reaction to form (-Si-O-Si-) bonds that are covalent. Upon bonding the 'top half' is placed on a hot plate at 75°C to help complete bonding.

To seal the microchannels a microscope coverslip (thickness #1.5, 0.16 – 0.19 mm thick) is bonded by plasma treating both the PDMS surface and the coverslip. The coverslip allows the use of high numerical aperture optics, thus allowing optical trapping, optical lysis and TIRF imaging to be performed within the microfluidic network. However, the procedure is more involved when fabricating a device for single cell pulldowns since the coverslip will have antibody patches printed on it in defined locations. Plasma treatment of such a surface would render the antibodies non-functional. Instead, only the PDMS surface is plasma treated. The interfacial bond is not as strong as plasma treating both surfaces but is much stronger than a seal produced by simple contact bonding alone. A homemade alignment stage is used to align the antibody patches to the microfluidic chambers (figure 3.2).

The final stage of fabrication involves interfacing the microfluidic channels via the access holes drilled or punched earlier with external plumbing in order to introduce fluid, reagents, cells, etc. Off-chip plumbing typically includes tubing, shut-off valves and gas-tight syringes. The actual chip-to-world interface may be made by glass capillary, here inner diameter 100 μm and outer diameter 375 μm , or NanoPorts. NanoPorts are commercial connectors that are robust, easy to fit and are able to connect to standard tubing sizes. They are particularly useful when particular connections are

swapped or removed frequently since the tubing connector may be screwed off rather than pulled off, as is the case with capillary-tubing interfaces that result in drawing fluid through the device. Syringes contain media that is to be pumped through the channels. Syringes containing a suspension of cells are unable to be shook to prevent sedimentation; instead, a small stirrer bar magnet is sterilised and placed within the syringe and is periodically used to agitate the cell suspension by action of another magnet, external to the syringe. In addition, sedimentation may occur along the tubing and so tubing length is kept to a minimum.

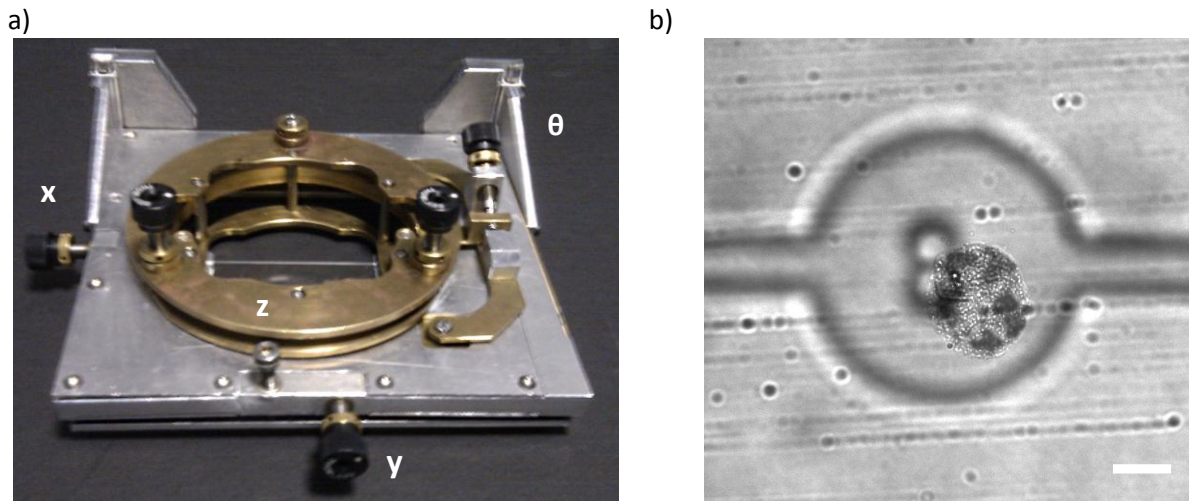


Figure 3.2: a) Homemade alignment stage (x , y , z , θ) which allows the orientation of antibody spots printed on a coverslip relative to the microfluidic chambers in PDMS. b) By closing the microscope condenser iris, contrast and depth of focus are increased allowing the antibody spot and channels (held in position $z = 750 \mu\text{m}$ above the coverslip to be bonded) to be simultaneously visualised. Once spots are aligned, z is decreased and the two surfaces are bonded. Scale bar $100 \mu\text{m}$.

Soft-lithography represents a low capital cost and very cheap per unit production cost. The technique requires simple training and allows devices to be rapidly prototyped within a few hours.

3.3 Trapping design

As discussed in §2.2, cells will be sequestered from flow by hydrodynamic trapping.

The design requires single cells to be trapped at defined locations that are separated by a distance determined by the diffusion length of the protein over the duration of the experiment. In order to promote diffusion around the antibody patch and minimise loss, the trap location and antibody patch are contained within a chamber that has a diameter much greater than that of the channels

feeding and leaving it. Loss of analyte by diffusion out of the chambers is proportional to the ratio of these diameters.

The diameter of trypsinised BE cancer cells transfected with cytoplasmic GFP (see §7.4) in flow is $16.1 \pm 2.0 \mu\text{m}$. The main channel running through the device will have a diameter of $50 \mu\text{m}$, which will allow single cells to be easily flowed while also accommodating for cell clumps that may otherwise block thinner channels. The chamber diameter is dictated by the diameter of the antibody patch and the precision to which they can be aligned throughout the device. Antibody patch diameter can vary depending on the antibody printed but for the antiGFP antibody will be between $100 - 150 \mu\text{m}$ (see §5.5). The homemade alignment stage is capable of aligning patches to $\pm 5 \mu\text{m}$ in the x- and y-direction but tends to drift as the ‘top half’ of the device is lowered onto the antibody coverslip below. This drift results in the patches being misaligned by as much as $50 \mu\text{m}$; therefore, the chamber requires a diameter greater than $200 \mu\text{m}$ and in order to minimise volume should be circular such that each chamber is cylindrical.

A design based on that of Tan & Takeuchi [32], Di Carlo [33, 35] and Kim et al. [37] for trapping single cells is proposed. The specific trapping point will be made by placing two pillars a distance less than the minimum cell diameter apart in order that they sieve cells from flow. There are important flow considerations that must be taken into account.

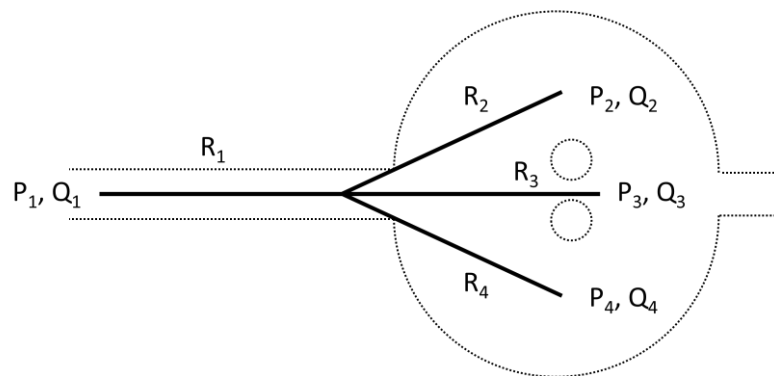


Figure 3.3: The fluid flow through the trap may be reduced to fluid flowing through different paths of a branching network. The flow rate and therefore trapping efficiency is proportional to the relative resistances of each branch in the network. Based on channel network from [164].

The pressure drop over a channel is given by

$$\Delta P = QR , \quad [3.9]$$

where Q is the flow rate through the channel and R is the hydrodynamic resistance. The flow through the pillars in the chamber may be modelled as a branching network (figure 3.3). Knowledge of the absolute flows isn't necessary since we wish to know the relative flow through the centre and around the trap. The flow network branches into 3 upon entering the chamber and by conservation of mass

$$Q_1 = Q_2 + Q_3 + Q_4 . \quad [3.10]$$

By applying eq. 3.9 to 3.10 the flow rate through a branch may be obtained

$$Q_1 = Q_2 \left(1 + \frac{R_2}{R_3} + \frac{R_2}{R_4} \right) , \quad [3.11]$$

with similar equations obtained for each branch. Assuming the trap is placed at the centre of the chamber then $Q_2 = Q_4 = Q_{nt}$, where the subscript nt denotes 'not trapped' since objects flowing down these branches will bypass the sieve and exit the chamber. Conversely, $Q_3 = Q_t$, where the subscript t denotes 'trapped' since fluid flowing down this central branch will pass through the sieve and so objects in this flow will become trapped. The trapping efficiency of each chamber will then be

$$TE = \frac{Q_t}{2Q_{nt} + Q_t} = \frac{2R_{nt} + R_t}{R_t} . \quad [3.12]$$

For a channel with rectangular cross-section with width w and height h , the hydrodynamic resistance is given by

$$R = \frac{12\mu L}{wh^3(1-0.630h/w)} , \quad [3.13]$$

where L is the length of the channel. Substituting eq. 3.13 into 3.12 the trapping efficiency may be expressed as a function of the relative widths of the network branches

$$TE = \frac{w_t}{2w_{nt} + w_t} , \quad [3.14]$$

where w_t is the pillar separation and w_{nt} is the width between the outside of the solid pillar and the chamber edge. The maximum size w_t can be is $d_{cell} - \delta$ and the minimum size w_{nt} can be is $d_{cell} + \delta$, where d_{cell} is the diameter of the cell being trapped and δ is a small finite width. The maximum trapping efficiency is obtained in the limit $\delta \rightarrow 0$ in eq. 3.15, which is 33%.

$$TE_{max} = \frac{d_{cell}}{3d_{cell}} . \quad [3.15]$$

The chamber diameter is constrained by the antibody patch to be around 500 μm . Cell diameter, d_{cell} , is $16.1 \pm 2.0 \mu\text{m}$ (human BE carcinoma cells; see §7.4) and so a pillar separation of 12.5 μm will comfortably trap all cells. The maximum trapping efficiency is approximately 2.5%. This assumes that cells are evenly distributed along the width of the channel that supplies the chamber. Since flow is laminar, fluid follows well defined field lines so only cells on field lines that pass through the trap width will be trapped.

Trapping efficiency may be enhanced by hydrodynamic focussing, which essentially allows inlet flow lines carrying cells to be concentrated more centrally that then pass through the trap. The greater the focussing the greater the trapping efficiency, which becomes

$$TE = \frac{w_t}{w_{ft}} \quad , \quad [3.16]$$

where w_{ft} is width of the focussed stream at the trap and $w_{ft} \geq w_t$. The width of the focussed stream in the channel feeding the trapping chamber, w_f , will increase by a factor dictated by the ratio of the chamber to channel diameters, i.e.

$$w_{ft} = w_f \frac{w_{ch}}{w_i} \quad , \quad [3.17]$$

where w_{ch} and w_i are the chamber and channel inlet widths, respectively. Figure 3.4a shows how TE varies as a function of focussed width for $w_t = 12.5 \mu\text{m}$. By combining eq. 3.16 and 3.17, a plot of TE as a function of chamber diameter, for $w_f = d_{\text{cell}} = 16.1 \mu\text{m}$, $w_i = 50 \mu\text{m}$ and the same trap width, is shown in figure 3.4b.

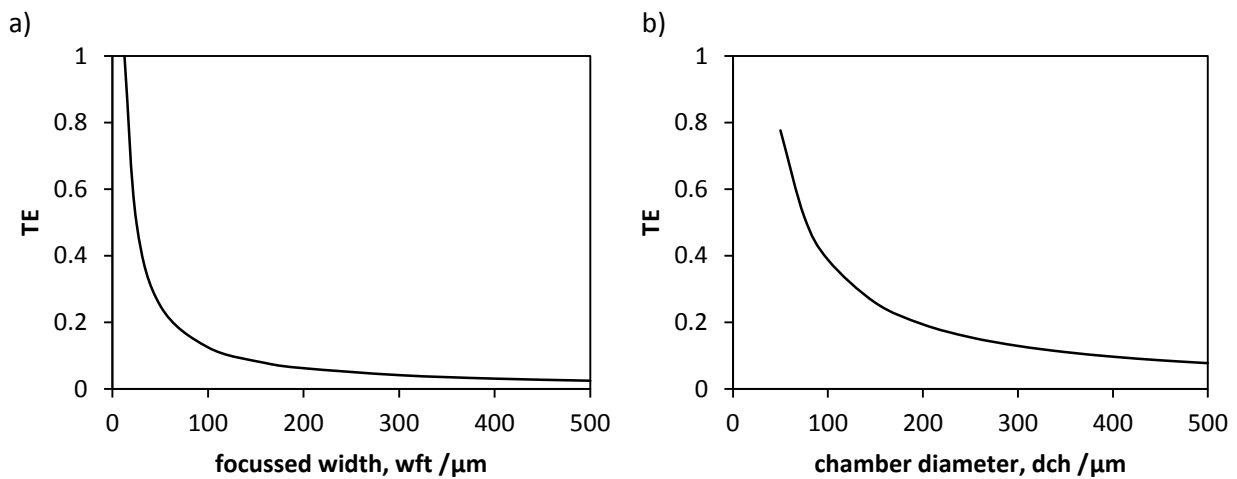


Figure 3.4: Plots showing how trapping efficiency of a 12.5 μm wide trap varies as a function of a) focussed width within a 500 μm chamber and b) chamber diameter with a focussed width of $d_{\text{cell}} = 16.1 \mu\text{m}$.

Trapping efficiency is not enhanced by any significant degree until flow focussing produces a stream $< 75 \mu\text{m}$ in the trapping chamber. Of course, the cell diameter, here $16.1 \pm 2.0 \mu\text{m}$, imposes a lower limit on the width of the focussed stream of d_{cell} in the channel and $d_{cell} w_{ch}/w_i = 161 \mu\text{m}$ in the chamber. The trapping efficiency is then enhanced to 7.8%, with hydrodynamic focussing from 2.5%, without focussing. This is only a modest increase but owing to the simplicity by which hydrodynamic focussing may be implemented it would be beneficial for it to form part of the design.

By decreasing chamber width, the relative flow rate outside the trap will be decreased, by eq. 3.12 & 3.14. Due to the constraints described previously, i.e. antibody spot diameter and alignment precision, the chamber diameter must be a minimum of $200 \mu\text{m}$ for multilayer fabrication or $500 \mu\text{m}$ for single layer fabrication. Again, only a modest increase of a factor of 2.5 in TE is gained by reducing chamber diameter from 500 (7.8%) to $200 \mu\text{m}$ (19.4%). Since the increase in efficiency did not, in my opinion, outweigh the added complexity in fabrication, the chamber diameter was fabricated at $500 \mu\text{m}$.

In symmetric focussing, two focussing flows are used to squeeze the central flow with the central flow width dependent on the relative flow rate through the focussing channels. The fluid is incompressible and so flow velocity increases. Lee et al. [165] have developed a theoretical model to predict the width of the focussed stream in symmetric hydrodynamic focussing in a rectangular microchannel (figure 3.5a). According to mass conservation, all fluid in the central channel must flow through the focussed dimension (w_f)

$$Q_i = w_f \bar{v}_f h, \quad [3.16]$$

where terms are expressed in the notation of Lee et al.; Q_i is the volumetric flow rate of the inlet channel, the $\bar{v}_f$ is the average velocity of the focussed stream and h is the channel height. Similarly, all fluid flowing passing through the outlet must have been supplied from the inlet and focussing channels

$$Q_o = Q_i + Q_{s1} + Q_{s2} = w_o \bar{v}_o h, \quad [3.17]$$

where Q_o , Q_{s1} and Q_{s2} are the volumetric flow rates of the outlet and side channels, respectively, w_o is width of the outlet channel and $\bar{v}_o$ is the average velocity of the outlet channel.

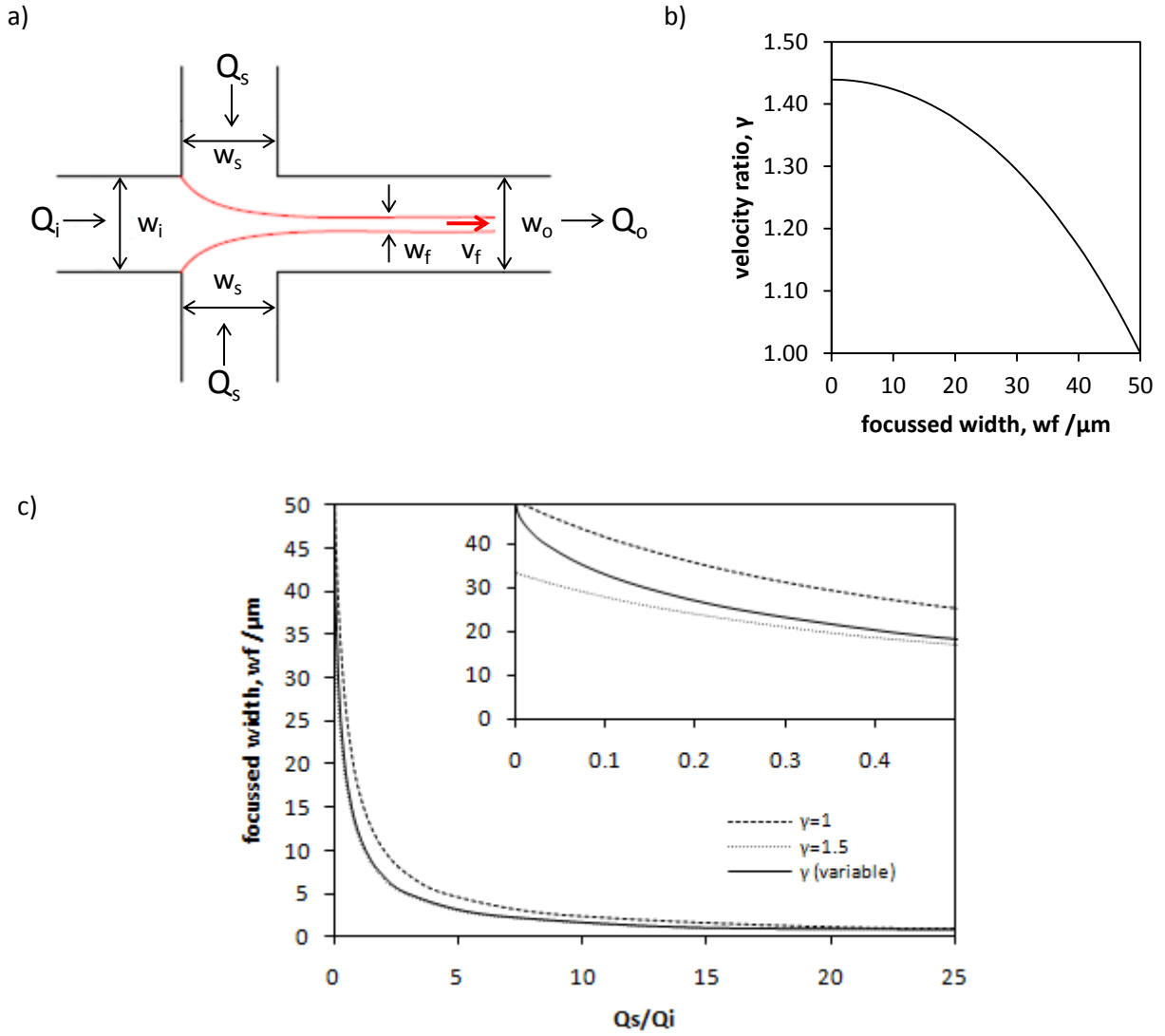


Figure 3.5: a) Schematic of symmetric hydrodynamic flow focussing, reproduced from [165]. b) Velocity ratio of the flow in the focussing to outlet channels. It is evaluated using eq. 3.19. c) Comparison of the width of the hydrodynamically focussed stream for different velocity ratios. It can be seen that for $Q_s/Q_i \gg 1$ the focussed width follows the profile set by the limiting case of $\gamma = 1.5$ whereas this is no longer appropriated for $Q_s/Q_i \ll 1$.

Combining eq. 3.16 and 3.17 gives an expression of the focussed flow width in terms of the input and focussing channel flow rates

$$\frac{w_f}{w_o} = \frac{Q_i}{\gamma(Q_i + Q_{s1} + Q_{s2})}, \quad [3.18]$$

where the velocity ratio $\gamma = \bar{v}_f / \bar{v}_o$ is an unknown quantity. It is important to note that the focussed width does not depend on the absolute flow rates. γ can be evaluated by integrating the Poiseuille velocity profile of the channels and the result calculated by Lee et al. is

$$\gamma = \frac{\left\{ 1 - (192h/\pi^5 w_f) \sum_{n=0}^{\infty} \frac{1}{(2n+1)^5} \frac{\sinh[(2n+1)\pi w_f/2h]}{\cosh[(2n+1)\pi w_o/2h]} \right\}}{\left\{ 1 - (192h/\pi^5 w_o) \sum_{n=0}^{\infty} \frac{1}{(2n+1)^5} \frac{\tanh[(2n+1)\pi w_o/2h]}{(2n+1)^5} \right\}} \cdot \quad [3.19]$$

For a symmetric focussing (i.e. $Q_{s1} = Q_{s2}$) channel network shown in figure 3.5a with dimensions $w_i = w_o = 50 \mu\text{m}$ and $h = 50 \mu\text{m}$, for a range of w_f , the velocity ratio may be calculated by evaluating eq. 3.19 and varies as shown in figure 3.5b. The velocity ratio is dependent on the channel aspect ratio h/w_o where two limiting cases exist for pressure-driven flow in rectangular channels. As $h/w_o \rightarrow 0$, the velocity profile across the outlet channel becomes plug-like and $\gamma = 1.0$. Conversely, as $h/w_o \rightarrow \infty$, the velocity profile becomes parabolic and $\gamma = 1.5$.

Thus, the volumetric flow rates required to produce a desired focussing width may be calculated using eq. 3.18. A plot of focussed width w_f against the ratio of the inlet and side channels volumetric flow rates, Q_s/Q_i , is shown in figure 3.5c. It can be seen that only modest ratios of 1 – 3 are required for a reduction in width by factors 1 – 10, whereas due to non-linearity, to achieve a width less than a micron requires significantly larger flow ratios. The factor γ becomes significant only at $Q_s/Q_i < 0.5$ (inset fig. 3.5c).

3.4 Trapping simulations

To better understand trap behaviour and how that behaviour depends on specific geometry, computation fluid dynamic simulations are performed.

Simulations are performed with the COMSOL Multiphysics 3.5a software. COMSOL uses finite element analysis with 2D and 3D capabilities. 2D modelling is simpler and allows analysis to be performed on computers with modest resources but tends to yield less accurate results. 3D modelling does produce more accurate results but requires significant computational resources.

The process involves first modelling the geometry, setting up the modelling physics and boundary conditions, meshing the geometry and finally solving the model. The geometry may be modelled in any CAD software and imported to COMSOL or created with the in-built CAD tools. An example of the geometries to be modelled is shown figure 3.6a. The geometry is not solid but the faces map out the boundaries of the channels.

In order to analyse fluid flow, the flow domain, i.e. the volume as defined by the modelling geometry, is split into smaller discrete subdomains made up of geometric primitives like triangles

and quadrilaterals in 2D or hexahedra and tetrahedra in 3D. This is called the mesh (figure 3.6b) and fluid flow is solved inside each mesh element as part of a finite element analysis to give a complete picture of fluid flow in the entire domain. The finer the mesh the more accurate the solution obtained; however, finite memory resources dictate a limit on accuracy.

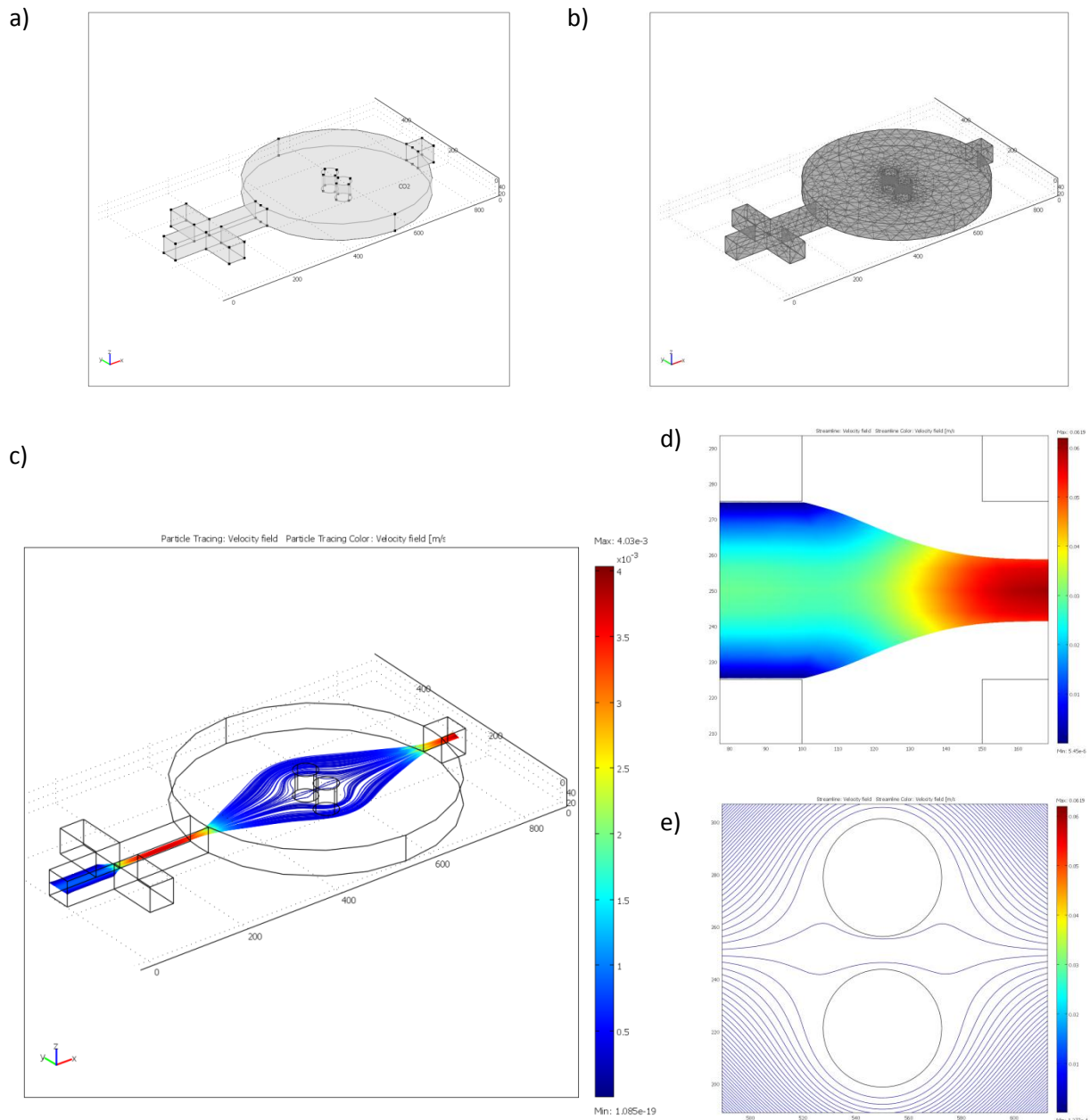


Figure 3.6: CFD simulations are performed in COMSOL Multiphysics. a) The geometry of the device is modelled using CAD drawing tools. b) The fluid domain is discretised by generating an unstructured mesh. c) Fluid flow through the device is solved and flow lines visualised. The streamline colour is set to the flow velocity. d) A close-up 2D view of the flow focussing region and e) the trap region. Cells on flow lines passing through the trap will become trapped.

The fluid flow is described by the Navier-Stokes equations outlined above, specifically eq. 3.3 and 3.5. The modelled fluid is water with a viscosity of 1×10^{-3} kg/s.m and a density of 1000 kg/m³.

The amount of memory required to solve a specific model is dependent on the number of node points, the number of dependent and independent variables, the element order and the mesh size. It is important to choose the correct solver settings. For problems with few degrees of freedom such as in 1D and 2D models, a direct solver (*PARDISO*, *UMFPACK*, *TAUCS Cholesky*) is preferred. For 3D models, that tend to have high degrees of freedom, direct solution is not possible due to memory limits and in this case, iterative solvers are required. Care must be taken in iterative solutions as they may not converge and rely on appropriate initial values. Here, the *PARDISO* solver is chosen since it is highly efficient for symmetric and non-symmetric systems and often uses less memory than *UMFPACK*. If the geometry to be modelled cannot be simplified, or a coarser mesh will not suffice and still exhausts system memory, then the *PARDISO out-of-core* solver is chosen due its ability to store solutions on disk in a swap file. Once a solution is obtained, flow within the fluid domain may be visualised by a variety of plots including surface, contour and streamline plots. The most useful of these to determine the TE of a specific trapping geometry is the streamline plot since it provides a quantifiable estimate of the proportion of flow lines that pass through the trap (figure 3.6c-e).

The prediction of the focussed width, w_f , afforded by the treatment of the channel resistances agrees well with simulation (figure 3.7a) whereas there is a significant discrepancy with respect to TE as a function of w_f . This is not surprising considering the simplicity by which the ‘theoretical’ approach was made. The CFD simulation takes account of more complex consideration, such as the finite size of the traps, which provides a more accurate efficiency. For $w_f = d_{cell}$, TE is 0.4% by simulation.

CFD simulations allow the trap design to be rapidly investigated over the course of hours, rather than weeks if each device were to be rapidly prototyped and efficiency tested. The full geometry is the same as that in figure 3.6c with the following boundary conditions. The central and focussing channels are set to inlet boundary type with a laminar inflow boundary condition and flow rates 1 $\mu\text{L min}^{-1}$ and 1.5 $\mu\text{L min}^{-1}$, respectively. This produces a focussed width on the order of the average cell size. The channel exit is set to outlet boundary type at zero pressure. All other boundaries are set as walls with the no slip boundary condition.

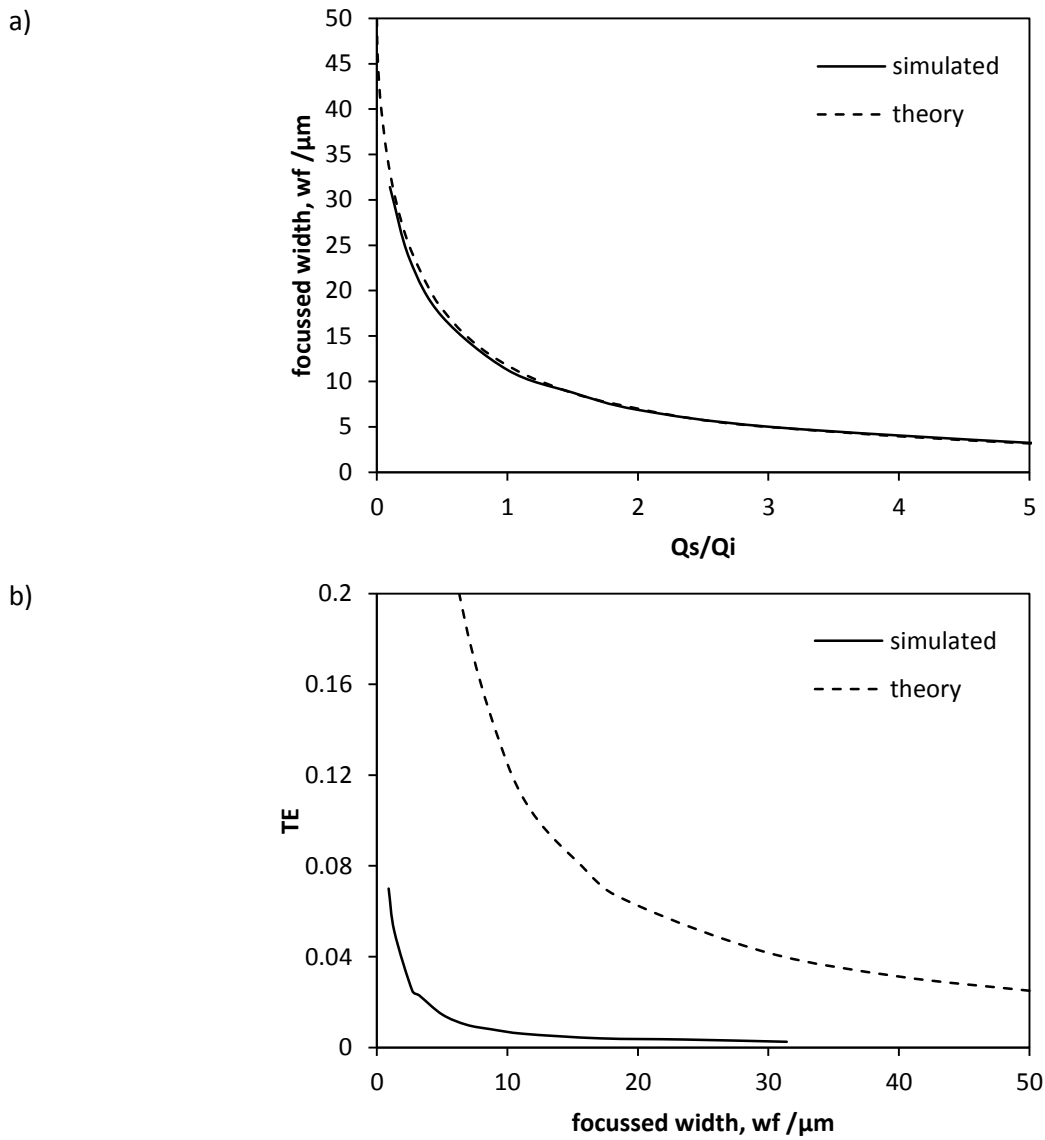


Figure 3.7: Comparison of how theory and simulations predict how a) the focussing width varies with flow focussing and how b) the trapping efficiency varies with focussed width for the geometry described in figure 3.6. Note that the focussed width is measured in the channel feeding the trapping chamber.

Figure 3.8 and figure 3.9 show simulations of several trapping variations, whereby variations upon the pillar design of figure 3.6 are simulated and compared to trapping structures which appear in the literature (figure 3.9c-f). The central aperture of all traps is $12.5\ \mu\text{m}$. The efficiency is determined by the proportion of stream lines originating at the inlet channel that eventually pass through the centre of the trap. The efficiencies are not changed by modifications in trap structure suggesting that this is not the crucial factor in these designs. The dominant factor is the chamber diameter and the ratio of the chamber diameter to the width of the channels feeding them. These are constrained by the antibody spot alignment and minimising diffusion out of the chambers. It is straightforward

to see that to increase trapping efficiency the length of the pillars in figure 3.8a may be increased to over 200 μm . However, this would effectively partition the chamber into two and significantly increase the diffusion time of protein from one half of the chamber to the other.

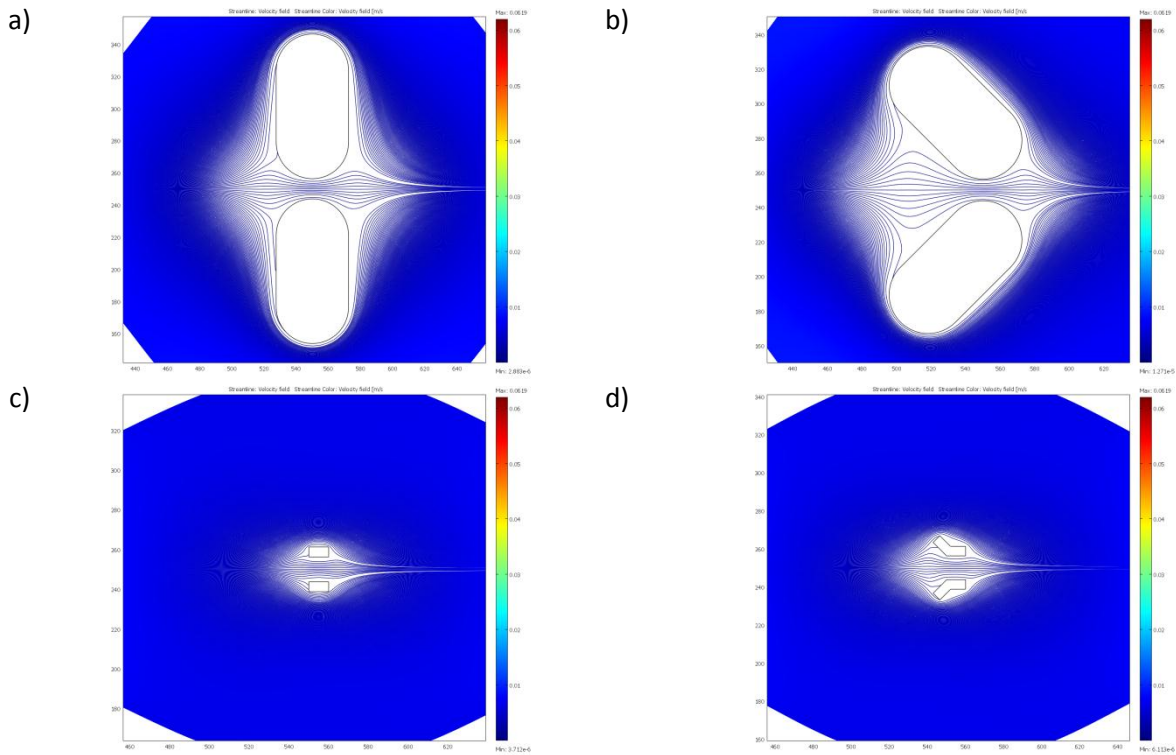


Figure 3.8: Streamlines in CFD simulations of different cell trapping geometries. Increasing the width of the trap to 90 μm a) or orientating it at 45° b) produces the same TE of 0.5%. Reducing the overall size to 5 $\mu\text{m} \times 10 \mu\text{m}$ c) and adding lips to serve as a funnel d) also produces a TE of 0.5%. Hydrodynamic focussing is made to produce a focussed width of $d_{\text{cell}} = 16.1 \mu\text{m}$ in all simulations.

Kim et al. [166] and Di Carlo et al. [35] have demonstrated highly efficient single cell trap and culture arrays and their designs are remodelled in figure 3.9c-f. Kim's U-type sieve (figure 3.9c & d) is individually just as efficient as the designs proposed here. However, in order to load cells within a matter of a few minutes, the chamber is packed with 9 trapping structures each. Trapping efficiency is increased much more than 9-fold since the flow resistances have been greatly altered within the chamber. Di Carlo's cup-shaped trap has no aperture but does not extend the full height of the channel. Originally, they hang 2 μm short of the channel bottom but here they are simulated hanging 12.5 μm short (figure 3.9e & f). The modified Di Carlo trap designs are not fully self-sealing as is the case with the pillar designs (figure 3.9b). This means that once a cell is trapped it blocks the trap aperture. Central fluid field lines re-establish themselves around the trap and cells on these flow lines flow to the next trap. This may be scaled to as many traps in series as is possible to fit on the device.

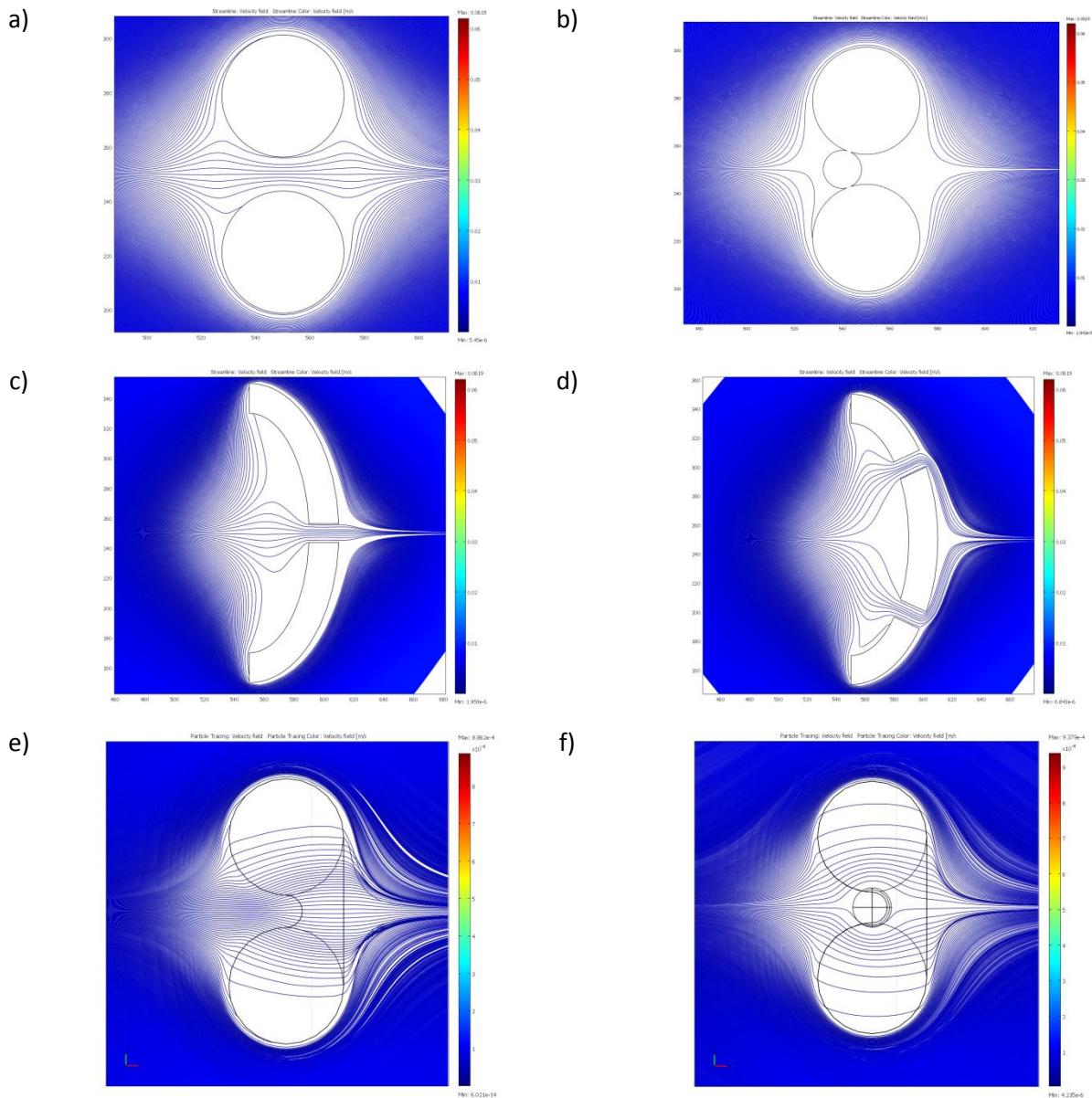


Figure 3.9: Streamlines in CFD simulations of different cell trapping geometries. a) The pillar design is 0.5% efficient and is self-healing as demonstrated in b) by modelling a cell as a solid sphere of radius 7.5 μm . Flow lines are redirected around the trap in the presence of a cell. Geometries in c), d) and e) are traps based on structures from literature that form part of devices that are reported to become fully loaded in a matter of a few minutes. According to simulations here, their trapping efficiencies are 0.4 – 0.9% with the geometry in e) being 2.25% efficient. The trap in e) does not extend the full channel height and so cells become trapped under the trap. The self-healing ability for e) is not as strong as the pillar design. Hydrodynamic focussing is made to produce a focussed width of $d_{\text{cell}} = 16.1 \mu\text{m}$ in all simulations.

3.5 Final design

Despite the low trapping efficiency the pillar-type design, with pillar radius $22.5\ \mu\text{m}$ and trap aperture $12.5\ \mu\text{m}$, is chosen for subsequent single cell experiments. This is due to the requirements of the single cell chamber; it must contain a single cell, have a surface footprint large enough to accommodate one to several antibody spots and be designed such that loss of material by diffusion is minimised. The full channel design is shown in figure 3.10. The prototype design comprises 1 inlet and 2 focussing channels of $100\ \mu\text{m}$ width. After 2.5mm the channel tapers to $50\ \mu\text{m}$ which feeds 10 trapping chambers in series of diameter $500\ \mu\text{m}$. The channels connecting the chambers are $50\ \mu\text{m}$ wide. The chambers are separated by 1mm , which prevents interference from adjacent chambers due to diffusion of protein from one chamber to the next (see §3.1). For GFP in water at 295K with a diffusion coefficient of $87\ \mu\text{m}^2/\text{s}$, this limits the experiment to 2.6 hours post-lysis.

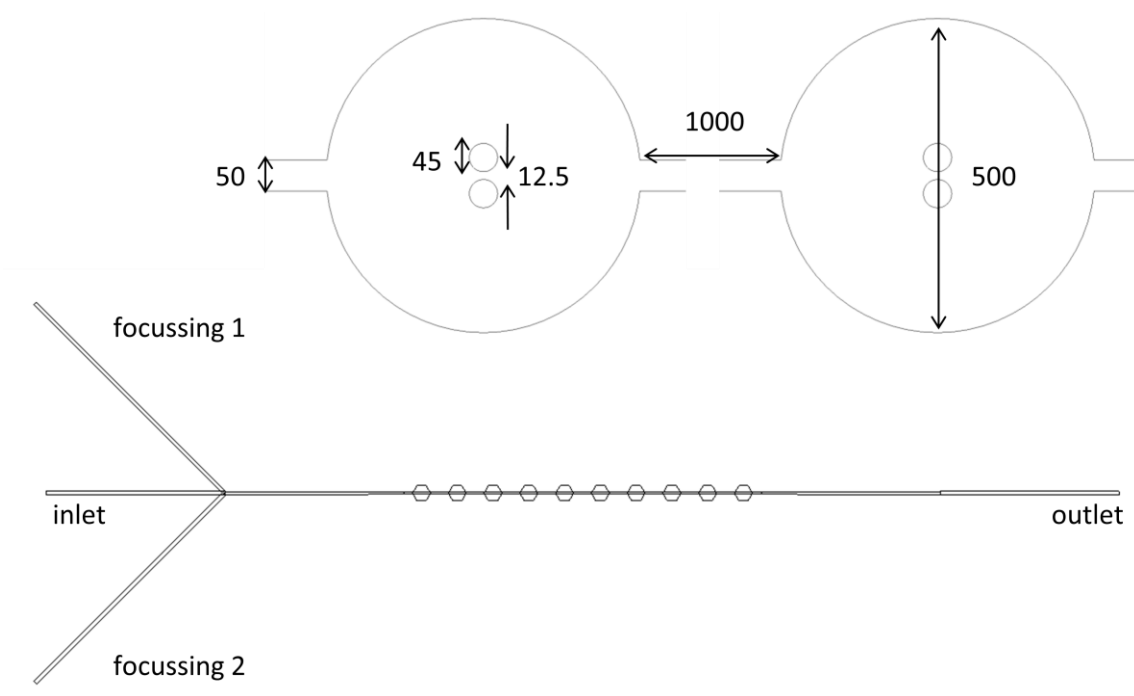


Figure 3.10: Schematic of the final channel design for hydrodynamically trapping single cells into separate chambers for subsequent proteomic analysis. Dimensions are in microns and the full channel network is drawn in proportion.

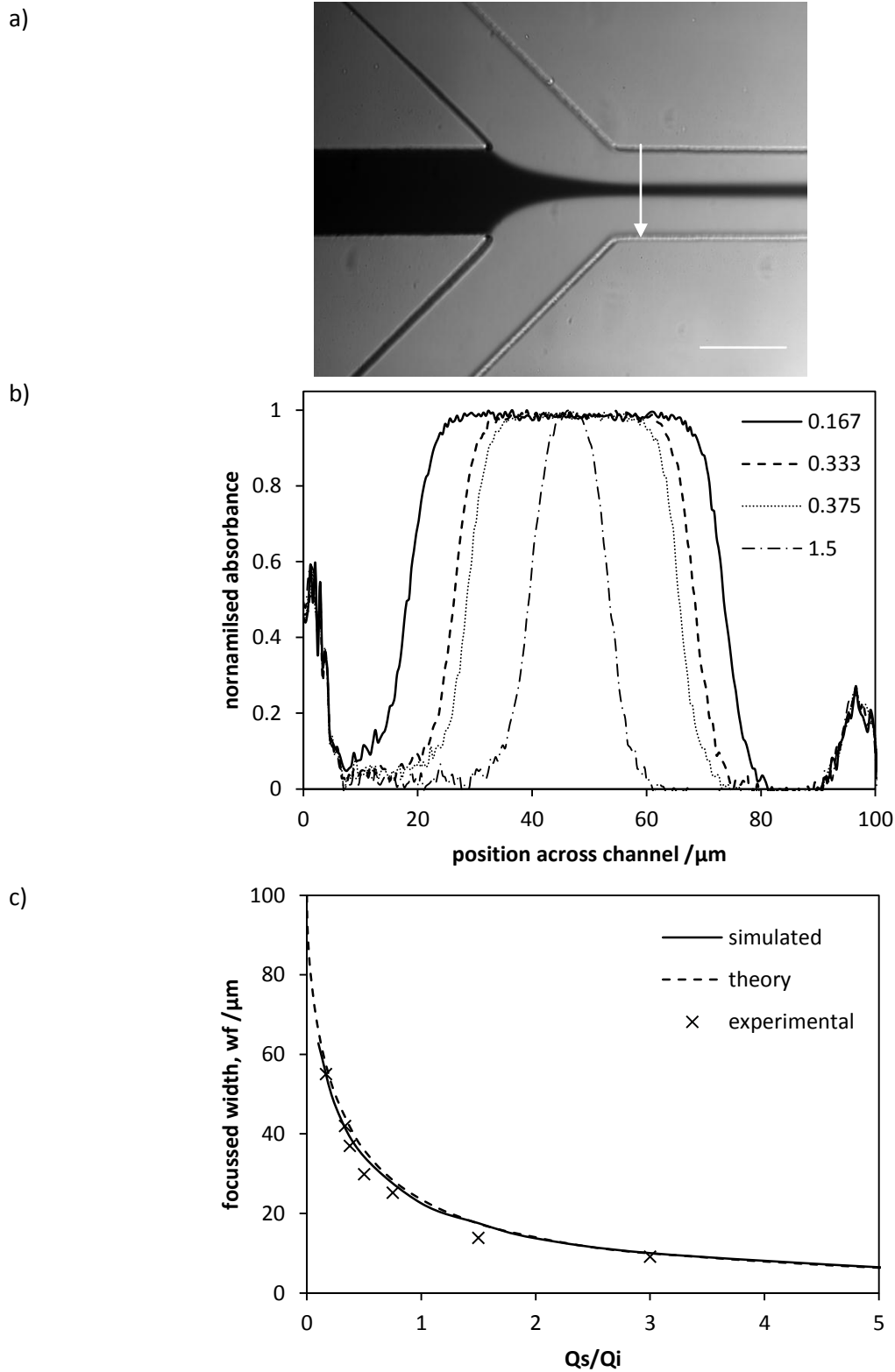


Figure 3.11: Results of testing flow focussing with red dye in the single cell trapping device. a) Brightfield image of the focussing junction. Scale bar: 100 μm . b) Absorption profile across the channel for different focussed to inlet flow ratios (Q_s/Q_i). The peaks near 0 and 100 μm are due to light scattering by the channel walls. c) The full-width half-maximum of each absorption line profile is plotted against simulated and theoretical values for the focussed width for 100 μm wide inlet and focussing channels.

3.6 Results

3.6.1 Flow focussing

PBS is flowed down the focussing channels and in order to visualise the compressed flow red food dye is flowed down the inlet channel (figure 3.11a). Only symmetric focussing is required and so flow from a single syringe pump is proportioned in off-chip tubing by a T-junction union. A separate pump controls the flow rate of the inlet channel. The flow rate through any input channel is never lower than $1 \mu\text{L min}^{-1}$. At flow rates below $0.25 \mu\text{L min}^{-1}$ the flow becomes unstable because of back-pressure from the device and the discrete steps of the stepping motor of the syringe pump. Flow ratios < 1 are therefore achieved by increasing Q_i . The relative flow rate (Q_s/Q_i) is varied and a line profile (direction of white arrow, figure 3.11a) of the normalised absorbance, achieved by taking the inverse of the intensity, is made (figure 3.11b). The focus width is taken as the full-width half-maximum of the absorbance peaks and is plotted with simulated and theoretical results based on the same focussing junction geometry (figure 3.11c). There is good qualitative and quantitative agreement demonstrating good control of the system. So close to the junction, there is little time for diffusion to broaden the focussed width stream. Further along the channel, however, the stream broadens until the dye is evenly distributed across the channel. Cells have diffusion coefficients orders of magnitude smaller than molecular dyes and so diffusion for micron-sized objects in flow needn't be considered. The focussed flow will remain tight throughout the device allowing cells on central flow lines to remain so until trapped.

3.6.2 Non-diffusive flow

When intending to stop flow, the syringe pumps are turned off. However, it is observed that small debris ($< 1 \mu\text{m}$) and even cells continue to flow even when there is no pumping. It is important to have a stationary body of fluid upon performing lysis such that cellular contents are not transported out of the chamber by flow. Mass transport must occur by diffusion alone and any such non-diffusive flow must be minimised.

Once the syringe pumps are stopped, there must exist a pressure difference in the system for fluid to continue flowing. This may be due to the bulging of PDMS, bulging of tubing to a lesser degree, trapped air in the device and the flexing of glass capillary inputs and tubing. In order to visualise the behaviour of the flow, 100 nm fluorescent beads (excitation 365 nm/ emission 415 nm) are added to the fluid in order to trace out the path of the stream lines. The motion of the beads is monitored

under fluorescence using a standard Nikon DAPI filter set. For precise measurements of the flow a video sequence is captured and particle displacement as a function of time, and therefore the flow rate, is calculated.

Devices using 100 μm inner diameter and 375 μm outer diameter glass capillaries as chip-to-world connectors are observed to be sensitive to flexing of the tubing induced mostly by translation of the microscope stage. The effects are small and would not disturb regular flow to a significant degree. By tracking beads, it is observed that the flow rate reduces from an intended flow rate of 1 $\mu\text{L min}^{-1}$ to $\sim 100 \text{ nL min}^{-1}$ upon stopping the syringe pump. 15 minutes later, the flow rate significantly reduced to $\sim 0.75 \text{ nL min}^{-1}$. This is very slow flow, however, the chambers have a volume of 9.8 nL and the chamber-chamber channel has a volume of 2.5 nL. At 0.75 nL min^{-1} the chamber volume would be replaced by fluid from the connecting channel and a portion of an adjacent chamber within $\sim 16.4 \text{ min}$. Flow in this device settles to zero, as determined by beads moving under only Brownian motion, after around an hour. Steps may be taken to ensure that cells remain viable over this time.

Introducing in-line flow valves to off-chip tubing and minimising tubing length helps to reduced non-diffusive flow to zero. Glass capillaries are replaced with Nanoport connectors, which are rigid and cannot flex and be held under tension when tubing is fixed to the microscope stage with adhesive tape.

3.6.3 Cell trapping

The trapping devices were successfully constructed and tested with human BE carcinoma cells. The trapping structures were two pillars of nominal radius 22.5 μm and separation 12.5 μm , centred in a chamber of radius 250 μm . The device comprises 10 such trapping sites separated by 1 mm with 50 μm wide channels. The channel and chamber height is constant throughout the device at 50 μm .

Cells are detached from culture flasks using trypsin which is inactivated by addition of 10% culture medium in PBS. A suspension of single cells was created by gentle agitation with a pipette before sieving through a nylon mesh of 40 μm pitch to minimise any cell clumps. Cells are loaded into a syringe containing a small magnetic stirrer bar [167] at a concentration of $1\text{-}5 \times 10^5 / \text{mL}$. By gently moving the stirrer bar with an external magnet, the problem of cells settling out of suspension during the experiment is minimised.

PBS is used as the fluid for flow focussing. The small refractive index change between the cell fluid containing 10% media in PBS and the focussing fluid of PBS only allows the central stream to be visualised, albeit with low contrast, near the focussing junction. Flow rates are $1.0 \mu\text{L min}^{-1}$ and $1.5 \mu\text{L min}^{-1}$ in the inlet and each focussing channel, respectively. This corresponds to a Q_s/Q_i ratio of 1.5 and a focussed width of $\sim 15 \mu\text{m}$.

The device is left for 15 – 30 min in order for a significant amount of cells to be trapped. Examples of trapped cells are shown in figure 3.12. Predominantly, single cells are counted within each trap, however, some traps may contain 0 or 2 cells. Sometimes, cell clumps may be found in the traps but this is only after a prolonged time whereby cells are given chance to aggregate in the off-chip tubing and in the syringe. For 5 different trapping runs the number of cells trapped in each chamber is counted. The data is compared with a Poissonian distribution for single cell loading (figure 3.13). There is an enhancement of single cell loading and a suppression of 0 cells and > 2 cells. This demonstrates that cell loading in these devices is not purely random due to the self-healing nature of the traps.

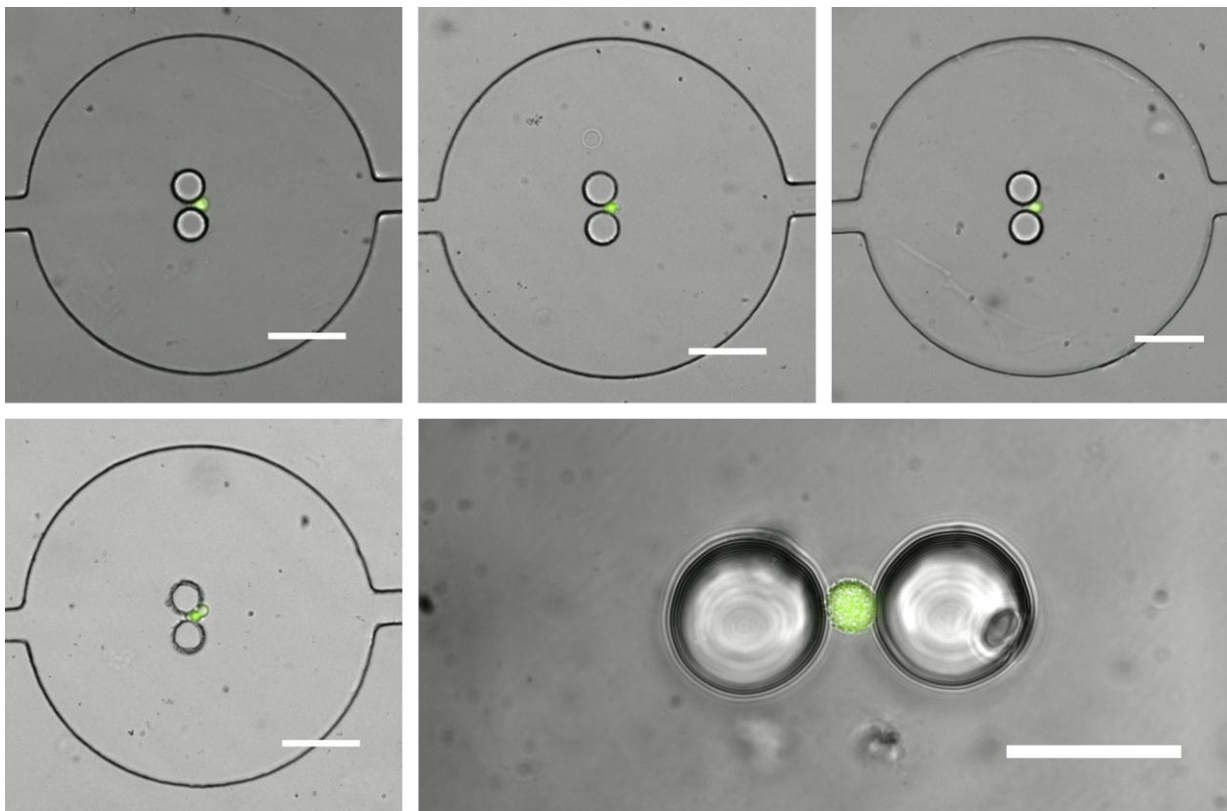


Figure 3.12: Combined brightfield and EPI fluorescence (FITC filter set) of trapped BE cells transfected with cytoplasmic GFP. Scale bars: $100 \mu\text{m}$. Note direction of flow from right to left.

Over the 15 – 30 min run, an average of 10 cells was trapped across all 10 chambers. At a constant flow rate and homogenous concentration of cells, approximately $3 - 15 \times 10^3$ cells flowed through the device per run. This suggests a maximum trapping probability per trap of 0.3 % and compares well with the value of 0.4% predicted by simulation.

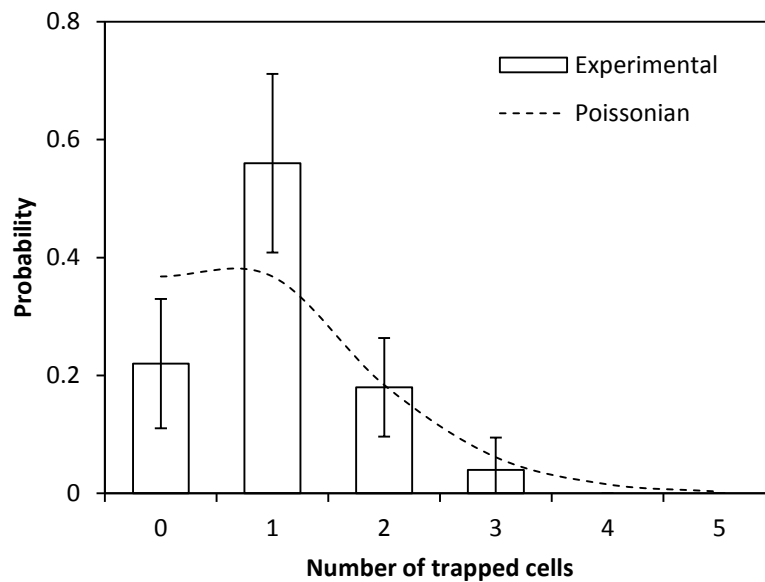


Figure 3.13: The distribution of the number of trapped cells in each chamber compared to a Poisson distribution. Error bars from 5 different runs.

3.7 Conclusions

A microfluidic device has been designed and constructed to facilitate the trapping of single cells in defined locations in separate chambers in order that an independent measurement may be made upon them. The device employs flow focussing to concentrate cells along central flow lines that pass through the trapping structures. The trapping structure is comprised of two pillars of radius $22.5 \mu\text{m}$ that extend the full channel height and are separated by $12.5 \mu\text{m}$, which is less than the cell diameter of the cell line used to develop the technology.

The device has a maximum measured trapping efficiency of 0.3% and requires a minimum of 3000 cells to fill all traps with a single cell each on the timescale 15 – 30 min. The experimental performance agrees well with that predicted by simulation (0.4%). Other works have reported more rapid loading times on the order of a few minutes [33, 35, 37]. This is achieved through dense packing of cell trapping structures within a single chamber but the design here is required to trap a

single cell per chamber. The trapping structures individually show similar efficiencies by CFD simulation to ones used here.

Variations of trapping structure were investigated using CFD simulations. The detail of the trapping structure has limited influence on the ability to physically sequester cells from flow in these devices. If the trapping chamber is modelled as 3 branches, one central branch that passes through the trap aperture and 2 symmetrical branches which pass around the trap, then the overriding factor in trapping efficiency is the ratio of the width of the two sets of branches. By decreasing the chamber radius the relative proportion of flow that passes through the trap aperture increases. However, the required presence of one or several antibody patches in the vicinity of the trap imposes constraints on the chamber geometry and, therefore, that of the trap, in this case.

4 Laser Lysis

The second stage of the technology to perform single cell proteomics requires cells be rapidly lysed. The concept of cell lysis afforded by the delivery of a single nanosecond laser pulse has been introduced in §2.3 while this chapter focuses on the experimental platform and results of employing laser lysis as a cell lysis strategy.

- Experimental platform

The physical platform upon which experiments are performed is described.

- Laser lysis

The requirements of the laser pulse energy and wavelength for lysis are determined. By tuning the energy it is possible to partial or fully disrupt cells. To avoid photobleaching of fluorescent proteins pulses of wavelength 1064 nm are used.

4.1 Experimental platform

A fully motorised inverted Nikon Ti-E microscope was used as the experimental platform (figure 4.1).

The microscope is capable of widefield white-light or fluorescence imaging and total internal reflection fluorescence (TIRF) microscopy. Widefield light sources are provided by halogen and mercury lamps. The objective turret is populated with achromat 10× 0.25 NA dry, plan fluor 20× 0.5 NA dry, plan fluor 40× 0.9 NA dry, and apo TIRF 60× 1.49 NA oil-immersion Nikon objectives. The objective turret may be translated in z to alter the object focal plane and incorporates automatic focus correction or a perfect focus system (PFS). The PFS uses an infra-red LED at 770 nm which is focussed on the sample and the upper coverslip surface interface (figure 4.2). A portion of the beam is reflected and imaged onto a line CCD which determines whether the interface is exactly in focus. The PFS may be offset such that any plane above the interface may be maintained in focus. The PFS is an active system and eliminates focal drift during experiments, allowing stable time-lapse imaging.

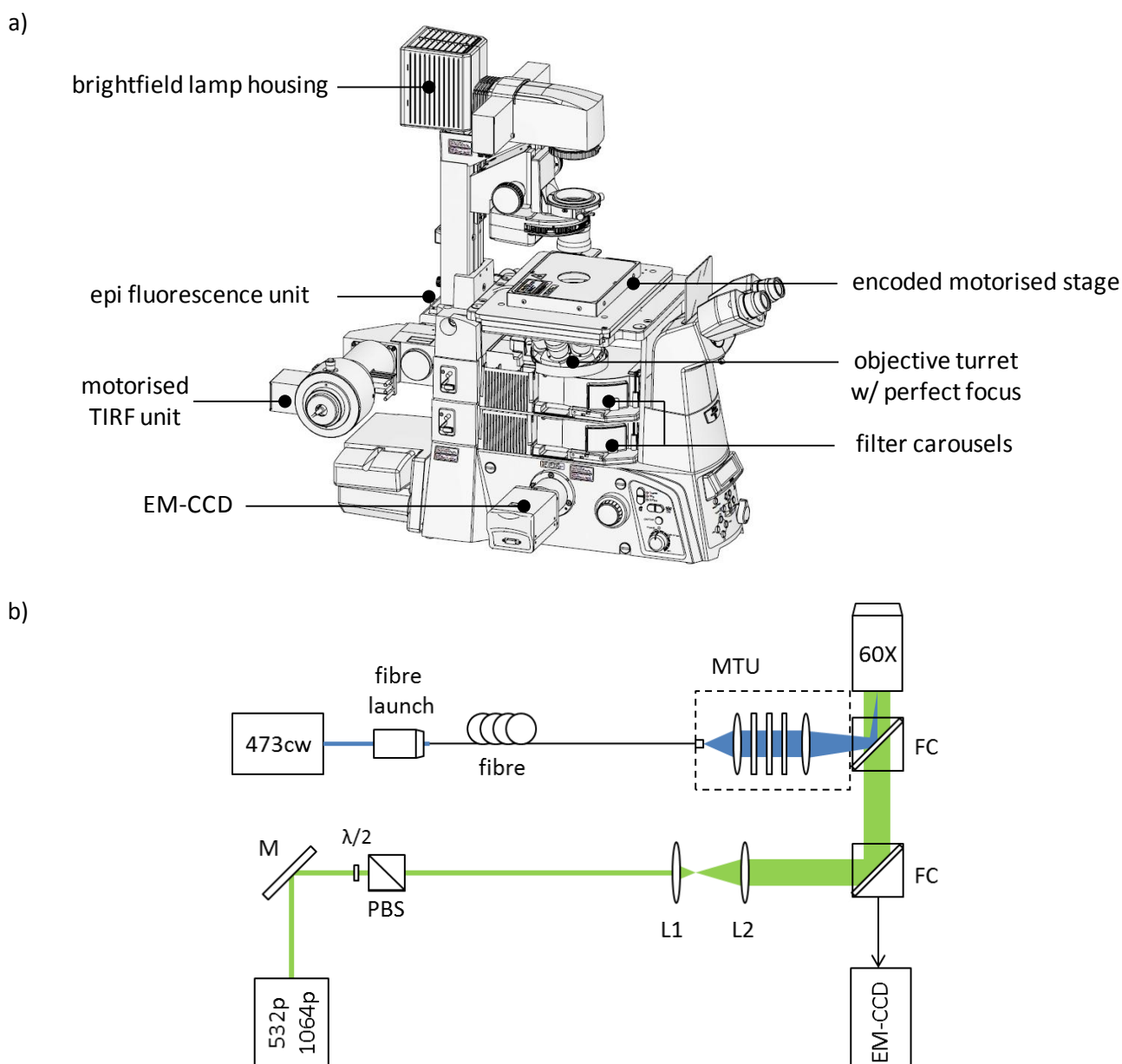


Figure 4.1: a) The experiment is based around a Nikon Ti-E microscope. External optics are coupled in via the back port of the chassis. Adapted from Nikon literature. b) Schematic of the optical setup. 473cw = cw TIRF laser at $\lambda = 473$ nm, 532p or 1064p = pulsed Nd:YAG laser at $\lambda = 532$ nm or 1064nm, EM-CCD = electron multiplying charge-coupled device camera, M = mirror, $\lambda/2$ = half-wave plate, PBS = polarising beam splitter, L1 & L2 = lenses in beam expander, MTU = Nikon motorised TIRF unit, FC = filter cube.

The microscope body contains two filter carousels, each with 6 dichroic filter cube positions. The upper carousel contains standard Nikon DAPI, FITC and TRITC filter sets in addition to a TIRF illumination dichroic (z488RDC; Chroma, USA). The remaining positions are empty to allow white-light imaging and external light sources reflected from the lower carousel. The lower carousel contains a filter set appropriate for coupling in the pulsed laser source required for laser lysis. It

comprises a single-edge dichroic (LPD01-532R-25; Laser 2000, UK) and long pass filter (LP03-532RS-25; Laser 2000, UK), which is placed on the emission side or at laser launch for lysis at $\lambda = 532$ nm or 1064 nm, respectively. The transmission profiles are shown in figure 4.3.

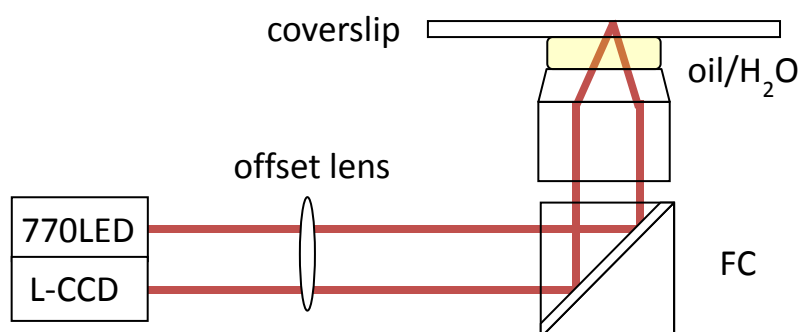


Figure 4.2: Schematic of the perfect focus system in use with an oil/water immersion objective. 770LED = LED emitting at 770 nm, L-CCD = line CCD, FC = filter cube.

The pulsed laser source is a high energy solid-state Nd:YAG (Surelite SL I-10; Continuum, USA), or neodymium-doped yttrium aluminium garnet laser, capable of delivering a single pulse of 6 ns duration at $\lambda = 532/1064$ nm. This type of laser is popular in applications requiring the breakdown of media such as in machining metal, ablation, ophthalmology and, of course, biological investigations such as the lysis of cells. The Nd:YAG crystal serves as the lasing medium which is optically pumped by a flashlamp. The laser may be operated at a maximum repetition rate of 10 Hz or may fire a single pulse by operation of a hand-held joystick. This button controls an active Q-switch. The pulse has a maximum energy of 1.43 ± 0.13 J, a nominal pulsewidth of 4-6 ns, a linewidth of 1 cm^{-1} or 28 pm, a beam diameter of 6 mm and is vertically polarised at 532 nm.

The laser pulse is passed through a half-wave ($\lambda/2$) plate and polarisation-sensitive beamsplitter. The reflected beam is terminated by a beam dump and the light passing through the beamsplitter is expanded 4 $\times$ by two plano-convex lenses, focal length 50 mm and 200 mm, in a Kepler arrangement. The expanded beam is introduced into the lower level (2 levels; one upper, one lower) back-port of the microscope and reflected upward into the back aperture of the objective by a dichroic mirror placed in a filter cube. The filter cube contains an edge filter placed in the emission path to prevent reflected or scattered laser light reaching the detector. The edge filter is placed at the laser source when employing a lysis beam at $\lambda = 1064$ nm. Pulse energy is reduced by placing neutral density filters at the laser source with fine adjustments made by rotating the $\lambda/2$ plate.

Pulse energy is measured near the focus of a 10× objective since the meter head of the energy detector is too small to measure the fully expanded beam at the back aperture. For laser lysis a 60× NA=1.49 oil-immersion objective is used and pulse to pulse energy variation was found to be $\pm 3\%$. Pulses are focussed to 10 μm above cells or the coverslip surface.

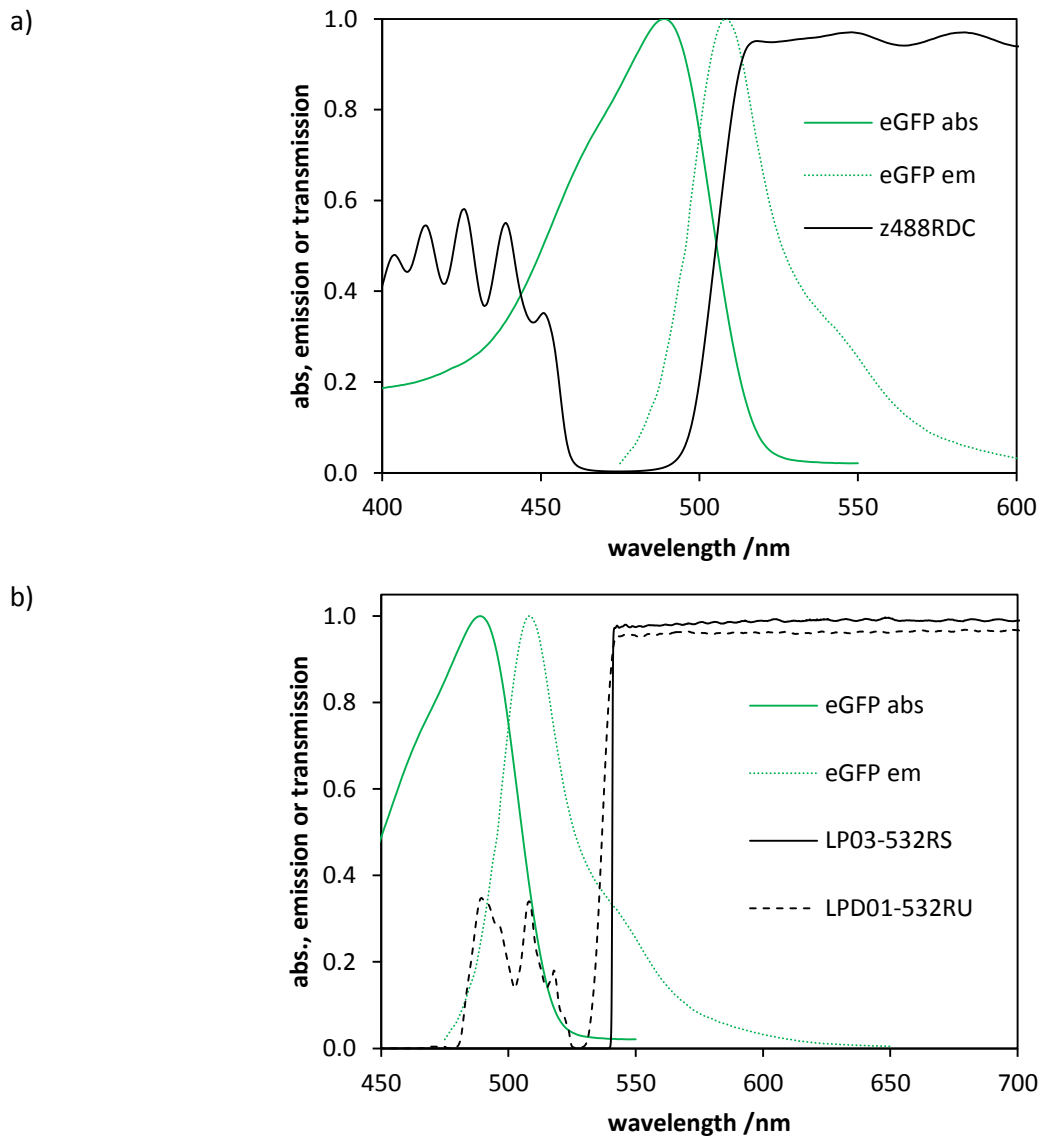


Figure 4.3: Transmission spectra of a) the TIRF dichroic (z488RDC) and b) the lysis filter set containing a single edge dichroic (LP03-532RS) and long pass filter (LPD001-532RU). Overlaid are the normalised absorption (abs) and emission (em) spectra of eGFP.

For illuminating antibody spots, a solid state cw laser (MBL-473-200; Laser 2000, UK) at $\lambda = 473 \text{ nm}$ is coupled into a single-mode optical fibre that is connected to a motorised TIR illumination unit (Nikon, Japan) situated at the rear of the microscope body. The unit contains a translatable fibre

mount and condenser lens that may be translated normal to the fibre exit. The laser beam is brought to focus at the objective back aperture plane which in the object plane forms parallel rays i.e. the objective performs a Fourier transform. When the beam is translated, the focus position is translated laterally toward the edge of the back aperture causing the exiting parallel rays to tilt. The tilted rays are refracted and reflected at both of the coverslip interfaces (oil-coverslip and coverslip-media). Beyond a critical angle, the TIR condition is met at the coverslip-media interface. From Snell's law,

$$\theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right) \quad [4.1]$$

where θ_c is the critical angle and n_1 and n_2 are the refractive indices of the glass coverslip and the liquid media, which is taken as water, respectively. For $\lambda = 473$ nm and at 25°C, the indices are $n_1 = 1.52$ and $n_2 = 1.34$ and, by eq. 4.1, $\theta_c = 61.8^\circ$. The laser is translated a distance from the centre of the back aperture plane r .

$$r = \frac{f n_1 \sin \theta}{2M} \quad [4.2]$$

where f and M are the focal length and magnification of the objective, respectively. For θ_c , $r = 1.97$ mm. TIR is judged at $r = 2.10$ mm to produce higher contrast images with an incident angle of 65.2° . The distance above the reflecting surface, which is the coverslip-media interface, at which the evanescent wave is 1/e the energy at the surface is given by

$$d = \frac{\lambda}{4\pi} [n_1^2 \sin^2 \theta - n_2^2]^{-1/2} \quad [4.3]$$

where λ is the laser wavelength used for TIRF. At an incident angle of 65.2° the penetration depth is ~ 105 nm. This means that only fluorophores located within a distance of the coverslip of 105 nm will be imaged. The laser has a maximum nominal output of 200 mW and the power at the back aperture is adjusted by neutral density filters to 1.5 mW.

Fluorescence detection and all imaging experiments are realised using an IXON DU-897E electron-multiplying charge-coupled device (EM-CCD; Andor Technologies, Ireland). Basic CCD operation involves the accumulation of charge in a pixel proportional to the light intensity imaged onto the pixel. The readout process may be summarised as shifting pixel charge to a readout register which is subsequently amplified. The main sources of system noise in CCD detectors are the dark current noise and the read noise. The dark current noise may be reduced to negligible levels with enhanced thermoelectric cooling. The read noise increases with pixel read rate so for high sensitivity the

devices are read very slowly to minimise noise. EM-CCDs apply a low-noise gain process using a gain register before the conventional charge amplifier. The effective readout noise is reduced by the gain factor and results in sub-electron effective readout noise. The gain register is a chain of pixels across which signal charge is transferred but which is clocked at a higher voltage, around 20 – 40 V, than is necessary to shift the charges from pixel to pixel, which is roughly 10V. The higher voltages are sufficient to cause impact ionisation as electrons are transferred from pixel to pixel resulting in generation of new electrons. Thus, the number of electrons increases as it passes through each element of the gain register. The probability of impact ionisation is very low with the gain per element only about $g = 1.01 - 1.015$. However, the number of elements N in the gain register can be high and so the total gain $G = g^N$ can easily exceed several thousand [168]. The background discrimination of TIR fluorescence and the high sensitivity of the EM-CCD easily allows the detection of single molecules.

4.2 Results

4.2.1 Plasma threshold

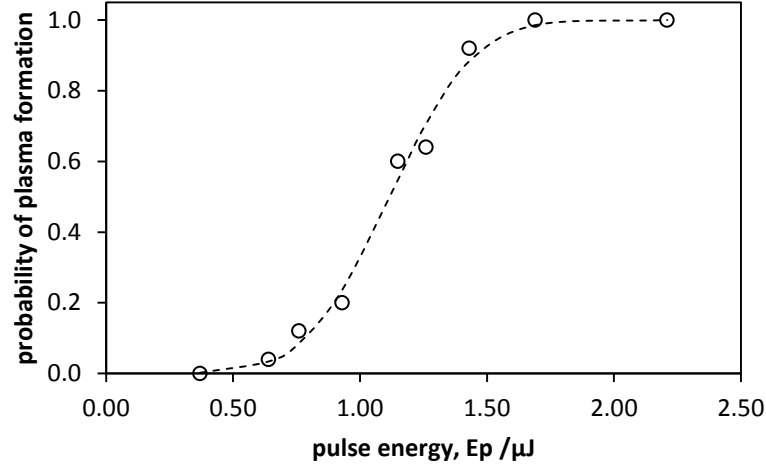
To determine the pulse energy required to induce breakdown of the media, a set of 50 pulses at discrete pulse energies was delivered into a droplet of 4% (w/v) bovine serum albumen (BSA) in phosphate buffered saline (PBS) on a coverslip. Plasma formation was recorded by noting the incidence of a bright flash after the delivery of each pulse. The flash is due to the recombination of free electrons as the plasma rapidly cools. The flashes were either observed on the camera or by eye via the microscope binocular. As in [104], the probability of plasma formation p as a function of pulse energy E_p was fit to a Gaussian error function,

$$p(E_p) = \frac{1}{2} \left\{ 1 + \operatorname{erf} \left[\frac{1}{\sqrt{\sigma^2}} (E_p - E_{th}) \right] \right\}, \quad [4.1]$$

$$\operatorname{erf}(x) = \frac{2}{\sqrt{\pi}} \int_0^x e^{-\eta^2} d\eta, \quad [4.2]$$

where the fitted parameters are $1/\sqrt{\sigma^2}$, which dictates the sharpness of the function and E_{th} , which is the threshold energy for plasma formation defined as the energy that results in a 50% probability of plasma formation.

a)



b)

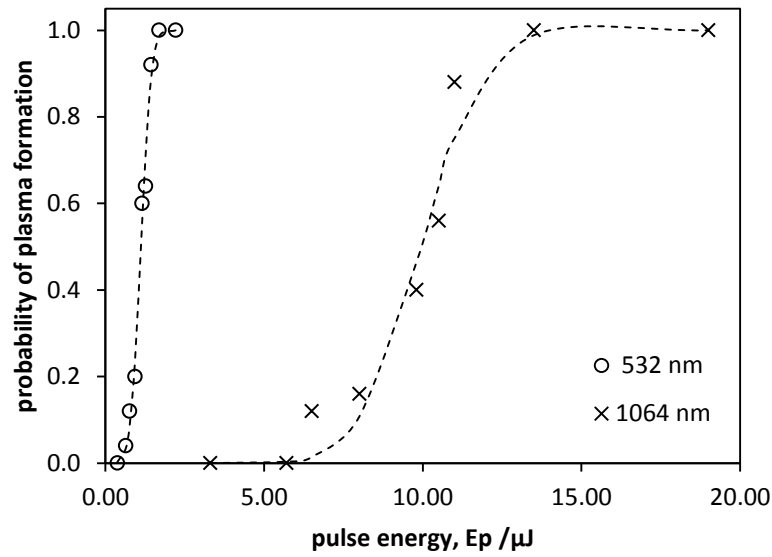


Figure 4.4: Probability of plasma formation as determined from 50 pulses at discrete energies for a) $\lambda = 532$ nm and b) $\lambda = 1064$ nm. A Gaussian error functions are fit to the data with $R^2 > 0.99$.

Results for $\lambda = 532$ nm are shown in figure 4.4a along with the fit for $1/\sqrt{\sigma^2} = 2.70 \pm 0.55 \mu\text{J}$ and $E_{th} = 1.12 \pm 0.04 \mu\text{J}$. Assuming a diffraction limited spot radius of 218 nm for an NA = 1.49 objective and with sech^2 shape pulses, the threshold irradiance is $3.46 \times 10^9 \text{ W/mm}^2$. This agrees well with literature in terms of order of magnitude but is nearly 4.5 times higher than a similar system ($I_{th} = 0.77 \times 10^9 \text{ W/mm}^2$) delivering 532 nm 6 ns pulses from a Q-switched Nd:YAG via a 0.9 NA objective into a distilled water filled cuvette by Venugopalan et al. [105]. Rau et al. [104] delivered pulses via a 0.8 NA objective and reported a threshold irradiance of $2.27 \times 10^9 \text{ W/mm}^2$, nearly 3 times that of Venugopalan. They suggested the reason for this to be poor spatial beam quality of the laser. If this is the case, and assuming a threshold irradiance of $I_{th} = 0.77 \times 10^9 \text{ W/mm}^2$, then the focussed spot radius may be 261 nm. This is only ~20% larger than the diffraction limited case but results in

approximately 4.5 times higher threshold irradiance. The higher threshold may be due to the significant amount of protein present in the media.

In addition, results for $\lambda = 1064$ nm are shown in figure 4.4b along with the fit for $1/\sqrt{\sigma^2} = 0.45 \pm 0.24 \mu\text{J}$ and $E_{th} = 9.94 \pm 0.45 \mu\text{J}$. The higher pulse energy is required due to the lower photon energy of 1064 nm wavelength. To ensure that each pulse results in plasma formation and subsequently cell lysis the pulse energy is set to nearly 3 $\times$ threshold at $3.0 \pm 0.06 \mu\text{J}$ for $\lambda = 532$ nm and nearly 1.5 $\times$ threshold at $14.1 \pm 0.3 \mu\text{J}$ for $\lambda = 1064$ nm.

4.2.2 Cell lysis

The dynamics produced by the delivery of a pulse which results in the formation of a plasma has been studied on time-scales spanning many orders of magnitude, tracking plasma formation, expansion, shock wave and cavitation expansion and contraction. The EM-CCD is capable of recording 45 frames/s with 8×8 pixel binning resulting in a frame every 22.2 ms. According to [103, 104, 109], most processes are completed within 50 μs , beyond which only the gas content of the collapsed bubble is seen to diffuse into the surrounding liquid (see figure 2.8). The minimum exposure time is 10 μs and so even if the camera acquisition was triggered by the laser pulse, a flash lamp or short lived illumination source would be required to probe early (ns $\rightarrow$ μs) dynamics at sufficient resolution. However, the dynamics on such timescales is of little importance for this work.

Figure 4.5 shows time sequences of single cells being lysed by laser lysis. BE cells stably transfected with cytoplasmic eGFP are harvested from culture flasks by trypsinisation, suspended in PBS and pipetted upon glass coverslips (thickness #1.5). The laser focus is aligned to the centre of the field of view and the motorised microscope stage is translated in order to move objects relative to the beam focus position. Depending on the pulse energy and the focal location relative to the cell, lysis may be partially (figure 4.5a) or fully disruptive (figure 4.5b) to the cell. Full cell disruption is where the nuclear and cell membranes are compromised and all cell contents are liberated into the solution whereas partial cell disruption occurs when only the cell membrane is compromised and the nuclear membrane is left largely intact. Full cell disruption occurs at pulse energies greater than threshold and focussed 10 μm above the centre of the cell. Focussing off the centre results in catapulting the cell without disruption. Partial lysis is best effected at lower pulses energies below threshold. Figure 4.5c shows that upon partial lysis the cell stains strongly for the nuclear stain DAPI indicating the membrane has been compromised and the nucleus remains intact. Cellular GFP is shown in the green FITC channel and is observed to diffuse rapidly out of the cell.

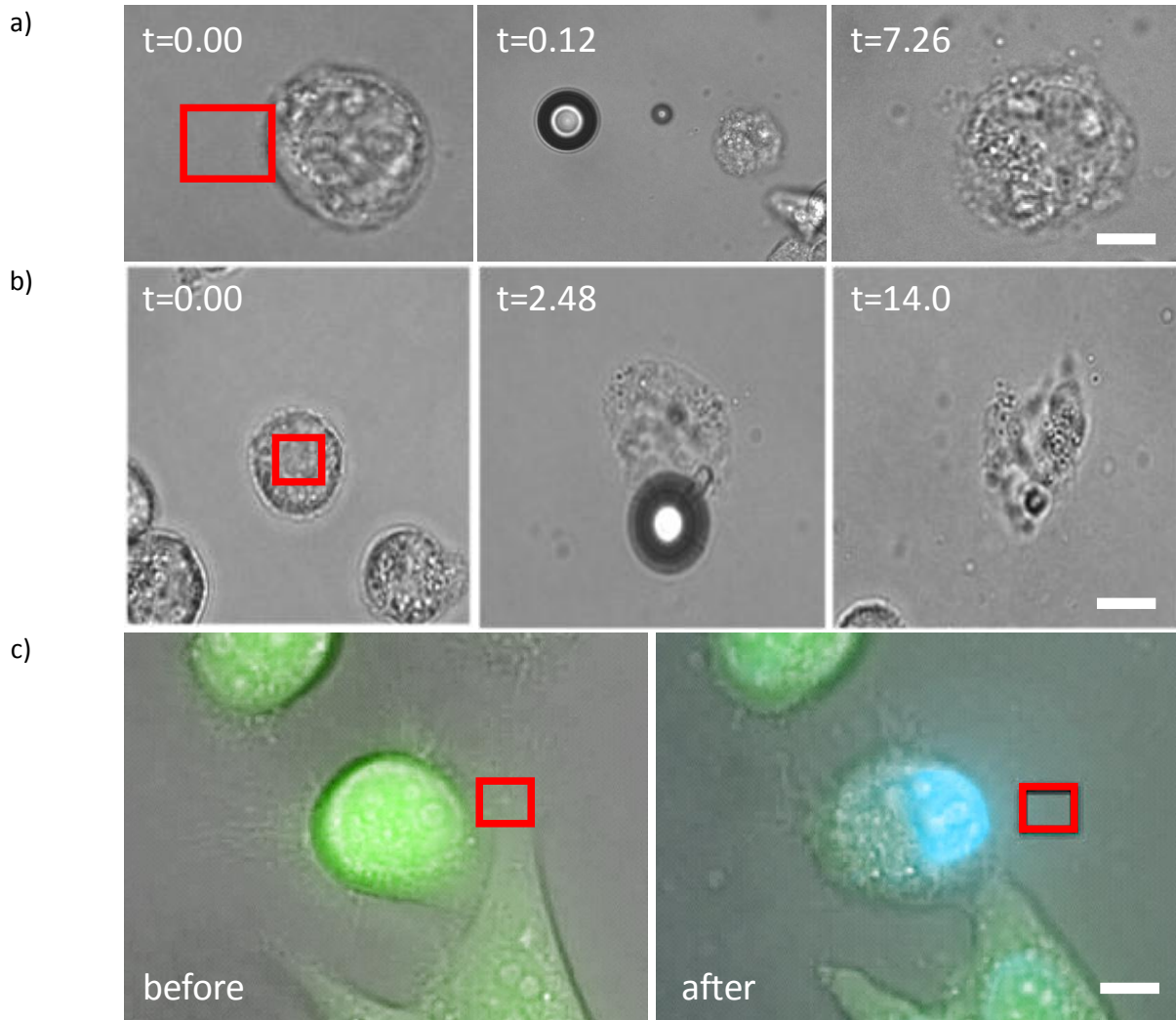


Figure 4.5: Time-resolved (seconds) sequence of images showing the result of laser lysis. Demonstrating a) partial (scale bar 5 μm) or b) full (scale bar 10 μm) lysis depending on the location of delivered pulse relative to the cell. c) The nuclear stain DAPI is rapidly included in partially lysed cells indicating that the nucleus is still largely intact (scale bar 5 μm) after 1 min.

4.2.3 Lysis wavelength

For single cell pulldowns it is important that as much protein present in the cell be preserved during the lysis process. There is no observed difference between using pulses at 532 nm or 1064 nm to lyse cells as observed in brightfield – cells are compromised and constituents are liberated into the surrounding volume. Pulldowns will be developed with cells containing cytoplasmic GFP. The rationale behind this is mainly due to the ease by which the GFP proteins themselves may be tracked and quantified. Figure 4.6 shows how GFP fluorescence in a cell is effected by the delivery pulses with energy at 0.5 $\times$ threshold at $\lambda = 532$ or 1064 nm and is compared to a cell to which no pulses are

delivered for comparison. The energy at 0.5 $\times$ threshold has a probability of 0.25 per pulse of creating a plasma and so this enables several pulses to be delivered before lysis occurs.

As is to be expected, each pulse at 532 nm leads to photobleaching by the pulse itself, which is roughly 0.62% of the initial cellular fluorescence per pulse. GFP absorption at 532 nm is 2.6 % of the maximum at ~ 508 nm. At $\lambda = 1064$ nm there is no photobleaching caused by the delivery of a pulse. Therefore, for single cell pulldowns lysis will be performed with pulses of $\lambda = 1064$ nm.

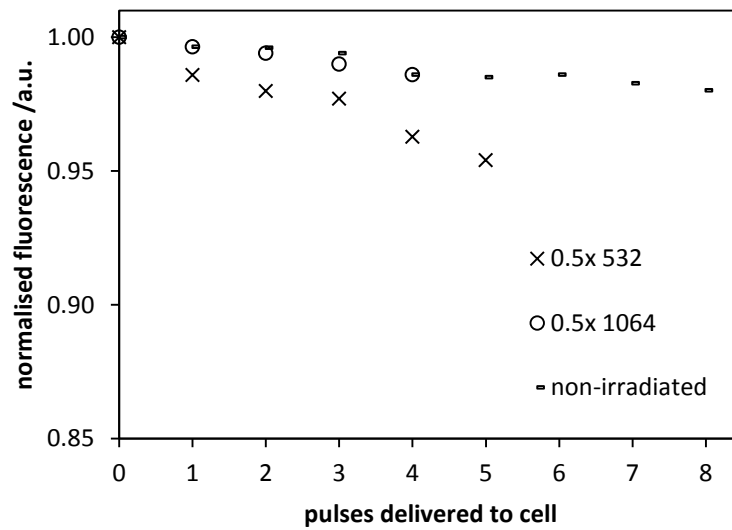


Figure 4.6: Monitoring eGFP fluorescence from single cells by widefield EPI-fluorescence in response to the delivery of pulses at 0.5 $\times$ threshold energy at $\lambda = 532$ nm and $\lambda = 1064$ nm. Cell fluorescence is also taken from a cell with no pulses delivered to it to account for photobleaching. Fluorescence is normalised to the level prior to delivery of pulses.

4.3 Conclusions

Cell lysis using a pulsed laser source has been demonstrated. The threshold pulse energy required for plasma formation in 4% BSA in PBS (w/v) was measured to be 1.22 ± 0.04 μ J at 532 nm and 1.22 ± 0.04 μ J at 1064 nm. It was determined that delivering several pulses under threshold could partially disrupt cells whereas a single pulse at or exceeding threshold 10 μ m above the cell could fully lyse cells. To avoid photobleaching of fluorescent proteins, such as GFP, the pulse wavelength is set to 1064 nm. The laser lysis method and microscope platform described in this chapter will be used with microfluidic devices designed in §3 to lyse trapped cells.

5 Proteomic Analysis using Antibody Microarrays

DNA microarray technology found widespread development and use in the 1990s owing to the efforts of whole-genome projects, particularly the human genome project. At the turn of the 21st century, microarrays proliferated beyond the realm of DNA and gene expression to lipids, carbohydrates and especially proteins. Unfortunately, the one-size-fits-all ethos of DNA microarrays was not applicable for protein microarrays. Significant development of printing, surface chemistry, and capture probes has been made since. Different capture agents perform optimally upon different immobilisation surfaces, by being printed with certain buffer additives and by different post-printing treatment strategies. Given the broad diversity of the proteins themselves, in terms of solubility, structure hydrophobicity/philicity, it comes as little surprise that each protein microarray must be individually optimised. This section deals with how cellular protein is captured upon a surface by protein antibodies and detected using total internal fluorescence microscopy, which will form the third and final part of the platform to perform single cell proteomics. A significant amount of work has dealt with optimising microarray production parameters and is described herein.

- Affinity capture

Affinity capture agent origin and their generation are described.

- Antibody validation

The proteins eGFP and p53 are used to develop and demonstrate the single cell proteomics platform. They are captured using antiGFP and anti-p53 antibodies, which must undergo strict validation to determine their affinity and reactivity.

- Model of protein binding

A simple diffusion and binding model is introduced to aid understanding of the dynamics of the pulldown chamber. The model may also be used to estimate antibody on or off-rates and binding affinity.

- Optimisation of antibody microarray production parameters

Antibodies are printed on functionalised glass coverslips using a microarrayer robot. The optimisation of printed spots by investigating printing buffer and glass surface chemistries is made for maximising capture density and sensitivity.

5.1 Affinity capture

The choice of sensor molecule may be monoclonal or polyclonal antibodies or antibody mimetics such as aptamers and affibodies.

Biologically, the function of an antibody is to recognise and bind foreign or non-self molecules in order to confer immune protection against invading agents such as bacteria and viruses. The structure of antibodies is responsible for their binding versatility, binding specificity and biological activity.

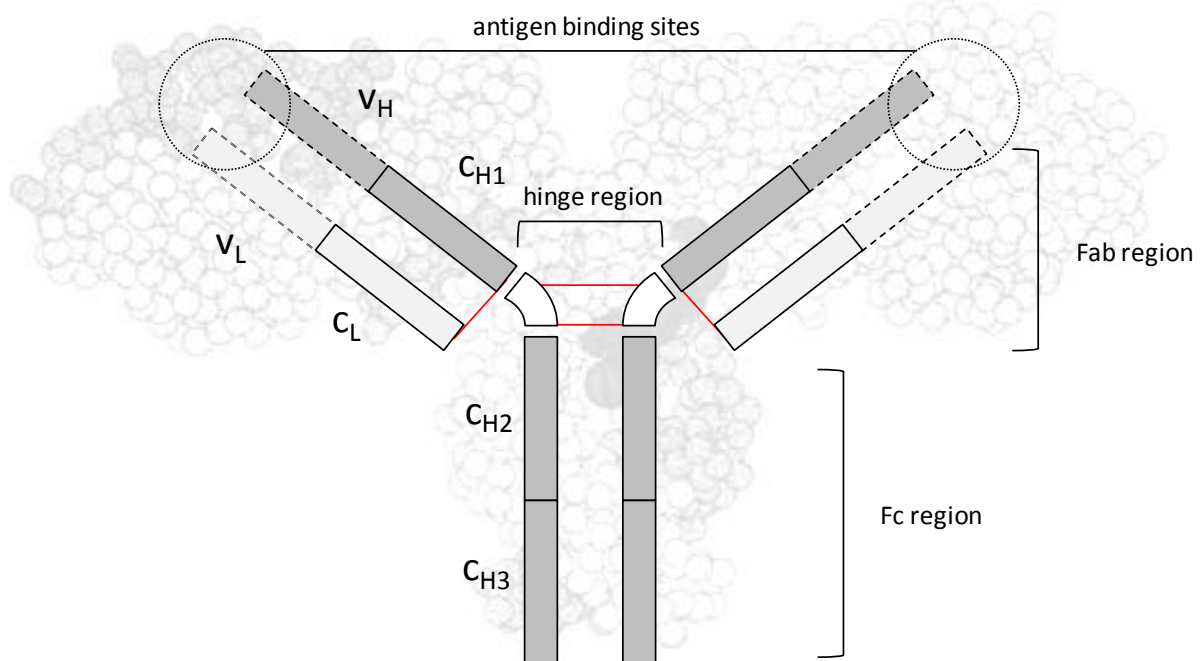


Figure 5.1: Schematic cartoon of IgG antibody structure depicting the light and heavy chains in the Y-shaped molecule. v_L and v_H , variable region of light and heavy chains, respectively; c_L and c_H , constant region of light and heavy chains, respectively. Red lines indicate disulphide bridges. The background image is the 3D structure as determined from X-ray crystallography and is adapted from [169].

Immunoglobulins, or antibodies (figure 5.1), are composed of two identical light chains (23 kDa each) and two identical heavy chains (50 kDa each). The chains pair (heavy to heavy and light to light) and are linked by covalent interchain disulphide bonds and non-covalent interactions. Each chain comprises a variable (amino-terminus) and constant (carboxyl terminus) region. The areas of increased variability in the variable regions are called hypervariable regions or complementary-determining regions (CDR). CDRs are interspersed by invariant segments known as framework residues. The CDRs of both the light and heavy chains form a cleft that serves as the antigen-binding site of an antibody as they are joined and fold. To recognise a different antigen the amino acid

sequence of the CDRs may be altered which will in turn alter the shape of the binding site, and this is how antibody specificity is conferred. The antigen binds to the antibody, specifically to the CDRs, through weak, non-covalent interactions. Per antibody there are two identical binding sites making it bivalent. The region of the antigen that is recognised by the binding site is called the epitope and may be based on the tertiary structure of the protein in conformational epitopes or the primary amino acid sequence in linear epitope recognition.

Antibodies are widely produced by immunisation of a mammal, such as a mouse, rabbit or goat, with the antigen to which the desired antibodies will be made. In response to the foreign proteins, the immune system is stimulated to produce B-lymphocytes which generate antibodies specific for the antigen. The antibodies present in the blood serum of the animal are called polyclonal antibodies since they will be derived from many different B-cells and be specific for different epitopes. Monospecific antibodies, or monoclonal antibodies, may be made by identical B-cells. Monoclonal antibodies are produced by the immunisation of an animal, typically a mouse, and spleens from hosts with the highest concentration of serum antibody are harvested. B-cells are terminally differentiated so do not proliferate and cannot be kept indefinitely in culture. Kohler and Milstein [170] mixed mouse antibody producing spleen-derived B-cells with immortal myeloma cells in the presence of polyethylene glycol (PEG), which altered membrane permeability and allowed cell fusion. This produces hybrid-myeloma, or hybridoma, cells which inherit both properties of specific antibody production and immortality of the parent cells. Cell fusion is random and so cell culture contains a mixture of all cell fusion variants. Cells are grown in hypoxanthine-aminopterin-thymidine (HAT) medium. Aminopterin serves to deplete nucleotide precursor purines and pyrimidines required for DNA, RNA and protein synthesis. Purine nucleotides may be synthesized from hypoxanthine through the purine salvage pathway using the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Myeloma cells lack HGPRT but B-cells do not. Therefore, only hybridoma cells are selected for in prolonged culture in HAT medium since the primary B-cells do not grow in culture. Hybridoma cells are separated and individual clones are grown indefinitely, producing monoclonal antibodies that may be harvested from the culture medium.

The generation of monoclonal antibodies by the hybridoma technique allows the murine immune response to be immortalised in vitro but is expensive and time-consuming. But, crucially, mouse monoclonal antibodies for therapeutic purposes will induce an anti-mouse response and are unsuitable. Recombinant technology is an alternate method of monoclonal antibody production, which, instead of immortalising the cells from which the antibodies are derived, immortalise the DNA which encodes the antibodies themselves. The DNA may be reverse transcribed from the

mRNA of primary cells of an immunised animal or generated by genetic engineering from an artificial, so-called naïve, library. The DNA is ligated into vectors and co-transfection of *Escherichia coli* is made with bacteriophage for the method of phage-display [171, 172]. Phage particles released will display antigen-binding fragment (Fab; figure 5.1) protein on the virion surface and carry Fab DNA internally. Fab/phage particles are then incubated with immobilised target antigen and the unbound fraction is washed away after incubation. The Fab/phage particles may be dissociated and allowed to infect further populations of *E. coli* so that, after several rounds, only Fab/phage specific to the antigen are amplified and propagated. These particles may then be genetically manipulated to express full antibody molecules free of phage that may be used in conventional immunoassays [173].

The development cost for producing polyclonal antibodies is low and produces large amounts of non-specific antibodies that are able to recognise multiple epitopes of a single antigen. This can have the effect of amplifying the signal from an antigen with low expression level and is tolerant of post-translational modifications whereby an epitope may become altered or unreadable. Polyclonal antibodies can display high cross reactivity, especially to proteins of high homology. The main drawback of using polyclonal antibodies is the batch to batch variability, which makes absolute quantification methods difficult.

Monoclonal antibodies are much more labour intensive and expensive in production. However, once hybridomas or DNA are made, the source of monoclonals is renewable and all batches will be identical. Monoclonal antibodies recognise only a single epitope and display less cross-reactivity than polyclonal antibodies but are more vulnerable to a loss of epitope.

The drawbacks and expense of antibodies have led to the development of antibody mimetics, such as affibodies [174] or affilins [175]. These are artificial peptides or proteins that can specifically bind antigens. Advantages of such molecules are extreme stability due to reversible folding and low-cost recombinant production. Antibody mimetics hold great promise but the technology is in its infancy and as such has limited target libraries. Currently, antibodies are the most prominent capture agents used in microarrays and have the largest target database of all such sensor molecules. Projects, such as the Human Protein Atlas [176], to produce antibodies for every human protein are underway.

5.2 Proteins investigated and their antibodies

Several antibodies were tested from laboratory and commercial sources. Below are results from the validation of the enhanced green fluorescent protein (eGFP) and p53 antibodies which were tested and worked in microarrays produced in this work. Antibody validation by Western blotting is performed by Heather Rada (Institute Cancer Research, London).

The protein p53 is not autofluorescent and so a labelling strategy must be followed. In the microarray experiments p53 will be pulled down using a primary antibody and detected by the use of a secondary antibody which is fluorescently labelled. The secondary antibody must therefore recognise a different epitope than the primary. Such a detection method is called a sandwich assay.

5.2.1 eGFP

To develop the technology an eGFP-transfected cell line is used. eGFP may be captured using only primary antibodies that are surface immobilised. Detection is afforded by the autofluorescent property of eGFP. AntiGFP monoclonal antibody was raised in BALB/c mice and donated by Pr. P. Parker (Cancer Research UK, Lincoln's Inn Fields Laboratories). It was dialysed into PBS, aliquoted and stored at -80 °C before use. To validate the specificity of the antibody for eGFP, Western blots (WBs) were made under non-native conditions on 12.5% sodium-dodecyl sulphate (SDS) gels for the recombinant protein and cell lysate (figure 5.2).

Recombinant eGFP was commercially sourced (Biovision, USA) and has a molecular weight of 32.7 kDa. BE lysate was made by harvesting cells and lysing in HARMS buffer (250 mM sucrose, 10 mM triethanolamine, 10mM acetic acid, 1mM EDTA, pH 7.45 + Roche Complete protease inhibitor cocktail) and using a mechanical ball buster to break open the cells. The sample was centrifuged and the supernatant containing the cytoplasmic fraction was extracted.

A secondary donkey antimouse antibody conjugated to horse-radish peroxidase and enhanced chemiluminescence reagent permits detection of the bands. The eGFP WB was made using 9 µg/mL antiGFP on 1 µg of eGFP and worked well with short film exposure. Lysate WBs were made on 8.5 µg of lysate prepared from GFP-transfected and non-transfected cells. As expected, there is no band for non-transfected lysate and the WB for the transfected lysate shows the antibody is specific for GFP and not cross-reacting to other proteins in the lysate. The band for GFP derived from lysate occurs at ~29 kDa. Native WBs were conducted but no bands were detected.

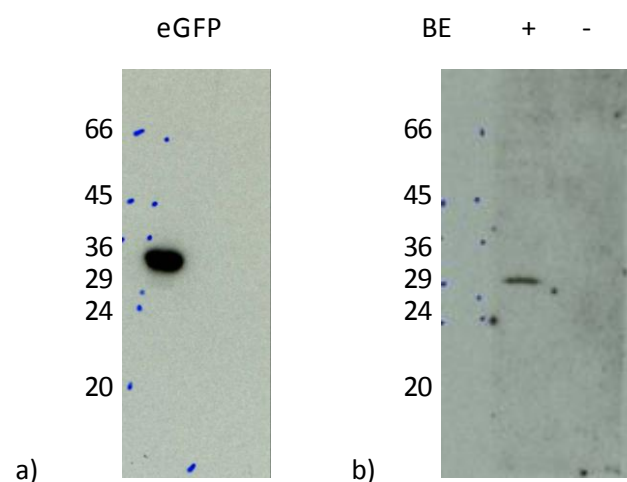


Figure 5.2: Western blots performed on a) pure eGFP protein and b) cell lysate prepared from GFP-transfected (+) and non-transfected (-) BE cells. The molecular weight markers are shown on the side of the blots.

5.2.2 p53

The transcription factor p53 is detected using an antibody sandwich assay. The anti-p53 primary monoclonal antibody is taken from a commercial test kit (p53/Mdm2; Enzo Life Science, UK) whose epitope is at the C-terminus of the protein. The detection antibody is the DO1 monoclonal antibody recognising amino acids 21-25 at p53's N-terminus and is fluorescently labelled with Alexa-Fluor 488 (sc-126; Santa Cruz, USA). The primary antibody is validated against recombinant p53 protein under non-native conditions (figure 5.3a), 3 cell lines under native conditions (figure 5.3b) and lysates, prepared by two different methods, from 5 cell lines under non-native conditions (figure 5.3c). The secondary anti-p53 antibody was validated against recombinant protein under non-native conditions (figure 5.3a).

Native and non-native WBs were conducted as with antiGFP validation and run using the same buffer (25 mM Tris, 190 mM glycine). Cell lysates were prepared either with HARMS buffer (denoted H; native) using the same procedures as before, or with RIPA buffer (denoted R; non-native). Primary and secondary antibodies were used at concentrations 0.6 µg/mL and 0.05 µg/mL, respectively.

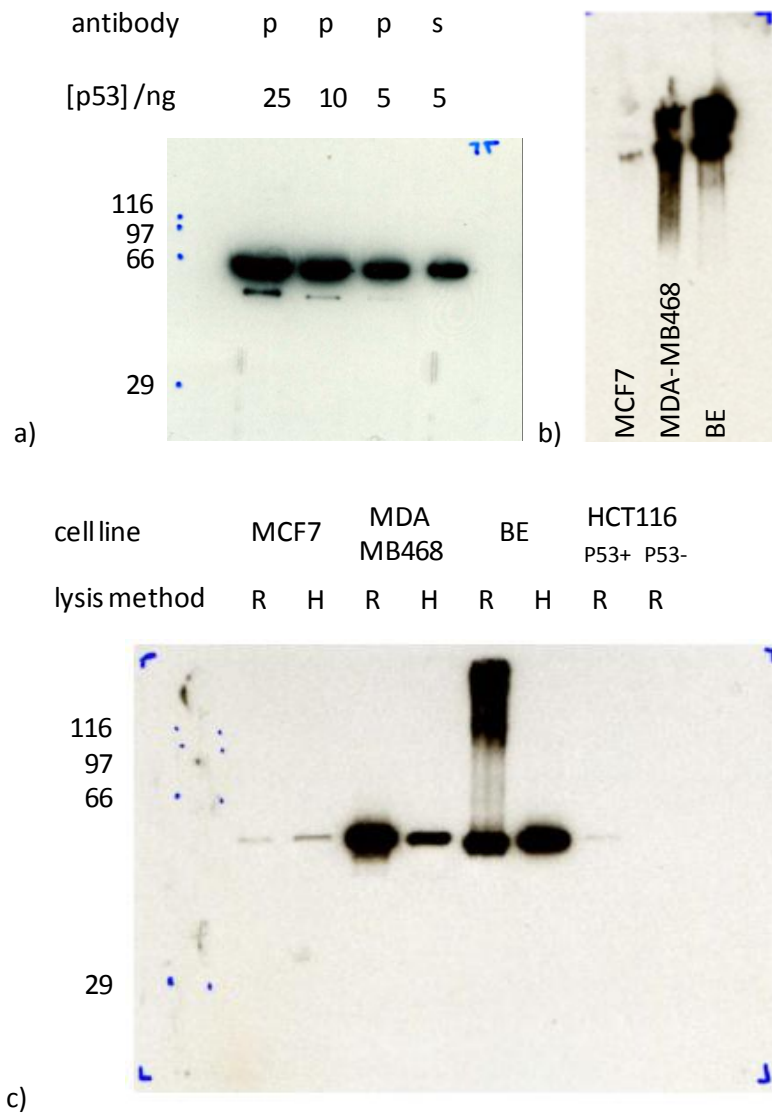


Figure 5.3: Western blots performed for the validation of p53 primary and secondary antibody. a) Non-native WB performed with varying concentrations of p53 recombinant protein with primary (p) antip53 antibody and secondary (s) antip53 antibody. b) Native WB performed on lysates prepared by HARMS lysis method from cell lines denoted. c) Non-native WB performed on lysates from the cell lines denoted prepared by RIPA (R) or HARMS (H) buffer. The molecular weight markers are shown on the side of the blots.

The mass of the p53 protein 43.7 kDa but runs as a 53 kDa protein due to the high number of proline residues in the protein, which slows migration [177]. Both antibodies are found to be specific to the recombinant protein. There is a band of slightly lower molecular weight visible in the gel on which antibodies were run with recombinant p53 protein and a protein fragment present in the sample may be responsible.

The cell lines from which lysates are derived are MCF-7(human breast adenocarcinoma), BE (human colorectal carcinoma), MDA-MB468 (human breast adenocarcinoma) and HCT116 (human colorectal carcinoma). There are two HCT116 lines that either express p53, HCT116 p53+/+, or are p53 null, HCT116 p53-/- . BE and MDA-MB468 cells show strong levels of p53 in comparison to MCF-7 and HCT116 p53 +/+. As expected, no expression is observed with HCT116 p53 -/- lysate. The following conclusions may be drawn. Either, the expression levels of p53 is higher in BE and MDA-MB468 or may be due to the cell lines p53 status. MCF-7 and HCT116 cells have been shown to express wild-type p53 [178, 179] whereas MDA-MB468 expresses mutant p53 [179]. This may be verified by protein sequencing or running lysates with mutant, wild-type and pan-specific anti-p53 antibodies [180].

MCF-7 cells were chosen since there exists published results on p53 expression levels as determined by an ELISA kit on bulk lysate for which to compare to single cell pulldowns [181, 182]. Since the antibody shows high specificity to p53 in BE and MDA-MB468 lysates, these cell lines will be appropriate to demonstrate native protein pulldowns of p53.

5.3 Binding kinetics and theory

Antigen binds to a surface immobilised antibody to produce an antigen-antibody complex according to the law of mass-action,



where $[Ab]$, $[Ag]$ and $[AbAg]$ are the concentrations of free antibody, free antigen and antigen-antibody complex, respectively. The reaction proceeds at rate k_a in the associative forward direction and k_d in the dissociative reverse direction. Therefore the rate of complex formation is

$$\frac{d[AbAg]}{dt} = k_a[Ab][Ag] - k_d[AbAg]. \quad [5.2]$$

The total amount of antibody and antigen remain constant throughout and so

$$[Ag]_T = [Ag](t) + [AbAg](t) \quad [5.3]$$

$$[Ab]_T = [Ab](t) + [AbAg](t). \quad [5.4]$$

It follows that

$$\frac{d[AbAg]}{dt} = k_a[Ab]\{[Ag]_T - [AbAg]\} - k_d[AbAg], \quad [5.5]$$

which has the solution,

$$[AbAg](t) = \frac{K[Ab]}{K[Ab]+1} (1 - e^{-(k_a[Ab]+k_d)t}), \quad [5.6]$$

where $[AbAg](t=0) = 0$ and $K = k_a/k_d$. As $t \rightarrow \infty$, $[AbAg] \rightarrow K[Ab]/(K[Ab] + 1)$ and is the maximum concentration of complex formed. Therefore,

$$[AbAg](t) = [AbAg]_{MAX}(1 - e^{-k_{obs}t}), \quad [5.7]$$

where $k_{obs} = (k_a[Ab] + k_d)$ and is the observed reaction rate. Since the antigen may be fluorescent or be bound to a fluorescent secondary antibody, photobleaching will occur to bound fluorescent species at a rate λ . So,

$$\frac{d[AbAg]^*}{dt} = -\frac{d[AbAg]}{dt} - \lambda[AbAg]^*, \quad [5.8]$$

where fluorescent species are denoted with a *. Eq. 5.7 now becomes

$$[AbAg]^*(t) = \frac{[AbAg]_{MAX}}{1-\lambda/k} (e^{-\lambda t} - e^{-k_{obs}t}). \quad [5.9]$$

The signal from the antibody patch will be proportional to $[AbAg]^*$ and may be fit to extract the rate constants. The preceding formulation does not take into account the transport of antigen to the antibody surface and therefore assumes homogeneous concentration and the binding kinetics are reaction-rate limited. In the single cell pulldown case, the antigen is initially contained within a volume determine by the cell radius. Upon lysis the antigen is then free to diffuse into the chamber volume, which is bounded by the chamber walls. As it diffuses it may encounter the antibody patch as which point it reacts to form the antigen-antibody complex which will associate and dissociate at rates k_a and k_d , respectively.

Diffusion occurs from regions of high concentration to regions of low concentration with a magnitude proportional to the concentration gradient. The antigen will diffuse in solution according to Fick's second law

$$\frac{\partial[Ag]}{\partial t} = D\nabla^2[Ag], \quad [5.10]$$

where D is the diffusion coefficient and ∇ is the gradient operator. The walls are impermeable and the antigen flux normal to these boundaries is zero.

$$\frac{\partial [Ag]}{\partial n} = 0, \quad [5.11]$$

where n is the spatial coordinate normal to boundary. The antibody patch represents a special boundary case since the flux normal to the spot surface is determined by the antigen-antibody binding reaction given by

$$D \frac{\partial [Ag]}{\partial n} = k_a [Ag] \{ [Ab]_T - [AbAg] \} - k_d [AbAg] \quad [5.12]$$

and the amount of antigen bound to the surface is given by

$$\frac{d[AbAg]}{dt} = k_a [Ag] \{ [Ab]_T - [AbAg] \} - k_d [AbAg]. \quad [5.13]$$

The initial conditions are given by the following expressions:

$$[Ag](cell, t = 0) = [Ag]_0 \quad [5.14-a]$$

$$[Ag](rest\ of\ chamber, t = 0) = 0 \quad [5.14-b]$$

$$[AbAg](spot, t = 0) = 0 \quad [5.14-c]$$

The above equations are solved numerically with the stated boundary conditions to generate model data for the concentration of bound antigen as a function of time. For comparison, protein is modelled diffusing from the chamber where at $t = 0$ it is contained homogeneously throughout the chamber. The geometrical model and each modelled scenario are presented in figure 5.4 – 5.6. The model assumes linear diffusion.

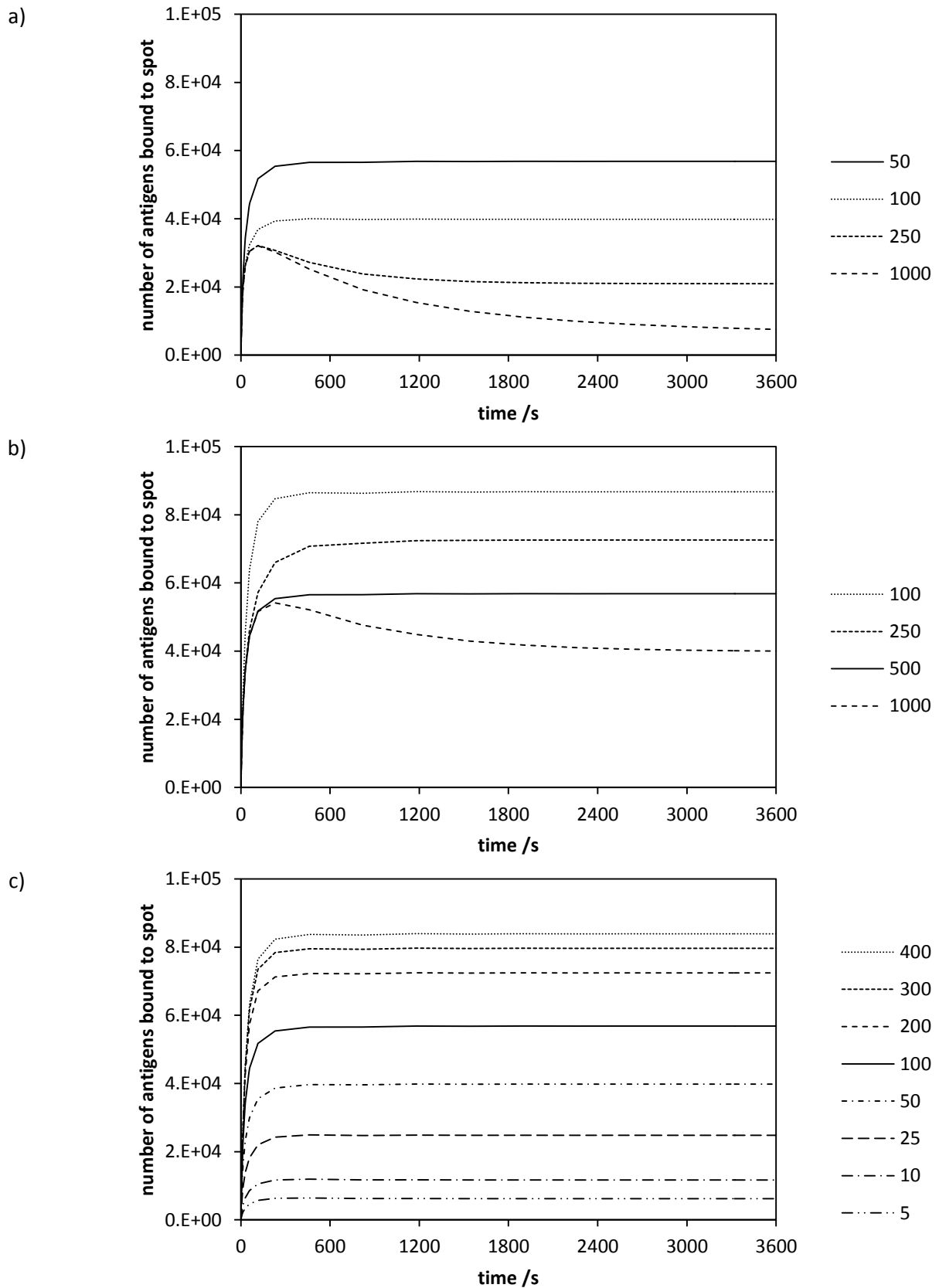


Figure 5.4: Protein diffusing from cell: model kinetic data showing the effect of changing the chamber a) height (constant width 500 μ m, spot diameter 100 μ m), b) width (constant height 50 μ m, spot diameter 100 μ m) and c) spot diameter (constant width 500 μ m, height 50 μ m). The dimensions in the legends have units of μ m.

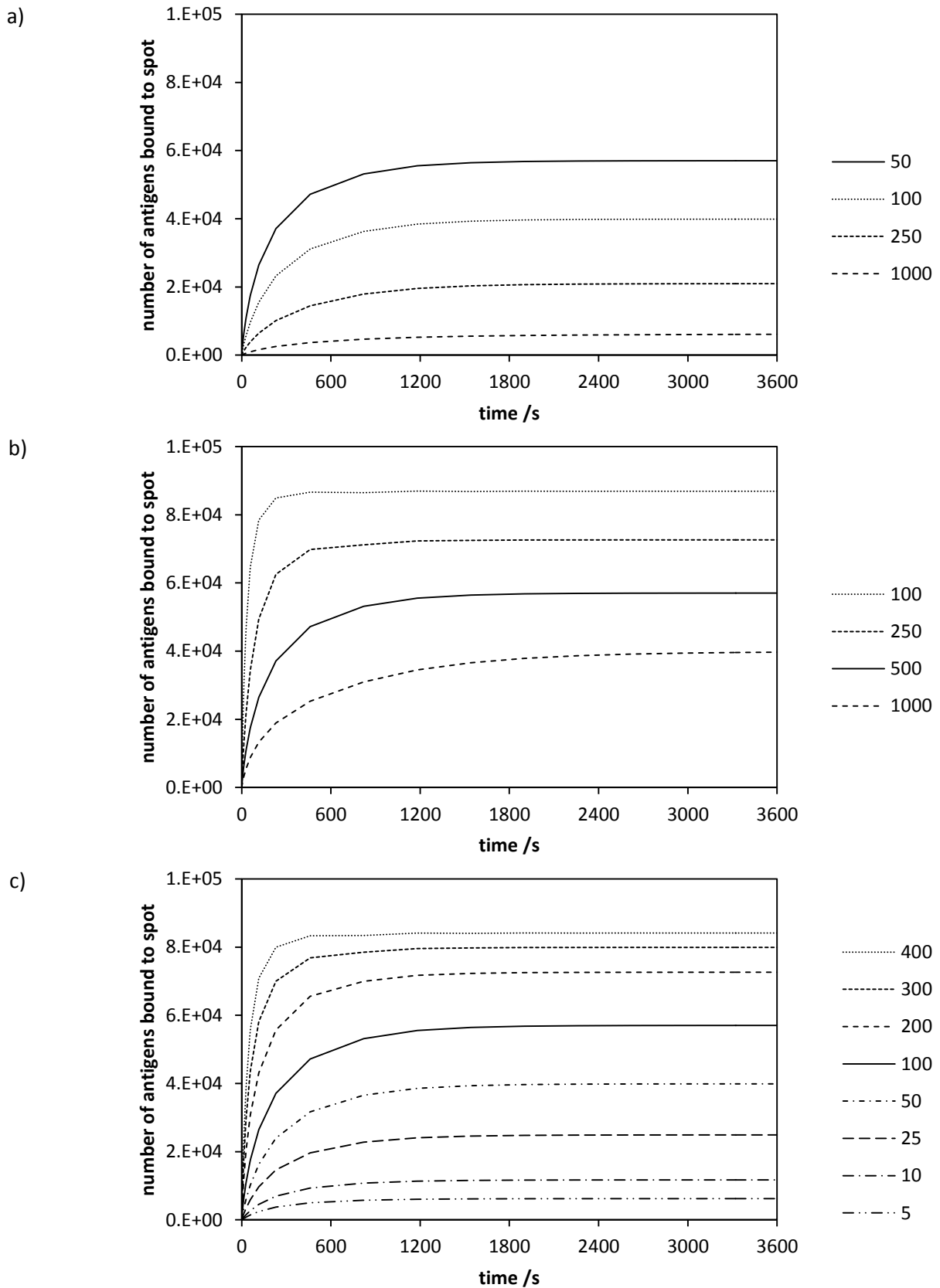


Figure 5.5: Protein diffusing from chamber: model kinetic data showing the effect of changing the chamber a) height (constant width 500 μm , spot diameter 100 μm), b) width (constant height 50 μm , spot diameter 100 μm) and c) spot diameter (constant width 500 μm , height 50 μm). The dimensions in the legends have units of μm .

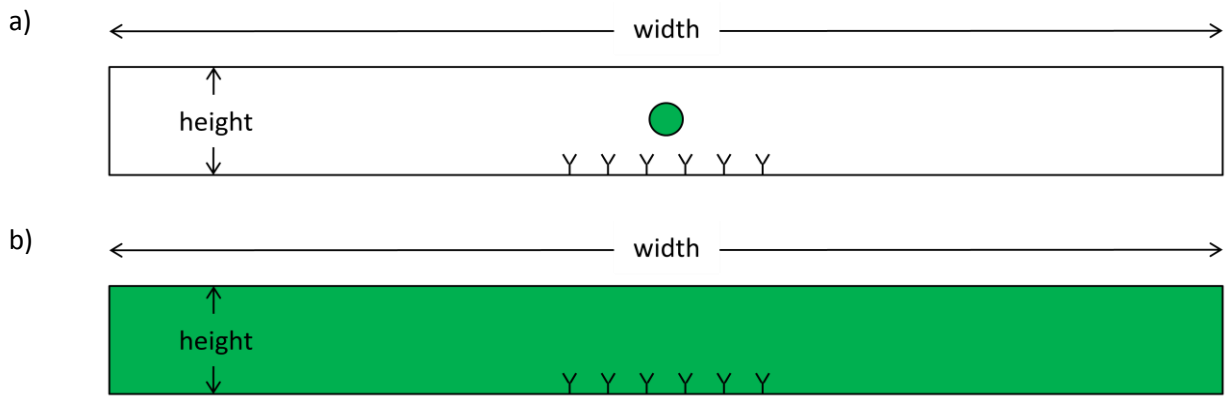


Figure 5.6: Geometry of modelled chamber showing both modelled scenarios. a) Protein diffusing from a cell: 10^5 copies of protein antigen are contained at $t = 0$ s in a cell of radius $7.5 \mu\text{m}$ with homogenous concentration. b) Protein diffusing from chamber: 10^5 copies of protein antigen are spread homogenously throughout chamber.

The model parameters are informed as follows. The structure of an intact monoclonal antibody was determined by X-ray diffraction [183] and was approximately $16 \text{ nm} \times 6.5 \text{ nm} \times 11 \text{ nm}$ giving a surface area of $\sim 104 \text{ nm}^2$. Antibody surface density is determined by assuming these dimensions are typical and antibodies are regularly packed across the surface of the spot as a monolayer. The binding site density is 16.0 nmol/m^2 and for a spot of $100 \mu\text{m}$ diameter there are approximately 0.8×10^8 antibodies present assuming a perfect monolayer. Values for k_a and k_d are set to $1.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $0.5 \times 10^{-2} \text{ s}^{-1}$, respectively, and are of the order reported for typical antibodies. The diffusion coefficient D is taken as that of GFP in water, measured experimentally to be $87 \mu\text{m}^2/\text{s}$ [156]. The modelled cell is assumed to have 10^5 copies of GFP antigen with homogeneous initial concentration $[Ag]_0$ of 0.94 pM .

The binding curves are qualitatively as expected. The binding curves for the microfluidic chamber geometry described in §3 are used as a reference and are shown consistently as a solid black line in the figures. Interestingly, due to the proximity of the cell to the patch, protein in chambers of larger volume, either from being deeper (figure 5.4a) or wider (figure 5.4b), initially binds at a rate dictated by the associative rate constant, k_a . At times $> 1/k_d$, the amount of protein bound decreases until equilibrium is reached, where protein has diffused to fill the chamber. The amount of protein bound at equilibrium decreases as chamber height or width, i.e. volume, increases. For small enough volume, the time taken for protein to diffuse to fill the chamber is of the order or $< 1/k_d$ and so no drop is observed following initial binding (e.g. heights 50 and $100 \mu\text{m}$ and widths 100 , 250 and $500 \mu\text{m}$; figures 5.4b & c). This is not observed in the case where protein concentration is homogenous

in the chamber at $t = 0$ (figure 5.5). The antiGFP antibody may have different rate constants and with a lower k_d or higher k_d the binding curves will exhibit such peaks and decays to equilibrium.

For the case of protein diffusing from the cell, varying the spot diameter has no effect on the time to reach equilibrium in a chamber of diameter 500 μm and height 50 μm , but does determine the amount of analyte bound from solution at equilibrium (figure 5.4c). For the case of protein diffusing from the chamber the equilibrium concentration of bound protein is the same as that in the other case. However, the observed rate constant decreases as spot diameter decreases (figure 5.5c). The model antibody spot binds 57% of the total cell protein at equilibrium for a spot of diameter 100 μm and as expected this does not change for either starting case. The effect of doubling the antibody patch diameter to 200 μm increases this to 73%. The antibody spots used in this work are between 100 and 150 μm . For a spot radius of 125 ± 25 μm , 60 ± 3 % of total protein is pulled down. Therefore, in order to minimise errors, spots of uniform size are desired.

The analysis time can be expected to be of the order of 1000 s.

5.4 Production of antibody microspots

A crucial aspect of employing antibodies as the sensor molecule in a microarray system is the reproducible production of antibody spots. Many of the techniques developed in DNA microarrays are applicable to protein and antibody microarrays but with much more complex requirements.

DNA and proteins differ most significantly in terms of their structure. DNA is unstructured and its function is contained within the primary nucleotide sequence. Antibodies, however, are globular macromolecules with secondary, tertiary and quaternary structure and all contribute to protein function. This has consequences for using microarrays. Proteins must be prevented from denaturing during all steps of production and use. Proteins may be denatured by changes in temperature pH, buffer additives such as detergents, solvents and steps must be taken to avoid or minimise such insults. Moreover, there is no simple method of protein amplification such as with DNA using polymerase chain reaction.

Several methods exist to deposit nanolitre droplets of antibody on solid surfaces in order to produce a microspot. By far the simplest is microcontact printing. A stamp moulded from an elastomeric material such as PDMS using such techniques as those used to create microfluidics, is treated and dipped in an antibody solution. Antibodies adsorb to the surface which are then transferred to the

solid surface by direct contact. Microcontact printing has the ability to be massively parallel but the amount of deposited antibody is not well controlled and deposition efficiency is low. Spots may be printed using lithographic techniques, whereby a surface is photoactivated in a pattern dictated by a mask and antibody may bind from solution to these regions. The high resolution capable by patterning light allows spots on the order of a micron but the method suffers due to low deposition density.

More widespread techniques include microspotting and non-contact ink jetting. Ink jet printing methods are able to produce spots by dispensing droplets from an orifice to the surface. It offers more precise control over the sample volume delivered to the surface than microspotting but cross contamination of samples is a main drawback owing to the nozzle and entire sample delivery system requiring extensive cleaning. Typical spot sizes are several hundred microns in diameter and depend on the wettability of the surface.

Microspotting is a contact printing method that transfers antibody solution from a sample reservoir to the substrate by a pin that essentially serves as a quill. The pin is wetted by submerging the tip which transfers a small volume of sample to the tip. The wetted tip is then brought into contact with the surface and a volume of sample is transferred to the substrate. As the pin is withdrawn, a liquid tether between the pin and the surface drop is generated and the lifetime of the tether determines the volume of fluid deposited. Thus the spot size is determined by many factors such as fluid viscosity, surface tension, humidity, temperature, pin velocity and, of course, pin diameter [184].

Pins may be solid-type or have a hollow bore which acts as a sample channel that permits many spots to be printed per sample loading. The sample channel is filled by capillary action upon submersion in the sample reservoir. Deposition efficiency is very high in microspotting and pin diameters as small as 62.5 μm are available, however, ultimately, spot size will be larger given the volume deposited and can be as large as $\sim 200 \mu\text{m}$ for such a pin. Pin diameters ranging from 62.5 μm to 600 μm are available.

All antibodies microspots will be printed by an OmniGrid Micro microarrayer (Digilab, UK) using 946MP2 pins (ArrayIt, USA). The microarrayer is essentially a precise long-range translation stage which moves slides relative to a stationary print head. The apparatus is housed as a closed unit with an access port. A humidifier unit controls the microenvironment humidity and is set to 75% for all antibody printing. The printing process involves cleaning the pins using an on-board wash station, priming the pin with sample and printing upon the substrate at predefined locations. The substrate carriage is designed for 75 mm $\times$ 25 mm $\times$ 1mm (l $\times$ w $\times$ t) glass microscope slides. The thickness is

not compatible with a 60× 1.49 NA microscope objective and so 50 mm × 25 mm × 0.16 - 0.19 mm (thickness #1.5) are held in place with the use of a vacuum pump. Microarrayer robot functions and operation are made automatic by the use of control software. The AxSys software (Digilab, UK) allows a program of operation to be compiled. Typically arrays are formed from a rectangular grid of spots but here spots are printed on coverslips in locations which correspond to the single cell pulldown chambers.

The dispensing pin is a 946MP2 which has a 62.5 µm spot diameter and sample loading bore which bisects the pin. The nominal uptake and dispensed volumes are 0.25 µL and 0.5 nL, respectively, and greater than 200 spots may be printed per single loading. To ensure optimal printing the pin surface must be free of contaminants such as dust and chemical contaminants such as dried proteins or detergent. Prior to printing and before the printing of a different antibody, the microarrayer is programmed to clean the pin. This involves immersing the pin in a distilled H₂O for 30s and then drying by vacuum. The water may be continuously flowed for more vigorous washes but this has been found to be inadequate to completely clean the pin of all contaminants.

Off-board wash steps were conducted and tested after printing 100 spots with a test solution of 5% glycerol in PBS containing a FITC conjugated antibody and DAPI dye. The pin was suspended such that the pin tip was fully submerged in cleaning solutions. The following was tested: stirred dH₂O using a magnetic stirrer, sonication in dH₂O, sonication in 70% ethanol (EtOH) in water and sonication in 5% Microcleaning solution (MCS) in dH₂O – a commercial detergent (ArrayIt, USA). Cleaning steps were conducted for 5 minutes before pin is washed copiously with dH₂O. After drying using filtered forced air the pins were observed under epi-fluorescence using standard Nikon DAPI and FITC filters to determine the degree by which each treatment cleaned the pin (figure 5.7). It is observed that sonication improves pin cleaning over that of just stirred water. It is not known whether treatment in ethanol actually removes protein or merely crosslinks and denatures it on the pin. MCS is a commercial detergent containing agents to solubilise proteins and by judgement of the fluorescence it fully cleans the pin. The detergent may result in poor downstream printing if it is not cleaned itself from the pin adequately. As directed by the manufacturer, pins are sonicated in dH₂O for 5 min following 5 min sonication with 5% MCS then dried using filtered forced air. Therefore, before each antibody is printed the pin must be removed from the microarrayer and cleaned off-board. This increases the risk of pin damage and also increases the time to create a microarray.

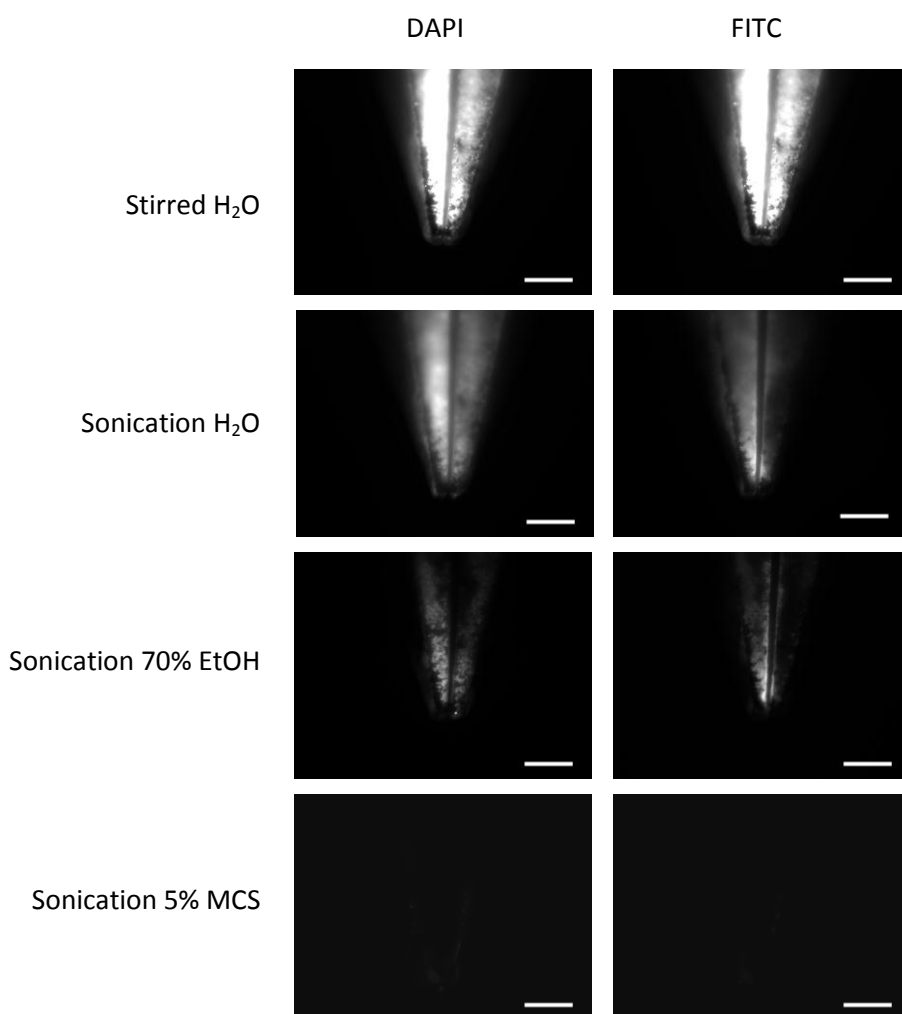


Figure 5.7: An array of 100 spots of FITC-conjugated antibody and DAPI dye is made using the 946MP2 microspotting pin. Fluorescence images show residual material on the pin after the cleaning steps indicated are made. Each cleaning step is conducted for 5 minutes before pin is washed copiously with dH₂O. Scale bar 100 μ m.

5.5 Optimisation of production parameters

Printed antibody spots are not so much judged based on their general morphology but their binding density and how this varies from spot to spot and intra-spot. The binding density may be measured with a fluorescently labelled secondary antimouse antibody to detect the printed antibody. However, this will detect functional and non-functional antibody (in the Fab region) alike, since it binds to the Fc region. To test antibody that is present and functional, testing binding of the antigen is the best way to do this.

5.5.1 Printing buffer

Even under high humidity, as nanolitre droplets of solution containing antibodies is deposited upon glass it will rapidly evaporate. This will cause a rapid increase in solute concentrations, shifts in pH, dehydration of proteins, and osmolarity as ionic strengths change. Printing buffer is perhaps the most crucial aspect of antibody printing since it must protect antibodies from these phenomena.

Glycerol, bovine serum albumin (BSA) and polyvinyl alcohol (PVA) have been used previously as protectants during freeze drying, also known as lyophilisation. Several mechanisms of protection have been proposed how proteins may be stabilised [185, 186]. During the dehydration process water that stabilises protein structure is removed and so additives which replace water will prevent denaturation. This is known as the water-replacement hypothesis [187]. On the other hand, the preferential-hydration hypothesis describes protection by additives that are excluded from the protein [188]. Such additives include osmolytes, which protect proteins from high osmotic pressure. The mechanism is believed to result from an unfavourable free energy of interaction between the osmolyte and the unfolded state of the protein therefore the protein retains its folded state and thus activity. Protection may be afforded by additives that form a glass in the dry state which provides a matrix to immobilise and stabilise proteins. This is known as the vitrification hypothesis [189, 190].

Printing buffer is optimised to print the antiGFP antibody and is present in buffer at 90 µg/mL. Assuming an antibody weight of 150kDa, the concentration of antibody in the buffer and therefore the deposited spot is 0.42 µM. For a deposited volume of 0.5 nL, 3.0×10^{-16} mol of antibody will be printed upon the surface. As before, assuming a binding site density of 16.6 nmol/m² and a spot diameter of 100 µm, the amount of antibody in a spot comprising an antibody monolayer is 1.3×10^{-16} mol. Therefore over twice as much antibody is being printed as necessary and ensures this is not the limiting factor in antibody density. Apart from the number of antibodies available in solution there is no control over deposited thickness. Upon evaporation, such a spotted droplet will result in antibody aggregating in several layers. Therefore, surfactants that lower surface tension and allow spreading of the deposited droplet may produce more homogeneous spots.

Antibodies are printed onto GPTS derivatised coverslips (see §5.5.2). All printing buffers are based on PBS to maintain physiological pH and salt concentrations when antibodies are in solution. The following additives were tested: glycerol at concentrations 0.5% and 5% (v/v), BSA at concentrations at 4%, 0.4%, and 0.004% (w/v) as a supplement to buffers containing glycerol; and 0.005% (v/v) Tween20. In addition, the commercial PBS-based protein print buffer from ArrayIt was tested alone

and supplemented with 0.5% PVA (molecular weight 9000-10,000). Printed spots were blocked for 30 min using 4% BSA in PBS. Spots were incubated for 30 min in 74 pM eGFP in PBS before being imaged. For evaluation of printing solutions, coverslips are tested in an open format with a reaction volume of approximately 50 μ L. Spot morphology isn't necessarily required to be circular as long as spot properties are reproducible. To compare spots with different morphology it is helpful to determine their equivalent diameter, which is a size feature derived from the spot area. It determines the diameter of a circle with the same area as the non-circular spot. Pixel intensity was averaged across the spot and a background image. Signal to noise ratios (S/N) for spots were then calculated. Results are shown in figure 5.8.

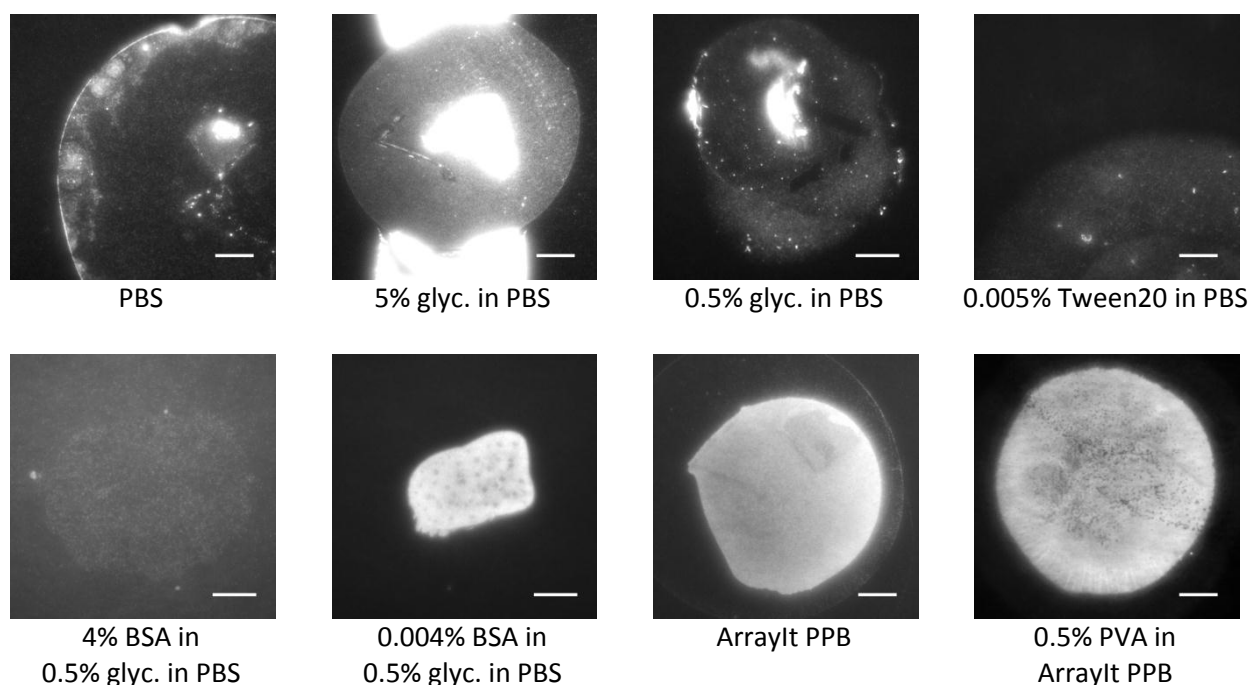


Figure 5.8: TIRF images of printed antibody spots using the printing buffer indicated. Spots are blocked and imaged after 30 min with 74 pM eGFP in PBS. Scale bar 20 μ m.

Printing with PBS alone produces spots with fluorescence congregated toward the spot periphery. This occurs due to Marangoni convection in the droplet produced by evaporation of H₂O in the PBS [191, 192]. Liquid evaporating from the edge is replenished by liquid from the interior and results in antibodies accumulating at the edges. The flow may be disrupted by limiting evaporation, such as with glycerol, or by the addition of surfactant to reduce surface tension and disrupt the flow. The use of glycerol at high concentrations produces the so-called “comet tail” effect, whereby upon washing of the spot antibodies bind at high concentration outside the perimeter of the undried spot.

The mechanism for this is unknown but the antibody may preferentially partition to glycerol until sufficiently diluted by the wash step to then bind to the surface. The comet tail effect can be mitigated by decreasing glycerol concentration to 0.5% but binding density within the perimeter of the spot remains low. The S/N for 5% and 0.5% glycerol is 5.3 ± 1.1 and 3.4 ± 0.5 , respectively. The spot to spot variation and intra-spot variation decreases from 23% and 25% to 17% and 15% as glycerol concentration is decreased from 5% to 0.5%, respectively.

The addition of BSA at concentrations 4% and 0.4% to 0.5% glycerol print solution reduced antibody binding density resulting in a S/N of 1.5 ± 0.1 and 1.1 ± 0.1 , respectively. At these concentrations, BSA ($0.3 - 3.0 \times 10^{-13}$ mol) is in excess to the antibody (3.0×10^{-16} mol) by a factor of 100-1000 and may physically prevent antibody from binding to the surface. This will have the effect of reducing antibody binding density by the same factor, assuming the affinity for the surface is similar for each protein species. At 0.004%, BSA is in a 1:1 molar ratio to antibody spots and results in small and irregular spots with a S/N of 5.1 ± 0.6 . The spot to spot variation was 19% with an intra-spot variation of 12%.

Tween 20 produced very large spots > 200 μm diameter and so only a portion of the spots may be imaged. Spots exhibited low signal to noise, 1.8 ± 0.3 , and a slightly blotchy morphology.

ArrayIt protein print buffer (PPB) produced spots with a region exhibiting high signal to noise, 9.6 ± 1.9 , and high binding density surrounded by a low binding halo. The uniformity within the high binding region is high with a variation of 13%. To encourage homogeneous coverage, ArrayIt PPB was supplemented with 0.5% PVA. As a surfactant, PVA promotes droplet surface spreading and limits Marangoni flow. PVA adsorbs to the droplet surface and so displacing protein from the surface and protects antibodies from surface energetics during solution evaporation. This resulted in the most reproducible spots with a spot to spot variation of 6% and an intra-spot variation of 7%. As visible from the TIRF image, material is evenly distributed across the entire spot area, with diameter $118 \pm 2 \mu\text{m}$.

5.5.2 Surface derivatisation

Surface derivatisation protocols are reproduced from the literature, in particular studies focussing on investigation production parameters for a wide range of surface derivatives [155]. Coverslips are prepared in batches in Hellendahl jars. Coverslips are sonicated in 96% EtOH for 5 min, washed with

0.5M NaOH and sonicated for 60 min. Coverslips were then rinsed 4 times in dH₂O then twice in 96% EtOH. For testing plain glass, coverslips are forced-air dried and used immediately.

The non-volatile silanes GPTS (3-glycidoxypropyl-trimethoxysilane), APTES (3-aminopropyl-trimethoxysilane) and MPTS (3-mercaptopropyltrimethoxysilane) were used to chemically modify the glass surface. Antibodies covalently attach to GPTS by epoxy chemistry. APTES is terminated by an amine group, which in neutral solution will be protonated and allows antibodies to electrostatically bond to the surface. However, the degree by which the antibody binds will be determined by its surface charges.

Once coverslips are prepared (as above) they are immersed for 1 hour in either 2.5% GPTS in 10mM acetic acid in 96% EtOH, 5% APTES in 96% EtOH or 1% MPTS in 10mM acetic acid in 96% EtOH, depending on the desired surface derivatisation. Coverslips are then rinse twice in 96% EtOH, sonicated in EtOH for 10 min and followed by a final rinse in 96% EtOH. APTES slides were baked at 110°C for 15 min following the final rinse. Coverslips are forced-air dried and transferred immediately to the microarrayer for printing.

In addition, a 2-dimensional APTES/PEG surface was tested. Polyethylene glycol (PEG) is a hydrophilic polymer which exhibits high resistance to protein adsorption [193]. Antibody capture efficiency has been shown to improve using PEG spacers and was postulated that this reduces steric interference during binding [194]. APTES slides were prepared and reacted with biotinylated polyethylene glycol (mPEGb). A droplet of 25% w/v PEG in 0.1M sodium bicarbonate is sandwiched between two coverslips and left to incubate for 3 hours in a humid chamber in the dark. Coverslip pairs were disassembled, rinsed with dH₂O and forced-air dried.

Arrays of 100 anti-GFP antibody microspots are printed using the 0.5% PVA in ArrayIt PPB on coverslips derivatised with APTES, GPTS, MPTS, APTES/mPEGb and are compared to plain glass. Surfaces are blocked for 30 min in 4% BSA in PBS then incubated with 74pM eGFP for 30 min, after which background and antibody spot signal is measured (figure 5.9a). For evaluation of the surface derivatives, coverslips are tested in an open format with a reaction volume of approximately 50 μ L. The mean pixel intensity for the background was calculated. All surface derivatives resulted in lowering non-specific adsorption of eGFP protein to the surface with the exception of MPTS. APTES and mPEGb surfaces were the most successful and resulted in 85 % and 71% less non-specific adsorption, respectively. Pixel intensity was averaged across the entire spot to calculate the S/N for spots printed on each surface.

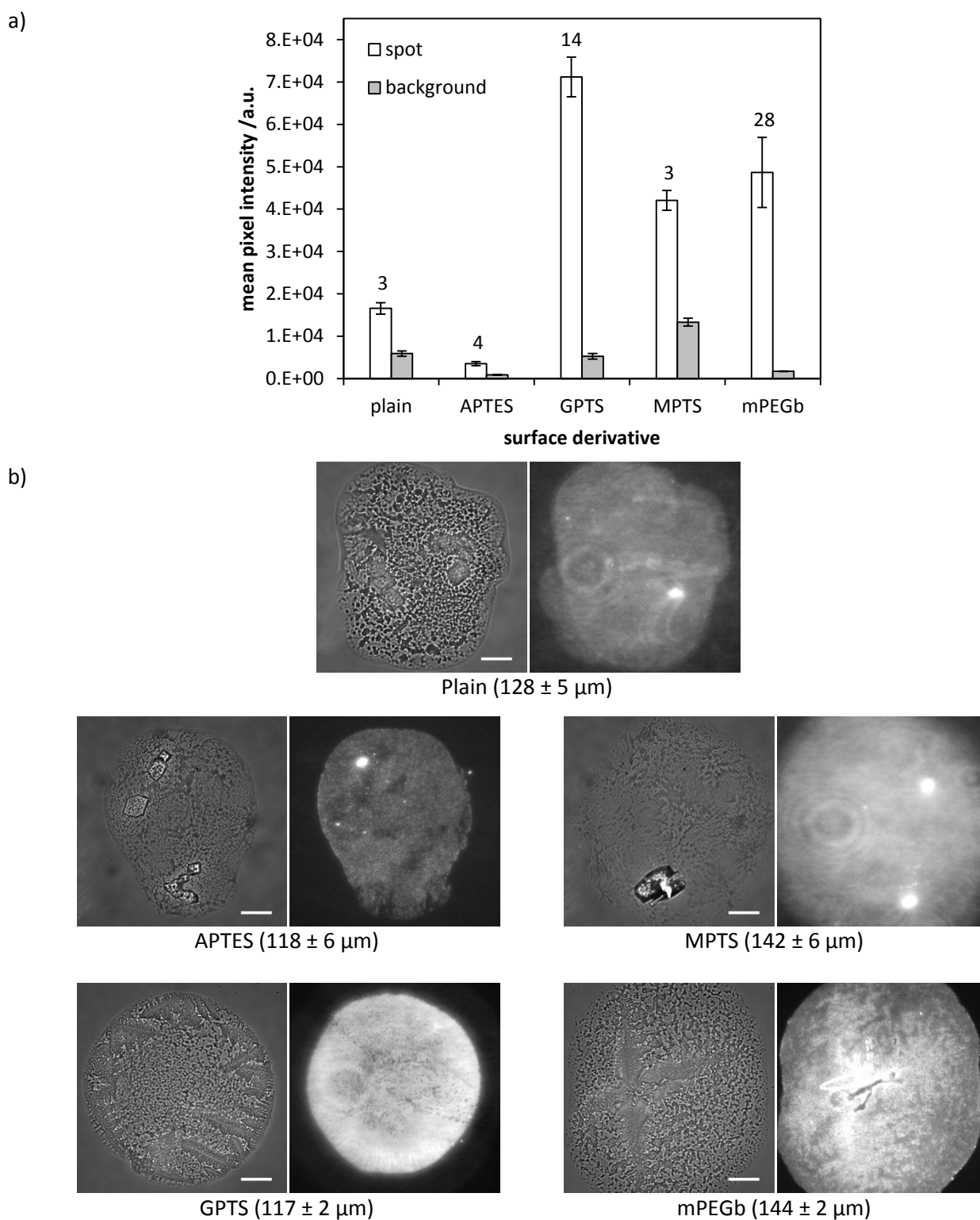


Figure 5.9: a) Comparison of background signal from coverslips modified as indicated. Slides were blocked then incubated for 30 min with 74 pM eGFP. Numbers on the chart indicate the signal to noise ratios measured from antibody spot signal against the off-spot background signal. b) Images of printed antibody spots on tested surfaces. Brightfield (left) images of printed antiGFP spots dehydrated before blocking and TIRF (right) images after incubation with eGFP. Lengths alongside surface derivative denote the equivalent diameter of spots for each surface. Scale bar 20 μm .

All surfaces produced largely uniform binding density across the spot. For a spot of constant area, an increase in spot intensity is concomitant with higher binding density. Strictly, this assumes that denaturation of antibody is constant upon each surface. This is a fair assumption given that the printing solution used is the same for all surfaces. The spot sizes are much larger than the pin diameter of 62.5 μm . This is due to the wettability of each surface and the surface tension of the printing solution. PEG is a hydrophilic polymer and results in the largest spots whereas the silane derivatives are mildly hydrophobic and generally result in smaller spots. It is possible to manipulate spot size by exploiting surface wettability.

APTES coated slides exhibit the lowest background but also the lowest binding density resulting in an S/N (4:1) similar to that of plain glass (3:1). The spot intensity on MPTS coverslips was much higher than APTES but so too was the background resulting in no improvement in S/N (3:1) over that of plain glass. The binding density of spots on mPEGb was similar to that of MPTS coverslips but due to the very low background the S/N (28:1) was the highest out of all the surfaces. The highest binding density was on GPTS coverslips, exhibiting greater than 4 times denser binding than that of plain glass. The background is similar to that of plain glass and so S/N (14:1) is not as high as that for mPEGb. A different blocking agent may perform better than BSA which would serve to increase the S/N but this was not investigated.

GPTS is the only surface tested that covalently bonds to the printed antibody and is the most likely factor in explaining the higher binding density. The other surfaces tested all rely on adsorption to the surface. As antibody is deposited, the droplet rapidly dries and promotes irreversible adhesion of printed antibodies [195]. It is not understood how antibodies specifically react with functionalised surfaces but van der Waals forces, electrostatic and interactions and irreversible surface energetics are likely to play a role. During the blocking step, weakly bound antibodies are washed away from the surface with only strongly adsorbed or covalently bound antibodies remaining [195]. The particularly low binding density and non-specific background adsorption upon APTES coverslips may be attributed to the presence of surface charges which electrostatically inhibit strong adsorption.

The spot to spot variation on GPTS coverslips is 7% whereas upon mPEGb is it 17%. Despite the high S/N on mPEGb coverslips, GPTS will be used to functionalise antibody coverslips that will form part of the microfluidic antibody devices. A more directed coupling method to the mPEGb surface, such as employing biotin binding chemistry by conjugating antibodies with avidin or streptavidin, may increase binding density further and reduce spot to spot variation. Unfortunately, the antibodies investigated here are not conjugated as such.

Roughly, 50% of print runs showed a 7% spot to spot variation or lower; those print runs that did not achieve this target were rejected.

A high S/N is necessary for developing antibody microarrays that have low limits of detection. The limit of detection in the open chip format is 5 pM (3.7 fmol) as determined by the background level. As the volume is reduced from 50 μ L for the open chip format to 9.8 nL of the individual microfluidic single cell chambers the limit of detection in terms of absolute quantity will reduce to 22.5 zmol, which corresponds to 1.36×10^4 molecules per chamber. This assumes that the background signal is concentration independent.

5.6 Conclusions

In order to detect proteins, antibodies are printed into 2-dimensional spots/patches using a microarrayer. The microarrayer uses a quill-type pin to transfer antibody from a 384-well plate to the coverslips that will form part of the microfluidic device designed in §3. The microarrayer allows antibodies to be printed at defined locations such that, when the coverslip is bonded to PDMS, the antibody spots are contained within the single cell analysis chambers. Material liberated from the cell by laser lysis may be captured and detected with the combination of the antibody spots and TIRF microscopy.

The antibodies for single cell pulldowns in §6 will be printed using a buffer containing ArrayIt PPB supplemented with 0.5% PVA. This buffer resulted in the most reproducible spots with a spot to spot variation of 6% and an intra-spot variation of 7% with an average diameter of 118 ± 2 μ m. Coverslip surfaces are functionalised with GPTS, which of the coatings investigated resulted in the highest S/N of spots.

The final concentration of antiGFP and anti53 in spotting solution were 90 μ g/mL and 62.5 μ g/mL, respectively. Spot to spot variation was determined by incubating antibody spots with purified antigen protein and 50% of print runs show a 7% spot to spot variation or lower; those print runs that do not achieve this target are rejected.

6 Single Cell Pulldowns

This chapter describes the integration of work described in the preceding chapters culminating in a microfluidic antibody capture chip with TIRF detection.

- Alternative trapping strategy

By flowing cells through the analysis chambers a problem of background protein in the media was encountered. Despite attempts to mitigate the problem it is easier to achieve cell trapping by a simple redesign of the microfluidic and employing an optical trap. A discussion of why an optical trap may be better suited to rare cell types than hydrodynamic trapping is made.

- Results with eGFP

Precision of the method is determined to be 88% using BE cells stably transfected with eGFP present in the cytoplasm.

- Results with p53

The capability to measure unlabelled proteins is demonstrated with the p53 sandwich-system and a breast-cancer cell line MDA-MB468. It is estimated that these cells contain 1500 ± 200 copies of free p53.

6.1 Free eGFP in solution

Microfluidic antibody capture devices are constructed as described in previous sections. PBS is flowed through the device and any bubbles are removed by degassing in a desiccator. The location of the antibodies are recorded relative to the trapping posts using the encoded XY stage and used to realign spots to the camera field of view upon returning the device to the microscope. Connections via appropriate tubing are remade and 4% BSA in PBS is then flowed through the device to block surfaces to reduce non-specific adsorption and was incubated for 30 min before the introduction of cells. Cells are then harvested from culture flasks by trypsinisation and inactivated by suspension in 4% BSA in PBS. The coating of BSA adsorbed onto the surfaces of the chamber will diffuse into solution and so BSA present with the cells will help to maintain the BSA coat. A suspension of single cells was created by gentle agitation with a pipette before sieving through a nylon mesh of 40 μm pitch to remove any cell clumps.

4% BSA in PBS is flowed down each focussing channel at $1.5 \mu\text{L min}^{-1}$ and the cell suspension is flowed down the inlet channel at $1 \mu\text{L min}^{-1}$ ($Q_s/Q_i = 1.5$), therefore producing a focussed stream of cells of minimum width $\sim 15 \mu\text{m}$. The device is allowed to trap cells for 30 min.

Antibody spots are imaged prior to the addition of cells and before lysis events. It is observed that spots become fluorescent as GFP+ cells are introduced to the device. The control whereby GFP negative cells are flowed is performed and no such spot fluorescence is observed. The GFP must be present in the cell solution and may be attributed to cell membranes being compromised during the harvesting process, during flow through the device or possibly GFP is being excreted from the cells in solution; however, the latter seems highly unlikely. PBS is flowed down the inlet channel at $1.0 \mu\text{L min}^{-1}$ and spot fluorescence is monitored over time. A decay in fluorescence is observed and fit to a single exponential decay function with a decay constant, which is the antiGFP antibody dissociation constant, of $0.54 \pm 0.08 \times 10^{-3} \text{ s}^{-1}$.

Using the kinetic model introduced in §5.3, an estimate of the amount of GFP bound to the spot under flow conditions may be made and from this how many cells become compromised, while either in flow or during cell harvesting, may be estimated:

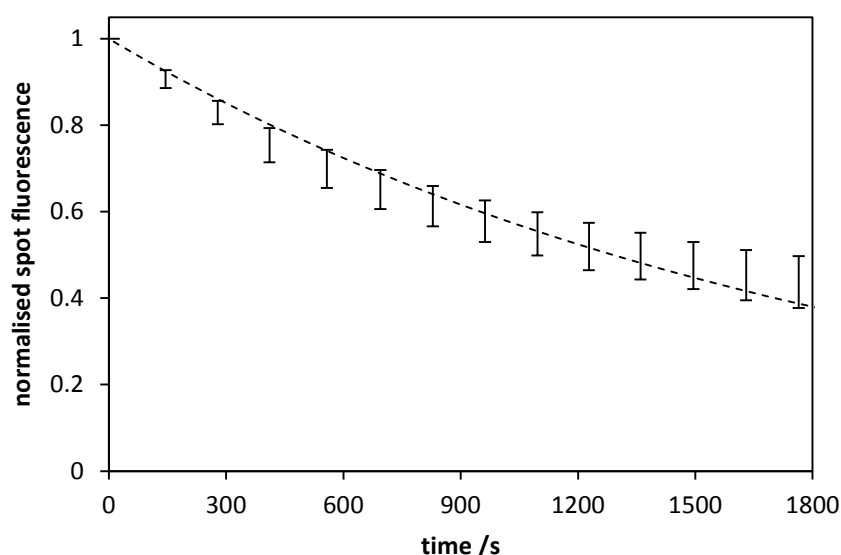


Figure 6.1: Averaged fluorescence decay of antibody spots under flow of PBS at $1 \mu\text{L min}^{-1}$. Measured data is averaged all 10 spots in the single cell pulldown microfluidic and a single exponential decay is fitted (dashed line) with $k_{\text{off}} = 0.54 \pm 0.08 \times 10^{-3} \text{ s}^{-1}$.

AntiGFP antibody spots are printed upon a coverslip and incubated with 50 μL PBS solution containing 74 pM recombinant eGFP. To contain the solution and to prevent spreading across the coverslip, a pen is used to draw a circle of hydrophobic ink around all the antibody spots. This results in an approximately cylindrical liquid volume of radius = 2.8 mm and height = 2mm. According to the kinetic model, 0.3 % antigen is bound to the antibody spot from the bulk volume in this 'open-chip' format. For a 74 pM concentration there are approximately 2.2×10^9 GFPs in a 50 μL volume and therefore suggests 6.6×10^6 GFPs (0.3%) bound to the spot.

The antibody spot fluorescence in the microfluidic chambers after flow of cells for 30 min is 0.13 ± 0.05 % of the open-chip format spot fluorescence that is incubated with 74 pM eGFP. Assuming similar fluorescence intensities, this suggests that there are $8.6 \pm 3.3 \times 10^3$ GFPs bound to the antibody spots in the microfluidic chamber. However, the kinetic model does not take account of conditions where antigen is under flow. Therefore, the kinetic model must be modified. Mass transport of the antigen must now include a convective term to account for the GFP which is in flow and so eq. 5.11 becomes

$$\frac{\partial[Ag]}{\partial t} = D\nabla^2[Ag] - [Ag]\nabla \cdot \underline{u} , \quad [6.1]$$

where $\underline{u}$ is the velocity of the antigen in the laminar flow. The concentration of the modelled fluid was 10^5 copies of GFP per chamber volume. In previous simulations where the chamber was modelled as a closed volume and mass-transport was facilitated by diffusion only, 56.8% of the antigen in the chamber is bound to the spot at equilibrium. In simulation, when fluid is flowed through the chamber at a rate of $1 \mu\text{L min}^{-1}$ and mass-transport is facilitated by diffusion and predominantly by convection then the amount bound to the spot at equilibrium can be much higher. Indeed, it can exceed the number of antigens present in the chamber at that time (figure 6.2). Of course, the absolute amount bound to the spot depends on the concentration in the flowing fluid; however, the amount bound to the spot relative to the concentration in the flowing fluid is dependent on the antibody association/dissociation constants. The antibody binding density is also a factor but this only becomes important towards spot binding saturation. Since there is an estimated $8.6 \pm 3.3 \times 10^4$ GFPs bound to the spot in the microfluidic chamber then this is 3 orders of magnitude lower than the total number of antibodies in the simulated spot (0.785×10^8) and so can be neglected here. The antibody dissociation constant has been measured ($0.54 \pm 0.08 \times 10^{-3} \text{ s}^{-1}$) and so the problem is effectively reduced to variation in only the antibody association constant, k_a .

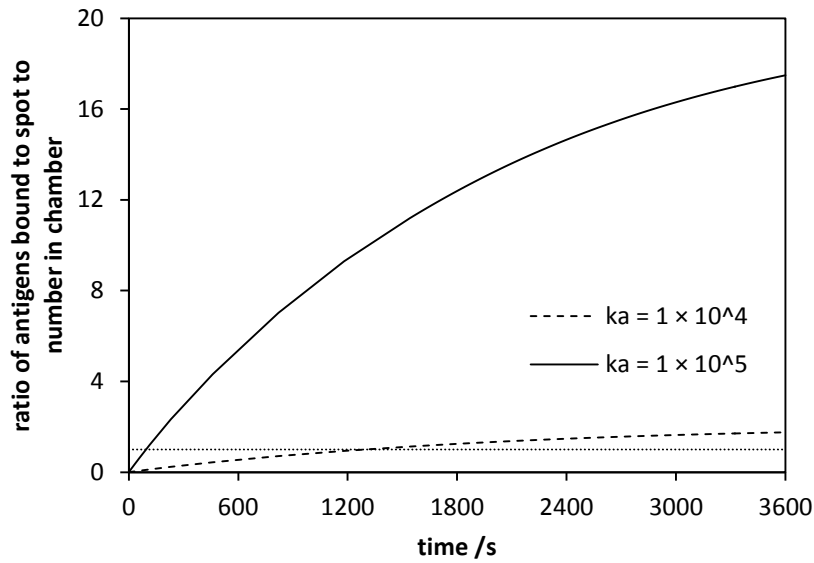


Figure 6.2: Protein binding under flow: model kinetic data showing how antibody spots may bind an amount greater than that present in the chamber when fluid is flowed at $1 \mu\text{L min}^{-1}$. The effect of altering the association constant k_a is shown. Modelled geometry is that as in §5.3. Horizontal dotted line indicates the number of antigens present in the chamber at any time t .

The relative amount of antigen bound to the spot as is present in the chamber at any time t under flow is modelled for 2 different k_a s (figure 6.2) and can be as high as 1250 % after 1800 s. Therefore, $8.6 \pm 3.3 \times 10^3$ GFPs bound to the spot suggests $6.9 \pm 2.6 \times 10^2$ GFPs flowing through the chamber volume at any time t at a concentration of 0.12 ± 0.05 pM. The number of cells compromised, assuming 1×10^5 GFPs/cell and a homogeneous background concentration, is $704 \pm 301/\text{mL}$ or $\sim 0.14 - 0.7\%$ of the total flowed through. Or for every 30 min run, the volume flowed ($30 \mu\text{L}$) would contain 21 ± 9 compromised cells. This assumes a k_a of $1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. For a k_a of $1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ the percentage of compromised cells per mL increases to $\sim 3 - 14\%$. For a k_a of $1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ the percentage increases such that it requires more cells than are present to be compromised.

The concentration of cells may be reduced but this will increase the trapping time; and by the same factor. GFP free in the cell media may also be reduced by washing cells through several rounds of centrifugation and resuspension in PBS upon harvesting. Several attempts at this saw no improvement in reducing background. This is most likely due to additional cells being compromised during the wash steps and further cell handling. This may have been quantified better by incubation of antibody spots with supernatant tested in the open-format; however, this was not performed.

The spot fluorescence may be reduced under flow as chambers are flushed through with 4% BSA in PBS. To expedite fluorescence decays of the spots, TIR beam power is increased to 10 mW and the

spot is continuously imaged under flow. It is observed that after 30 min of flow and continuous photobleaching the spot fluorescence is reduced to background. Upon ceasing flow and photobleaching, spot fluorescence starts to reaccumulate and must reach steady state before proceeding with lysis. A more effective photobleaching strategy would be to set the TIRF angle to 0° such that the TIR condition is no longer satisfied and the beam deeply penetrates the channel and PDMS walls. The contents of the entire chamber may be photobleached instead of the volume within the evanescent volume. However, the contents of the trapped cell will be undesirably bleached.

6.2 Alternative cell trapping strategy

To avoid the problems of free GFP in solution, non-diffusive flow and trapping efficiency an alternative strategy is made. An obvious solution is to remove the chambers from the main channel such that the antibody spots are no longer in direct communication with cell solution.

Fluid will only flow through a channel if there is a pressure difference, and the analogy has been made with voltage (pressure) and current (fluid flow) of an electrical circuit. The design shown in figure 6.3a satisfies this but free GFP in solution may diffuse into the chamber. This design is perhaps worse for free protein in solution as spot fluorescence would not be able to be reduced by flow removing dissociating antigen but only by photobleaching. Only the TIRF volume may be photobleached to avoid cellular photobleaching and would be time-consuming to photobleach fluorophores diffusing through the chamber volume. Furthermore, such a design is only applicable to an immunoassay employing a primary antibody and labelled antigen. Running a parallel channel dedicated to introduce secondary would now put a potential across the chambers and therefore a portion of flow from the main channel would redirect through the chambers (figure 6.3b). The design shown in figure 6.3c illustrates a design whereby there is no flow from the main channel and is compatible with a sandwich immunoassay. This design is based on that of Bang et al. [196] and has been used in the group for effecting cell sorting with the goal of applying this to enrich populations of circulating tumour cells from patient blood samples (Joseph Kaplinsky; Single Cell Proteomics Group). Here, a secondary antibody inlet is connected to the sorting reservoir via single cell pulldown chambers that are arranged in parallel.

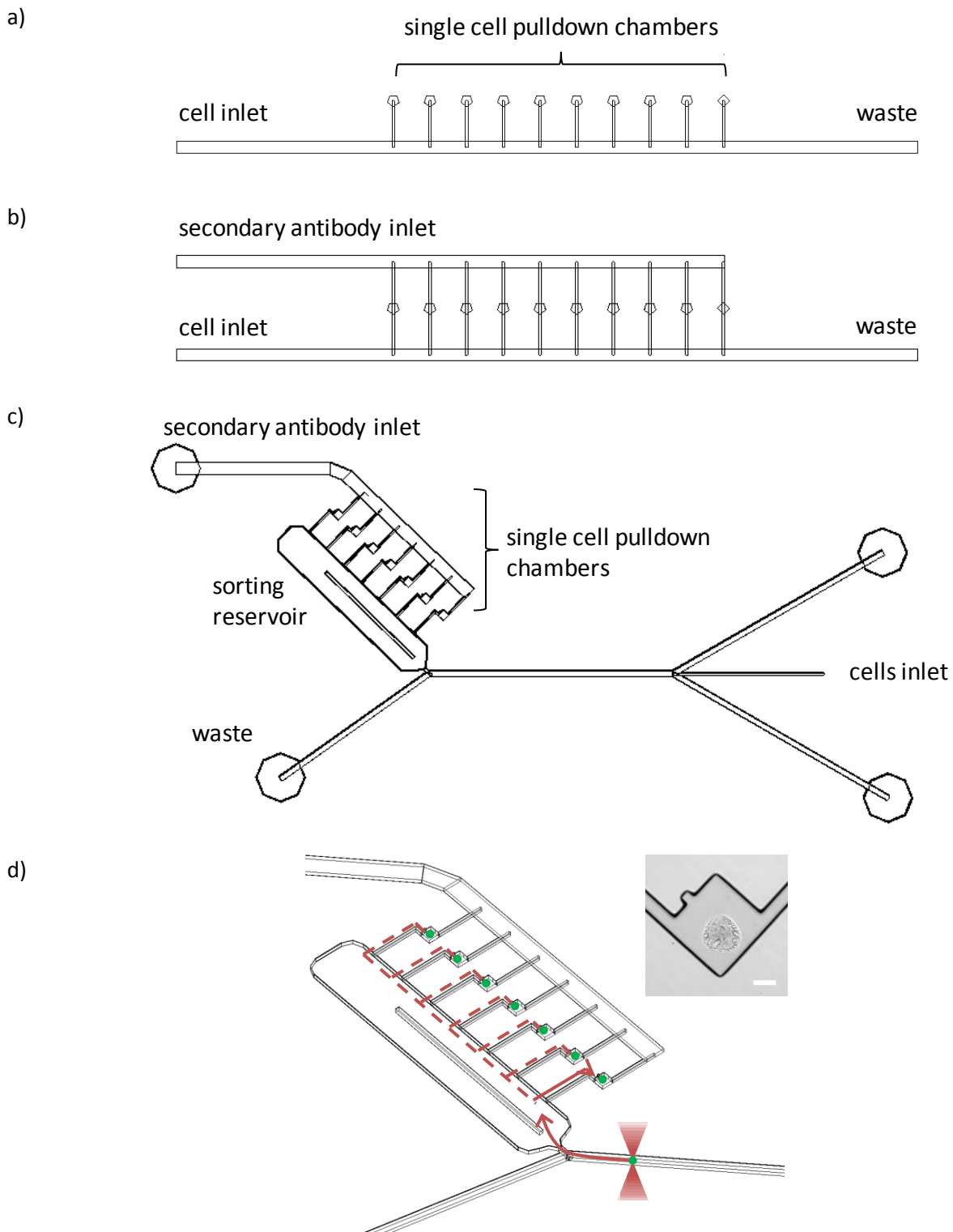


Figure 6.3: Alternative microfluidic device designs whereby single cell pulldown chambers are removed from the main channel. a) No pressure across each chamber therefore mass-transport occurs by diffusion only. b) By connecting the chambers to a common channel to introduce secondary antibody produces a pressure drop across each chamber branch and therefore a portion of flow will redirect through the chambers. c) Schematic of the device used for single cell experiments. d) An optical trap is used to move cells (green circles) from flow to analysis chambers. Inset: brightfield image of an antibody spot aligned within a chamber. Scale bar = 100 μm .

In order to trap cells into chambers that are not in direct communication with the main flow an optical trapping method is employed. Cells flow non-focussed through the main channel of the device at $1 \mu\text{L min}^{-1}$ and upon nearing the entrance to the sorting chamber flow is stopped and a cell is moved to each analysis chamber (figure 6.3d). The optical trapping beam is delivered via the same 60x NA = 1.49 oil-immersion objective and is integrated into the existing optical setup for the delivery of the lysis and TIRF beams. The integrated beam paths are shown schematically in figure 6.4.

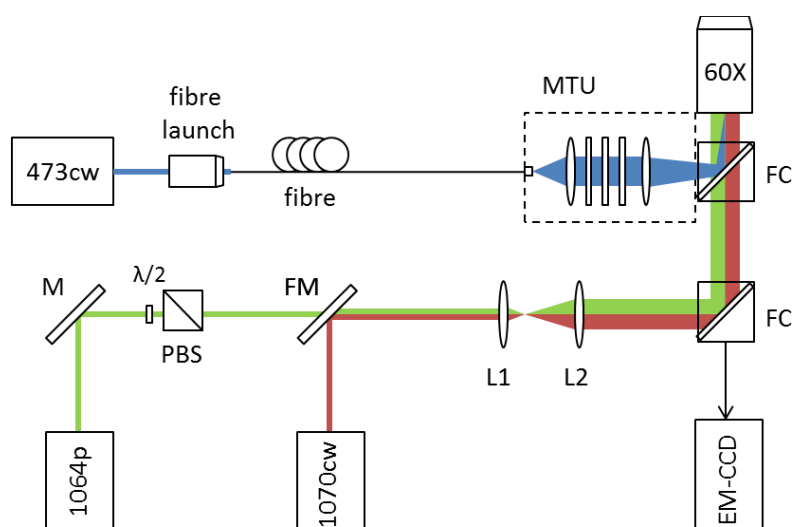


Figure 6.4: Schematic of the optical setup. 473cw = cw TIRF laser at $\lambda = 473 \text{ nm}$, 1064p = pulsed Nd:YAG laser at $\lambda = 1064 \text{ nm}$, 1070cw = cw Ytterbium fibre laser at $\lambda = 1070 \text{ nm}$, EM-CCD = electron multiplying CCD, M = mirror, FM = flip mirror, PM = periscope mirrors, $\lambda/2$ = half-wave plate, PBS = polarising beam splitter, L1 & L2 = lenses in beam expander, MTU = Nikon motorised TIRF unit, FC = filter cube.

The optical trap is formed of a single beam produced by an Ytterbium fibre laser (YLM-5; IPG Photonics, UK) at $\lambda = 1070 \text{ nm}$, with the objective focal and trapping planes being coincident. The objective lens back aperture is overfilled to ensure stable trapping at low powers, typically 50 – 75 mW, measured at the back aperture. A trapped cell is effectively moved through the microfluidic channels by translating the motorised stage. Before operation, the device is degassed and allowed to fill with 4% BSA in PBS in a desiccator chamber. Here, the role of BSA is two-fold: BSA serves to block the antibody spots from non-specific binding and to critically prevent cells sticking to the glass while being transported by the optical trap. The electrostatic forces between the glass coverslip and the cell cannot be overcome by the optical trap even at powers exceeding 200 mW and so the cell may become lost.

Cells may be translated at a typical speed of $15 \mu\text{m s}^{-1}$. The maximum time a cell is spent in the trap as it is being translated from the main channel is when a cell is being loaded into the chamber furthest from the main channel and is approximately 9 min; with the minimum time being around 4 min. Here, cellular photodamage is assessed qualitatively by any blebbing of the cell membrane and the impact the optical trap has on GFP fluorescence. GFP fluorescence was imaged every 120 s for 3 cells under the influence of the optical trap and 1 control cell for a duration of 10 min. Over the course of 10 min no blebbing was observed and the action of the trap did not affect GFP fluorescence (figure 6.5).

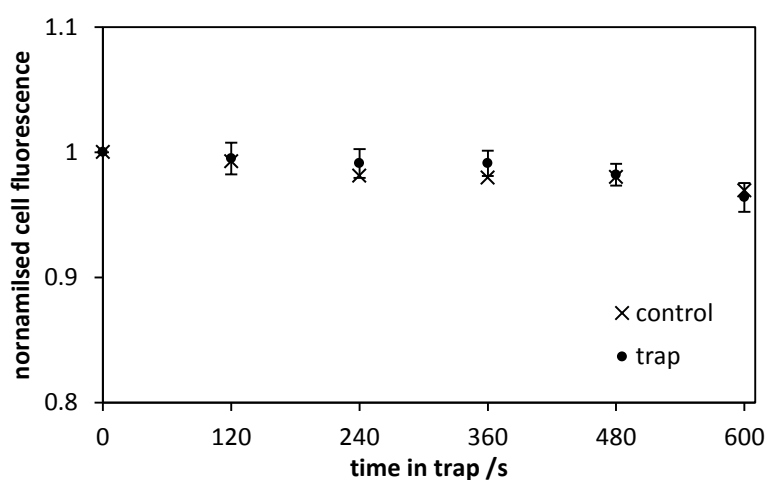


Figure 6.5: Normalised GFP cell fluorescence of cells in an optical trap ($n=3$) compared to a control cell that is not showing that the trap has no effect on GFP fluorescence. Of course, stricter viability tests for other proteins and their networks will need to be undertaken.

Single cell pulldown chambers comprise a main compartment of $300 \mu\text{m} \times 300 \mu\text{m} \times 50 \mu\text{m}$ (width $\times$ length $\times$ height) and a small cubicle measuring $40 \mu\text{m} \times 40 \mu\text{m} \times 50 \mu\text{m}$ with a total volume of 4.6 nL. Cells are loaded into each chamber and placed in the cubicle to protect cells from being removed from the chamber by the introduction of secondary antibody (figure 6.6a). The flow velocity profile through the chamber under $1 \mu\text{L min}^{-1}$ flow is simulated (figure 6.6b) and there is no flow that will penetrate the cubicle and displace the cell. The fluid velocity normal to the cubicle aperture within the cubicle is $\pm 1 \text{ nm s}^{-1}$ which indicates fluid is stagnant, follows closed flow lines and is of a magnitude insufficient to displace cells (figure 6.6c).

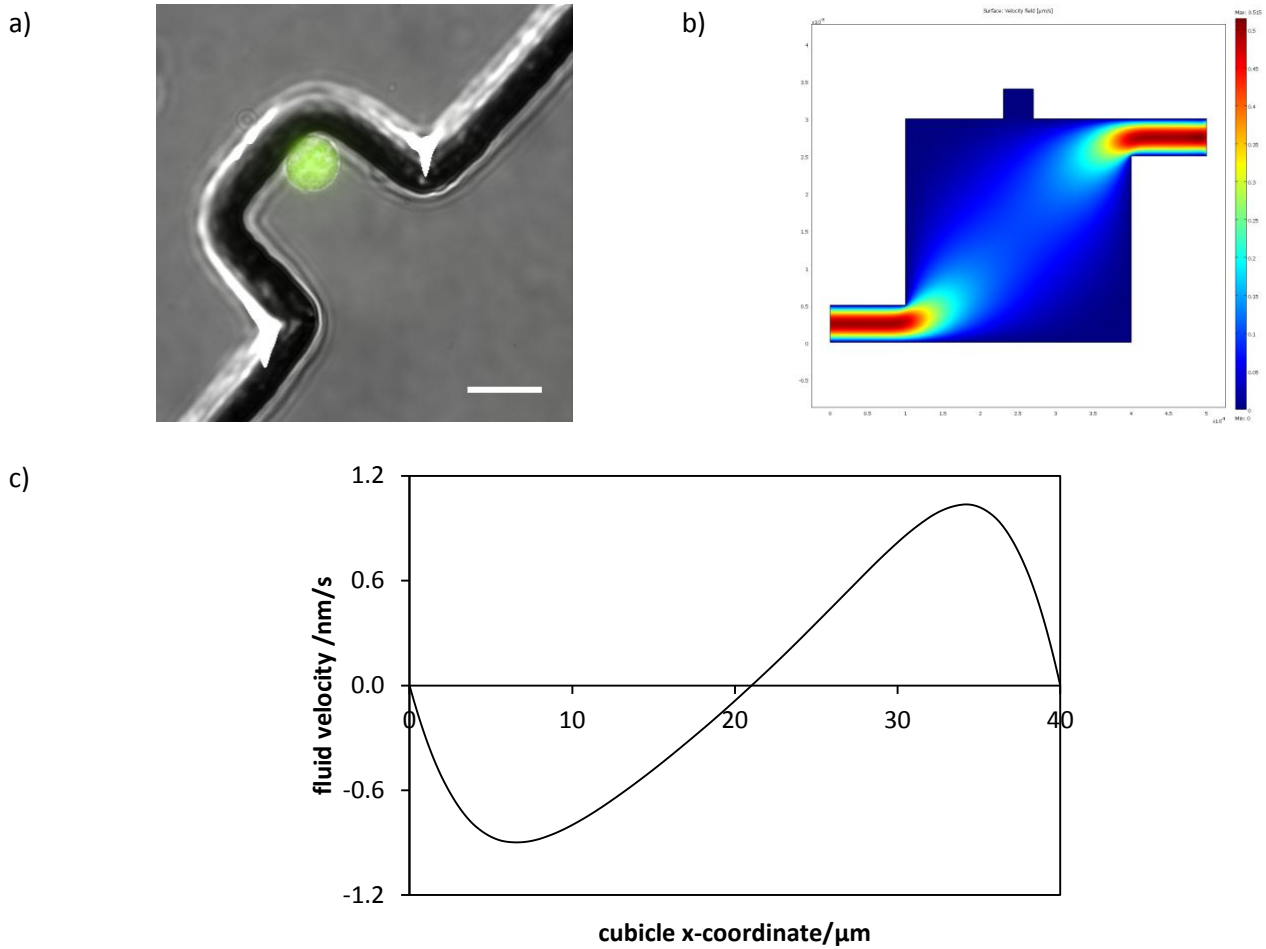


Figure 6.6: Profiling the ‘optical trap’ chamber. a) Cells are loaded into a cubicle, as depicted in figure 6.3d, measuring $40\ \mu\text{m} \times 40\ \mu\text{m}$ which protects cells from displacement under flow. Scale bar $15\ \mu\text{m}$. b) Simulation of the velocity flow profile through the chamber showing that there is no flow into or out of the chamber indicating the strong containment of the trap. c) A velocity line profile is made perpendicular to the cubicle entrance aperture $20\ \mu\text{m}$ into the cubicle. The flow is symmetric and opposed indicating that fluid follows closed flow lines and mass transport occurs only by diffusion. Therefore, the cubicle serves well as a trap for the cell.

6.3 Single Cell Pulldowns

6.3.1 eGFP pulldowns

Upon cell lysis, an automated time-resolved acquisition is made, managed by software macros which control microscope stage position and the auto-focussing system. Before degassing and wetting the device with 4% BSA in PBS, the coordinates of dehydrated antibody spots are determined relative to a chamber vertex and stored in an acquisition macro so that antibody locations are not lost upon removing the device to a desiccator. BE cells stably transfected with GFP cells are flowed through

the main channel and each chamber is loaded with a single cell. Antibody spot fluorescence is not increased by the flowing of cells through the device or by the loading of a cell into the chamber.

Prior to lysis, cells are imaged using widefield epi-fluorescence as an indicator of the amount of GFP in the cell. It follows that the greater the number of GFP proteins in a particular cell, the brighter that cell will be. Upon lysis, antibody spots are imaged by TIRF in two phases. In phase 1, where accumulation is at its highest rate, spots are imaged every 5 min for 30 min followed by phase 2, where equilibrium is, or close to being, established and spots are imaged every 20 min for a further 40 min. Relative GFP fluorescence is compared prior to and following lysis in order to determine that the single cell pulldown method can faithfully measure the relative levels of GFP in lysed cells and therefore variation in protein level. The precision by which the single cell pulldown method can measure protein level may then be determined.

To minimise photobleaching, low excitation power and a short duty cycle of 0.075 - 0.3 % are employed; however photobleaching effects are effectively cancelled out when making relative measurements so long as the experimental conditions are always the same.

First controls experiments are performed. In the 7 chambers, 6 are loaded with single BE/GFP cells and 1 is not. Of the 6 chambers loaded with single cells, 3 undergo lysis and 3 do not. Only the antibody spots in chambers in which cells are lysed accumulate cytosolic GFP which is freed upon lysis to bind to an antiGFP antibody spot within the same 4.6 nL chamber. No such accumulation is observed when cells are not lysed or present (figure 6.7a). The background is monitored up to 10 hours and no accumulation is observed.

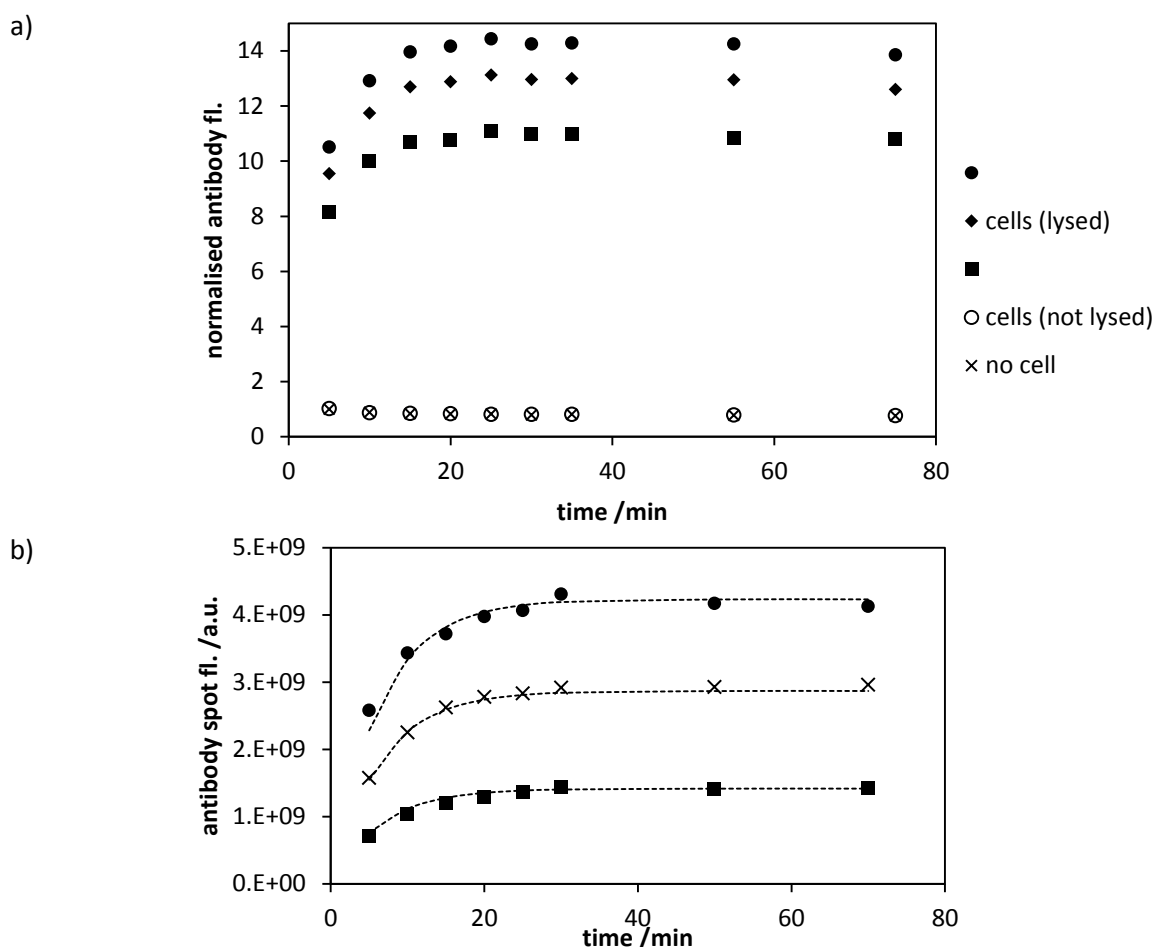


Figure 6.7: a) Time resolved plots of antibody fluorescence showing accumulation of bound GFP onto the spot in chambers where a cell is lysed ($n=3$). The symbols, squares (■), diamonds (◆) and circles (●) represent 3 separate measurements of protein from a single cell binding to an antibody spot. In chambers where a cell is present but not lysed ($n=3$, error bars too small to be shown) or if a chamber does not contain a cell at all ($n=1$) then no accumulation is observed. Antibody fluorescence is normalised to spot fluorescence prior to lysis. b) 3 more example plots of GFP binding to antibody spots over time. Dashed lines are fits to the data with $k_{\text{obs}} = 2.58 \times 10^{-3} \text{ s}^{-1}$. The symbols, squares (■), crosses (X) and circles (●) represent 3 separate measurements of protein from a single cell binding to an antibody spot. The variation between the curves reflects the varying levels of GFP from cell to cell.

Figure 6.7b shows accumulation curves, each from single BE cells. The binding kinetics are approximately described by equation 5.7. Curves are fit globally with an observed antibody binding constant of $k_{\text{obs}} = 2.58 \times 10^{-3} \text{ s}^{-1}$. Simulated kinetic data is fit to experimental data in order to estimate the association constant k_a . In §5.3, a maximum binding density was estimated to be 16.0 nmol/m^2 and assumes that all antibodies are functional and perfectly packed across the spot. It is safe to assume that not all antibodies will be orientated correctly relative to the substrate or be functional. This may be taken into account by reducing the binding density. The association rate constant is inversely proportional to the binding density i.e. a unit magnitude decrease in binding

density is concomitant with a unit magnitude increase in k_a . For a spot comprising antibodies at maximum binding density having $k_d = 0.54 \pm 0.08 \times 10^{-3} \text{ s}^{-1}$, the association rate constant is estimated to be $2.25 \pm 0.75 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ by simulation. Therefore, the antibody affinity, K , defined as the dissociation rate constant divided by the association rate constant, is $24 \pm 9 \text{ nM}$. If only 10% of the antibody in the spot is functional, i.e. binding density is reduced 10-fold, then the association rate constant and antibody affinity are $2.25 \pm 0.75 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $2.4 \pm 0.9 \text{ nM}$, respectively. Furthermore, at 1% functionality the antibody affinity becomes sub-nanomolar at $0.24 \pm 0.09 \text{ nM}$. These are reasonable estimates for the antibody parameters.

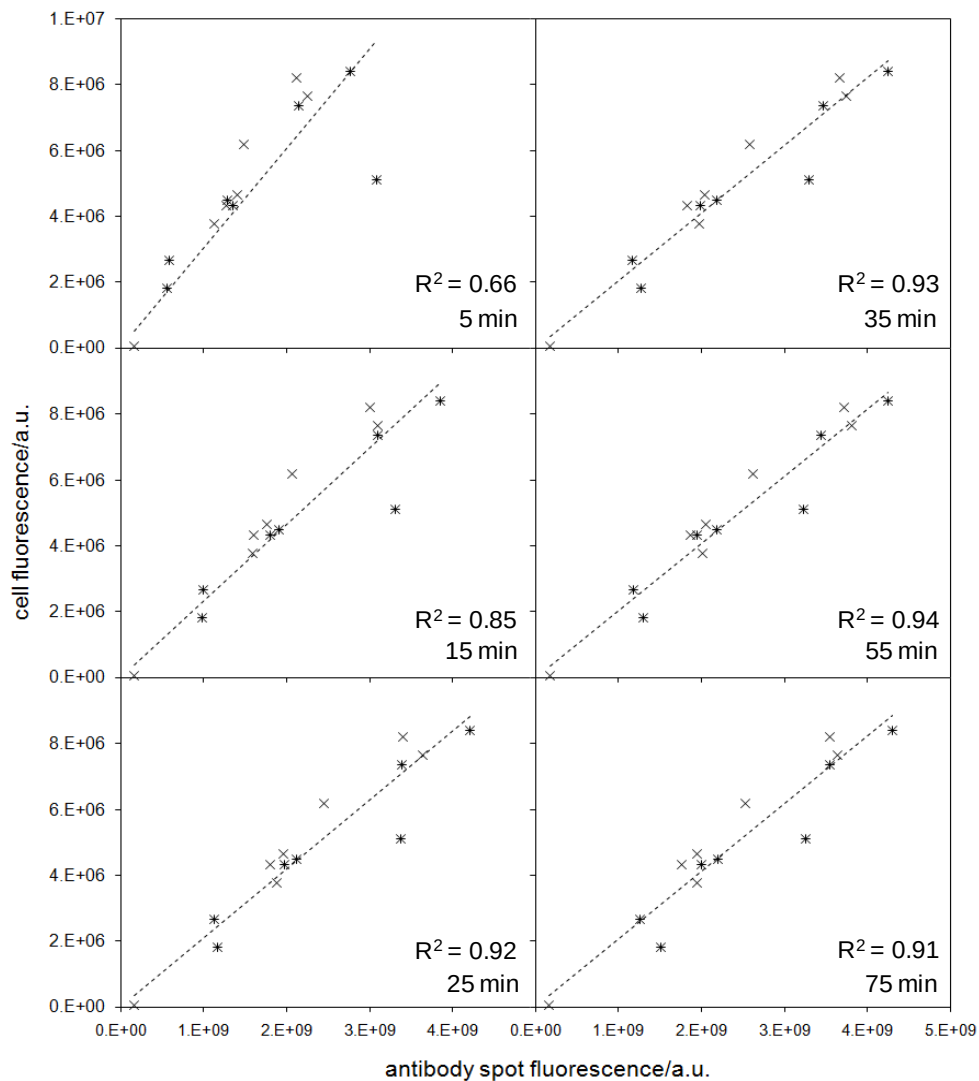


Figure 6.8: Correlation between the amount of GFP in cells, as measured by widefield fluorescence, and the amount of GFP bound to each antibody spot, as measured by TIRF, over the time-course of the experiment. Widefield fluorescence levels are on the vertical axis and fluorescence from TIRF measurements of protein bound to the antibodies on the chip surface is on the horizontal axis. The stars and crosses represent data from two different experiments to give a feel of the reproducibility of the methodology. The straight lines are fits to the data and the high degree of correlation (R^2) shows that the TIRF measurements faithfully recover the level of protein as measured by widefield microscopy.

Plots of cellular GFP fluorescence prior to lysis as measured by widefield epi0fluorescence against antibody spot fluorescence post lysis as measured by TIRF is shown in fig. 6.8 for six sequential time points. The stars and crosses represent data from two separate experiments to give a feel of the reproducibility of the methodology. The straight lines are fits to the data and the high degree of correlation shows that the TIRF measurements faithfully recover the level of protein as measured by widefield microscopy. It is clear that the binding is not saturating the antibody spot as if it were there would be no correlation between the GFP levels measured by TIRF and those measured using wide-field microscopy. As long as the antibody patch has a significantly higher density than the copy number then saturation will not occur.

The number of GFPs captured per cell can be estimated from dividing the image intensity by the known average intensity of a single GFP molecule (figure 6.9). Images of 10 single GFP molecules bound to an antibody spot are fit to a two-dimensional symmetrical Gaussian function. The parameters from all fits were averaged and used to estimate the number of GFPs bound to each antibody spot at steady state after 35 min. This ranged from 0.41×10^5 , for the lowest intensity spot, to 2.7×10^5 , for the highest. According to simulation, the amount of GFP bound to the antibody spot in a $300 \mu\text{m} \times 300 \mu\text{m} \times 50 \mu\text{m}$ chamber is 81 – 89 % of the total amount present in the chamber. Upon lysis, protein may be adsorbed onto the PDMS surface and diffuse out of the chamber so the level of cellular GFP will be higher on account of such losses. The total capture efficiency will need to be determined if the method is to be absolutely quantitative.

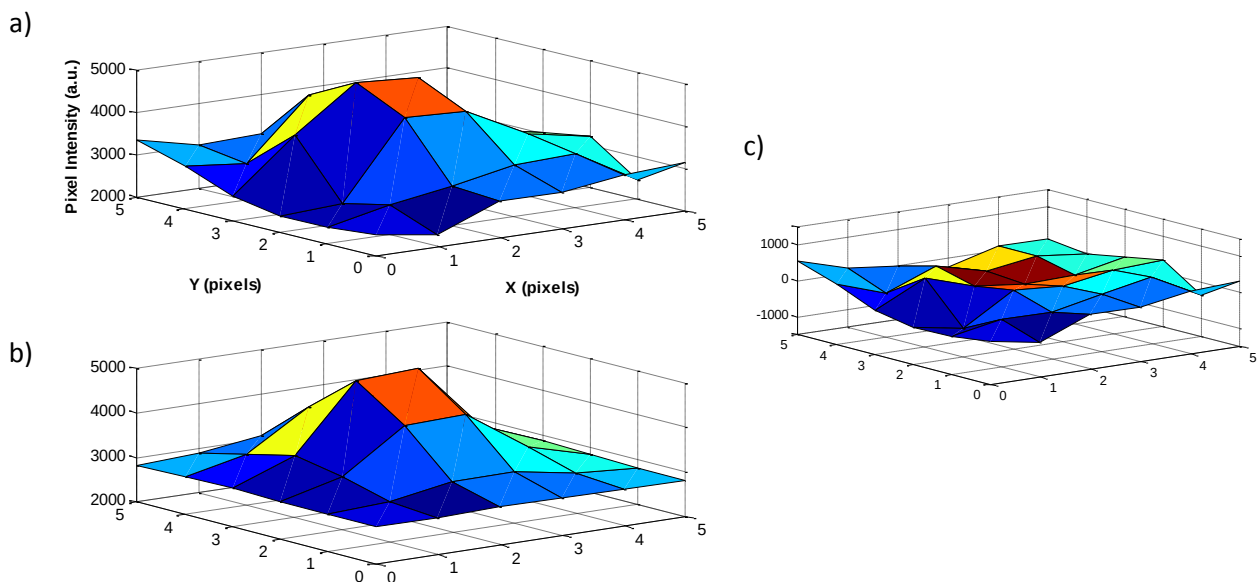


Figure 6.9: (a) The fluorescence point-spread function of a single GFP molecule measured by the EM-CCD is (b) fitted to a 2D Gaussian function and is used to estimate the number of GFP molecules bound to an antibody patch. (c) The residuals of the example fit.

Nevertheless, the method currently serves as a measure of the relative abundance of protein. The precision of the method in measuring the relative levels of protein can be given a lower limit by making the assumption that the widefield fluorescence measurements are perfectly precise. In this case the standard deviation between the protein levels measured in the cell by widefield epifluorescence and bound to the antibody spot by TIRF is found to be 12 %. This means that the precision of the single cell pulldown method is 88 % at worst and could in fact be better than this depending on the actual precision of the widefield fluorescence measurement methodology.

6.3.2 p53 pulldowns

In order to make measurements on unlabelled proteins and to demonstrate the generality of the methodology we have also employed a sandwich assay format where a primary antibody printed onto the glass surface captures the protein of interest while a secondary antibody carrying a fluorescent label, present in the chambers at 50 ng/mL, also binds to the captured protein in order to make each protein detectable by TIRF. To demonstrate this capability the amount of p53 is measured in the breast-cancer cell line MDA-MB468.

Using the same protocols as for the preparation and printing of antiGFP antibody devices, anti-p53 devices are constructed. The anti-p53 antibody affinity was observed to be much lower when tested the antibody with recombinant p53. The background transient count rate in one image frame is 100 proteins due to transient non-specific binding of the secondary antibodies so that in each frame approximately 100 of the counts are due to this background. Therefore, in our setup the limit of detection for anti-p53 is 100 proteins.

Using single molecule counting as for GFP, it is estimated that 1500 ± 200 proteins are captured on average from each cell. In order to demonstrate the linearity of the fluorescent signal with protein copy number, 1 – 4 cells were deposited in chambers and lysed (figure 6.10). As can be seen, the fluorescence increases linearly with the number of cells lysed. Those deviations from linearity that are visible from the straight line fit to the data almost certainly reflect genuine variation in protein copy number from cell to cell. Those deviations from linearity that are visible from the straight line fit to the data almost certainly reflect genuine variation in protein copy number from cell to cell. No replicates were performed and therefore is representative data.

Hu et al., using an ELISA kit, measured p53 protein levels in a cell line where p53 expression was regulated by a tetracycline promoter [181]. Minimum levels were measured at 0.57×10^5 which

increased to 5.9×10^5 upon complete withdrawal of tetracycline. Ma et al. have made similar measurements [182] in 7 different cell lines and report p53 levels from $2 - 20 \times 10^4$ per cell, 5 lines of which with levels less than 5×10^4 . It is highly likely that each cell contains significantly more p53 than the 1500 copies captured in these devices. This relatively low number almost certainly reflects the high affinity of p53 for DNA and for other proteins, the binding of which is likely to obscure the recognition epitope for either the primary or secondary antibodies. For now therefore, and in the absence of any further information, the 1500 copies captured are probably representative of the fraction of p53 unbound to DNA or other proteins. Nevertheless it proves the principle that we can reliably determine relative levels of protein in single cells without the need to resort to labelling of the proteins.

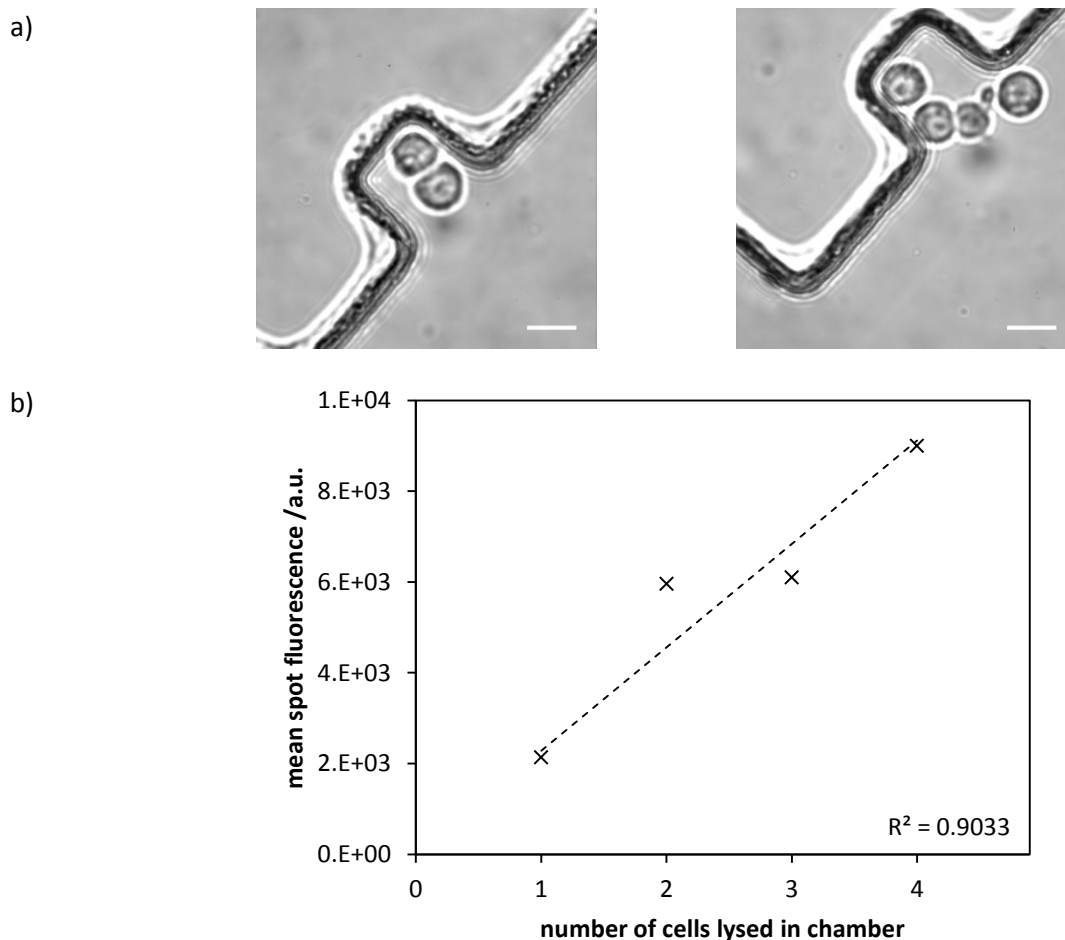


Figure 6.10: a) Brightfield images of multiple cells deposited into the chamber cubicles by the optical trap. Scale bar 20 μm . b) One, two, three and four MDA-MB468 cells are loaded into separate chambers followed by lysis, capture of the protein and analysis. The figure shows that the p53 signal increases linearly with cell number and that the response is not saturating.

6.4 Conclusions

A microfluidic platform that is able to isolate and trap single cells in individual analysis chambers and perform a proteomic measurement at single cell resolution has been demonstrated [197].

The hydrodynamic trapping device that is designed and characterised in §3 was replaced by device which removed the analysis chambers from the path of cell flow. This change was made in order to circumvent the problems with protein background in the flow that bound to antibody spots before any single cell pulldown experiment commenced. In order, then, to trap single cells in the chambers, a single-beam optical trap was used. The use of the optical trap can make trapping many cells time-consuming and is best suited for investigations where only a few cells are available such as with circulating tumour-cells and stem cells.

Once cells were trapped the single cell pulldown experiments proceeded as expected from the results in §4 (lysis) and §5 (readout). To demonstrate the ability of the method, BE cells stably transfected with cytoplasmic eGFP were used. GFP is autofluorescent and therefore does not require a labelled secondary antibody to afford a fluorescent readout. The amount of eGFP pulled down by an antiGFP antibody and measured by TIRF microscopy was shown to strongly correlate ($R^2 > 0.9$) with the widefield fluorescence of the cell prior to lysis. From this the precision of the system in measuring the relative levels of protein was calculated to be $\geq 88\%$.

Once the method was determined to accurately readout levels of protein using a transfected cell line, a non-transfected cell line was used to demonstrate the capability to measure unlabelled proteins. The protein p53 was measured in the breast-cancer cell line MDA-MB468 using an antibody sandwich system. It is estimated that these cells contain 1500 ± 200 copies of free p53.

PART II

AUTOFLUORESCENT DETERMINATION OF METABOLIC PHENOTYPE

7 Redox metabolism

The study of rare cells such as stem cells or circulating tumour cells does not lend itself to high-throughput population-based methods. Therefore, the development of techniques that allow multiple measurements to be conducted on the same cell to maximise information on cell state is vital. This project also aims to develop multidimensional fluorescence imaging technology for the monitoring and analysis of the metabolic phenotype of single cells, which will inform a proteomic measurement of a cell. This chapter introduces the concept of metabolic imaging and how it may be applied to certain models of disease.

- **Metabolic imaging**

Metabolic imaging exploits the autofluorescence the key cofactors in metabolic pathways, NADH and FAD, in order to determine the metabolic state of a cell. To understand how a change in a fluorescence parameter reflects a change in metabolic state the basic metabolic pathways of the cells are described with particular relevance to NADH and FAD. This will be referred to heavily in §9 when interpreting results.

- **Cell models**

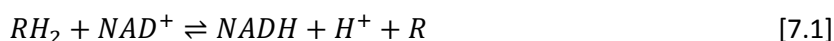
In certain diseases, metabolism becomes altered and could serve as a biomarker. It has the potential to inform a proteomic measurement of a cell enabling one to know the disease state of the measured cell. The cell models of cancer, stem cell differentiation and heart failure are discussed in terms of altered metabolism and are investigated using human BE cells, H7 embryonic stem cells and adult rat cardiomyocytes, respectively.

7.1 Autofluorescence

Autofluorescence originates from fluorescent molecular species which are intrinsic to a tissue or cell. Often it is considered a low level background that can interfere with detection of specific fluorescent signals particularly when the signal level is low. Methods exist to reduce or eliminate autofluorescence but an understanding of its molecular origins can provide key insight into cellular processes. Most notably is with the case of nicotinamide adenine dinucleotide (NADH), as it serves as a non-invasive method to measure metabolism without the use of exogenous species that may perturb or, in some cases, poison the cell. At the tissue level, collagen and elastin, which make up the extracellular matrix and provide rigidity to the lumen of blood vessels, are a significant source of

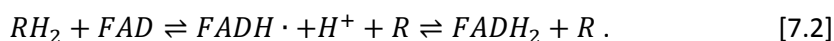
autofluorescence. At the cellular level, NADH, flavin adenine dinucleotide (FAD) and flavin mononucleotides (FMN) are the principal autofluorescent species.

NAD⁺ is synthesised from nicotinamide, the niacin form of vitamin B3, which is joined with ribose and adenosine diphosphate (ADP). In redox metabolism it is key to the electron transfer reactions that ultimately deliver electrons from the oxidation of substrates such as glucose to the electron transport chain to synthesise ATP (adenosine triphosphate). Electrons are transferred as part of glycolysis, the tricarboxylic acid cycle and through to oxidative phosphorylation. NAD⁺ is the oxidised form and accepts electrons from metabolic substrates that are oxidised resulting in the reduced form NADH, which in turn is used as a reducing agent to donate electrons. Although, NAD⁺/NADH may serve other roles in the cell, its principal function is in electron transfer. The general reaction is as follows



where the reactant RH₂ may be glucose, pyruvate or a metabolic intermediate or substrate. The reaction may proceed in the reverse direction allowing NAD⁺ to be regenerated without being consumed and is continuously cycled between oxidised and reduced forms. The ratio of NAD⁺/NADH is known as the redox ratio and determines the relative environment of the cell to be reducing or oxidising and permits the metabolic state of a cell to be known.

Similarly, the flavins, FAD and FMN, are cofactors involved in redox metabolism, but exclusively in the mitochondria. FMN consists of a flavin moiety attached to a single phosphate group via ribitol and an adenosine monophosphate group attached to the phosphate group makes FAD. FAD and FMN are collectively referred to as FAD. FAD is the oxidised form and accepts electrons from metabolic substrates that are oxidised or NADH resulting in the reduced form FADH₂, which in turn is used as a reducing agent to donate electrons. The general reaction is similar to that of NADH. However, FAD can carry one or two electrons,



NADH and FAD absorption and emission spectra are shown in figure 7.1. NAD⁺ and NADH both exhibit peak absorption in the deep UV at around 220 nm and 260 nm but only NADH has an absorption peak at 340 nm with a corresponding broad emission band peaking at around 450 nm [198] (figure 7.1a). The phosphorylated form of NADH, i.e. NADPH, is spectrally indistinguishable from NADH and although methods exist to resolve the two [199], the vast majority of studies do not and treat NADH and NADPH fluorescence as an ensemble measurement, sometimes referred to as

NAD(P)H. The minimal involvement of NADPH in energy metabolism makes its fluorescence a minor and roughly constant background signal [200].

Similarly, FAD and FADH₂ exhibit absorption peaks at around 220 nm and 270 nm but only FAD has absorption peaks at around 370 nm and 450 nm with a corresponding broad emission band peaking around 515 nm (figure 7.1b).

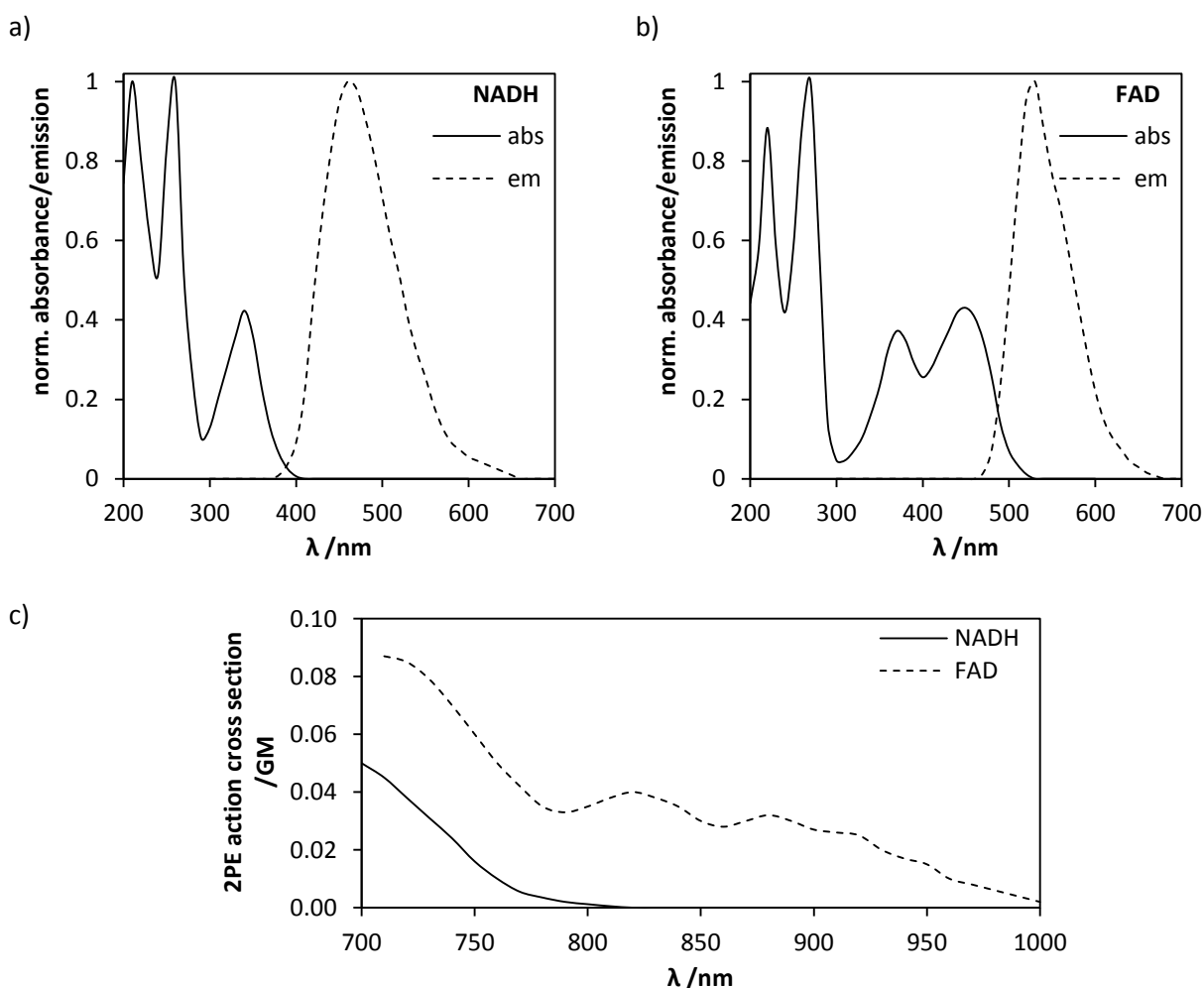


Figure 7.1: Normalised absorption and emission spectra for a) NADH and b) FAD. Emission spectra of NADH and FAD are obtained at excitation wavelengths of 340 nm and 450 nm, respectively. c) 2PE action cross section ($1 \text{ GM} = 10^{-58} \text{ m}^4 \text{ s}$) spectra of NADH and FAD. Adapted from [201].

FAD and NADH may be co-excited at 330 – 370 nm; however, there is significant overlap in their emission spectra. NADH is therefore excited at 330 – 370 nm and FAD at around 450 nm. Figure 7.1c shows the two photon excitation (2PE) cross sections for FAD and NADH (see §8.1). 2PE excitation of NADH and FAD is therefore made at 740 nm and 850 nm to maximise signal and to minimise spectral overlap and photodamage.

In addition to fluorescence intensity, the fluorescence lifetime (see §8.1) of NADH and FAD has been shown to change depending on their local environment. These changes predominantly depend on whether the cofactors are free in solution or bound to a protein and to which protein they are bound. Typically, free NADH has a lifetime of 0.3 – 0.5 ns which increases to around 2.0 – 3.0 ns when bound whereas free FAD exhibits a lifetime of around 3.0 ns which decreases to around 0.1 – 0.5 ns when bound [202, 203].

So, FAD and NADH are fluorescent whereas FADH_2 and NAD^+ are not and also their fluorescence lifetimes change when bound to different enzymes. By measuring these parameters and their ratios of the autofluorescence of NADH and FAD it has been shown that the metabolic state of a cell may be determined.

7.2 Metabolic pathways

Living organisms require a continual input of free energy through cellular metabolism for the performance of mechanical work, active transport of molecules and ions and the synthesis of macromolecules and proteins from their amino acid precursors. The carrier of free energy is adenosine triphosphate (ATP), which is derived from the oxidation of metabolites such as carbohydrates and fatty acids. This does not happen directly but is mediated by the oxidised forms of nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD), which feed the mitochondrial electron-transport chain (ETC) for ATP synthesis.

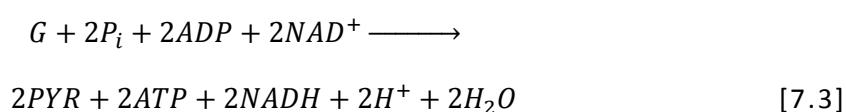
7.2.1 Glycolysis

Overall, the pathway of glycolysis, formally the Embden-Meyerhof-Parnas pathway, is the cleavage of six-carbon glucose into three-carbon units of pyruvate. The sequence involves a net generation of ATP and NADH whilst also providing energy and intermediates which may be removed for use in other metabolic pathways. The process is present within all cells of the human body and occurs within the cytosol. Glycolysis is unique amongst metabolic pathways as it has the ability to produce ATP under both aerobic and anaerobic conditions and for many tissues becomes an emergency energy producer when oxygen supply is a limiting factor.

Glucose enters the cytosol via facilitated diffusion mediated by membrane bound glucose transporters and is immediately phosphorylated by hexokinase to glucose-6-phosphate (G6P). This

serves to restrict glucose within the cell due to lack of G6P transporters and acts to maintain the glucose concentration gradient, promoting continuous transport of glucose into the cell. The full pathway of glycolysis is shown in figure 7.2. Glycolysis comprises two phases: an energy investment phase where ATP is used to activate and increase the energy content of the intermediates, phase I, and an energy generation phase where ATP is generated, Phase II.

ATP can be synthesised from ADP by either substrate-level phosphorylation or oxidative phosphorylation. Substrate-level phosphorylation is the direct transfer of a phosphate group to ADP from a reactive intermediate and is performed in glycolysis. The net reaction in the oxidation of glucose to pyruvate is



NAD^+ is the primary oxidising agent of glycolysis and an important cofactor; however, it is limited in availability and must be regenerated from NADH. The energy yield of glycolysis depends on the fate of pyruvate which in turn depends whether the cell is respiring aerobically or anaerobically. Pyruvate may be anaerobically metabolised to lactate by lactate dehydrogenase, with NADH being oxidised to NAD^+ in the cytosol. This is a reversible reaction in which the direction is determined by the ratio of NADH to NAD^+ .

Under aerobic conditions, pyruvate is transported to mitochondria where it enters the tricarboxylic acid (TCA) cycle. NAD^+ is regenerated by the electron transport chain by oxidising NADH.

The amount of glycolysis, sometimes referred to as glycolytic flux, is adjusted in response to intra- and extracellular conditions. The main regulatory sites are the irreversible reactions (red arrows, figure 7.2) catalysed by hexokinase, phosphofructokinase-1 (PFK-1) and pyruvate kinase. PFK-1 is the most important of these controls which is allosterically inhibited by high levels of ATP. This lowers its affinity for fructose-6-phosphate. Citrate, produced in mitochondria, enhances the inhibitory effect of ATP on PFK-1 since an increased concentration in both indicates an abundance of metabolic intermediates and products. Adenosine monophosphate (AMP), indicative of a low energy charge in the cell, reverses the inhibitory action of ATP. Therefore, glycolysis is stimulated or inhibited as the energy charge falls or rises, respectively. Additionally, pyruvate kinase is also inhibited by high levels of ATP and hexokinase is inhibited by high levels of glucose-6-phosphate.

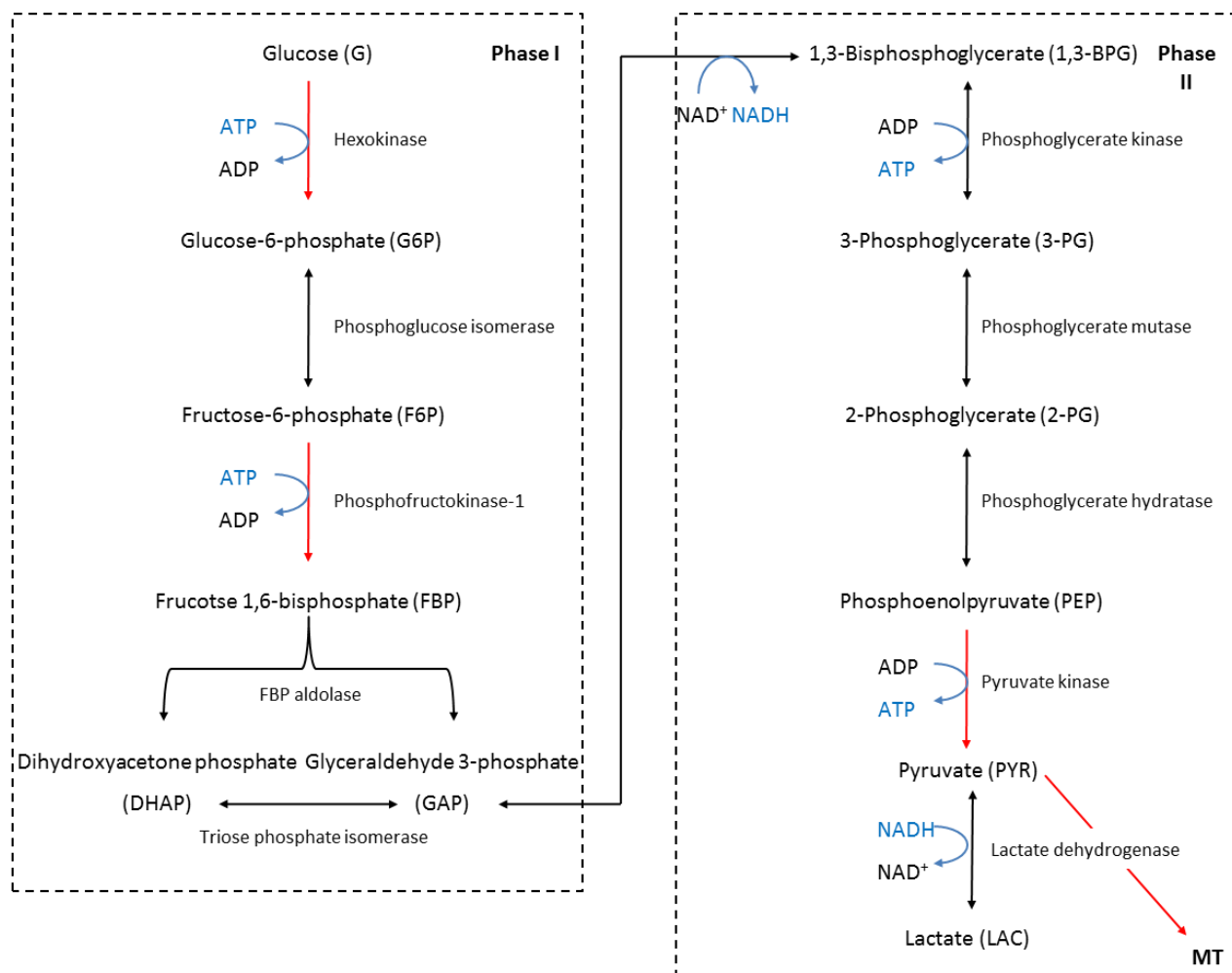
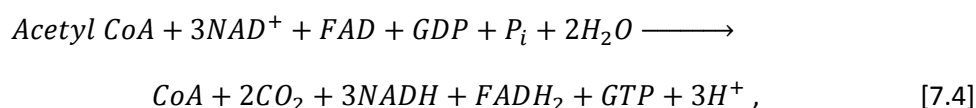


Figure 7.2: The pathway of glycolysis highlighting the redox reactions involving NADH. Red arrows indicate irreversible reactions and serve as points of regulation.

7.2.2 The tricarboxylic acid cycle & oxidative phosphorylation

Under aerobic conditions, an alternative fate of pyruvate is entry into the TCA cycle. Pyruvate is transported into mitochondria by monocarboxylate transporter (MCT) where pyruvate dehydrogenase catalyses the irreversible oxidative decarboxylation to acetyl co-enzyme A (acetyl CoA). The TCA cycle proceeds exclusively within mitochondria and provides a final common pathway for the oxidation of carbohydrate (glucose), fat and protein and is also an important source of biosynthetic precursors. It is a cyclical series of reactions (figure 7.3) that oxidises acetyl CoA completely to produce CO₂, generating energy, either directly as guanosine triphosphate (GTP) or in the form of the reducing equivalents NADH and FADH₂. The overall reaction may be written as



where GDP is guanosine diphosphate. GTP may be easily converted to ATP and is used primarily in protein synthesis. The TCA cycle itself neither generates large amounts of ATP nor includes oxygen as a reactant. The main function of the TCA cycle is to harvest electrons to form NADH and FADH₂. In oxidative phosphorylation, high transfer potential electrons are released from NADH and FADH₂ and are used in the ETC. These electrons are used to reduce molecular oxygen to water via a series of inner mitochondrial membrane-bound electron carriers and the free energy liberated is used to generate ATP. This electron flow leads to the pumping of protons out of the mitochondrial matrix which produces a pH gradient and a transmembrane electric potential that creates a proton-motive force. ATP is synthesised when protons return to the mitochondrial matrix via ATP synthase.

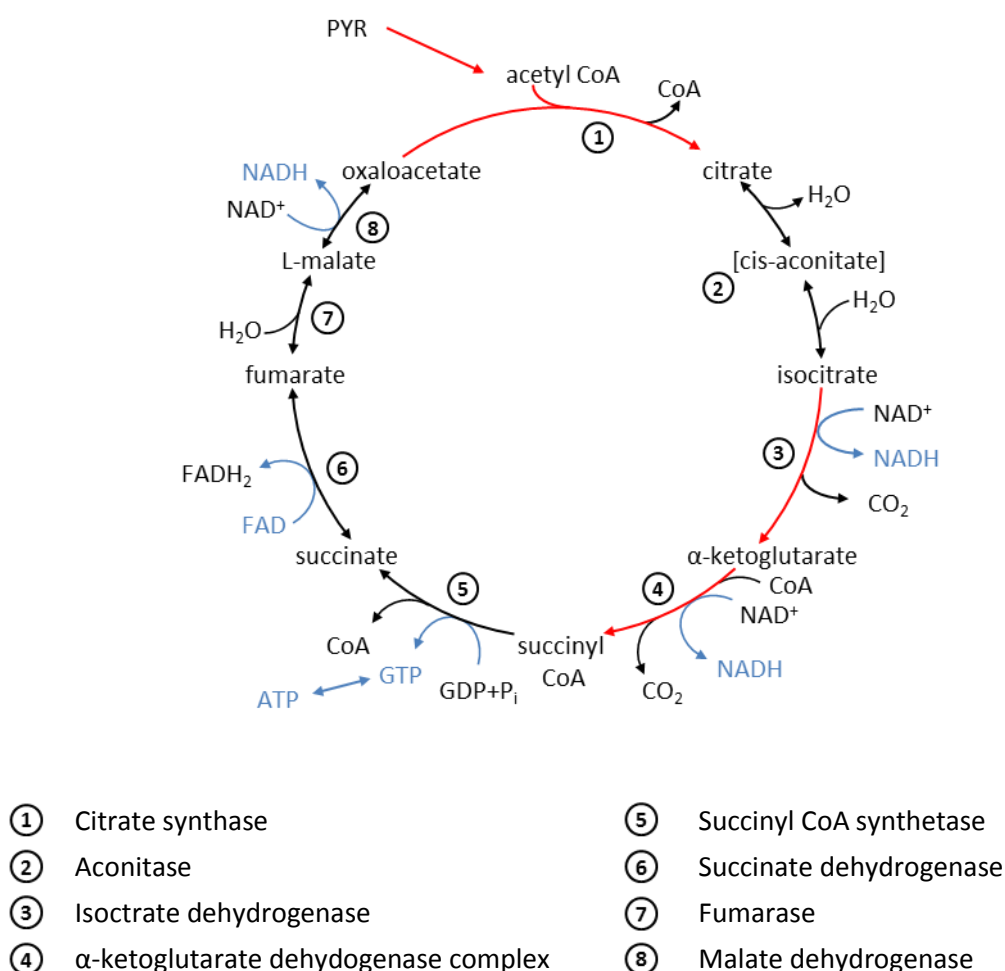


Figure 7.3: The tricarboxylic acid cycle, which is spatially compartmentalised within the mitochondria. The cycle produces energy rich NADH and FADH₂ that are used to generate ATP.

The control of the cycle, like that of glycolysis, is made by allosteric regulation and additionally, by respiratory control. The main sites of regulation are, again, irreversible reactions within the cycle i.e. those catalysed by citrate synthase, isocitrate dehydrogenase and α -ketoglutarate dehydrogenase (red arrows, figure 7.3). Isocitrate dehydrogenase is allosterically stimulated by ADP being mutually cooperative with NAD^+ , Mg^{2+} and the substrate isocitrate. NADH and ATP inhibit iso-citrate dehydrogenase by directly displacing NAD^+ and ADP, respectively. Control of α -ketoglutarate dehydrogenase is similar to that of isocitrate dehydrogenase and is inhibited additionally by succinyl CoA, the reaction product. Several steps in the TCA cycle require NAD^+ or FAD, which are abundant only when the energy charge is low. Thus, the rate of the cycle is reduced when ATP levels are high. Since oxidative phosphorylation and the ETC require oxygen, a deficiency during hypoxia leads to a total or partial inhibition of the cycle.

The TCA cycle, in conjunction with oxidative phosphorylation, provides the vast majority of energy used by aerobic cells. Oxidation of 1 molecule of glucose produces 2 ATP under anaerobic conditions whereas under aerobic conditions ~30 ATP are produced [204], depending on the shuttle mechanism employed to transport glycolysis-generated NADH into the mitochondria.

7.3 Cell models & adapted metabolism

Several tissues within the human body have adapted metabolism in order to respond to specific functions. For example, skeletal and cardiac muscles have a high mitochondrial density due to the energetic demands placed upon these tissues. A more subtle adaptation is present within hepatocytes. The liver maintains blood-glucose levels and possesses a specialised isozyme of hexokinase, glucokinase, which is not inhibited by glucose-6-phosphate. This enables the liver to continually synthesise glycogen when blood-glucose levels are high. Glucokinase has a lower affinity for glucose and so uptake into tissues with hexokinase, such as the brain, where glucose is virtually the sole fuel, is favoured.

7.3.1 Cancer

Cancer is a class of disease whereby normal somatic or germline cells develop abnormalities which produce a malignant phenotype. Malignant cells may display any of the following: uncontrolled proliferation, invasion of adjacent tissues and metastasis, where cells mobilise to form secondary

tumours at other sites. Tumours are classified as to their cell type and almost any mammalian organ and cell type can succumb to oncogenic transformation.

The causes of cancers are varied but all deregulate a wide spectrum of regulatory and downstream effector pathways. Genetic defects are the primary cause of this and may even be effected by viral agents, such as human papillomavirus infection which can cause cervical cancer [205].

Cancer cells have been shown to have modified glucose metabolism contrasting that of normal tissue where they perform a significant increased level of aerobic glycolysis. In metabolically active cancer, pyruvate is metabolised to lactate when oxygen is not in limiting supply to inhibit mitochondrial function. This is called the Warburg effect and is one of the hallmarks of carcinogenesis [206, 207]. Although the Warburg effect is not present in all cancers, it is the most common phenotype in rapidly growing cancers. This does not mean that cancer cells rely predominantly or exclusively on glycolysis for ATP production, which is a common misconception [208]. Cancer cells can obtain approximately the same amount of energy from anaerobic glycolysis as from aerobic mitochondrial respiration whereas normal cells obtain much more energy from aerobic respiration than anaerobic glycolysis.

The exact mechanisms responsible for this metabolic alteration remain to be fully elucidated but mitochondrial defects are suspected to play an important role [209]. Recent studies reveal that mutations in the mitochondrial DNA (mtDNA) are present in cancer cells [210]. This “injury” to the respiratory chain is thought to force compensatory upregulation of glycolytic enzymes. The inherent inefficiency in ATP production by glycolysis when compared to oxidative phosphorylation would require a high consumption of glucose to fulfil cellular requirements.

7.3.2 Embryonic stem cells

Work on embryos by T. Boveri and E. B. Wilson [211] in the late 19th century identified cells that possessed the capacity to give rise to either germ or somatic cells and distinguished them from other cells that had already become specialised into one or the other. Specifically it was E. B. Wilson [212] who introduced the term “stem cell” and captured their biological characteristics with the analogy to stems in plants: largely undifferentiated but proliferating cells capable of branching out and specialising in response to environmental cues.

Embryonic SCs (ESCs), defined by their origin, are derived from the preimplantation stage of the embryo at the time when they have reached the blastocyst stage. The totipotent zygote (sperm fertilised oocyte) undergoes rapid division, or embryonic cleavage, and produces the morula. This collection of approximately 12-32 blastomeres (early embryonic cells) will go on to differentiate and represents the first steps in mammalian embryonic development. The individual blastomeres will now start to organise into three distinct locations, each encompassing a different cell type. These are the trophoblast comprising of outside epithelial cells, the primitive endoderm comprising of cells at the blastocoele surface of the inner cell mass (ICM), and cells inside the ICM which make up the primitive ectoderm.

ESCs originate from the primitive ectoderm, which is a transient group of cells existing as the embryo enters the next stages of development. These cells were first isolated from mouse embryos [213, 214] and under specific culture conditions were found to self-renew. The differentiation program of the cells was able to be blocked and also to be reactivated if placed into a differentiation promoting environment.

The discovery of SCs in human systems lagged significantly behind their murine counterparts; it wasn't until 1998 that the first human ESCs were isolated by J. Thomson [215]. The isolation techniques and the necessary markers to identify human ES and EC cells were all available [215, 216] but the reasons lay in the difficulties and stigma with working with human embryonic material. SC research now is driven by the goal of complete regenerative medicine in replacing whole tissues and organs that are damaged mechanically or by disease.

The ability to self-replicate and the ability to differentiate along a somatic cell lineage are the two most important and basic characteristics of SCs. The process by which SCs sequentially develop into more specialised cells or particular lineage within an organism is known as differentiation. It is still poorly understood. However, it is necessary that SCs must up-regulate expression of a set of genes, down-regulate others in order to specialise or alter their phenotype into that of the differentiated cell. As a result, differentiated cells develop specific structures which enable new functions, such as the dendrites of the neuron or the sarcomeres of cardiomyocytes. A stem cell may be manipulated to become specialised through directed differentiation under the presence or absence of certain growth factors or molecular signals.

ESCs naturally exhibit preference toward glycolysis for the generation of ATP [217] which is thought to result in life span extension - a decrease in usage of mitochondrial oxygen may protect the cells from oxidative damage [218]. ESCs are harvested from the ICM which is not vascularised at this

point in development and so oxygen supply is limited so a reliance on or upregulation of glycolysis would serve to be a metabolic advantage. ESCs are also known to have a very low mitochondrial density and a high nuclear-to-cytoplasm ratio [215, 219]. Upon commitment to a specific lineage during differentiation, it has been observed that mtDNA is extensively replicated, leading to an expansion of mitochondrial number [219]. This would serve to cope with the increased levels of ATP required for differentiation and begin to adapt the mode of metabolism to that of the target cell type. The increasing engagement of mitochondria as ESCs differentiate may be investigated using autofluorescent monitoring of NADH and the cells response to specific substrates.

The current methods of determining pluripotency of a stem cell population rely on cell staining, fluorescent labelling or gene expression. Human embryonic stem cells (hESCs) stain positive for the enzyme alkaline phosphatase and exhibit low Hoechst nuclear dye staining due to their increased efflux capacity. Immunofluorescent staining is also performed; a typical set of cell surface markers of pluripotency includes SSEA1 (stage-specific embryonic antigen-1), SSEA3, SSEA4, TRA-1-60 and TRA-1-81 [215]. Genes known to be upregulated or involved in maintaining pluripotency include Oct4 (octamer-binding transcription factor 4), FGF4 (fibroblast growth factor 4), Sox2 (SRY-box 2) and Nanog [220].

Loss, or change, of these markers means that pluripotency is being lost and the ESCs are differentiating [221]. To which lineage they are committing will require further testing, as above, with a new suite of markers that are hallmarks of a particular cell type. For example, differentiation into the neuronal lineage is concomitant with expression of Pax6 (paired box gene 6), Sox1, Sox3 and NCAD (neuronal cadherin) [222]. The expression or regulation of many of these proteins, especially in early differentiation, is transient and it is important for methods aiming to track differentiation to account for this.

Morphologically, ESCs have a high nuclear to cytoplasmic ratio, prominent nucleoli and form tightly packed, rounded colonies. As cells differentiate into specific cell types their morphology changes but determining the preservation or loss of pluripotency in this way is not reliable. However, it is a practice used in almost every stem cell lab for day to day housekeeping of cultures.

7.3.3 Heart failure

The heart consists of pacemaker and conducting tissue, blood vessels, extracellular space and muscle cells known as cardiomyocytes. Cardiomyocytes are responsible for the contractile nature of

the heart and are generally cylindrical in appearance. Atrial cardiomyocytes are approximately 10 μm in diameter and 20 μm in length whereas ventricular cardiomyocytes are much larger at approximately 25 μm in diameter and 50 – 100 μm in length. The cardiomyocyte itself is comprised of primarily contractile filaments and mitochondria, including a sarcoplasmic reticulum and nucleus, amongst other organelles. The striated structures in cardiomyocytes are formed by myofibrils, which are organelles of interdigitated actin and myosin filaments organised into subunits called sarcomeres. When the cell contracts, the actin is pulled along myosin towards the centre of the sarcomere until they are completely overlapped. The mitochondria occupy around 30 % of the cardiomyocytes volume and are located in proximity to the myofibrils such that energy production is close to the site of energy consumption.

The basic function of the heart is to provide peripheral tissue with oxygen via the circulation of blood. As the oxygen demands of the body increase, so must the cardiac output in order to meet these requirements. Heart failure is the inability of the heart to supply sufficient blood flow to meet demand. The common causes of heart failure include: ischaemic heart disease, whereby the myocardium receives insufficient blood flow; myocardial infarction, an interruption of blood supply to a region of the heart; hypertension, or high blood pressure; and cardiomyopathy, which is the deterioration of the myocardium leading to heart failure.

Metabolic substrate availability and myocardial workload are continually changing and the heart has a limited capacity for substrate storage. The heart is, therefore, a metabolic omnivore and is able to derive ATP from the oxidation of fatty acids, glucose, lactate and other substrates such as ketone bodies. Cardiomyocytes are oxygen demanding and produce more than 90 % of their energy from mitochondrial respiration. The predominant fuels of the heart are free fatty acids (FFAs) and glucose and the relative contribution of each is dependent on the substrate's availability and the energetic demands of the heart [223]. The switch from one substrate to another is regulated allosterically in the short term under acute stress. For example, fatty acid oxidation inhibits glycolysis and vice versa as the relative abundance of each substrate changes. Additionally, increasing workload such as with exercise increases the oxidation of lactate. In the longer term, as with chronic stresses such as those leading to heart failure and diabetes, the regulation is made at the transcription level where enzymes specific to particular pathways are upregulated [224]. The loss of metabolic flexibility is considered an early feature of metabolic dysregulation in the failing heart [225].

Mechanisms leading to heart failure are able to be compensated to a degree by remodelling cardiac structure and metabolism [226]. As heart failure progresses, enzymes involved with fatty acid oxidation are downregulated and there is a switch to glucose oxidation [227, 228]. This is initially a

compensatory mechanism provided there is adequate oxygen supply since glucose oxidation generates more ATP per mole of oxygen than fatty acid oxidation [229]. Conversely, during cardiac foetal development, the chief metabolic pathway is glycolysis which switches to fatty acid oxidation after birth [228]. Indeed, the response of failing hearts has been shown to include a reactivation of foetal genes [230]. As heart failure progresses further, a decrease in oxygen delivery results in downregulation of oxidative metabolism and a reduced ability for energy production. Although, adaptive in reducing oxygen consumption the lack of energy leads to disruption of contractile function and calcium handling [231].

7.4 Cell culture

7.4.1 Human BE cells

Human BE carcinoma cells are an established tissue culture line derived from a colon cancer donated by the Institute of Cancer Research. They are the standard, workhorse cell line for much of the work by the SCP group due to their robust nature and simplicity of culture.

BE cells contain an activated Ki-Ras - an oncogene shown to be activated along with overexpression of cell surface growth factors in colorectal cancers. The cells used in autofluorescence experiments in Part II (§9) of this thesis contain no viral sequences or transfected transforming genes. Stably transfected BE cells containing cytoplasmic GFP are used for experiments in Part I (§3-6) of this thesis.

The cells are mycoplasma-free and are cultured at 37°C in a 5% (v/v) CO₂ humidified incubator in Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented with 10% (v/v) foetal bovine serum (FBS, Sigma).

7.4.2 H7 Embryonic stem cells and ESC-derived cardiomyocytes

Human ESCs (H7 line) are propagated in the undifferentiated state and also used to obtain human ESC-derived cardiomyocytes (hESCMs). hESCs are maintained under feeder-cell free conditions on Matrigel-coated six well plates in mouse embryonic fibroblast conditioned hESC medium (MEF-CM), supplemented with 8ng ml⁻¹ recombinant basic human fibroblast growth factor. hESC media contained 20% knockout serum replacement, 1 mM L-glutamine, 10 mM non-essential amino acids

(1% of stock), 0.1 mM mercaptoethanol, antibiotics (50 U ml⁻¹ penicillin 50 g ml⁻¹ streptomycin) and 4 ng ml⁻¹ basic human fibroblast growth factor. Cells were fed daily by complete media change.

Differentiation into hESCMs was made by embryoid body (EB) formation. H7 EBs were made by mechanically breaking the hESC colonies, followed by culturing for 4 days in suspension (in low-adherence six-well plates) in hESC differentiation medium (20% foetal calf serum without basic human fibroblast growth factor). The differentiation medium contained 80% knockout Dulbecco's modified Eagle's medium, 1 mM L-glutamine, 0.1 mM -mercaptoethanol, 10 mM non-essential amino acids and 20% non-heat-inactivated foetal calf serum. After 4 days in suspension, 1–3 EBs were transferred onto 0.5% gelatin-coated glass coverslips (10 mm in diameter) in the centre of 35 mm dishes (MatTek Corp., Ashland, MA, USA). From days 9–12 following induction of differentiation, spontaneously beating cardiomyocytes emerge as rhythmically contracting clusters in the outgrowths of differentiating EBs.

The embryonic tubular heart begins to beat 28 days from fertilisation, where the movement of blood is initially an ebb and flow. The tubular heart folds and subdivides into the four chambers characteristic of the adult heart. The newly formed contractile tissue is limited to roughly 70 contractions/minute and increases up to 170–190 beats/minute by day 54. As young hESCMs mature it is predicted that their metabolism shifts from reliance upon anaerobic to aerobic metabolism, concomitant with increased mitochondrial biogenesis. This is due to increased energetic demands of the myocardium in line with embryonic development, where demands for 4/oxygen and nutrients are met by simple diffusion early on then the cardiovascular system develops to cope with demand.

It is difficult to match embryonic development stage with that of cultured differentiation; it is unknown how closely the artificial differentiation programme mimics natural embryonic development, if at all.

Stem cell material is kindly handled and donated by Dr N. N. Ali (National Heart & Lung Institute, Imperial College London).

7.4.3 Adult rat cardiomyocytes

Female adult rats are sacrificed and the heart is rapidly excised from these animals. The heart is dissected and cells are dissociated. Freshly isolated cells are suspended in *physio-cm* solution,

consisting of 0.2 mM CaCl_2 , 5.4 mM MgSO_4 , 5.4 mM KCl, 120 mM NaCl, 6.3 mM pyruvate, 20 mM glucose and 20 mM taurine buffered by HEPES at pH 7.4. Cells are tested within 6 hours of being isolated.

Myocardial infarction was induced in adult female rats by left coronary ligation. Six weeks after myocardial infarction the rats were sacrificed and the heart rapidly excised. The heart is dissected and cells are dissociated and suspended in *physio-cm* solution.

Adult rat cardiomyocytes samples are kindly donated by Prof. S. E. Harding (National Heart & Lung Institute, Imperial College London).

7.5 Summary

The role of NADH and FAD in the major metabolic pathways of glycolysis, oxidative phosphorylation and the electron transport chain has been described. Metabolism has an important role in certain diseases. The altered metabolism of cancer, differentiating embryonic stem cells and in heart failure is described and 3 different cell types corresponding to these models are also described: Human BE cancer cells are a stable cell line from the Institute of Cancer research; H7 embryonic stem cells are differentiated using a directed programme to beating ESC-derived cardiomyocytes; and primary cardiomyocytes are sourced from failing or non-failing hearts of adult rats.

By investigating how metabolism differs in these cell types and how it responds to appropriate stimuli, it is hypothesised that the metabolic state of the cell and, potentially, its disease state be determined by metabolic imaging.

8 Two-photon Microscopy

Microscopy has had a profound effect upon biology and although methodologies such as electron microscopy can produce images at much finer detail, fluorescence microscopy is unique in its ability to reveal, and importantly maintain, dynamic processes such as signalling, intracellular transport, and metabolism. Upon lysis these processes are terminated and it is difficult to elucidate dynamic behaviour based on a single snapshot of the proteome. The complementary role of fluorescence microscopy to single cell proteomics will be explored and fluorescence emission intensity and lifetime will be measured in order to derive cellular information. In particular, two-photon microscopy to enable long-term metabolic imaging.

- Fluorescence parameters

In order to maximise the information gathered from during metabolic imaging, several fluorescence parameters may be measured. This includes fluorescence intensity and lifetime, which are discussed in terms of their theoretical basis and how they are measured in practice.

- Microscopy

The types of microscopy relevant to metabolic imaging are described with particular focus on two-photon excitation microscopy, which is used in this work. Two-photon excitation allows the excitation of UV-fluorophores, such as NADH, with light typically in the near-IR. This reduces photodamage to cells and removes the requirement of UV-optics.

8.1 Fluorescence parameters

Fluorescence is the emission of light from a species that has undergone optical or electronic excitation (figure 8.1). Ground state electrons can be excited to singlet states via absorption of a sufficiently energetic photon and occurs on the order of 10^{-15} . Excitation may promote electrons to vibrational energy levels within S_x ($x = 1, 2, 3 \dots$). Since fluorescence lifetimes are on the order of $10^{-9} - 10^{-7}$ s and vibrational state lifetimes are on the order $10^{-14} - 10^{-11}$ s the electrons are able to relax to the lowest energy excited state within S_x . The electron can then decay either radiatively or non-radiatively to the ground state, S_0 .

Radiative decay results in the emission of a photon i.e. fluorescence emission. If the electron has lost energy the emitted photon will be Stokes shifted to a longer wavelength relative to that of the absorbed photon. Non-radiative decay defines those processes which compete with the emission process. They include photochemical or dissociative processes in which the species undergoes a defined chemical change, collisional quenching or resonant energy transfer and dissipation of energy as heat.

Electrons may undergo spin conversion into an excited triplet state via intersystem crossing, for example, from S_1 to T_1 , instead of the lowest singlet excited state. The transition between T_1 and S_0 is forbidden due to spin considerations and results in a very long lived state lifetime; in this time (order of 10^{-3} (ms) to several seconds) the electron undergoes spin conversion and only then may decay in a process known as phosphorescence. The electron may also cross back to the lowest singlet excited state before decaying to the ground state.

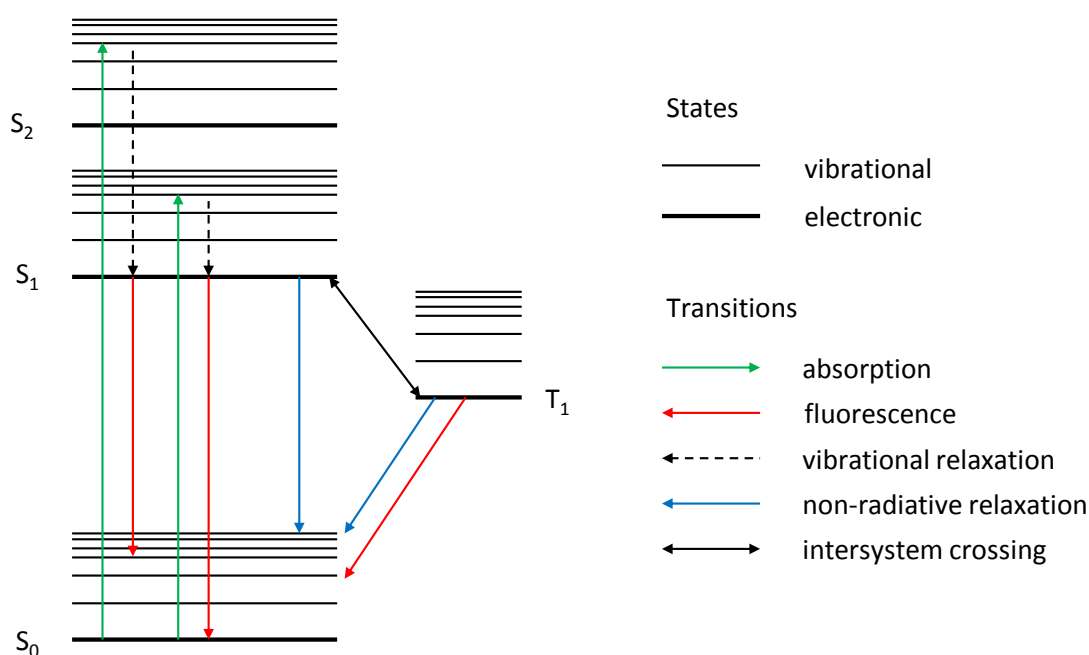


Figure 8.1: Jablonski diagram illustrating the available radiative and non-radiative transitions following excitation.

The wavelength λ of a photon capable of exciting an electron to an excited state of energy E above ground state is given by

$$\lambda = \frac{hc}{E} \quad [8.1]$$

where h is Plack's constant and c is the speed of light in a vacuum. However, this energy may be supplied by 2 or more photons such that

$$\lambda_{1PE} = \left(\sum_{n=1}^N \frac{1}{\lambda_n} \right)^{-1} \quad [8.2]$$

where λ_{1PE} is the wavelength of a photon needed to excite an electron in the 1-photon case. In 2-photon excitation (2PE) the energies must combine within a temporal window on 10^{-16} s, which is determined by the Heisenberg uncertainty principle. Typically photons at half the energy are made to combine as this is the most straight forward to implement practically,

$$\lambda_{2PE} = 2\lambda_{1PE} \quad . \quad [8.3]$$

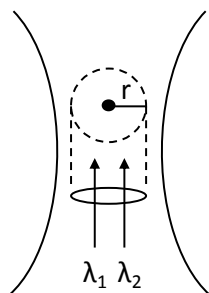


Figure 8.2: Illustration of a molecule of radius r within a beam of wavelength λ . Two-photon absorption will only occur if two photons are present within the molecular volume (assumed to be spherical of radius r) within the virtual state decay time.

The first photon is absorbed and an electron is excited to a virtual state. In order to be excited to a real state a second suitable photon must be absorbed before the virtual state decays. The probability of undergoing a 2-photon transition is therefore proportional to the probability of finding 2 photons within a volume occupied by a molecular species within the virtual state decay time (figure 8.2). For a molecular species in a beam comprised of photons at $\lambda = \lambda_{2PE}$ then the number of photons, n , within it's molecular volume is

$$n = \frac{E_n}{hc} \lambda_{2PE} \quad . \quad [8.4]$$

The intensity is the energy passing through the volume per molecular cross-section, A , per time, t .

$$I = \frac{E_n}{At} \Rightarrow n = \left(\frac{2\pi r^3}{hc^2} \right) \lambda_{2PE} I \quad . \quad [8.5]$$

For NADH, an estimate of r from typical molecular bond lengths is taken to be 500 pm. It is generally excited at 740 nm with peak intensities of the order GW cm^{-2} resulting in n being ~ 0.01 . The probability of finding k photons in the volume is therefore given by a Poisson distribution.

$$p(k = 2) = \frac{n^k}{k!} e^{-n} , \quad [8.6]$$

which simplifies to

$$p(k = 2) = \frac{n^2}{2} \quad [8.7]$$

since n is small. This suggests that 2PE is dependent on the square of the intensity. This has important optical consequences such as inherent sectioning capability, which will be discussed in §8.2.2.

8.1.1 Fluorescence intensity

The intensity of a fluorescence measurement is a function of species abundance and quantum yield (ϕ). The quantum yield is a measure of how efficient a species is at reemitting absorbed energy and is defined as the ratio of the number of photons emitted to the number absorbed. A perfect emitter is one with a maximum ϕ of 1. The quantum yield may be expressed as a relation between the radiative and non-radiative pathways,

$$\phi = \frac{k_r}{k_r + \sum k_{nr}} \quad [8.8]$$

where k_r and k_{nr} are the radiative and non-radiative decay rates, respectively. It can be seen that non-radiative processes serve to reduce ϕ . Experimental determinations of ϕ usually rely on comparison of the fluorescence intensity to that of the exciting radiation using known standards or making absolute measurements. The latter involves an integrating sphere which is a closed chamber with a reflective inner wall, inside which a sample is placed and a detector window replaces a small area of the wall. Photons entering the chamber may either be absorbed by the sample or detected allowing number of photons absorbed and number emitted for a particular species to be quantified directly. Variations upon the method of comparison exist, which even negate the need for a sphere, as well as calorimetric techniques that measures the thermal expansion of the illuminated solution.

Determination of ϕ from intensity measurements requires knowledge of the absorption and emission spectra and of fluorophore concentration. This is difficult in strongly heterogeneous media

such as biological tissue and so ratiometric parameters, such as fluorescence lifetime, that require no *a priori* knowledge of the fluorophore concentration or spectral profiles are measured instead in order to study species and biological systems.

8.1.2 Fluorescence lifetime

Fluorescence lifetime measurements may be affected by perturbations to the local fluorophore environment such as pH, viscosity, refractive index and temperature, which may therefore may be measured [232]. The fluorescence lifetime of an excited state, τ , is defined by the average time the species remains in the excited state before returning to the ground state. Strickler and Berg, using the Einstein A and B coefficients, derived a relationship, in the absence of any excited state deactivation processes, between the natural radiative lifetime, τ_0 , and the absorption and emission spectra of a fluorophore

$$\tau_0^{-1} = k_r = 2.88 \times 10^{-9} n^2 \frac{\int I(\bar{\nu}) d\bar{\nu}}{\int I(\bar{\nu}) \bar{\nu}^{-3} d\bar{\nu}} \int \frac{\epsilon(\bar{\nu})}{\bar{\nu}} d\bar{\nu} \quad [8.9]$$

where n is the refractive index, I is the fluorescence emission, ϵ is the extinction coefficient and $\bar{\nu}$ is the wave-number. The approximation does not take into account the effect of the non-radiative processes,, which is related to fluorescence lifetime through the quantum yield by

$$\tau = \frac{\phi}{k_r} = \frac{1}{k_r + k_{nr}} \quad [8.10]$$

The fluorescence lifetime, τ , like ϕ , depends on the radiative and non-radiative decay rates and thus can be used to contrast different species (via k_r) and local environments (via k_{nr}) [233].

In general, the behaviour of an excited population of fluorophores may be described by a rate equation. Fluorescence by spontaneous emission will decay according to the relation

$$I(t) = I_0 e^{-t/\tau} \quad [8.11]$$

where I_0 is the initial intensity at $t = 0$ and $1/\tau$ is the sum of the rates for each decay pathway. For a system containing i fluorescent species, or a single species expressing i decay pathways,

$$I(t) = \sum_i I_i e^{-t/\tau_i} \quad [8.12]$$

where I_i are the pre-exponential factors. Many autofluorescent species exhibit multiple exponential temporal decay profiles. Nicotinamide adenine dinucleotide has two distinct lifetimes depending on whether it is bound or unbound to a protein [234, 235]. This indicates that NADH fluorescence decay curves can be approximated to a double-exponential decay model:

$$I(t) = I_1 e^{-t/\tau_1} + I_2 e^{-t/\tau_2} \quad [8.13]$$

Fluorescence lifetime measurements can be made in the frequency or time domain (figure 8.3). In the frequency domain, the phase change of fluorescence with respect to a sinusoidally modulated excitation beam is measured. There will exist a delay between absorption and emission (i.e. τ) and so the fluorescence signal will exhibit a phase delay, the amount of which will depend on the modulation frequency of the excitation beam. The lifetime can be calculated from the phase delay and also from the demodulation factor [236] between the excitation and fluorescence signals.

The choice of time or frequency domain is a matter of application; however, the frequency domain is considered simpler due to the use of a C.W. laser source and has been applied to wide-field imaging of Föster energy resonance transfer [237]. Ultrafast lasers are required when working in the time domain, which make the approach more expensive. Considering, however, that biological systems often exhibit complex fluorescence decay profiles, they are more straightforward to analyse from direct measurements in the time domain.

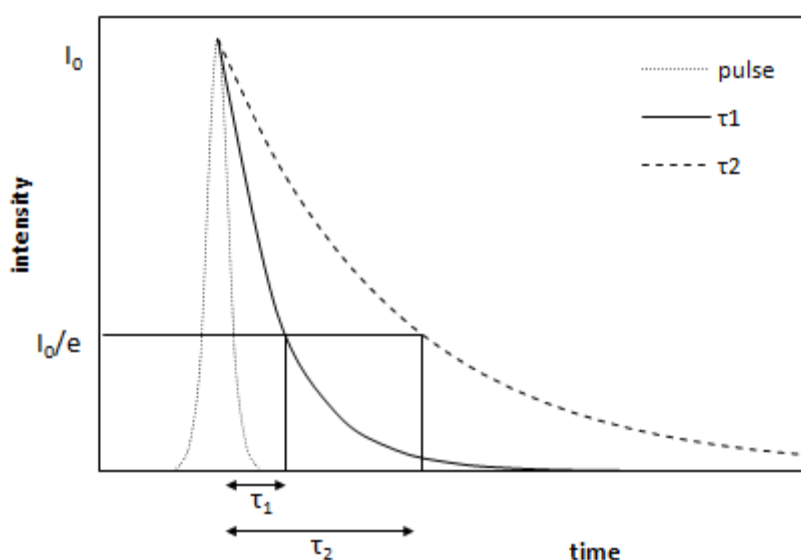


Figure 8.3: Time-domain fluorescence lifetime. Fluorophores are excited using a short pulse after which emitted photons are measured over time. The emission will decay depending on the fluorescence lifetime of the fluorophore.

In the time domain ultrafast lasers and detectors are employed since τ may range from pico- to nanoseconds and signals are measured with either gated optical intensifiers (GOIs) or by the technique of time correlated single photon counting (TCSPC). GOIs act as fast shutters when used with a CCD camera. The sample is illuminated by an ultrafast laser and the fluorescence intensity recorded at a series of delays after the arrival of the excitation pulse; the emissions follow exponential decay statistics and the intensity at each gate (t_1 , t_2 , t_3 , etc.) is used to calculate the lifetime, which is displayed using a false colour map.

In classic TCSPC the detector, typically a photomultiplier tube, records the arrival time of the first photon relative to the arrival of a single excitation pulse at the sample. The measurement relies on the concept that the probability distribution for emission of a single photon after an excitation yields the actual intensity decay profile (figure 8.4). Each measurement is binned according the arrival time and a histogram of photon arrival times is built up.

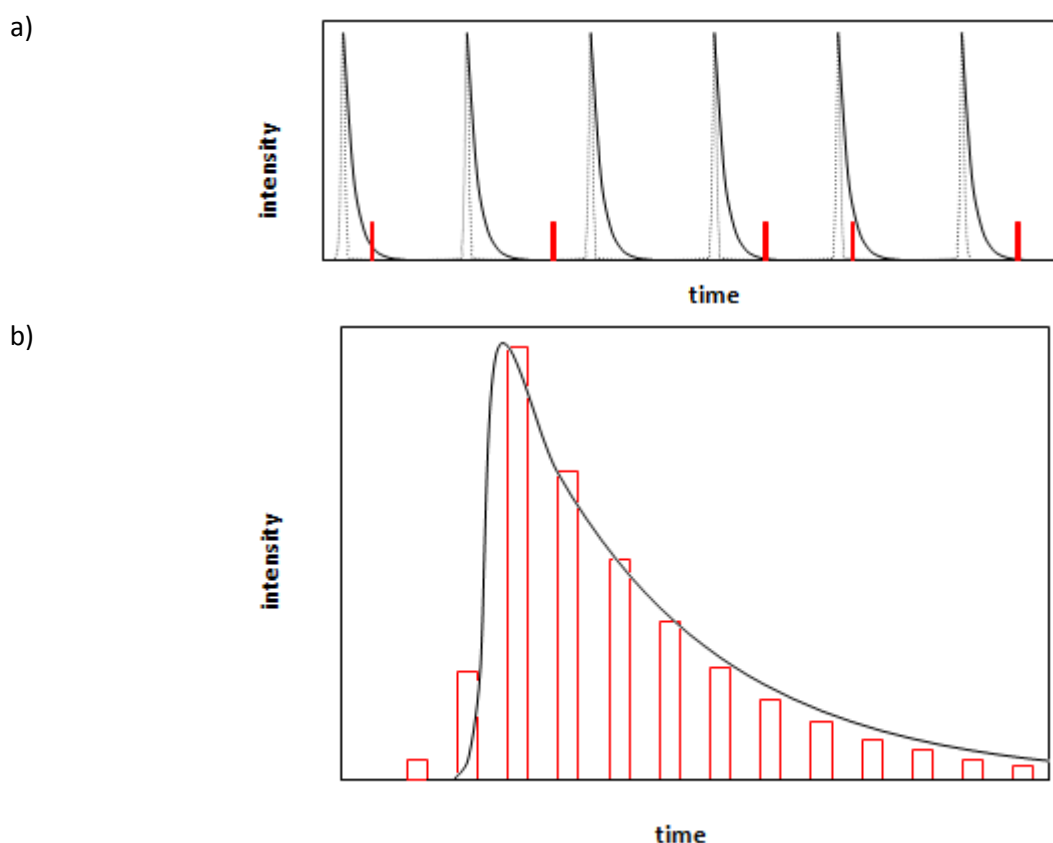


Figure 8.4: Principle of TCSPC. a) A pulse sequence (dashed line) is shown producing an expected signal waveform (solid line). In practice, single photons (red bars) are detected as randomly distributed pulses. b) A histogram of photon arrival times relative to the excitation pulse is built up to yield a fluorescence decay profile.

The excitation signal has low intensity in order that the probability of detecting more than one photon in one signal period is negligible. This makes measurements more accurate but can lead to long acquisition times. This was particularly problematic for early TCSPC systems, which suffered slow recording speed and long data acquisition times due to the low repetition rate, typically 10kHz, of the light sources used and the long ‘dead time’ and slow signal processing speed of the electronics. In principle, the count rate could be increased to the point that one photon is detected per signal period. This, however, drastically increases the probability that 2 photons per signal period are detected; in which case, the photon that arrives first will be counted and an inaccuracy in decay time will result due to the bias towards early photons. Nowadays, advanced TCSPC systems in conjunction with high-repetition-rate lasers, 80MHz typical, achieve count rates several orders of magnitude greater than early systems [238]. Their experimental convenience has led TCSPC to become the prevalent technique for FLIM microscopy since TCSPC involves a relatively low-cost and straightforward addition to a laser scanning microscope.

Fluorescence lifetime imaging (FLIM) temporally resolves fluorescence profiles and allows not only the location of molecular species in live cells to be imaged but also the biophysical environment in which they reside. Suppose a sample is composed of two chemically defined regions, each with equal fluorescence intensity. Assume further that the lifetime of a fluorescence probe is higher in the central region (τ_1) than in the surrounding regions (τ_2), differing due to the local chemical environment e.g. calcium concentration. Observation of the intensity image alone will not reveal the different environments and will provide no contrast. Fluorescence lifetime may also be used to contrast fluorophores which are spectrally inseparable but exhibit different lifetimes.

8.1.3 Errors in lifetime estimation

A TCSPC measurement produces a histogram of photon arrival times relative to an excitation pulse. A decay model, such as that in eq. 8.13, is fitted to the data set and the lifetime parameters are estimated. Assuming a perfect model function, the error in estimation will be proportional to the signal strength i.e. the number of detected photons.

The Rao-Cramér bound states that a lower bound for the variance of any unbiased estimator (one which produces the correct result) is the inverse of the Fisher information matrix i.e. it cannot be smaller than a specific limit. The matrix is defined, in this case,

$$F_{hj} = \sum_k \frac{1}{y_k} \frac{\partial y_i}{\partial \alpha_h} \frac{\partial y_i}{\partial \alpha_j} \quad [8.14]$$

where y_i is the average number of photons in channel i , as predicted by the model function y , which depends on the parameters α_h and α_j that are to be estimated i.e. lifetime, and h and j run from 1 to M , where M is the number of parameters to be estimated.

The number of counts observed in a single channel is a random sample from the Poisson distribution of the expected number of counts for that channel. The histogram comprises of N photons distributed over k channels and is a multinomial distribution

$$P(n, \alpha_1, \alpha_2, \dots) = \frac{N!}{n_1! \dots n_k!} p_1^{n_1} \times \dots \times p_k^{n_k} \quad [8.15]$$

$$N = \sum n_i; \quad \sum p_i = 1$$

where p_i is the probability that a photon will fall into channel i and so the expected number of counts is Np_i . The Fisher matrix can thus be constructed and so the required number of photons for a desired variance is

$$N \geq \frac{var_1(\tau)}{desired\ variance(\tau)} \quad [8.16]$$

Where $var_1(\tau)$ is the variance of one photon. The derivation is reproduced, in part, from [239] and therein the case of a background-free monoexponential decay is considered

$$var_N(\tau, T, k) = \frac{1}{N} \tau^2 var_1(r, k) \quad [8.17]$$

$$var_1(r, k) = \frac{k^2}{r^2} [1 - e^{-r}] \times \left(\frac{e^{r/k} [1 - e^{-r}]}{[e^{r/k} - 1]^2} - \frac{k^2}{e^r - 1} \right)^{-1}$$

where $r=T/\tau$ is the number of lifetimes in the measurement time window, T , and k is the number of channels (or bins). For example, to measure the average NADH lifetime of 2.0ns with a resolution of 256 channels distributed over a time window of 12.5ns would require 432 photons to achieve 5% accuracy in estimation.

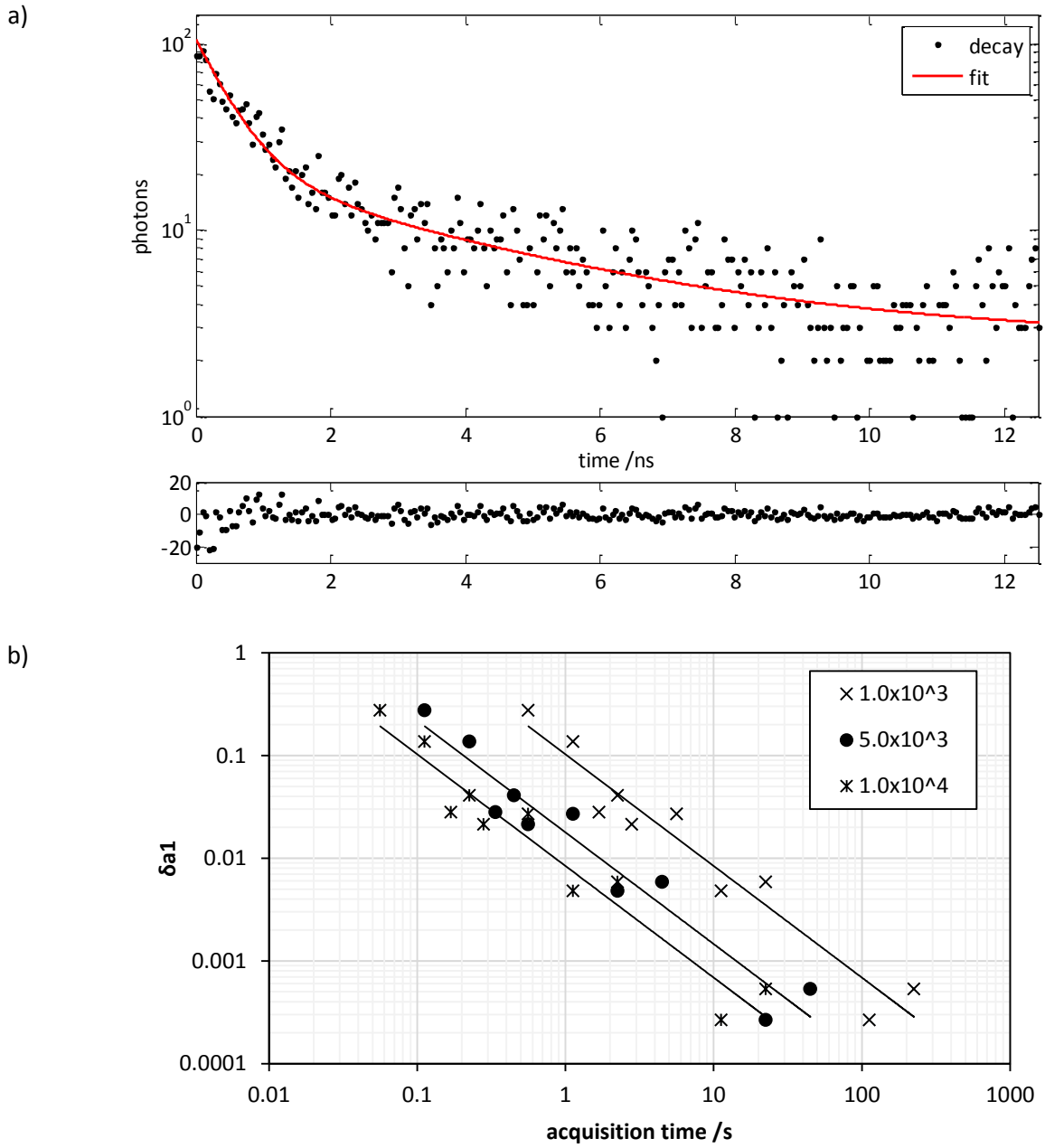


Figure 8.5: a) Model bi-exponential decay data including fit and residuals. Noise on the model data is Poissonian. Here, a decay with 100 photons in the peak is shown. b) Acquisition times are determined to achieve an accuracy δ for typical cellular NADH photon count rates (photons/s). An acquisition time of 1s will provide a relative error $0.01 < \delta < 0.1$ in the decay parameters.

The solution for multiexponential decay is more complex and so a biexponential decay model was generated:

$$N(t) = A(a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}) + B \quad [8.18]$$

where A is the peak amplitude of the decay, a_1 and a_2 are the normalised decay amplitudes and B is a background offset. The lifetime parameters were set to values typical of NADH: $a_1 = 0.75$, $a_2 = 0.25$, $\tau_1 = 0.5$ ns and $\tau_2 = 2.75$ ns. The background was set at 3 photons per channel with Poisson noise added to the decay and tail-fitted using a Levenberg-Marquardt algorithm to determine the error in the fit as a function of peak amplitude A . An example decay generated for $A = 100$ photons is shown in figure 8.5a. The total number of photons in each decay of amplitude A was calculated by

$$N_T = \frac{t}{k} \int_0^t N(t) dt \quad [8.19]$$

and is expressed as an acquisition time required for a given photon count-rate and plotted as a function of error. Figure 8.5b shows how the variation of a_1 reduces as a function of acquisition time. Similar results are obtained for the other fitting parameters. The conclusion that more photons provide greater accuracy is not unexpected. Variation in a_1 less than 1% can be achieved, depending on photon count rate, in a minimum time of approximately 1s. However, for a 256×256 image, comprising 65536 pixels this would take an abhorrently long 18.2 hours to achieve, whereas 10% variation per pixel would take approximately 30 min.

At the cost of spatial resolution the image may be spatially binned such that the relative number of photons per pixel increases. This yields improved accuracy with a concomitant decrease in the required acquisition time.

8.2 Microscopy

A microscope basically consists of a light source, some focussing optics and a detector. Depending on how these are arranged does the method by which the image is obtained changes. Regardless of method, the image acquired needs to possess as little aberrations as possible and be acquired in as little time as possible with sufficient resolution and as high a S/N as possible.

8.2.1 Confocal scanning

The simplest setup is the wide-field fluorescence microscope, which is able to acquire whole images at a time and has instantaneous 2D acquisition times. Wide-field imaging suffers from out of focus light creating noise and blurring the region of interest and becomes problematic for specimens having thicknesses greater than a few micrometres. Optically sectioned images are therefore desired. These can be obtained from a wide-field microscope by projecting a single spatial

frequency grid pattern onto the object, known as structured illumination [240]. However, these are not straightforward to implement and require mathematically complicated and computationally intensive holographic masks to achieve sectioning.

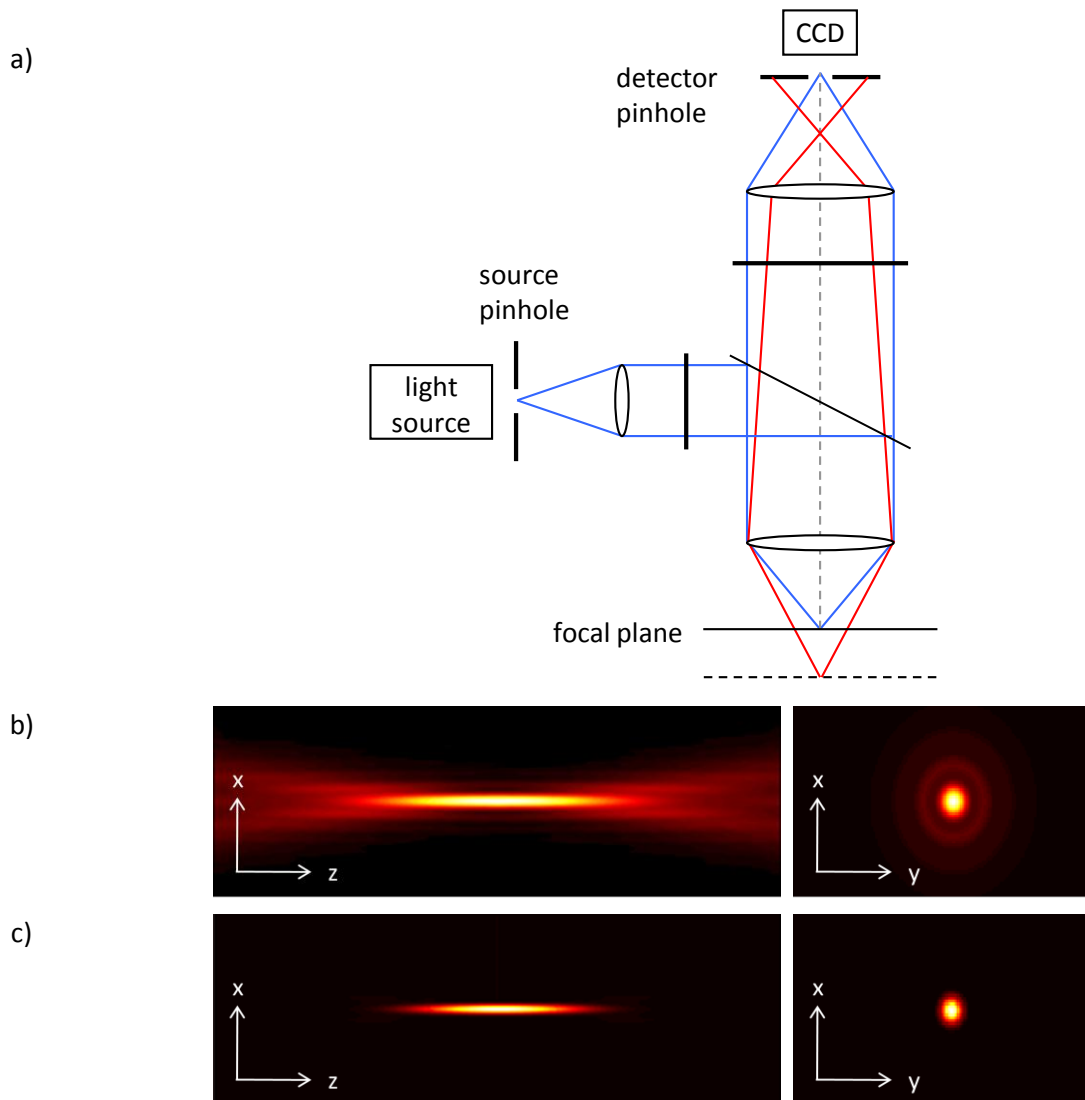


Figure 8.6: a) Conventional confocal fluorescence microscope illustrating the discrimination between light originating from the focal (blue) and out of focal (red) planes. The axial (x - z plane) and lateral (x - y plane) b) widefield and c) confocal point spread functions. [241].

Confocal microscopes are the most ubiquitous instruments for achieving optically sectioned images. The confocal optical system may be achieved by focussing a point-source of light onto a spot, or small region, of the sample and imaging the signal onto a point or pinhole detector [242]. This ensures that only light from the probed region is detected since out-of-focus light is rejected. This

principle is best understood from figure 8.6a. Simple ray-tracing illustrates the depth discrimination of the system, in particular the pinhole, against planes that do not lie within the focal region of the lens. This, therefore, requires that the sample be scanned, point by point, until a full image is obtained [243]. The confocal microscope is optically quite straightforward and is mainly constructed around electronic control boxes which manage point to point scanning and image formation.

Image formation has inherent resolution limitations due to diffraction that are fundamental to any optical system. The resulting resolution limits can be described by the microscope's point spread function (PSF). The PSF of an optical device is the image of a single point object and the degree of spreading (blurring) in the image of the point object, i.e. the PSF, is a measure of the quality of an optical system. In widefield systems the lateral (Δr) and axial (Δz) resolutions are given by

$$\Delta r \approx \frac{0.61\lambda}{NA}; \Delta z \approx \frac{2\lambda n}{NA^2} \quad [8.20]$$

where λ is the fluorescence emission wavelength, n is the refractive index of the medium and NA is the numerical aperture of the objective.

In confocal microscopy the light emerging from the sample plane is spatially filtered through the detector pinhole which is on a plane conjugate to the focal plane (figure 8.6a). Thus the effect is to apply the PSF twice resulting in a confocal PSF that is the square of the widefield PSF. This results in improved lateral and axial resolution,

$$\Delta r \approx \frac{0.61\lambda}{\sqrt{2}NA}; \Delta z \approx \frac{2\lambda n}{\sqrt{2}NA^2} . \quad [8.21]$$

The improvement in lateral resolution is only slight. However, if the integrated intensity of the PSFs is considered it is easy to see that the sectioning capabilities of the confocal setup are far superior (figure 8.6b). The integrated intensity in the widefield case does not reduce away from the focus whereas there is a clear maximum at the focus for the confocal PSF. This results in increased contrast and suppression of out of focus light.

8.2.2 Two-photon excitation

One of the major drawbacks of fluorescence confocal microscopy is that the whole thickness of a sample within the focussed beam path is affected with each scan. Even though the confocal system is physically rejecting the out-of-focus fluorescence it is still being generated. Photobleaching and

phototoxicity need to be seriously considered, especially for long-term imaging and the use of fluorophores that require UV excitation. Two-photon excitation (2PE), predicted by Maria Göppert-Mayer in 1930, helps to alleviate these problems.

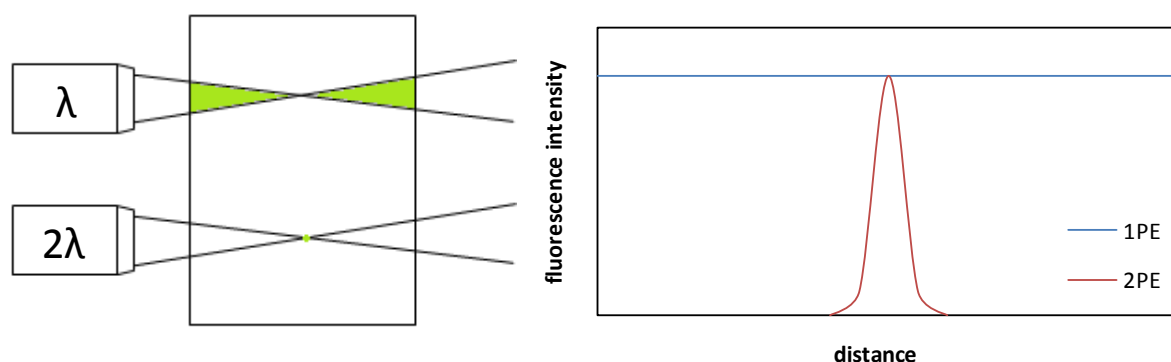


Figure 8.7: Comparison of 1PE and 2PE excitation exciting a sample through depth z . In 1PE the fluorescent species is excited throughout the cone of light passing through the sample. In 2PE, intensity is only sufficient at the focus to permit excitation owing to the significantly lower 2-photon absorption cross section of species compared to their 1-photon cross sections.

Absorption of a photon of sufficient energy by a species will promote it into the excited state. The same transition may be excited by simultaneously absorbing two less energetic photons (see eq. 8.3). These photons may not necessarily be identical but must combine their energies to effect an excitation that would otherwise require a single photon of equivalent energy. It was shown that the transition probability of two-photon absorption by a photon through an intermediate virtual state is proportional to the square of the intensity (see eq. 8.7). Such high intensities required will only occur in the focal plane of the objective which gives 2PE an intrinsic optical-sectioning ability (figure 8.7). This eliminates the requirement of a pinhole aperture at the detector since little fluorescence (in contrast to conventional wide-field & confocal systems) is generated out of the focal plane. The pinhole may be opened fully if using a confocal microscope or removed completely to increase collection efficiency.

The quantum-mechanical selection rules for 2PE differ from those for one-photon excitation (1PE) and as a consequence the two-photon cross-sections are considerably smaller than their one-photon counterparts, typically 30 orders of magnitude smaller [244]. A general rule of thumb is the 2PE cross-section peaks at twice the wavelength with respect to 1PE. The 2P absorption cross section is given by

$$\sigma_{2P} = \sigma_{iv}\sigma_{vf}\tau_v \quad [8.22]$$

where σ_{iv} and σ_{vf} are the 1P absorption cross sections and τ_v is the lifetime of the virtual intermediate state. τ_v is on the order of $10^{-15} - 10^{-16}$ s and the 1P absorption cross sections are typically $10^{-16} - 10^{-17}$ cm². Therefore, σ_{2P} is typically of the order 10^{-50} cm⁴ s/photon, which is equivalent to 1 GM (Göppert-Mayer). For example, fluorescein and wt GFP have 2P absorption cross sections of 38 GM (at 782 nm) and 6 GM (at 800 nm), respectively. The metabolic cofactor NADH has a σ_{2P} of 0.02 GM at 700 nm [245].

It has been shown that n_a , the number of photons absorbed per species per pulse, is given by

$$n_a \propto \frac{\sigma_{2P}\bar{P}^2}{\tau_p f_p^2} \left(\frac{\pi N A^2}{hc\lambda} \right)^2, \quad [8.23]$$

where τ_p is the pulse duration, f_p is the repetition rate and $\bar{P}$ is the average laser intensity. For a repetition rate of 80 MHz and a pulse width of 140 fs using an objective of NA = 1.32 to excite NADH at $\lambda = 740$ nm (taking $\sigma_{2P} = 0.02$ GM), n_a is $1.54\bar{P}^2$. At saturation n_a is equal to unity and the average power is approximately 25 mW.

The number of fluorescence photons collected by the system per unit time is given by

$$F(t) = \frac{1}{2}\phi\eta_{2P}N(t) \quad [8.24]$$

where $N(t)$ is the number of photons absorbed by the 2P process in the focal volume, ϕ is the collection efficiency of the system, η_{2P} is the quantum yield (~ 0.01 for NADH) and the factor of $\frac{1}{2}$ accounts for the requirement that for each emitted photon two must be absorbed. For pulsed excitation, Xu & Webb [246] derived the following expression for the time averaged fluorescence detected, $\langle F(t) \rangle$, as

$$\langle F(t) \rangle = \frac{1}{2}\phi\eta_{2P}\sigma_{2P}Cg \frac{8n\langle P(t) \rangle^2}{\pi\lambda}, \quad [8.25]$$

where C is the species concentration (cm⁻³), n is the sample refractive index, $\langle F(t) \rangle$ is the time averaged excitation power and the factor g is the degree of the second-order temporal coherence; it is expressed as

$$g = g_p / f_\tau, \quad [8.26]$$

where f is the pulse repetition rate of the laser and τ is the excitation pulse width. The factor g_p is a dimensionless quantity which depends on the shape of the excitation pulse and is calculated by

$$g_p = \tau \int_{-1/(2f)}^{1/(2f)} I_0^2(t) dt / \left[\int_{-1/(2f)}^{1/(2f)} I_0(t) dt \right]^2, \quad [8.27]$$

where I_0 describes the temporal distribution of the excitation pulse. For pulses with a Gaussian temporal profile, $g_p = 0.664$ [246]. For a laser with 80 MHz repetition rate and pulses of width 140 fs, g is approximately 0.6×10^5 . For a cell containing NADH at an average concentration of 100 μM , being excited with a saturation power of 25 mW at 740 nm results in a fluorescence count rate of approximately 7.15×10^5 photons s^{-1} . Assuming a collection efficiency of 1% then the measured count rate would be on the order 7150 photons s^{-1} .

2PE is particularly useful when imaging highly scattering and thick media such as tissue. Using longer IR wavelengths reduces absorption and scattering allowing for deeper penetration. A general advantage for thin and thick samples alike is that UV optics is not required to image UV fluorophores and simplifies optical design. Due to the higher intensities associated with 2PE, photobleaching and photodamage at the focal plane may be increased.

The 2PE radial, Δr , and axial, Δz , resolutions in the confocal microscope are

$$\Delta r \approx \frac{0.7\lambda}{NA}; \Delta z \approx \frac{2.3\lambda n}{NA^2} \quad [8.28]$$

where variables have their usual meanings. The resolution of two-photon microscopes for point-like objects is worse than confocal, or even conventional widefield, microscopes [247]. The use of 2PE in laser scanning microscopy is expected to be more compatible with long-term fluorescence imaging, such as metabolic monitoring, due to the localisation of photobleaching and photo-mediated damage to the focal plane [248]. Contradictory studies exist [249, 250] and illustrate the strong dependence upon excitation wavelength [251]. It is still unclear whether 2PE is a suitable technique for long-term imaging studies.

2PE microscopy uses the same scanning system as the confocal microscope and also requires sample scanning in order to obtain a full wide-field image. Due to the high intensities and long dwell times a degree of photobleaching and sample damage in the focal plane is inevitable and must be accounted for.

8.3 FLIM data acquisition

The most straightforward configuration of a TCSPC module is to build up an image whereby the contrast is the fluorescence lifetime. The TCSPC detection system begins with one or several photomultipliers (PMTs) which detect individual photons of a periodic light signal and measure their arrival time in relation to a laser pulse and the distribution is built up over many repetitions. TCSPC modules, such as the B&H SPC830 employed in this work, receive scan clock signals (frame sync, line sync and pixel clock) from the scanning unit of the microscope and determines the spatial location of the photon within the scanning area. Thus, for each pixel, fluorescence decay parameters may be determined and an image constructed. For typical count rates obtained from living specimens, the pixel rate is higher than the photon count rate and makes the recording process more or less random [238].

FLIM images maintain spatial resolution but average time information. For instance, an acquisition over time will average any dynamic processes. Photon arrival time may be determined in relation to the start of the experiment, the “macrotime” T , in addition to the signal period, the “microtime” t . The data is then continuously written to memory/file and is dubbed “timetag” or “FIFO” mode (first-in-first-out). Decay curves may be extracted by building up histograms of the microtimes or tracking intensity by binning photons based on their macrotime. Images may be reconstructed from FIFO mode by calculating the x-y coordinates of the photons from the macrotimes, which are kept in synchrony with the scan by markers in the data files [252, 253]. FIFO Image mode can also be used to record FLIM images but requires vast amounts of storage space.

FIFO is applicable to long-term cell monitoring but steps must be taken against the increased deposition of energy over such periods of time which increase photobleaching and photodamage. Excitation power must be kept at a minimum while maintaining reasonable count rates.

8.4 Summary

In order to investigate metabolism and how it becomes altered, multidimensional fluorescence imaging will be performed employing the methods of microscopy outlined in this chapter.

NADH and FAD will be excited by 2-photon excitation. The benefits of 2-photon excitation are that UV-fluorophores such as NADH, may be imaged without using UV radiation, which is more damaging to cells and requires special UV optics.

The fluorescence emission will be measured in terms of its intensity and lifetime. Fluorescence intensity is proportional to the concentration of a fluorophore but, as maybe the case with NADH and FAD, this may remain constant while other properties may change. These changes are related to the local environment such as pH and whether or not NADH or FAD are bound to an enzyme. The fluorescence lifetime becomes altered when NADH and FAD are bound or unbound to enzymes and by measuring fluorescence lifetime, information regarding the binding pattern of these cofactors may be gathered. For example, it may be determined whether a cell is respiring aerobically or anaerobically by the binding pattern of NADH and FAD.

9 Metabolic Imaging

This chapter presents the results of two-photon imaging of 3 cell types representing either development or disease. It is found that the imaging suffers from undersampling and induces spontaneous beating in adult rat cardiomyocytes; however, despite this it is determined that glycolysis and mitochondrial metabolism is functional for exogenous glucose and pyruvate in the BE cancer cell line and fluorescence intensity and lifetime serve as biomarkers for the differentiation of embryonic stem cells.

- Developing metabolic imaging

The instrumentation that performs 2-photon excitation and single-photon counting for fluorescence intensity and lifetime measurements is described. Using the BE cancer cell line (no transfected GFP) the method is explored and developed. Due to the inherent sectioning strength of 2-photon excitation cells are undersampled as determined by an investigation into the z-dependence of the fluorescence parameters. The alternative was to reduce magnification by employing a lower power objective. However, this would result in approximately 40-fold reduction in signal. Despite this, cells were investigated in their response to the metabolic substrates glucose and pyruvate.

- Type of measurements

Imaging was performed on either separate populations incubated with buffer containing different metabolic substrates or continuously in a time-resolved fashion to measure cellular response to metabolic stimuli. Cells were measured by their response to increasing levels of glucose (0 – 25 mM) and pyruvate (0 – 10 mM). It was found that cells could be contrasted by the parameters of either intensity, lifetime or both. The responses are discussed by drawing on information in §7 and results in the literature. However, a more detailed investigation is required to exactly determine the meaning of these responses. It was found that continuous time-resolved imaging of cellular response with the use of pharmacological agents enabled a more precise determination of how glucose and pyruvate were being metabolised by these cells.

- Adult rat cardiomyocytes

ARCMs were imaged using the same techniques that were developed with BE cells. It was found that this induced cells to spontaneously beat which prevented measurements. A

switch from 2-photon to 1-photon excitation did not alleviate the problem and was only resolved by employing a drug which disrupts intracellular Ca^{2+} signalling.

- Stem cell differentiation

Embryonic stem cells differentiating into beating ESC-derived cardiomyocytes were measured to be significantly different at 3 stages of development by the fluorescence parameters of intensity and lifetime.

9.1 Strategies

Before the strategies to determine what metabolic phenotype or state a cell currently displays, the term metabolic phenotype will be defined. The cell's metabolic phenotype determines which metabolic substrates it can or cannot metabolise and how they are processed. An aerobic metabolic phenotype is one whereby the mitochondria are active and cells engage in oxidative phosphorylation as an end-stage to glycolysis to produce ATP. On the other hand, an anaerobic phenotype is one where oxygen is not required by cells to produce ATP. This may be due to an injury to the mitochondria themselves or any shuttle system that feeds the products of glycolysis and other cytoplasmic metabolic products to the mitochondria. These are two very broadly defined examples of metabolic phenotypes and may apply only in part to diseased or malignant cells, if at all.

The parameters that may be measured by the autofluorescent metabolic cofactors, NADH and FAD, are the fluorescence intensity and the fluorescence lifetimes. For NADH and FAD there are 2 lifetimes corresponding to bound and free species of each along with the relative contributions of each lifetime component. For each parameter and sub-parameter, there are broadly 3 possible strategies to determining metabolic phenotype or cellular response to metabolic substrates. If a particular metabolic state may be described by a unique set of values or lie within a specific range of values for a set of parameters then single-point measurements may be made (figure 9.1a). For example, a decrease in the protein-bound lifetime of NADH is due to a shift from oxidative phosphorylation to glycolysis [254]. Given the complexity of the metabolic network and the vast array of injuries or processes that are able to result in a disease state or differentiation programme, it seems unlikely that an absolute coordinate to any set of parameters is able to sufficiently determine the metabolic phenotype. Therefore, single-point measurements may be inadequate and cells must be measured in response to a metabolic stimulus.

How a cell will respond to specific metabolic stimuli will be governed by the metabolic network's infrastructure. This includes enzymes, proteins, and small molecules. Metabolic substrates will only illicit a response if the cell expresses factors that are able to process them. The response may be modulated by several factors such as protein expression level or compartmentalisation of metabolic enzymes or cofactors. The cellular response to specific metabolic stimuli will allow individual metabolic pathways to be characterised by a two-point measurement (figure 9.1c). Of course, the time at which the second point is measured relative to the stimulus becomes important if the network response is transient or has features in the time-domain. Therefore, a strategy which achieves a fully time-resolved measurement of any response to a set of metabolic stimuli (figure 9.1e) is the one most likely to determine the cellular metabolic phenotype. In all 3 strategies, it is possible to produce contrast between states by measuring extra parameters. For example, intensity alone may not change whereas a parameter of fluorescence lifetime may (figures 9.1b, d & f).

A quantity that has been used to measure the metabolic state of the cell is the redox ratio. The intracellular space defines the redox environment (RE). The RE of a linked set of redox couples in a cell is the summation of the products of the reduction potential and reducing capacity of the linked redox couples present [255]. Changes in the cellular RE can alter signal transduction [256], protein synthesis [257] and regulation of the cell cycle [258] and determines whether a cell will proliferate, differentiate or apoptose [259, 260]. The primary cellular redox couples which maintain the RE are the NAD^+/NADH and $\text{GSSG}/2\text{GSH}$ (glutathione disulphide-glutathione) couples. The components of the glutathione couple are non-fluorescent and therefore inaccessible by optical techniques. Oxidised FAD and reduced NADH are fluorescent and are the major electron carriers in the primary metabolic networks. At specific points in the metabolic networks, these coenzymes are oxidised to NAD^+ and reduced to FADH_2 upon transferral of electrons to molecular oxygen via the ETC. The ratio of oxidised FAD to reduced NADH provides an indication of the redox state and the balance of NAD^+/NADH , of the cell [261].

$$\text{Redox Ratio (RR)} \propto \frac{[\text{FAD}]}{[\text{NADH}] + [\text{FAD}]} \propto \frac{I_{\text{FAD}}}{I_{\text{NADH}} + I_{\text{FAD}}} \quad [9.1]$$

Monitoring the RR and the fluorescence lifetime including the relative amounts of free and bound NADH and FAD may be used to detect changes in metabolism [262, 263]. For instance, this technique has been utilised to provide contrast between cancerous and non-cancerous tissue [264].

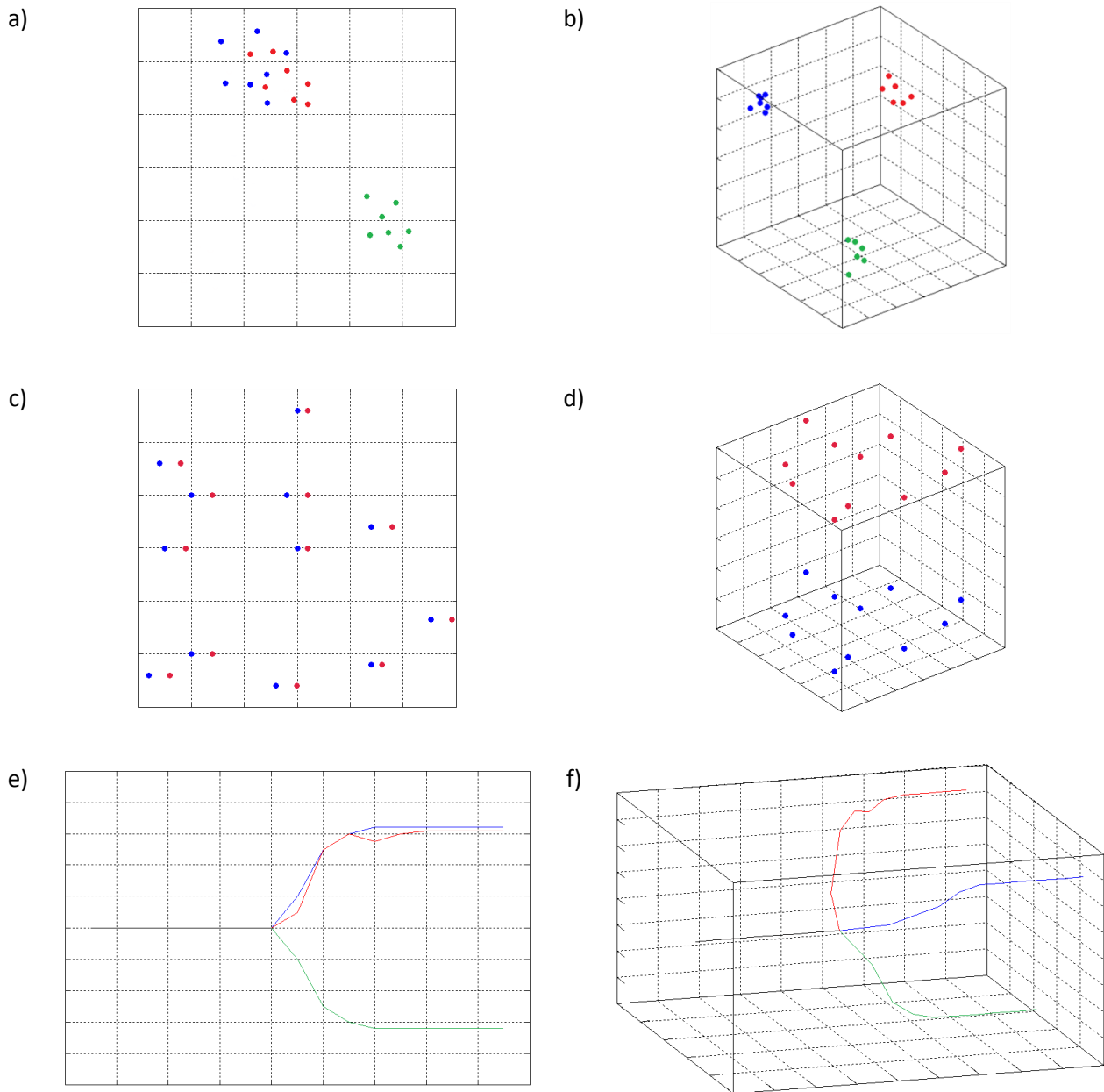


Figure 9.1: There are 3 possible strategies to measure metabolic phenotype using parameters associated with fluorescence intensity and lifetime. a) If cells have distinct values or ranges for a set of parameters S (here $n = 2$ parameters) then single-point measurements are possible. The red, blue and green points correspond to different phenotypes or states. b) Any congestion may be reduced by increasing the parameter set e.g. to $n = 3$. c) For a given phenotype or state there may not exist a single absolute value or range for a set of parameters. Therefore single-point measurements are inadequate and cells must be measured in response to metabolic stimuli. Again, increasing the parameter set may provide contrast, d). e) Having time as a permanent parameter by continually measuring the response will allow the determination of a fully time-resolved response to a metabolic stimulus. The point at which the possible responses (blue, red and green lines) flow from the black line represents the point at which a stimulus is applied. f) Of course, if the same or similar response is measured to two differing stimuli, an extra parameter may distinguish the response.

NADH binds to many enzymes in its role in TCA/OP and glycolysis, and as the preferred pathway shifts in the progression of cancer (Warburg effect), so does the binding distribution of NADH [265]. Similarly, albeit somewhat in reverse, pluripotent ESCs produce the majority of their ATP via glycolysis and metabolise predominantly anaerobically. Upon differentiation, mitochondria mature and increase in density, especially during differentiation down neuronal and cardiac lineages, and there is a shift to metabolise aerobically. Such redistributions may alter the fluorescence parameters to a degree that is measurable allowing disease, based upon metabolic alterations, to be diagnosed by observing cellular autofluorescence [254, 264, 266].

9.2 Instrumentation

The imaging system is built around a Leica TCS SP5 confocal laser scanning microscope. The Chameleon Ultra II is a diode pumped Ti:Sapphire femtosecond modelocked laser (Coherent, UK) with a tuning range between 680 nm – 1080 nm. The pulse width is nominally 140 fs and a maximum average power of > 1.6 W is produced between 700 nm - ~1000 nm. It is used as a tunable excitation source at 740 and 850 nm for 2PE FLIM of NADH and FAD, respectively. The excitation source is coupled into the Leica confocal laser scanner by the use of steering mirrors. Spectral selection may range from 380 nm – 850 nm with 5 nm resolution. Tuneable detection is afforded by a dispersing prism which is imaged onto a detector slit and is set to 380 – 500 nm for NADH and 500 – 650 nm for FAD. Fluorescence collection is operated in descanned mode since the detectors are internal (Leica, Germany). The detectors are photomultiplier tubes (PMTs) which are routed to a TCSPC SPC-830 module (Becker & Hickl; Germany). Transmitted 740 nm light is used to monitor the state of the cells during image acquisition. A 63× oil immersion objective lens (NA = 1.32) is used. Multiphoton excitation can be damaging to cells [267] and in order to maintain cell integrity the excitation source power is kept to a minimum. The average power, measured at the back aperture of the objective, was approximately 12 mW, and the scan area for each image was $82 \times 82 \mu\text{m}$ (256×256 pixels) with a scanning rate of 100 lines s^{-1} . Fluorescence lifetime acquisitions were limited by the photon count rate which was typically between $0.7 \times 10^3 - 1 \times 10^4 \text{ photons s}^{-1}$, depending on the cell type under excitation. Acquisitions were made for 20 s and images are background thresholded and binned globally using a Matlab script. The average fluorescence parameters are presented with their standard errors of the mean.

Cells are cultured as described in §7.4 and prepared as necessary on glass coverslips of thickness #1.5 (0.16 - 0.19 mm) and held in a simple water-tight custom made metal holder that is placed on the microscope stage during measurements.

9.3 Optically compatible solution

In order to minimise background fluorescence, a simple CO₂ independent HEPES buffered saline solution was used to replace BE cell growth media, which contains the dye phenol red, during imaging. *Physio* is buffered at pH 7.4 and contains 1.8 mM CaCl, 0.8 mM MgSO₄, 4 mM KCl and 135 mM NaCl in Milli-Q ultrapure water. Metabolic substrates and inhibitors may be added to the solution as required.

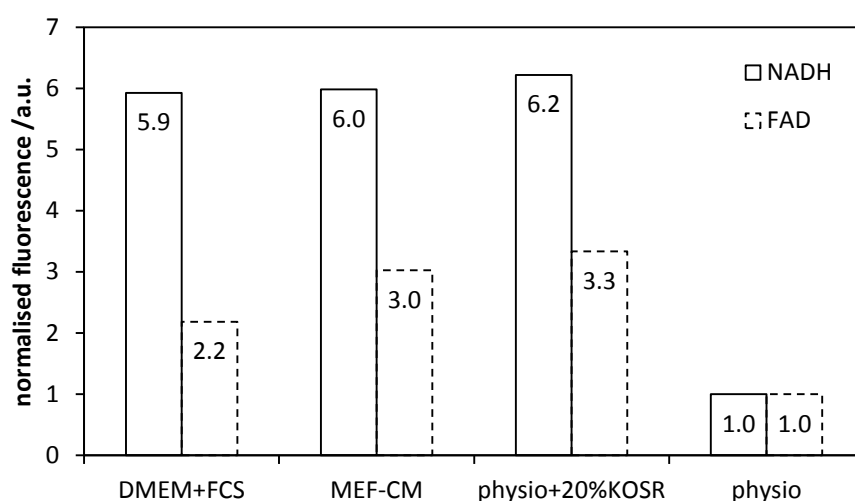


Figure 9.2: NADH and FAD excitation is made and fluorescence background is determined for several cell culture media. Counts are normalised to that for *physio* solution. DMEM, Dulbecco's Modified Eagle Medium; FCS, foetal calf serum; MEF-CM, mouse embryonic fibroblast conditioned medium; KOSR, knockout serum replacement.

Figure 9.2 shows that background noise is minimised when using *physio* solution. Cell growth media is supplemented with foetal calf serum (FCS). The media will invariably contain a significant concentration of proteins, which according to figure 9.2 is the main contributor of background noise – the addition of knockout serum replacement (KOSR), an FCS substitute in ESC culture, to *physio* increases the background fluorescence 6-fold and 3-fold in the NADH and FAD channels, respectively. Although, BE cells may be incubated in *physio* + 10 mM glucose solution for > 10 hours

in a CO₂ incubator at 37°C, long term culture is detrimental due to the lack of growth factors. Considered from the time growth media is removed from cells, experimental time scales are no longer than 60 min and so the solution is suitable for the metabolic experiments described below.

9.4 Photobleaching and substrate addition

Photobleaching was investigated by comparing results obtained by continuously imaging cells for a period of 25 min by FIFO acquisition (n = 6) to 10 s acquisitions at 5 min intervals (n = 4) (figure 9.3a). The low duty cycle of interval acquisitions helps minimise photobleaching. There is no significant drop in NADH photon numbers for interval acquisitions over 25mins indicating that the drop in NADH fluorescence intensity during continuous acquisition of cells in *physio* solution is due to photobleaching. NADH fluorescence intensity is fit to a single exponential decay,

$$I(t) = Ae^{-t/\tau} + S, \quad [9.2]$$

where τ is the photobleaching decay constant, A is a pre-exponential amplitude factor and S is the steady state level reached after time t. According to fit, the photobleaching decay constant is 181 ± 18 s (95% confidence bounds) for NADH (dashed red line; figure 9.3a). NADH fluorescence intensity reaches a steady state after 600 s.

According to measurement, photobleaching during 20 s of continuous excitation reduces NADH intensity by 4.9 ± 1.2 %; however, this is minimised when imaging cells for the same duration at 5 min intervals.

To test cellular response to a metabolic substrate media is added by syringe. It is fixed in position, angled over the coverslip holder, prior to commencing measurements and allows media to be added gently minimising the possibility of disturbing the microscope stage or cell holder. To test that the method of media addition does not affect cells, *physio* solution lacking any substrates is added. No response is observed (figure 9.3b) suggesting the method is adequate. Media is prepared as a 2× solution of their final desired concentrations.

The free (τ_1) and bound (τ_2) fluorescence lifetime components of NADH are shown to decrease during continuous 2P imaging of cells for 25 min (figure 9.3c). The decrease occurs over a shorter timescale to the drop in NADH fluorescence intensity (figure 9.3a) and reaches a steady state after approximately 300 s. There is no change in either fluorescence lifetime component over the course of 25 min when cells are imaged at 5 min intervals (figure 9.3d).

NADH fluorescence intensity and lifetimes are both at a steady state within 600 s of commencing acquisitions and therefore substrate and pharmacological agents are added after this point.

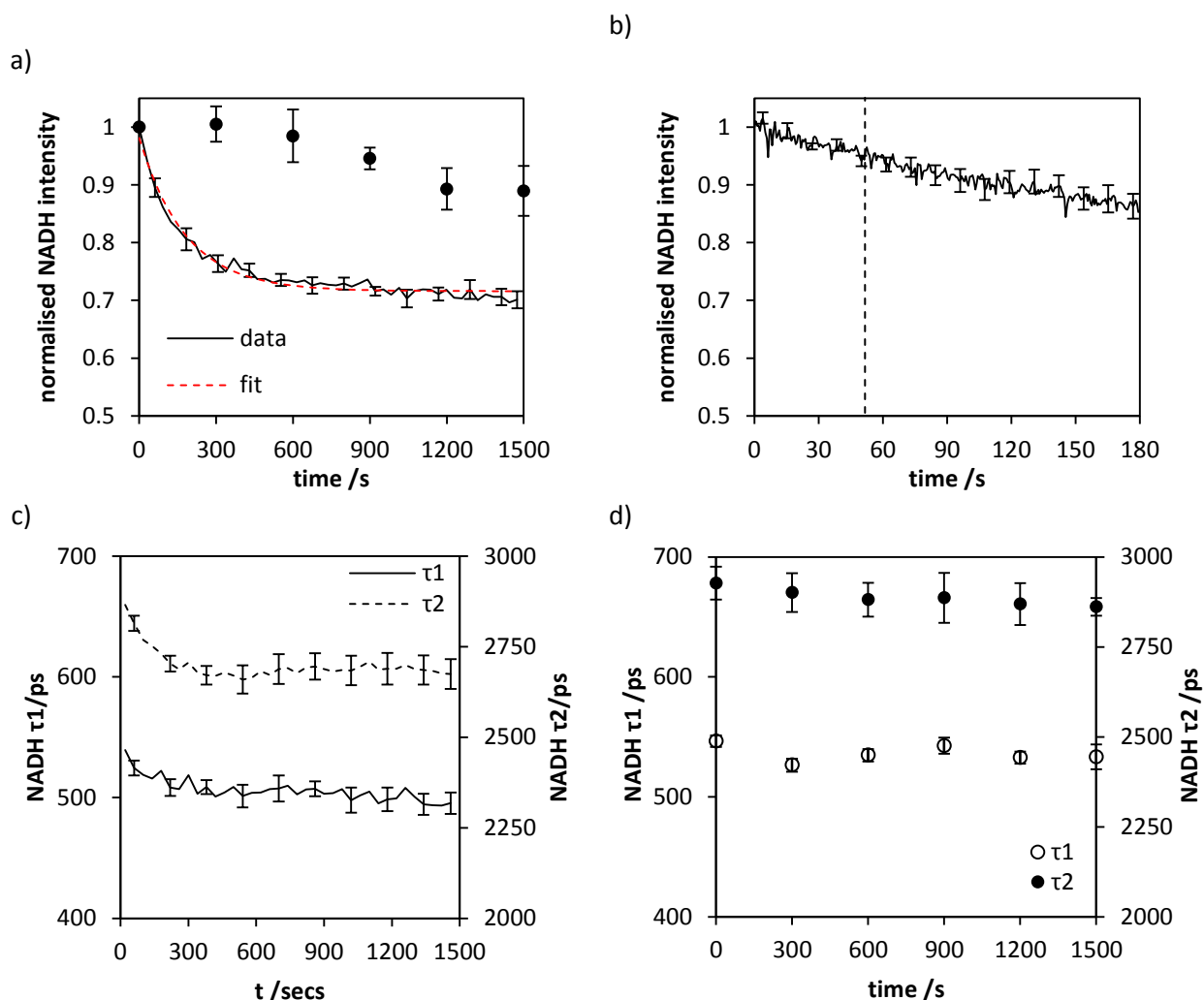


Figure 9.3: Control experiments for 2P metabolic imaging of BE cells acquisition. a) The drop in NADH signal during the initial 300 s while continuous FIFO acquisition (solid black line) is made is due to photobleaching since no such significant drop is observed when 10s acquisitions at 5 min intervals are made (black circles) ($n = 6$). b) Addition of *physio* solution at 50 s (vertical dashed line) by a fixed syringe does not illicit a response in NADH intensity ($n = 6$). c) and d) show the response in NADH lifetime over the course of the continuous and interval acquisitions in a), respectively. Note: cells incubated under room conditions for 30 min in *physio* solution prior to commencement of acquisitions i.e. $t = 0$ s. Traces in a) and b) are normalised to NADH intensity at $t = 0$ s.

9.5 z-dependence

The Leica SP5 microscope is not equipped with any focus maintaining apparatus, as is the case with the Nikon Ti-E microscope for TIRF measurements. The variation in focal position or the presence of focal drift may therefore be problematic if the fluorescence parameters exhibit z-dependence. This

would be the case if a cell is undersampled by the 2P focal plane and, for example, the cell's redox environment is heterogeneous or the mitochondria are not homogeneously distributed.

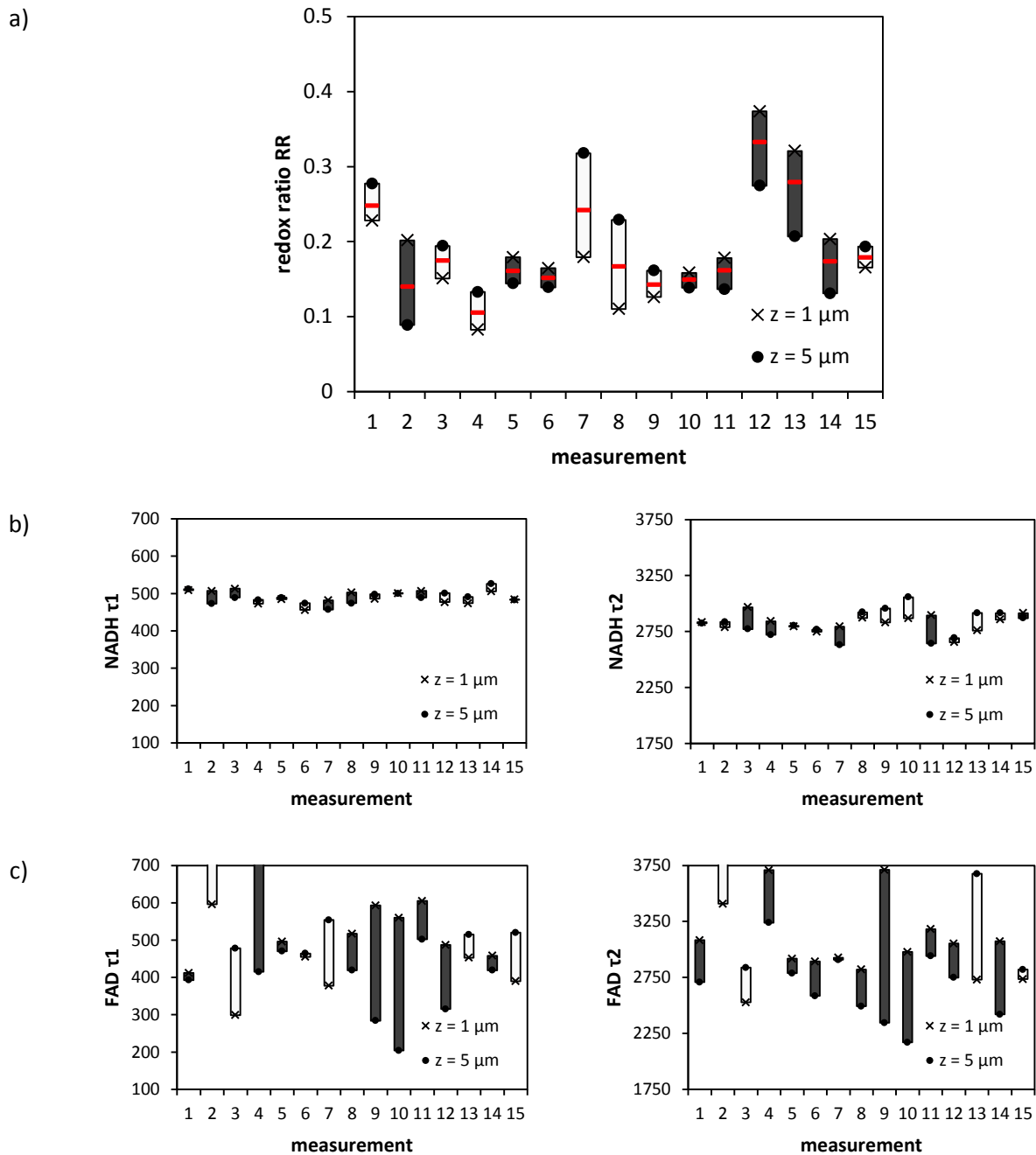


Figure 9.4: Investigating z-dependence of fluorescence parameters in BE cells by 2P imaging of NADH and FAD 1 and 5 μm above the coverslip. a) The redox ratio is calculated for each plane. White bars indicate an increase, whereas dark grey bars indicate a decrease, in the fluorescence parameter at the higher plane compared to the lower. The red dashes represent the RR calculated from the summed NADH and FAD intensities from both planes. b) The NADH fluorescence lifetimes at each plane are fit for each plane and compared to the c) FAD fluorescence lifetimes exhibit much less inter- and intra-cellular variation.

Fluorescence originates only from the focal plane in 2P imaging. The diffraction-limited axial resolution of the 2P beam is given by eq. 8.28 and for the excitation of NADH ($\lambda_{\text{ex}} = 740 \text{ nm}$) and FAD ($\lambda_{\text{ex}} = 850 \text{ nm}$) using a 1.32 NA oil-immersion objective to image cells of refractive index 1.36 [268] the axial resolutions are $1.33 \text{ }\mu\text{m}$ and $1.53 \text{ }\mu\text{m}$, respectively. This suggests the focal plane is approximately $2.66 \text{ }\mu\text{m}$ thick for NADH and $3.06 \text{ }\mu\text{m}$ thick for FAD imaging.

Single BE cells are imaged for NADH then FAD at 1 and $5 \text{ }\mu\text{m}$ (sequentially) above the coverslip to investigate the z -dependence of the fluorescence parameters. The position of the coverslip is determined as the point at which cell protrusions and processes are in focus. This is performed at room temperature in *physio + 10mM glucose + 10mM pyruvate* solution, in which cells are incubated under room conditions (21°C with atmospheric oxygen/ CO_2 concentration) for 30 min prior to imaging. The fluorescence intensity from each plane of each cell is normalised to the average area occupied by cells at the lower plane to account for variation in cell size and results are shown in figure 9.4. It is assumed that since each z -plane is separated by ~ 1.5 NADH focal planes ($4 \text{ }\mu\text{m} / 2.66 \text{ }\mu\text{m}$) and ~ 1.3 FAD focal planes ($4 \text{ }\mu\text{m} / 3.06 \text{ }\mu\text{m}$) there is minimal photobleaching in the $z = 5 \text{ }\mu\text{m}$ plane. This may, however, not be the case.

The average RR is calculated for each plane ($n = 15$) and the difference between RR ($z = 1 \text{ }\mu\text{m}$) = 0.19 ± 0.02 and RR ($z = 5 \text{ }\mu\text{m}$) = 0.18 ± 0.02 is statistically insignificant ($P = 0.72$, paired t-test); results are shown in figure 9.4a. The RR may be unchanged despite significant changes in the individual intensities of NADH and FAD. The normalised fluorescence intensity of FAD in each plane is measured to be $I_{\text{FAD}}(z = 1 \text{ }\mu\text{m}) = 4.97 \pm 0.82 \times 10^3$ photons and $I_{\text{FAD}}(z = 5 \text{ }\mu\text{m}) = 3.94 \pm 0.43 \times 10^3$ photons, which is statistically insignificant ($P = 0.18$, paired t-test). The normalised fluorescence intensity of NADH in each plane is measured to be $I_{\text{NADH}}(z = 1 \text{ }\mu\text{m}) = 6.49 \pm 0.35 \times 10^4$ photons and $I_{\text{NADH}}(z = 5 \text{ }\mu\text{m}) = 5.57 \pm 0.25 \times 10^4$ photons, which is statistically significant ($P < 0.05$, $P = 0.02$, paired t-test). If fluorescence intensity is not normalised to cell area then the difference in both NADH and FAD intensity between each plane is statistically significant ($P < 0.01$, paired t-test) which simply reflects the reduced cross section of the cell further from the coverslip. The fluorescence lifetimes of FAD are highly variable at and between each plane but do not significantly differ ($\tau_{1,\text{FAD}}$: $P = 0.48$, $\tau_{2,\text{FAD}}$: $P = 0.18$, paired t-test), whereas the fluorescence lifetimes of NADH are tightly distributed and are consistent between each plane ($\tau_{1,\text{NADH}}$: $P = 0.76$, $\tau_{2,\text{NADH}}$: $P = 0.84$, paired t-test); results are shown in figure 9.4b & c. A better sampled RR may be calculated from the summed fluorescence intensities of NADH and FAD at each plane (red dashes, figure 9.4a). The RR (summed) = 0.19 ± 0.02 and is not different to the RR at either individual plane.

The cells are heterogeneous in terms of their intracellular mitochondrial distribution, which can be observed by the fluorescence intensity images of NADH from each plane in figure 9.5a. The change in normalised NADH and FAD fluorescence intensity between each plane is shown in figure 9.5b. If the variation was due to photobleaching alone it would be expected that all measurements decrease. For the cells where the normalised NADH intensity is observed to decrease, photobleaching accounts for, on average, 5% of the decrease (see §9.4). The variation in the fluorescence parameters between planes is most likely due to intracellular variation. Furthermore, this variation is sufficient that the 2PE optical setup undersamples the cell. If the cell was homogeneous with respect to the redox ratio and the fluorescence parameters of NADH and FAD, undersampling would not be an issue. The only parameters that are observed to be homogeneous throughout the cell are the free and bound NADH fluorescence lifetimes (figure 9.4b).

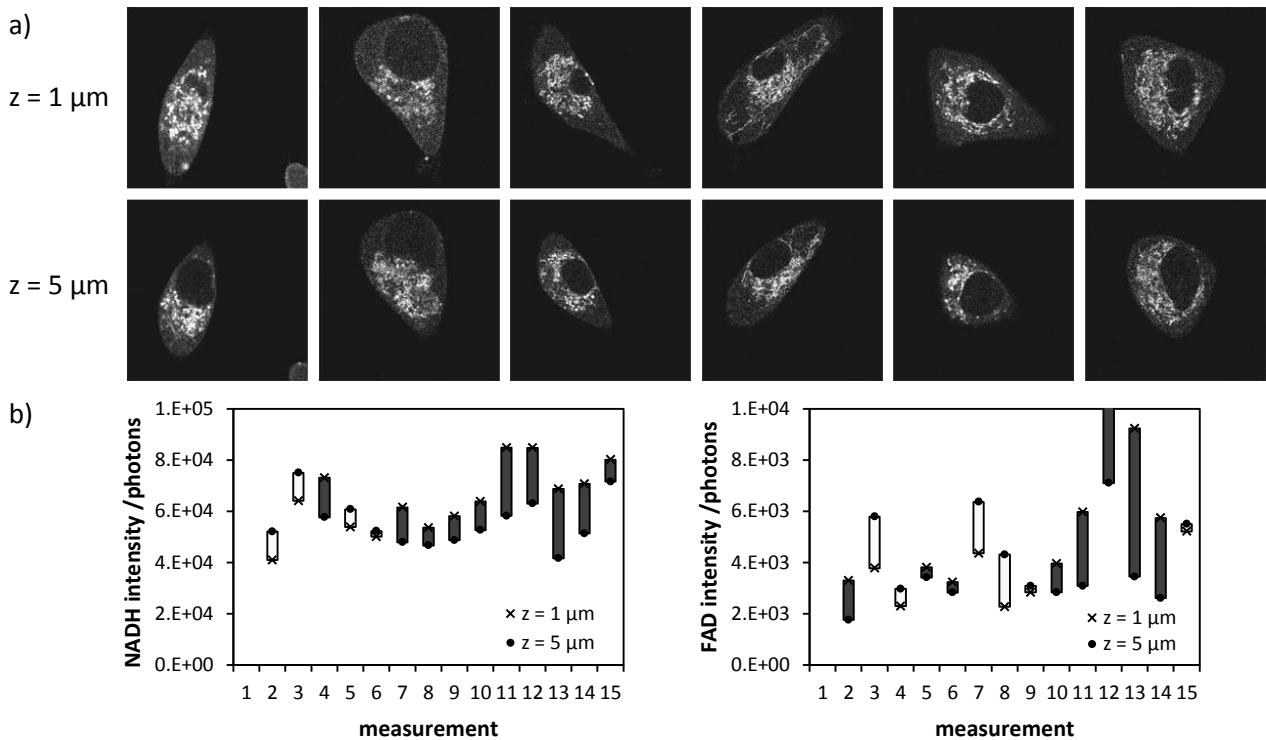


Figure 9.5: a) NADH fluorescence intensity images of cells acquired by 2PE. The mitochondria, seen here as punctuate perinuclear distributions of high fluorescence, are seen to vary between planes. This demonstrates that the optical setup undersamples the cell. b) Graphs showing how normalised NADH and FAD intensity changes between z-planes for each cell measurement ($n = 15$). NADH intensity is found to change significantly whereas the change in FAD is insignificant. White bars indicate an increase, whereas dark grey bars indicate a decrease, in intensity at the higher plane compared to the lower.

NADH fluorescence predominantly originates from the mitochondria. Furthermore, mitochondria are the only source of FAD fluorescence with BE cells exhibiting very low FAD photon count rates of $0.5 - 3 \times 10^3$ photons s^{-1} . Therefore, much of the variation may be attributed to the density variation and spatial configuration of the mitochondria within each cell. Any variation in these will produce a significant difference in the sampled intensities and as a consequence in the calculated RR.

To help eliminate the variation in NADH and FAD intensity due to the variation in mitochondrial number within cells the average intensity per pixel is determined for pixels corresponding to mitochondria. NADH and FAD fluorescence intensity images are thresholded and the average mitochondrial fluorescence signal per pixel is calculated and results are presented in figure 9.6.

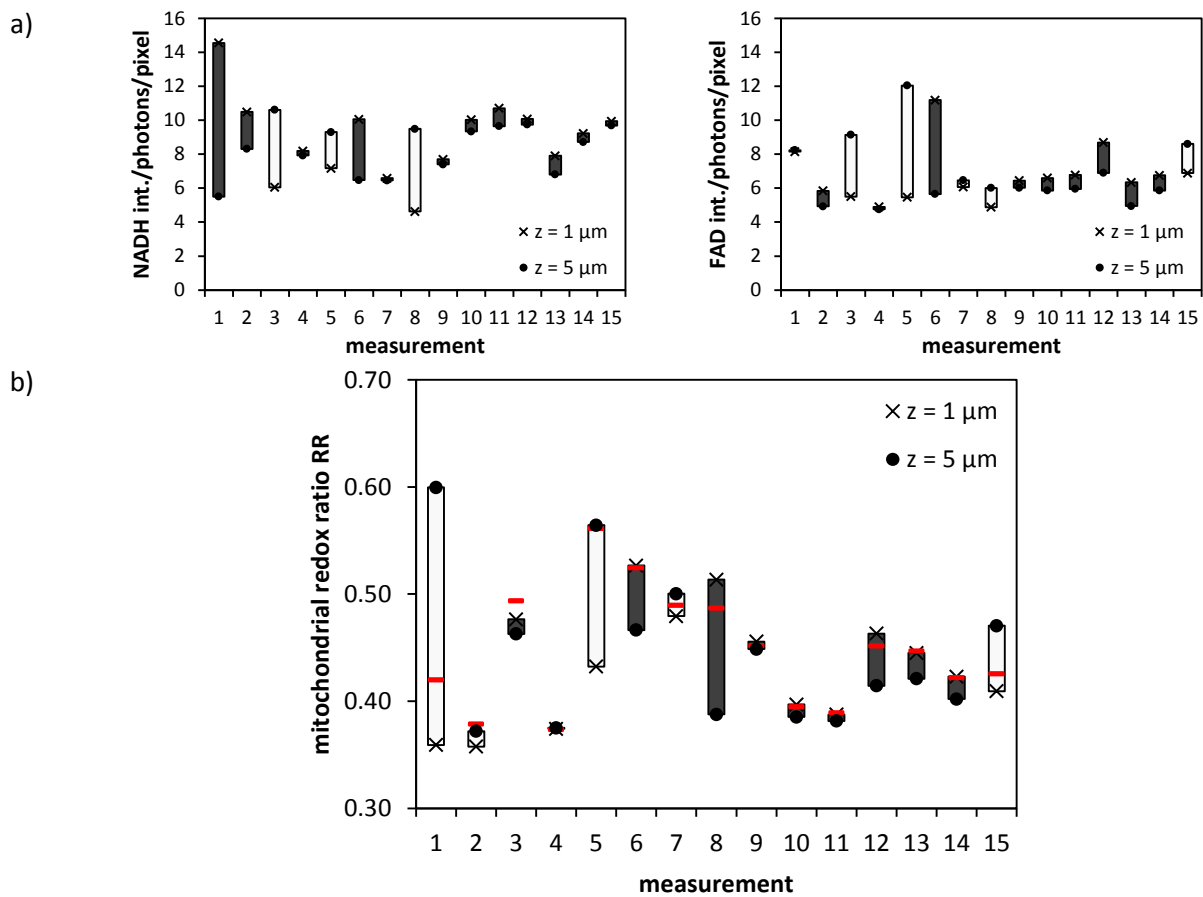


Figure 9.6: Investigating z-dependence of NADH and FAD fluorescence intensity in BE cell mitochondria alone 1 and 5 μm above the coverslip. a) The average NADH and FAD intensity per pixel is calculated for the mitochondrial regions in each cell measured. b) The mitochondrial redox ratio calculated for each plane. The red dashes represent the RR calculated from the summed NADH and FAD mitochondrial intensities from both planes.

The relative intra- and intercellular variation is reduced when considering the mitochondria alone compared to the entire cell (cf. figures 9.4 and 9.6). For instance, the average intra- and intercellular

whole-cell (figure 9.4a) variation in redox ratio are approximately 35 % and 10 %, respectively. Whereas, the average intra- and intercellular mitochondrial (figure 9.6b) variation in redox ratio are reduced by approximately a third to 12 % and 3.2 %, respectively. The average mitochondrial RR (RR_{mt}) is calculated for each plane ($n = 15$) and the difference between RR_{mt} ($z = 1 \mu m$) = 0.43 ± 0.01 and RR_{mt} ($z = 5 \mu m$) = 0.44 ± 0.02 is statistically insignificant ($P = 0.68$, paired t-test).

The mitochondrial redox ratio is significantly different from the whole cell ratio at both planes and also when integrating the signal from both planes ($P < 0.001$, paired t-test). A decrease in RR signifies an increase in metabolic activity [269]. This suggests that metabolic activity is higher when considering the entire cell than just the mitochondria alone. The mitochondrial RR is related to the whole cell RR simply by

$$\left[\frac{I_{FAD}}{I_{NADH} + I_{FAD}} \right]_{mt} = \alpha \left[\frac{I_{FAD}}{I_{NADH} + I_{FAD}} \right]_{cell}, \quad [9.3]$$

where the subscripts *mt* and *cell* signify the mitochondrial and whole cell parameters, respectively, and, in this case, $\alpha = 2.26 \pm 0.24$. Since FAD is exclusive to the mitochondria $I_{FAD,mt} = I_{FAD,cell}$ and eq. 9.3 becomes

$$\frac{I_{FAD}}{I_{NADH,mt} + I_{FAD}} = \alpha \frac{I_{FAD}}{I_{NADH,cell} + I_{FAD}}. \quad [9.4]$$

NADH intensity is typically at least an order of magnitude greater than FAD intensity so $I_{NADH} + I_{FAD} \rightarrow I_{NADH}$. Additionally, $NADH_{cell} = NADH_{cyt} + NADH_{mt}$, where *cyt* signifies the cytoplasmic parameter. Therefore, eq. 9.4 may be simplified to

$$NADH_{cyt} = (\alpha - 1)NADH_{mt}. \quad [9.5]$$

Here, the quantity $(\alpha - 1)$ is close to unity and the mitochondrial and cytoplasmic NADH intensities are similar and suggest a similar concentration of NADH within each compartment. However, this assumes there is no difference in the quantum yield of NADH in the cytoplasm to the mitochondria. Due to the much lower volume of the mitochondria, mitochondrial NADH concentration is, therefore, much higher.

The mitochondrial density may vary, and therefore NADH or FAD intensity, between intracellular planes; however, it is reasonable to expect that all mitochondria within the same cell to exhibit the same phenotype. If this is the case, the fluorescence lifetimes would be consistent between planes while the fluorescence intensity would vary. This is demonstrated by the NADH fluorescence lifetimes (figure 9.4b) but is not the case for FAD fluorescence lifetimes (figure 9.4c). Furthermore,

figure 9.4b suggests that the intra- and intercellular variation in NADH lifetime is low whereas figure 9.4c suggests this is not for the case of FAD.

The cell may be better sampled by either scanning in z or extending the field to encompass the entire cell. Scanning in z would allow the fluorescence intensity and lifetime signals to be integrated throughout the entire cell. This may be hard to practically implement due to the potential of overlapping planes in the stack, which would lead to some regions contributing more or less than others. Extending the depth of focus may be achieved by employing an annular lens aperture [270], which also has the benefit of increasing the 2P lateral resolution. This was not attempted but a more straightforward step would be to image cells using an objective with lower NA. The Leica SP5 objective turret, in addition to a 60× NA = 1.32 oil-immersion objective, is also populated with dry 40× NA = 0.75 and 20× NA = 0.5 objectives. The fluorescence intensity or count rate for epi-configurations is related to the objective numerical aperture and magnification given by

$$F \propto \left(\frac{NA^2}{M} \right)^2, \quad [9.6]$$

where NA and M are the objective numerical aperture and magnification, respectively. According to eq. 8.28, the focal planes of the 40× and 20× objectives for NADH are 8.23 μm and 18.5 μm thick, respectively. The 40× objective would be capable of fully sampling a single layer of cells grown on a flat coverslip but would lead to a 4-fold reduction in signal and using the 20× objective would lead to a 5-fold reduction. 2PE power would need to be approximately doubled (square dependence of fluorescence signal on excitation power) to achieve similar photon count rates and would lead to increased photodamage and disruption of cells. However, this assumes the 2P excitation efficiency remains the same for the lower powered objectives as for the 63× objective. According to eq. 8.23, the 40× objective is almost a factor of 10 less efficient at 2P excitation of NADH than the 63× objective. In this case, the compensatory increases in excitation power or acquisition time would not be reasonable.

2P Imaging is therefore best performed at a set distance above the coverslip of 1 – 2 μm employing a 63× oil-immersion objective. This results in a sampling efficiency of approximately 60% for cells of 5 μm thickness.

9.6 Cell confluence

It is known that the cellular redox environment can change depending on the synthesis of DNA and RNA and of proteins and during the regulation of the cell cycle [255]. MCF10A human breast cancer cells have been shown to exhibit confluence dependent metabolic behaviour [266]; the fluorescence lifetime and contribution of protein-bound NADH decreased as cells progressed from an early to logarithmic to confluent growth phase. Therefore, the behaviour of BE cells for increasing confluence is determined by measuring the fluorescence parameters of NADH and FAD for BE cells grown at increasing cell densities (figure 9.7).

A haemocytometer was used to determine the cell concentration per millilitre and counted in duplicate. The volumes required were then pipetted onto coverslips placed in the wells of a 6 well plate (6WP). The choice of cell concentration represents early (25×10^3 cells/well), logarithmic (100×10^3 cells/well) and confluent (1×10^6 cells/well) stages on the cellular growth curve for cells. Cells were imaged 24 hours after plating with 5 replicates performed at each cell density.

There exists no statistically significant differences in redox ratio or free to protein-bound ratios of NADH and FAD ($P < 0.05$, unpaired t-test). The study by Bird *et al.* [266] showed a clear increase in free to protein-bound lifetime contribution ratio of NADH for MCF10A cells but did not present any data on how the redox ratio since FAD was not imaged in the study. No change in the redox ratio (figure 9.7a) suggests possibly that cellular metabolic rate is not altered during growth, the NADH:FAD ratio is kept constant, or the changes in the fluorescence parameters are too subtle to be measured here. BE cells are a cancer cell line which do not undergo contact inhibition, as with many cancer cell lines. It is reasonable to assume that if the rate of mitosis does not change then the rate of metabolism does not either.

This shows that the requirements upon the culture of BE cells for metabolic imaging are not strict in terms of cell confluence.

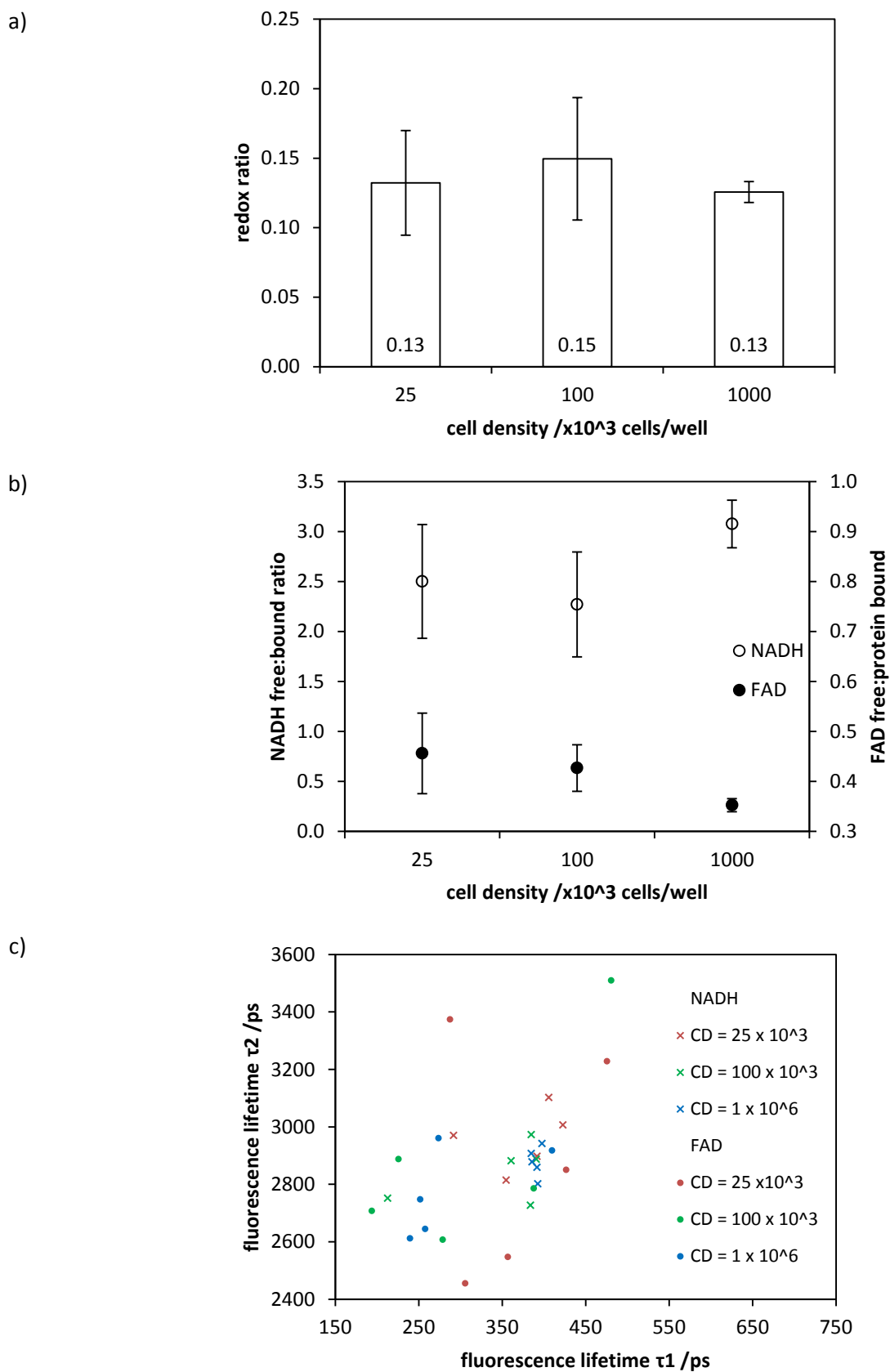


Figure 9.7: The parameters a) redox ratio, b) free:bound ratio and c) the fluorescence lifetimes of NADH and FAD are measured at increasing cell density showing no significant change as density is increased. CD = cell density in units of cells/well.

9.7 Single and two-point measurements

BE cells are tested for their response to the metabolic substrates glucose, at 1, 10 and 25 mM, and pyruvate, at 1 and 10 mM, in order to determine how they are used to maintain the redox potential of the cell. This is performed at room temperature after cells are incubated in the appropriate *physio + substrate* solution under room conditions (21°C with atmospheric oxygen/CO₂ concentration) for 30 min prior to imaging. Cells are also incubated in *physio* solution lacking any substrates and serves as a comparison.

As described in §7.2, glucose is metabolised by glycolysis, which involves the reduction of NAD⁺ to NADH, to produce pyruvate. Pyruvate may be further metabolised, either in the cytosol or the mitochondria. In the cytosol under anaerobic conditions, pyruvate is reversibly metabolised to lactate by lactate dehydrogenase, which concomitantly oxidises NADH to regenerate NAD⁺. Alternatively, under aerobic conditions, pyruvate is transported to the mitochondria where it undergoes irreversible oxidative decarboxylation to acetyl CoA by pyruvate dehydrogenase, and commits to the TCA cycle. The energy made available by the oxidative steps of the cycle are transferred to NAD⁺ as electrons to form NADH – for each acetyl CoA that enters the cycle, 3 NADH are formed. The TCA cycle serves to increase the pool of NADH which is oxidised to regenerate NAD⁺ and produce ATP by the ETC and oxidative phosphorylation. The cytosolic and mitochondrial pools of NAD⁺/NADH are separated since NADH cannot cross the mitochondrial inner membrane. Therefore, electrons from cytosolic NADH are transferred to the mitochondria via reducing agents using the glycerol-phosphate and/or malate-aspartate shuttles.

Therefore, whether the cell respire aerobically or anaerobically, an increase in the level of NADH is expected upon the exposure of cells to glucose. The exposure to pyruvate is expected to produce a response based on how cells are respiring. If cells are respiring anaerobically, the level of NADH is expected to decrease as pyruvate is reduced in the cytosol to lactate. However, if cells are respiring aerobically NADH is expected to increase as pyruvate is transported to the mitochondria and enters the TCA cycle.

Each intensity image is binned to a single pixel and the NADH and FAD summed intensity for each measurement ($n = 10$ per substrate concentration) is plotted (figure 9.8a). Cells that are deprived of substrate may act as a comparison to how cells respond. NADH concentration is observed to be increased in cells incubated for 30 min after the addition of glucose and pyruvate suggesting that both are utilised in BE cells as metabolic substrates (figure 9.8a). This increase in NADH is statistically significant for both substrates at all concentrations ($P < 0.0001$, unpaired t-test, $n = 10$).

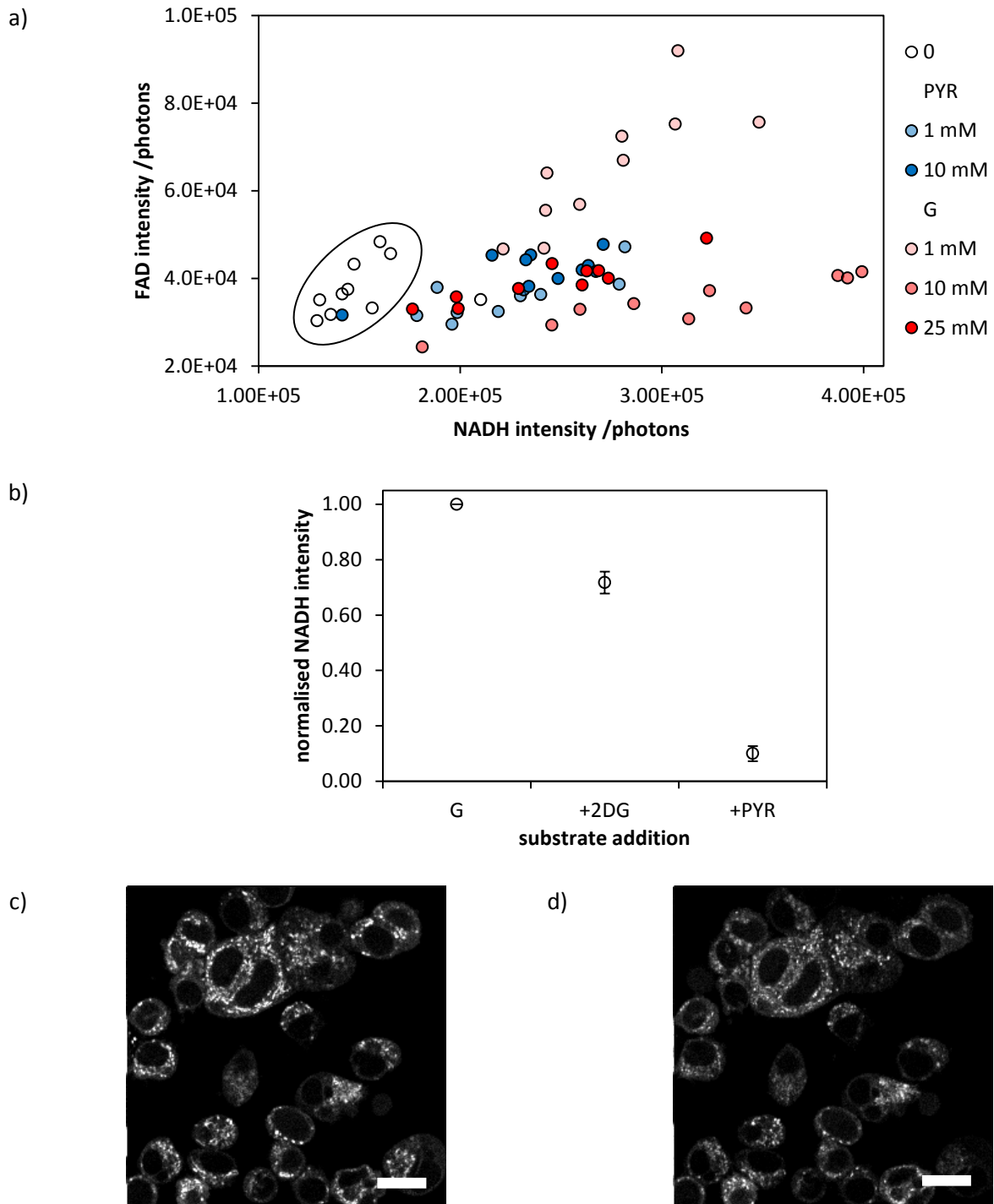


Figure 9.8: a) BE cells are investigated for their response to the metabolic substrates glucose (red circles), at 1, 10 and 25 mM, and pyruvate (blue circles), at 1 and 10 mM after 30 min incubation and compared to cells deprived of substrate (0, white circles). Both substrates produce an increase in measured NADH whereas there is little change in FAD intensity as compared to substrate deprived cells. b) Upon addition of an inhibitor of glucose (2DG) to cells incubated in media containing 10 mM glucose, NADH fluorescence decreases suggesting glycolysis is active in BE cells. Addition of pyruvate to 2DG treated cells provokes a sharp decrease in NADH (see §9.2.7 for discussion). c) and d) show NADH fluorescence intensity images before and after the addition of 2DG, respectively. NADH in mitochondria, bright punctate regions, decreases suggesting aerobic glycolysis is being performed. Scale bars 15 μm .

It is unknown from the intensity measurements alone whether glucose is being metabolised aerobically or anaerobically but pyruvate may be inferred to be metabolised predominantly in the mitochondria.

FAD is not observed to significantly change in cells incubated with pyruvate or with the concentrations of glucose tested > 1 mM ($P > 0.1$, unpaired t-test, $n = 10$) whereas for 1 mM glucose it increases significantly ($P < 0.0001$, unpaired t-test, $n = 10$) (figure 9.8a). This would seem to suggest that neither exogenous nor glucose-derived pyruvate is aerobically metabolised in the mitochondria.

BE cells were tested for their response to an inhibitor of glycolysis, 2-deoxy-D-glucose (2DG), to determine whether aerobic glycolysis was occurring. 2DG is a glucose analogue that is actively taken up by hexose transporters and phosphorylated. 2DG-6-phosphate accumulates in the cell and inhibits hexokinase and phosphoglucose isomerase thus inhibiting carbohydrate metabolism [271]. Growth media is replaced by *physio + 10 mM glucose* solution and incubated at room conditions for 30 min. Cells are imaged as previously described and solution is replaced with *physio + 10 mM glucose + 5 mM 2DG* solution and imaged after 10 minutes with a statistically significant measured decrease in NADH ($P < 0.01$, $P = 0.005$, paired t-test, $n = 3$) (figure 9.8b). As evidenced by comparing the NADH intensity images in figures 9.8c & d, the mitochondrial NADH intensity – seen as bright punctate regions surrounding the nucleus – is found to decrease. Since cells have no other exogenous substrate other than glucose, a reduction in mitochondrial NADH following an inhibition of glycolysis would suggest aerobic glycolysis was taking place. However, an inhibition of glycolysis will also disturb the glycerol-phosphate and/or malate-aspartate shuttles which effectively transport cytosolic NADH to the mitochondria. After imaging cells in *physio + 10 mM glucose + 5 mM 2DG* solution, pyruvate is added to the solution to a final concentration of 10 mM and cells are left to incubate for another 10 min. NADH is measured to dramatically decrease.

Interestingly, incubating cells in solution with increasing glucose concentration does not increase NADH further (figure 9.8a). It may be the case that the ATP requirement of cells observed in this particular state is met already by 1 mM glucose. Alternatively, it may be the case that the rate of glucose transport, facilitated by the membrane GLUT transporters, is already at a maximum for 1 mM glucose. Therefore, any additional glucose in the medium will not be able to be taken up in the cell. The response to glucose and pyruvate (figure 9.8a) may be such that the NADH response integrated over the cell remains the same whereas it differs within each compartment (cytosol and mitochondria). The intensity of a sample region of fixed area, approximately $0.5 \mu\text{m}^2$, is measured in regions containing mitochondria and regions not – this is determined by intensity thresholding the

image and observing the FAD intensity images (figure 9.9). The response by the cytosol and mitochondria when incubated with *physio* solution containing 1 mM glucose compared to 1 mM pyruvate is distinct (figure 9.9a & b). It can be seen that glucose promotes reduction of cytosolic NAD^+ i.e. increases cytosolic NADH, whereas pyruvate promotes reduction of mitochondrial NAD^+ .

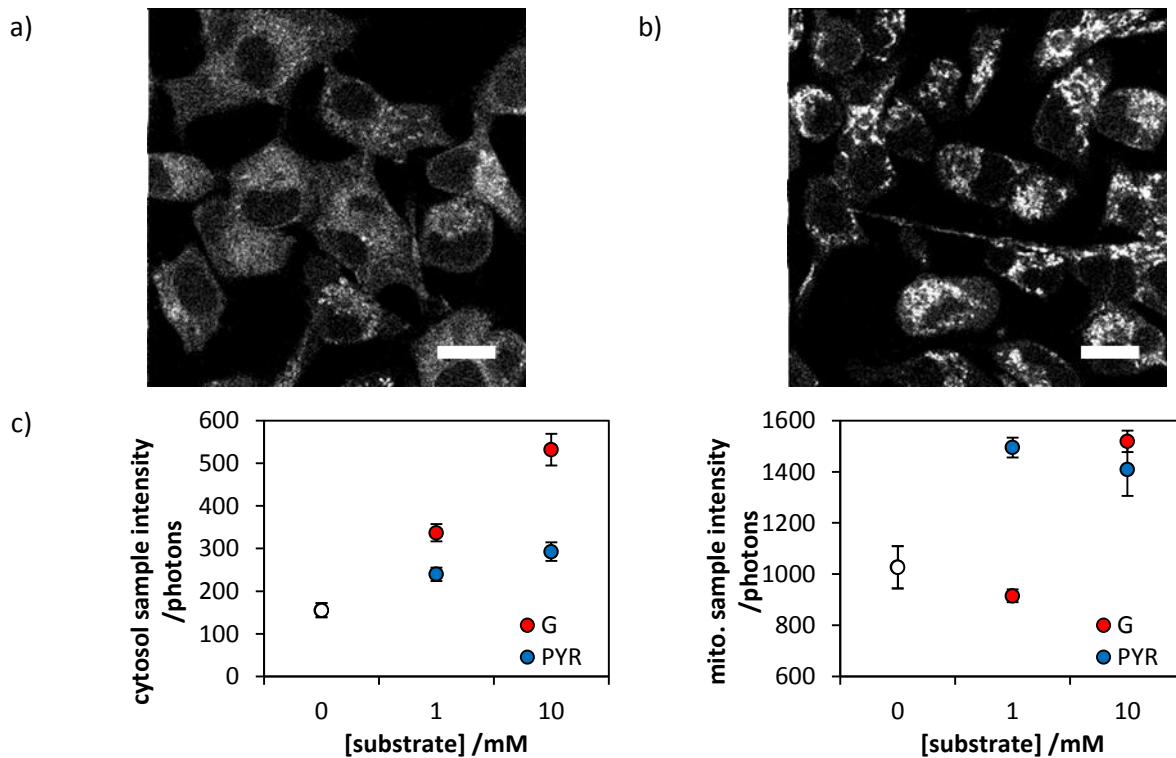


Figure 9.9: NADH fluorescence intensity images of BE cells after incubating cells in *physio* solution containing a) 1 mM glucose and b) 1 mM pyruvate. Scale bars 10 μm . c) The NADH response to increasing concentrations of glucose and pyruvate is spatially resolved to the cytosolic and mitochondrial compartments. This is done by measuring NADH fluorescence intensity in a sampled region ($n = 15$ cells) corresponding to the cytosol (diffuse) and the mitochondria (punctate).

The different response each compartment has for increasing glucose and pyruvate concentrations is shown in figure 9.9c. For glucose, the cytosolic NADH response increases markedly with concentration whereas a mitochondrial response is only observed at 10 mM glucose concentration. Pyruvate has a small but significant effect of increasing cytosolic NADH and increases mitochondrial NADH. Mitochondrial NADH is not increased further by increasing pyruvate concentration from 1 mM to 10 mM and may indicate a saturation of the mitochondrial metabolic pathways by exogenous pyruvate. A relatively low cytosolic NADH signal for cells incubated with pyruvate may be indicative

of LDH activity which metabolises pyruvate to lactate. Therefore, pyruvate may be metabolised both in the cytosol and mitochondria in BE cells.

The FAD intensity needn't be considered as above with compartmentally resolving NADH since it only originates from the mitochondria. The lack of change in FAD intensity may be due to how cells respond to substrate deprivation. Glycolysis is stimulated by low levels of ATP. Glycolytic ATP may be produced at a higher rate than ATP produced by the TCA cycle and oxidative phosphorylation in the mitochondria. Upon replacing growth media with *physio* solution lacking any substrates, it is reasonable to assume, therefore, that glucose is 'used up' at a higher rate and cytosolic NADH is either oxidised by lactate dehydrogenase to regenerate NAD^+ or is shuttled to the mitochondria. Mitochondrial NADH is increased when incubated with pyruvate suggesting that the mitochondrial level of NADH after substrate deprivation becomes relatively low. In mitochondria, NADH is oxidised to NAD^+ via complex I of the ETC with the concomitant reduction of FAD^+ to FADH_2 . Considering only the 'primary' reaction steps involved in the TCA cycle and the ETC, i.e. no shuttling mechanisms or side pathways, it follows that when mitochondrial NADH is low then FAD is comparatively high. This assumes that the metabolic pathways of the mitochondria, particularly the ETC, are fully intact and not impaired. However, this is typically not the case with the majority of cancer cell types (see §7.3). Nevertheless, it follows that FAD is expected to decrease when incubated with substrates that are metabolised mitochondrially compared to when cells are deprived of substrate. This is not the case and is difficult to interpret alongside the results of NADH.

Each intensity image is binned to a single pixel and the redox ratio is for each measurement ($n = 10$ per substrate concentration) is calculated using eq. 9.1. The RR for the substrate deprived cells serves as a level for comparison. Upon the addition of glucose or pyruvate the RR decreases, which is statistically significant for all substrate concentrations ($P < 0.0001$, unpaired t-test, $n = 10$) with the exception of 1 mM glucose (figure 9.10). A decrease in the redox ratio usually indicates increased cellular metabolic activity [264, 272] and is consistent with the discussion above. Considered in isolation, the RR suggests that metabolic activity does not change significantly ($P = 0.44$, unpaired t-test, $n = 10$) when cells are incubated in 1 mM glucose when compared with substrate deprived cells. However, it has already been seen that 1 mM glucose elicits a cytosolic increase in NADH, which does suggest a change and increase in metabolic activity. According to the RR the cells exhibit highest activity with 10 mM glucose.

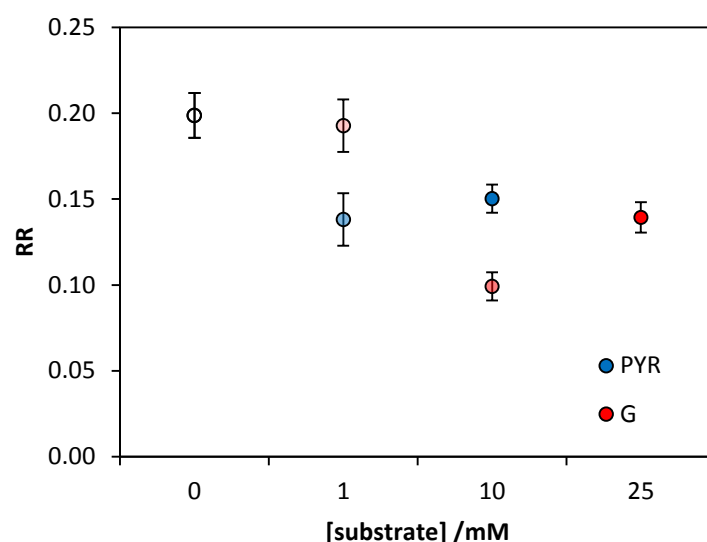


Figure 9.10: The average redox ratio is calculated for cells in figure 9.8a. A decrease in RR indicates increased metabolic activity. Therefore, the general decrease in RR is expected for cells incubated with metabolic substrates compared to substrate deprived cells.

Since the FAD response, or predominant lack thereof, has been difficult to interpret alone, it is not clear how reliable the RR is for cells that do not express a FAD based response to metabolic substrates.

The fluorescence lifetime serves as an additional parameter by which to interpret results (figure 9.11). Images are binned to a single pixel spatially whilst preserving decay information and imported into SPCImage software (Becker & Hickl, Germany). The data is fit to a model function (see §8.1.2) and all fitting parameters are free i.e. not fixed. SPCImage optimises the fit parameters using the Levenberg-Marquardt algorithm, minimizing the weighted chi-square quantity – the lower the quantity the better the goodness of fit. NADH and FAD were both best fit to a double exponential and all fits produced a $\chi^2 < 2$. The short (τ_1) and long (τ_2) lifetime components of NADH fluorescence correspond to the cofactor being unbound and bound, respectively, to metabolic enzymes for which it is a cofactor [234]. Conversely, short (τ_1) and long (τ_2) lifetimes of FAD correspond to the cofactor being bound and unbound, respectively [202]. The fluorescence lifetime of bound NADH has been shown to be dependent on which enzyme or protein to which it is bound. The bound FAD lifetime has been shown to decrease in the presence of NAD^+ due to quenching [273]. This suggests that changes in metabolic activity may be measured as changes in the fluorescence lifetimes of NADH and FAD.

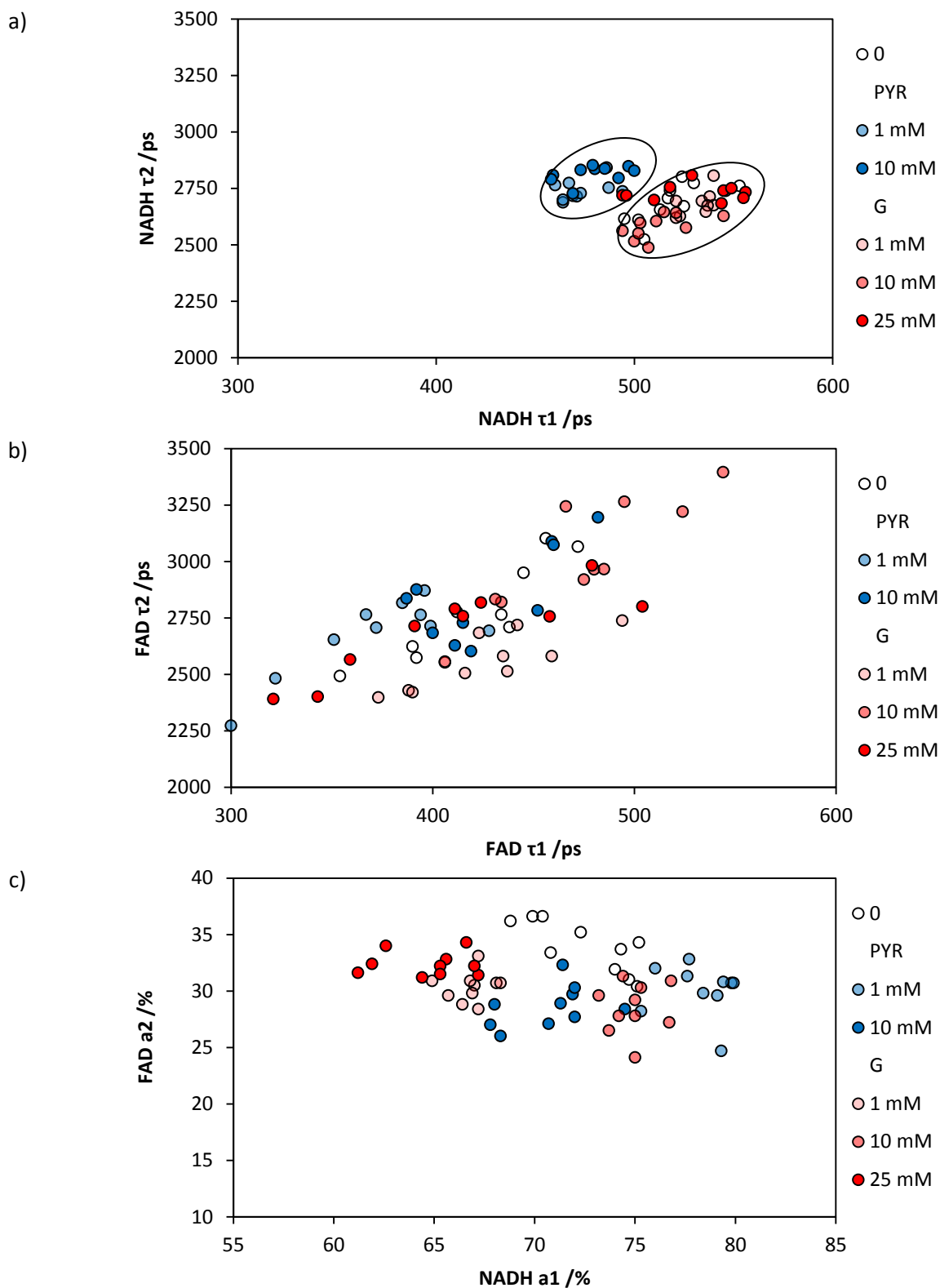


Figure 9.11: The fluorescence lifetime parameters of lifetime and lifetime contribution are determined for cells in figure 9.7a. Plots of free (τ_1) and protein-bound (τ_2) lifetimes of a) NADH and b) FAD for cells incubated under different metabolic conditions. c) Plot of the relative amount of free NADH (a_1) and free FAD (a_2). PYR = pyruvate, G = glucose.

The fluorescence unbound (τ_1) and protein-bound (τ_2) lifetimes are plotted for NADH (figure 9.11a) and FAD (figure 9.11b). For NADH, the plot shows a distinct contrast between cells incubated with glucose or undergone substrate deprivation and cells incubated with pyruvate. Lifetime parameters are collated for substrate deprived cells and cells incubated in glucose ($n = 40$) and for cells incubated in pyruvate ($n = 20$). For the collated results, there is a statistically significant increase in the protein-bound NADH lifetime ($P < 0.0001$, unpaired t-test) and a statistically significant decrease in the free NADH lifetime ($P < 0.0001$, unpaired t-test). From intensity measurements of the metabolic compartments of the cell (figure 9.9), it is expected that pyruvate is metabolised predominantly in the mitochondria whereas glucose may be metabolised via glycolysis and the mitochondria. Therefore, the shift in protein-bound lifetime may be indicative of increase mitochondrial metabolic activity for pyruvate as compared to glucose.

A shift in free NADH lifetime is not expected for any changes solely in binding pattern for different substrates or metabolic phenotype. The shift in the free NADH lifetime may reflect an increase in concentration of quenchers such as oxygen or changes in pH. A decrease in pH, may suggest elevated levels of lactic acid production which is corroborated by a relatively low cytosolic NADH signal (figure 9.9).

For FAD, the plot of the protein-bound (τ_1) and unbound (τ_2) lifetimes shows no difference between cells incubated with glucose or undergone substrate deprivation and cells incubated with pyruvate (figure 9.11b). The variation in both lifetimes is also much higher. FAD is quenched by NAD^+ and so a relative decrease in mitochondrial NAD^+ , observed here as a relative increase in NADH, suggests FAD lifetime should increase. This is not observed.

The pre-exponential factors of the fluorescence lifetimes (see §8.1.2) indicate the relative amounts of each species. Expressed as a percentage of the total fluorescence, the relative amounts of free NADH (a_1) and FAD (a_2) are plotted (figure 9.11c). Cellular NADH is predominantly in the free state whereas the majority of FAD is in the bound-state. In comparison to substrate deprived cells, cells incubated with metabolic substrates should exhibit a decreased level of free NADH and FAD, since it is expected they be bound to enzymes. Compared to substrate deprived cells, incubating cells with glucose or pyruvate resulted in an average decrease of free FAD (a_2) by $4.0 \pm 0.8 \%$, which is statistically significant ($P < 0.0001$, unpaired t-test, $n = 50$). The amount of free NAD (a_1) does not change in any discernable way.

The acquired FLIM images may be masked to investigate the intracellular changes in NADH or FAD lifetime. However, this not possible owing to the low signal to noise for each masked region – a

sample region of fixed area, $0.5 \mu\text{m}^2$ as before, typically contains 30 – 40 photons in the peak of the decay and a signal to noise ratio (S:N) of approximately 5. Clearly, increasing the sample region would increase the S:N but segregating signal from mitochondria and the cytosol becomes difficult.

9.8 Continuous measurements

As discussed above, it is difficult to fully interpret the results without the consideration of kinetic models. It is expected that continuously monitoring fluorescence may provide additional insights into cellular response. After 30 min incubation with a substrate, it is expected that cells are imaged in a steady state. This means that any transient or short lived responses upon substrate addition or removal are unable to be measured. By continuously measuring cell autofluorescence before, during and after the addition of metabolic substrates it is possible to determine a more complete time-resolved response and measure any transient responses that may be occurring. The time-resolved response of NADH fluorescence is measured in response to glucose and pyruvate.

Data was collected in the FIFO, or time-tag, mode (see §8.3) which not only time-resolves photons with respect to the laser-pulse but to the start of the experiment. This allows the recording of time-resolved intensity measurements which also provides fluorescence lifetime data. A classic FLIM image maintains spatial resolution but averages macro-temporal resolution whereas FIFO acquisition averages spatial resolution (effectively a single pixel image) while maintaining micro- and macro-temporal resolution. Unfortunately, therefore, spatial information is lost and does not permit the time-resolved response to be further resolved depending on its mitochondrial and/or cytosolic origin. In order to do so, pharmacological agents that inhibit particular pathways or transporters are employed. For instance, pyruvate may be either transported to the mitochondria by monocarboxylate transporter (MCT) or metabolised to lactate by lactate dehydrogenase (LDH) in the cytosol. Pyruvate transport to the mitochondria may be prevented or limited by the MCT inhibitor α -cyano-4-hydroxy cinnamate (CIN) [274]; CIN does not block mitochondrial respiratory complex I or complex II. LDH may be inhibited by oxamate. The alternative fates of pyruvate may therefore be discerned by measuring the cellular integrated NADH response with or without the addition of such agents.

BE cells grown on coverslips are removed from culture, growth media is aspirated and cells are incubated in *physio* solution lacking metabolic substrates for 30 min under room conditions prior to commencing imaging. Figure 9.3 shows the NADH intensity and lifetime response exhibited by the cells to continuous illumination over the course of 25 min (1500 s). The reduction of NADH signal

due to photobleaching reaches a steady state after 600s, therefore any substrate or pharmacological agent is added beyond this time. Inhibitors are added after 600 s of continuous imaging and allowed to incubate for 300 s before the addition of substrate. Addition of substrate is performed as before, whereby solutions are added as a 2× solution of their final concentrations, except CIN, which is added as a 5× solution. Pyruvate and glucose are tested at a final concentration of 10 mM.

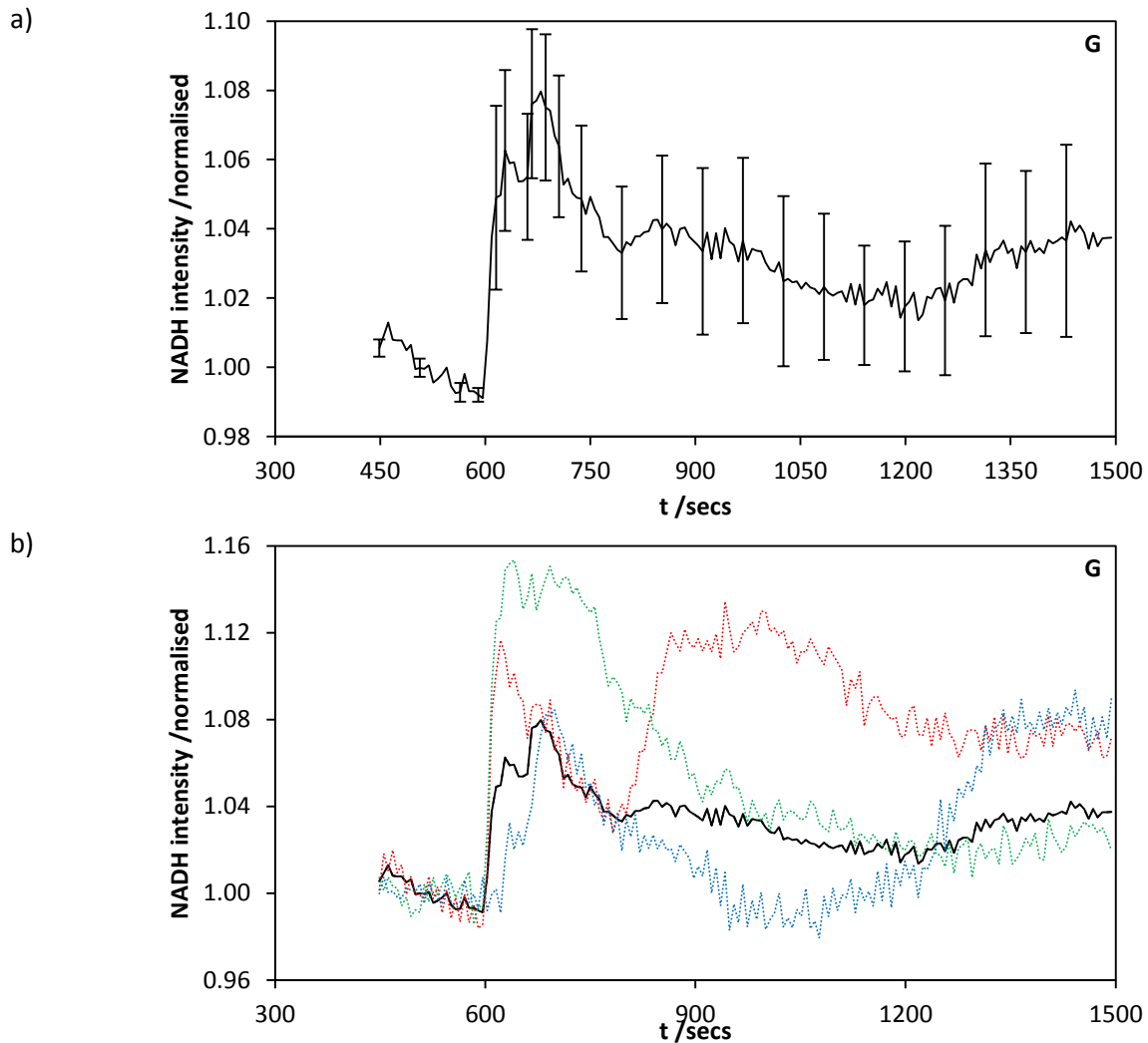


Figure 9.12: Testing BE cell response to glucose. a) Glucose is added to a final concentration of 10mM at 600s. Error bars represent the standard error of the mean for $n = 6$ replicates. b) The cellular NADH response to glucose varies and is exemplified by the 3 colour traces, exhibiting an initial spike, decay then secondary rise.

The addition of glucose provokes a transient rise in NADH by $8 \pm 2 \%$, which peaks between approximately 15 and 85 s subsequently after (figure 9.12a). The response is variable and seems to consist of an initial spike which decays followed by a second rise. Three typical responses are

compared to the averages response ($n = 6$) to illustrate this in figure 9.12b. The initial and secondary responses vary in duration, magnitude and time of onset. It has been observed that cytosolic and mitochondrial NADH increases when cells are incubated in solution containing glucose (figure 9.9).

The data of figure 9.8a and 9.9c shows that when cells are incubated in media containing only glucose cytosolic and mitochondrial NADH is increased compared to cells which are deprived of substrate. Since glucose must be first metabolised to pyruvate by glycolysis before it can enter the mitochondria, it is expected that the initial response originates from the cytosol. What controls the behaviour beyond the initial response is unknown. Indeed, although most likely given previous data, glucose may not be fully responsible for the initial response since glycolytic intermediates may be used in side pathways which generate NADH. Further investigation with inhibitors such as 2DG is required. However, this has not been performed.

The addition of pyruvate stimulates a sharp decrease of NADH intensity by $23 \pm 2 \%$ over approximately 60 s (figure 9.13a). NADH decreases by a further 10 ± 3 over approximately 9.5 min and remains constant for the remainder of the acquisition. A decrease in NADH is expected if pyruvate is being metabolised by LDH to lactate as NADH is concomitantly oxidised to NAD^+ in the cytosol. Anaerobic glycolysis is often predominant in tumours and causes elevated levels of lactic acid as well as increased LDH activity. Alternatively, pyruvate may enter the mitochondria. Pyruvate oxidation to lactate is inhibited by incubating the cells for 300 s with 5 mM oxamate before the addition of pyruvate (figure 9.13b). This provoked a very similar response – a sharp decrease of NADH by $19 \pm 6 \%$ over approximately 60 s. Similarly, pyruvate transport to the mitochondria is inhibited by incubating cells with 1 mM CIN, instead of oxamate. The addition of pyruvate subsequent to incubation with CIN produces a different response where the sharp decrease of NADH by 20 % no longer occurs (figure 9.13c). Instead, after approximately 140 s following addition of pyruvate, NADH decreases at a constant rate for the remainder of the acquisition. The sharp decrease in NADH is therefore due to pyruvate being transported to the mitochondria.

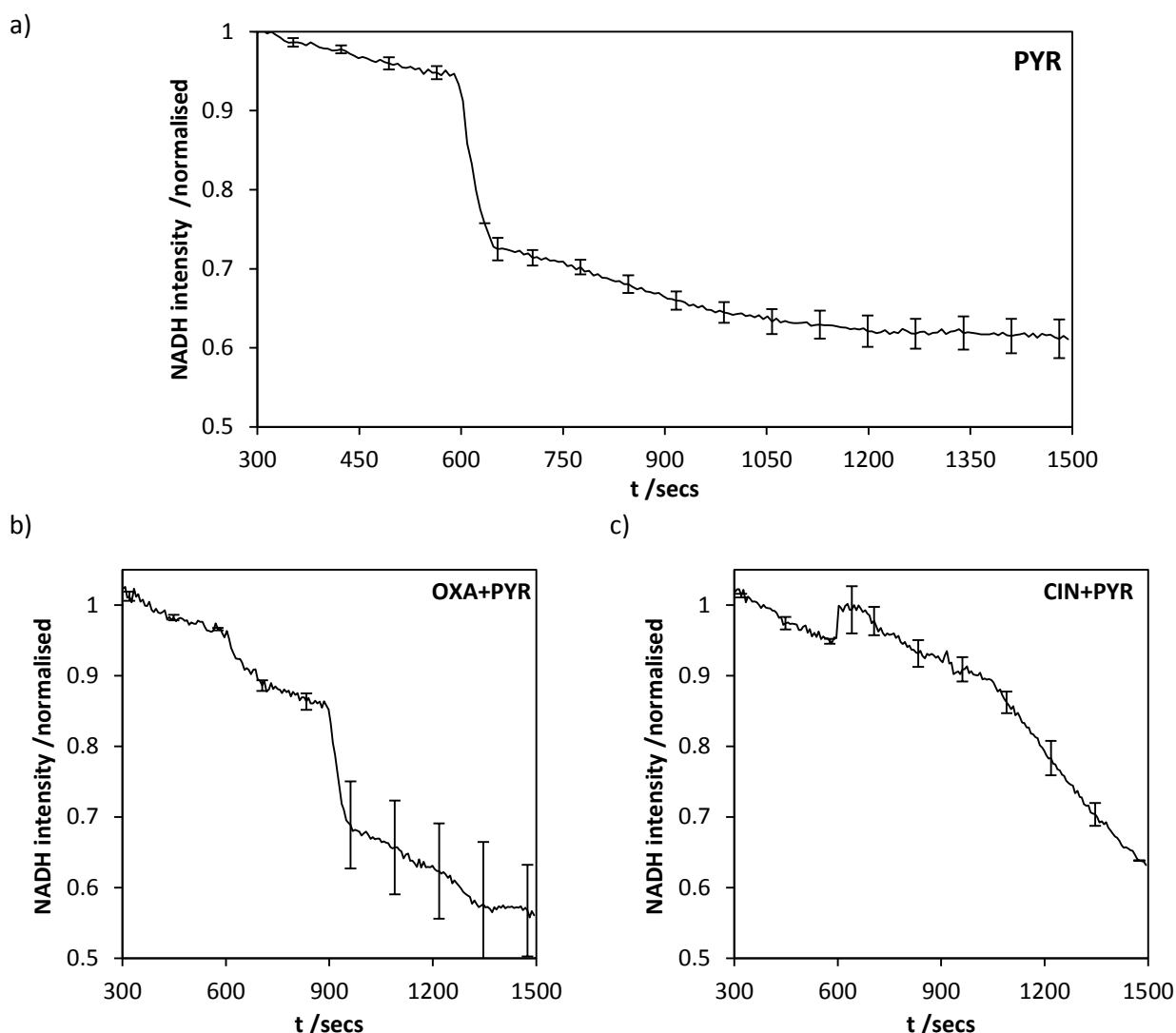


Figure 9.13: Testing BE cell response to exogenous pyruvate and inhibitors. a) Pyruvate is added to a final concentration of 10mM at 600s. b) Oxamate and c) CIN are added to final concentrations of 5mM & 1mM, respectively, at 600s and pyruvate subsequently added to a final concentration of 10mM at 900s. Error bars represent the standard error of the mean for n = 6 replicates.

Pyruvate is processed by the TCA cycle to produce metabolic intermediates by the reduction of NAD^+ to NADH. NADH is then oxidised by complex I of the ETC during aerobic respiration. It is expected that such a decrease in NADH is due to the aerobic respiration of pyruvate in the mitochondria. The results of inhibiting pyruvate transport to the mitochondria by CIN corroborate this. Interestingly, when uptake to the mitochondria is inhibited, NADH slowly decreases. This may be due to pyruvate pooling in the cytosol which is then metabolised by LDH.

The data of figure 9.8a and 9.9c shows that when cells are incubated in media containing only pyruvate, mitochondrial NADH is increased compared to cells which are deprived of substrate. The data of figure 9.13 suggests that mitochondrial NADH immediately decreases upon addition of

pyruvate to substrate deprived cells that have undergone continuous imaging. It is difficult to reconcile the observed differences without further tests involving additional inhibitors and controls.

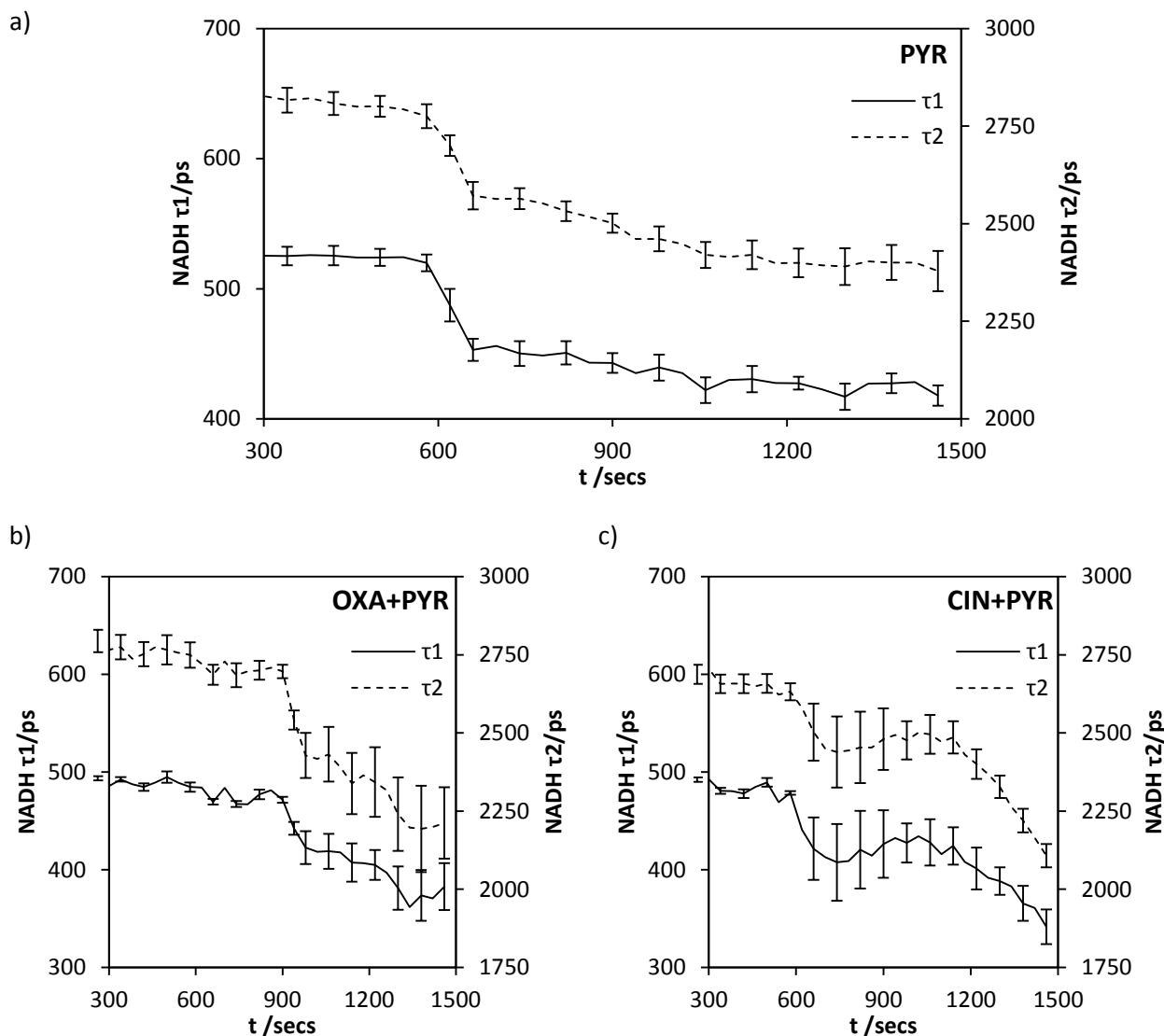


Figure 9.14: Fluorescence lifetime response of NADH in BE cells to exogenous pyruvate and inhibitors. a) Pyruvate is added to a final concentration of 10mM at 600s. b) Oxamate and c) CIN are added to final concentrations of 5mM & 1mM, respectively, at 600s and pyruvate subsequently added to a final concentration of 10mM at 900s. Error bars represent the standard error of the mean for n = 6 replicates.

FIFO acquisition allows the simultaneous measurement of NADH fluorescence intensity and lifetime. The change in NADH free (τ_1) and bound (τ_2) lifetimes in response to the addition of pyruvate with or without inhibitors and in response to the addition of glucose is shown in figures 9.14 and 9.15,

respectively. The NADH fluorescence lifetime data corresponds with the fluorescence intensity data presented in figures 9.12 and 9.13.

Upon the addition of pyruvate both the form of the change in NADH free and bound lifetimes mimics the form of how the changes in NADH intensity – initially, there is a sharp decrease followed by a slower one to a lower steady state level (figure 9.14a). This response is reproduced when inhibiting cytosolic reduction of pyruvate by inhibiting LDH with oxamate (figure 9.14b); however, upon inhibiting mitochondrial transport with CIN the response is different (figure 9.14c). The addition of oxamate does not result in a change in either lifetime component whereas the addition of CIN results in a reduction of both, which further decreases upon addition of pyruvate.

Figure 9.15 shows that upon the addition of glucose the free NADH lifetime remains unchanged whereas the bound NADH lifetime increases. The bound lifetime increases over approximately 100 s, which is similar to the time observed for the initial spike in NADH fluorescence intensity to peak and start to decay in figure 9.12. From results, at 10 mM, the incubation of cells with glucose increases cytosolic as well as mitochondrial NADH (figure 9.9c). Also, the addition or incubation of cells with pyruvate leads to a relative reduction in NADH fluorescence lifetime (figure 9.14). It is therefore expected that, if glucose-derived pyruvate is being metabolised in the mitochondria, a decrease in NADH fluorescence lifetime would be observed at some point after the addition of glucose in figure 9.15. This is not observed; instead, the bound lifetime remains constant for the remainder of the acquisition after the rise in bound NADH lifetime.

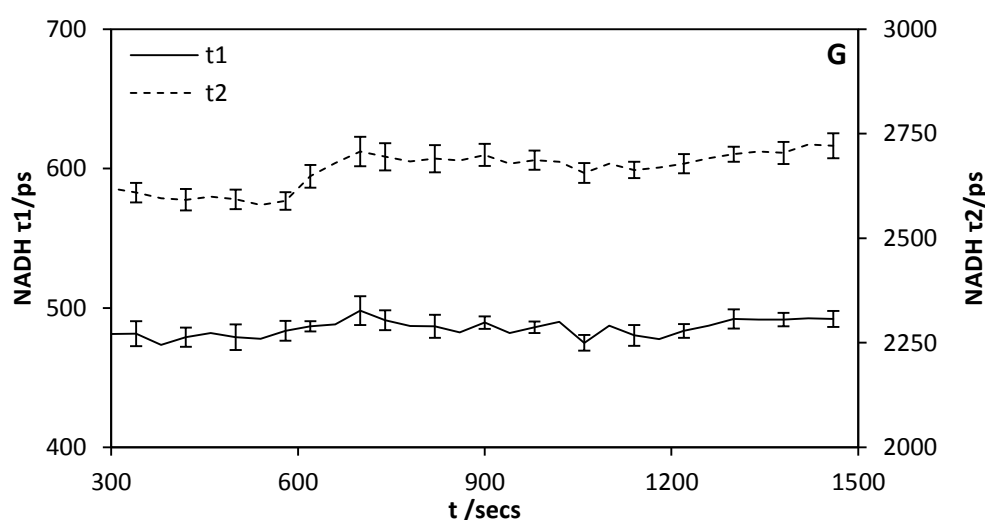


Figure 9.15: Fluorescence lifetime response of NADH in BE cells to exogenous glucose. Glucose is added to a final concentration of 10mM at 600s. Error bars represent the standard error of the mean for n = 6 replicates.

Fluorescence lifetime not only provides contrast between free and bound NADH it also provides the relative concentration of both forms. The ratio of free:bound NADH is calculated from the pre-exponential factors associated with each lifetime component (see §8.1.2). This ratio is expected to decrease upon the addition of substrate as NADH becomes bound to metabolic enzymes to which it is a co-factor. The bound form of NADH is associated mostly with the dehydrogenases of complex I of the ETC [275] such that any changes in the free:bound ratio may be predominantly attributed to the dynamics of complex I.

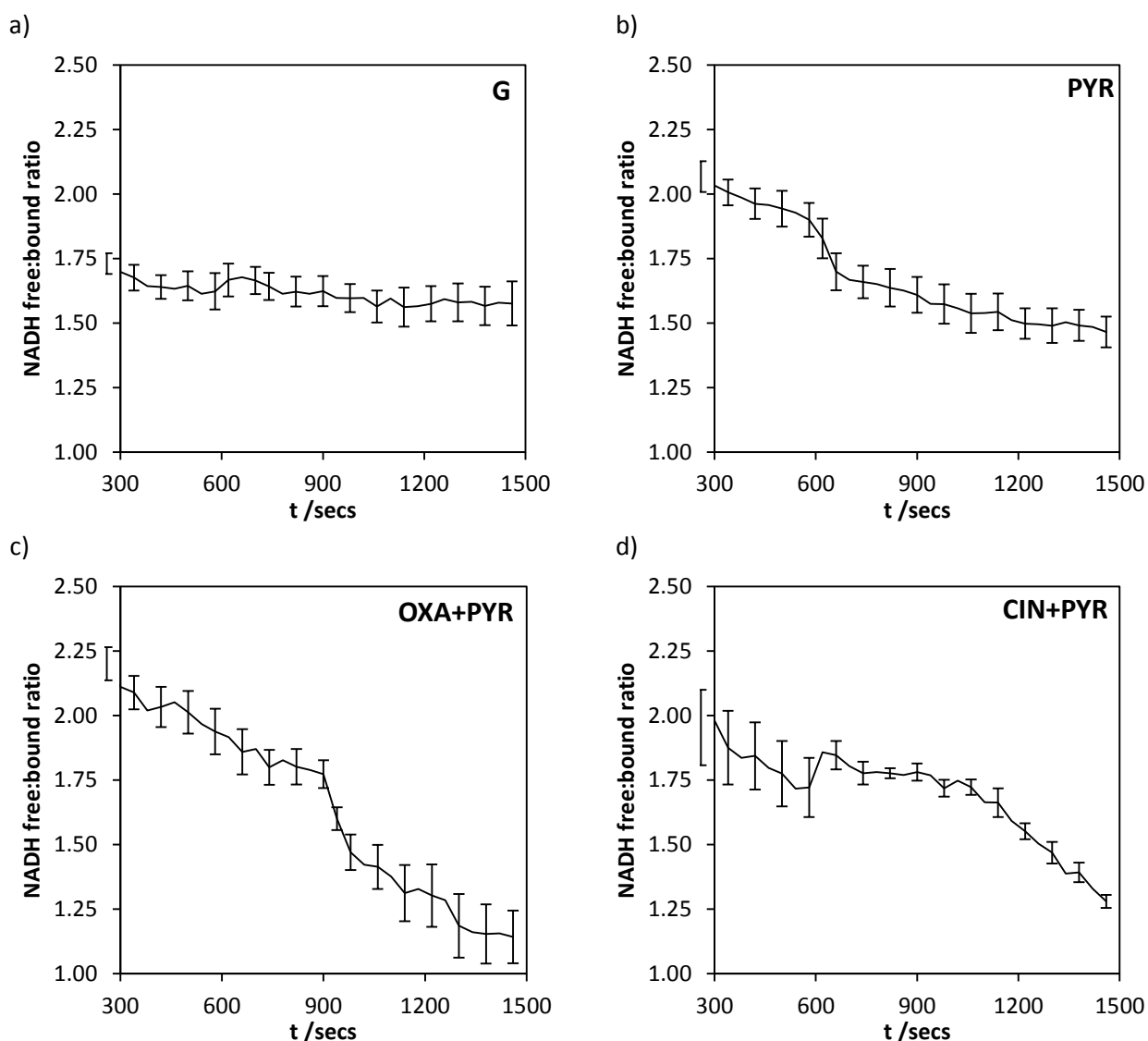


Figure 9.16: The NADH free:bound ratio is calculated from the relative contributions of the free (a_1) and protein-bound (a_2) fluorescence lifetimes. a) Glucose is added to a final concentration of 10mM at 600s. b) Pyruvate is added to a final concentration of 10mM at 600s. c) Oxamate and d) CIN are added to final concentrations of 5mM & 1mM, respectively, at 600s and pyruvate subsequently added to a final concentration of 10mM at 900s. Error bars represent the standard error of the mean for n = 6 replicates.

In response to the addition of glucose, the free:bound ratio does not change (figure 9.16a). If a change in free:bound ratio is, indeed, consistent with an engagement of substrate metabolism then this suggests glucose is not being metabolised in BE cells. However, this would be contradictory to the increase in cytosolic and mitochondrial NADH observed in figure 9.9 and the effect of 2DG, an inhibitor of glycolysis, on cellular NADH (figure 9.8b). A decrease in the free:bound ratio by $16 \pm 6\%$ was measured upon the addition of pyruvate (figure 9.16b). The inhibitors oxamate and CIN demonstrate that the drop originates from the mitochondria (figures 9.16c & 9.16d). This is consistent with previous results that suggest exogenous pyruvate may be metabolised in the mitochondria.

9.9 Preliminary measurements

9.9.1 Adult rat cardiomyocytes

In order to investigate and characterise the shift in metabolism that is reported as heart failure progresses, cardiomyocytes derived from healthy and infarcted adult rat hearts, described in §7.3 and §7.4, are imaged by 2PE.

Adult rat cardiomyocytes (ARCMs) from infarcts are imaged in *physio-cm* solution within 4 hours of isolation (figure 9.17). The solution contains 6.3 mM pyruvate and 20 mM glucose. The NADH and FAD fluorescence intensities are normalised to cell area and are 14 ± 1.2 and 11 ± 2.2 times higher per pixel, respectively, in ARCMs compared to BE cell mitochondria. The average ARCM RR is 0.20 ± 0.01 (figure 9.17b) and is similar to differentiated ($0.17 \pm 0.02 - 0.28 \pm 0.06$) and undifferentiated (0.15 ± 0.02) ESCs and BE cells (0.19 ± 0.02 , substrate deprived cells). The intensity images of NADH and FAD (figure 9.17c & d) show that the mitochondrial density is very high and contrasts with BE cells where the cytoplasmic volume is much larger.

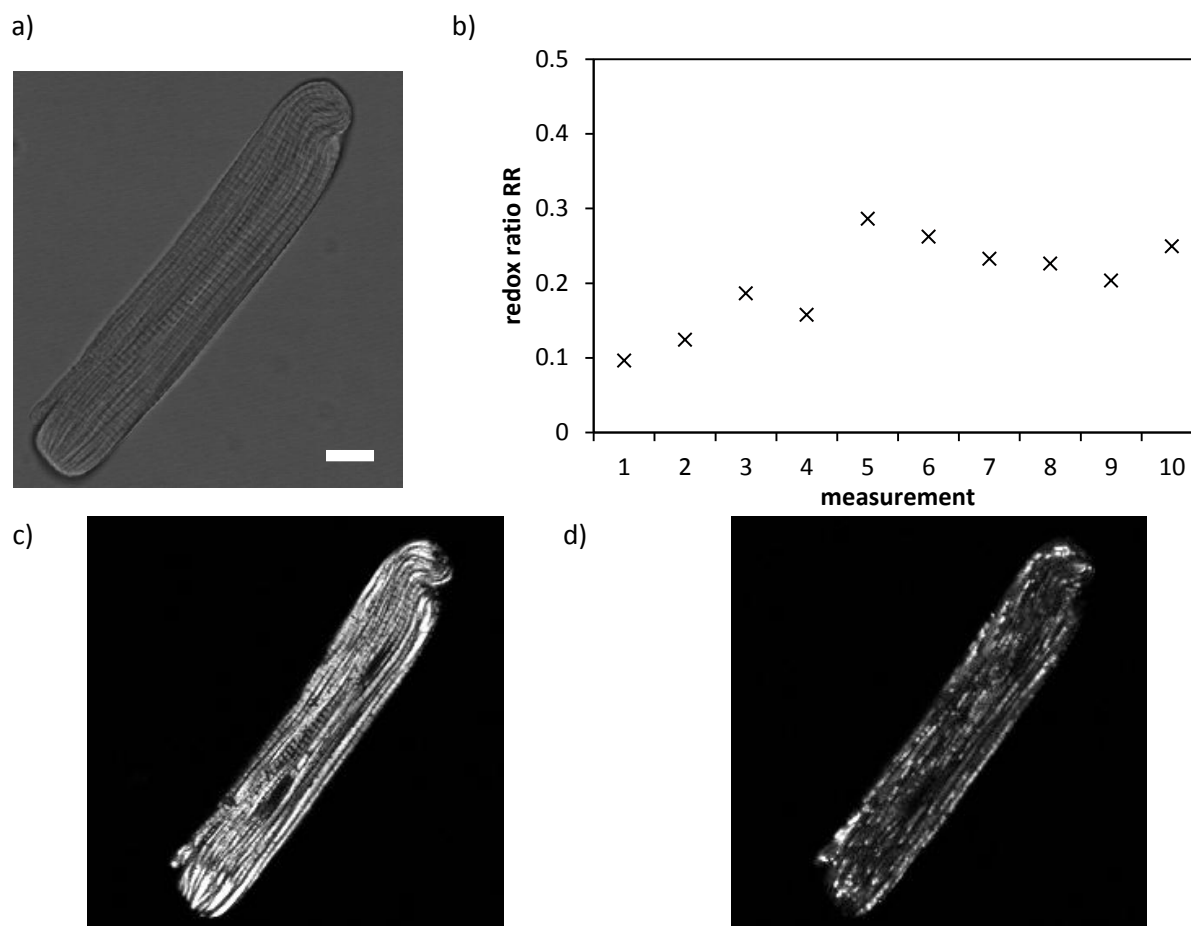


Figure 9.17: Imaging unhealthy rat cardiomyocytes. a) Bright-field image of a typical isolated ARCM where sarcomeric striations can be clearly observed throughout the entire cell. Scale bar 15 μm . b) Redox ratio of $n = 10$ ARCMs. Fluorescence intensity images of c) NADH and d) FAD of the ARCM in a).

The fluorescence lifetimes of NADH and FAD of the ARCMs imaged in figure 9.17 are presented in figure 9.18. The free NADH lifetime (τ_1) is similar to that in cells tested so far; however, the protein-bound lifetime (τ_2) is significantly lower ($P < 0.001$, unpaired t-test). The average protein-bound NADH lifetime of the ARCMs images is 2320 ± 13 ps. A decrease in protein-bound NADH lifetime has been used to differentiate normal and cancerous tissue [254]. It was suggested that the decrease was consistent with increased levels of aerobic glycolysis. Additionally, the contribution of free FAD (a_2) unhealthy ARCMs is 51 ± 2 % and is similar to that for undifferentiated hESCs (d0) 50 ± 2 %. ESCs are known to have high levels of glycolysis and reduced aerobic metabolism due to their low mitochondrial numbers. This suggests the unhealthy ARCMs are undergoing high levels of glycolysis and would be consistent with a shift to glycolysis seen in heart failure. However, the level of fatty acid oxidation and mitochondrial metabolism are unknown and further studies are required to test this.

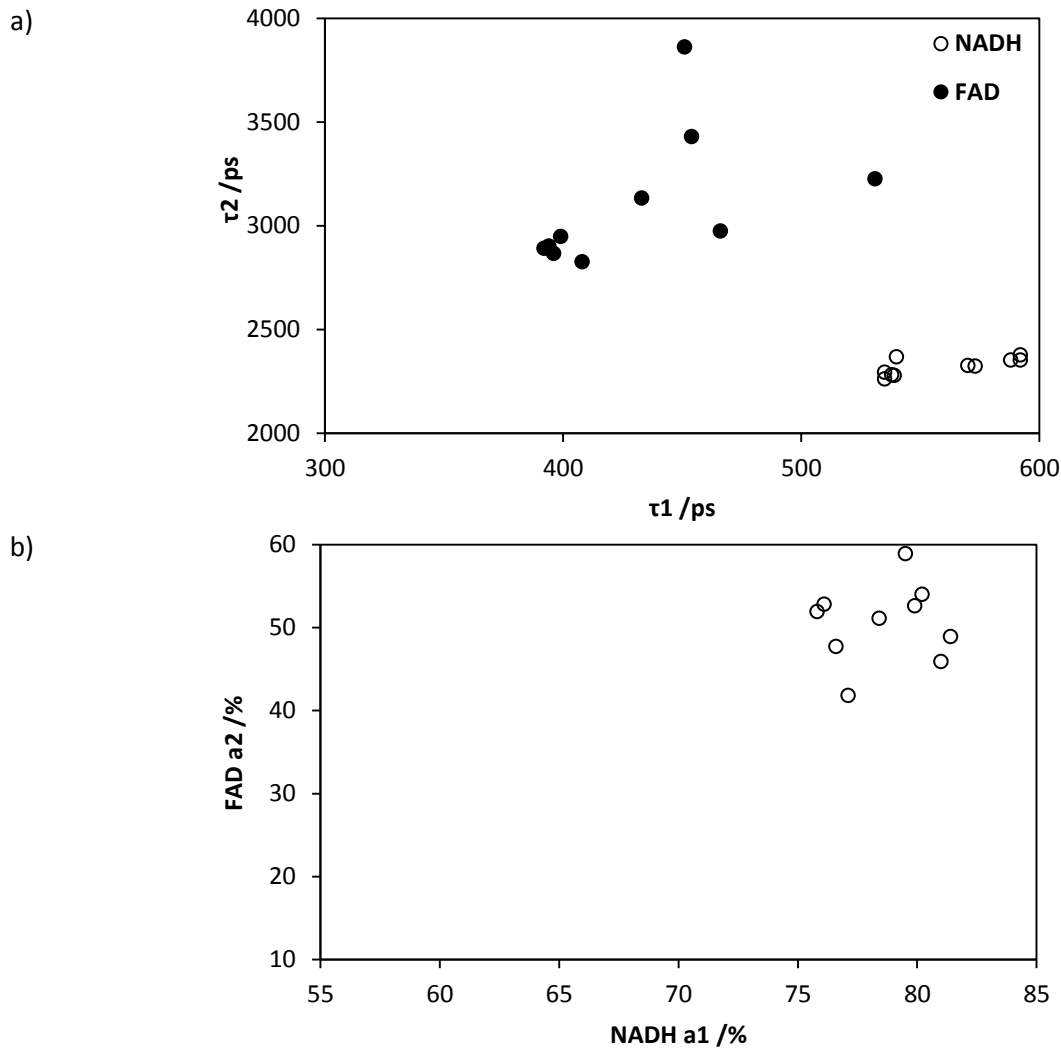


Figure 9.18: Fluorescence lifetime parameters of NADH and FAD for $n = 10$ ARCMs. a) Plot of NADH and FAD free and protein-bound fluorescence lifetimes. b) Plot of the relative amount of free NADH (a_1) and free FAD (a_2).

ARCM fluorescence is continuously monitored as in §9.8 by FIFO acquisition in order to determine the level of engagement of the mitochondria in healthy versus unhealthy cells. ARCMs are observed to contract after a few minutes of irradiation the cardiomyocytes began to contract. This beating begins with a period of a few seconds but increases in frequency and severity. The relaxation length decreases and the cell shrinks longitudinally until it eventually is observed to be destroyed (figure 9.19). Contraction is the result of calcium ions (Ca^{2+}) binding to the sarcomeres, released from the sarcoplasmic reticulum (SR). Several reports exist that document the use of pulsed femtosecond laser irradiation to induce cytosolic Ca^{2+} transients and excite cells and contractions via laser-triggered calcium effects [276-278]. The 2PE of NADH in ARCMs may be triggering the release of calcium which results in spontaneous beating and finally destructive contraction. The SR may be sustaining damage. As a result cytosolic Ca^{2+} does not rise and decrease periodically resulting in

contraction and relaxation but increases continually such that the contractions become irreversible leading to cell destructively contracting.

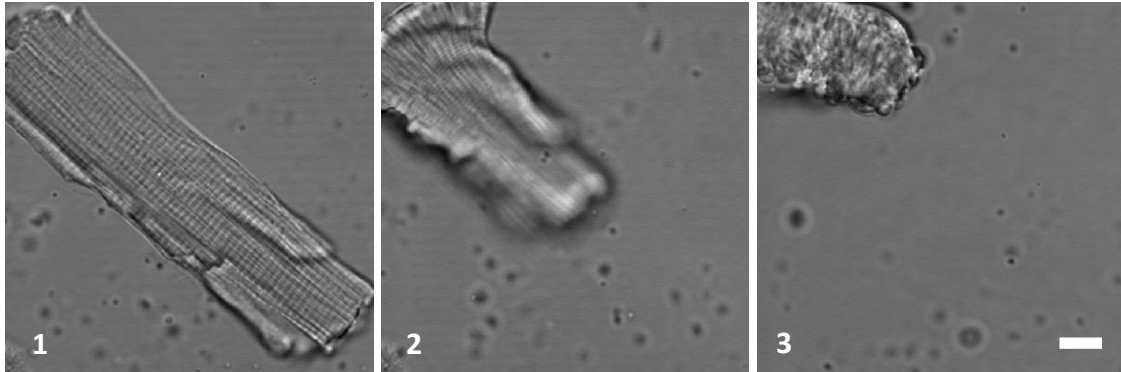


Figure 9.19: Brightfield images of an ARCM undergoing destructive contraction after a period of continuous 2PE at 740 nm. 1, cell prior to 2PE; 2, cell spontaneously beats and shrinks until 3, it fully buckles and is destroyed. Scale bar 15 μm .

Figure 9.20 shows the response of ARCMs to pulsed one photon (1PE) and 2P excitation as determined by NADH intensity. To minimise suspected photodamage of the sarcoplasmic reticulum to limit Ca^{2+} release, the power beam power at 740 nm is reduced from 12 mW to 5 mW at the back aperture. The NADH count rate is reduced as the square of the reduction in power and is reduced to a level similar to that measured for BE cells. ARCMs are prevented from destructively contracting for at least 10 min; seen as a sharp drop in fluorescence in the time-resolved fluorescence trace (figure 9.20a). On average, NADH fluorescence intensity drops by approximately 40 % within 250 s but is unlike the response of BE cells, which fits well to a single exponential photobleaching model. In order to contract, cells require ATP and so are incubated in *physio-cm* solution lacking any metabolic substrates in order to reduce laser-induced contractions. However, ARCM response does not change and cells still eventually undergo destructive contractions. A similar response is measured for ARCMs incubated in *physio-cm* solution lacking Ca^{2+} .

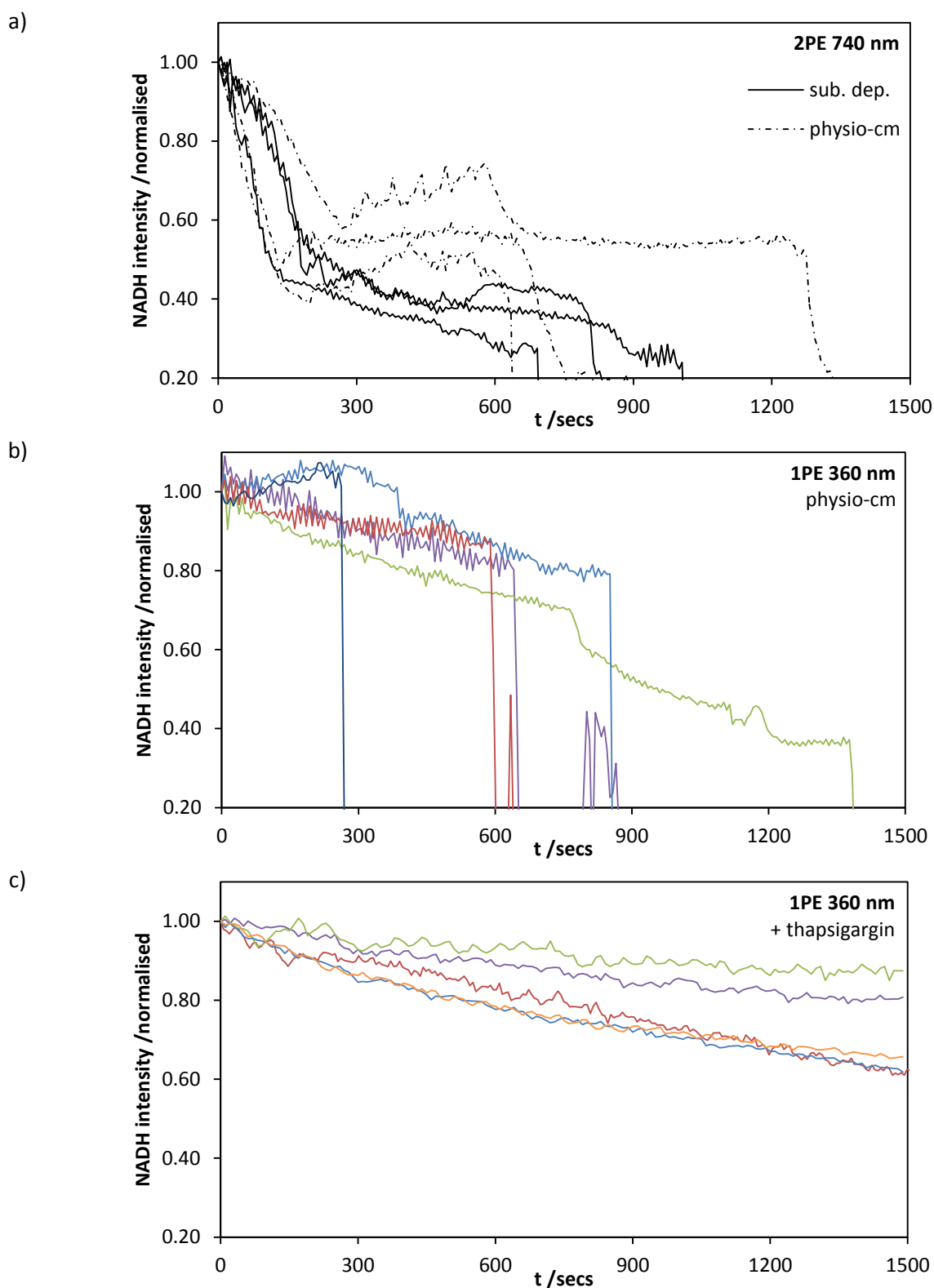


Figure 9.20: ARCM response to pulsed femtosecond irradiation. The response is shown to a) 2PE at 740 nm with cells incubated in *physio-cm* solution or cells incubated in *physio-cm* solution minus metabolic substrates; b) 1PE at 360 nm with cells incubated in *physio-cm*; and c) 1PE at 360 nm with cells incubated in *physio-cm* supplemented with 0.1 μ M thapsigargin. Each trace in all figures represents the response of a single ARCM.

Pulsed single-photon excitation is investigated for its compatibility with long term ARCM imaging. NADH single-photon absorption is made between 330 – 370 nm (see §7.1). The Ti:Sapphire laser is able to generate radiation from 700 nm – 1080 nm and in order to produce second-harmonic UV light, laser output is passed through a frequency doubling β -barium borate (BBO) crystal. The range of wavelengths able to be generated with the crystal is therefore 350 nm – 540 nm.

ARCMs are imaged at 360 nm with an average power of 0.6 – 1.0 μ W at the back aperture. At this power the measured NADH photon count rate is similar to that of BE cells. The onset of destructive contractions is not reduced for ARCMs incubated in *physio-cm* solution (figure 9.20b). The drug thapsigargin is a non-competitive inhibitor of SR Ca^{2+} ATPase [279], blocking Ca^{2+} uptake into the SR and causing SR Ca^{2+} stores to become depleted. The response of ARCMs imaged with 1PE at 360 nm incubated in *physio-cm* solution supplemented with 0.1 μ M thapsigargin is shown in figure 9.20c. Thapsigargin is a non-competitive inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase and disrupts Ca^{2+} dynamics within cardiomyocytes. ARCMs are no longer observed to undergo spontaneous contractions and respond in a reproducible manner. However, it remains to be seen how ARCM metabolism is altered by the interference of intracellular Ca^{2+} signalling.

9.9.2 Stem cell differentiation

Human embryonic stem cells (hESCs) from the H7 line are grown on Matrigel-coated glass-bottomed 35mm dishes and imaged after 30 min incubation in *physio + 10mM glucose + 10mM pyruvate* solution. Cells are shown in figure 9.21a. Typically, hESCs have a high nuclear to cytoplasmic ratio and exhibit low mitochondrial density. This contrasts a more differentiated cell, such as BE cells, whereby the relative volume of the cytoplasm is much larger and contains easily identifiable mitochondrial structures from NADH fluorescence intensity images. Here, granular-like objects pervade the hESC cytoplasm. 2P imaging of the hESCs for NADH reveals that the granules are highly fluorescent with a measured count rate exceeding 1×10^5 photons s^{-1} and completely overwhelms the NADH signal. The granules are not fluorescence when imaging the cells for FAD.

From the known autofluorescent species [280], the most likely origins of such fluorescence are lipopigments, folic acid (vitamin B9), retinol (vitamin A) and pyridoxine (vitamin B6). Folic acid and pyridoxine are important cofactors in methylation in stem cells. Retinol or retinoic acid may be used as a factor for neuronal differentiation but is not used in any protocol in this work. The granules may have an unknown physiological function in hESC cells but makes difficult and for the most part precludes NADH imaging of pluripotent hESCs. So far, only the H7 hESC stem cell line has been

tested and this phenomenon may not be universal to all hESC lines. For instance, all Sheffield University derived hESC lines (SHEF1-8) do not exhibit such granular features as determined by phase contrast imaging, but not by autofluorescence, (personal correspondence, Prof. P. Andrews, Sheffield University). Furthermore, granules may be a product of sub-optimal culturing conditions.

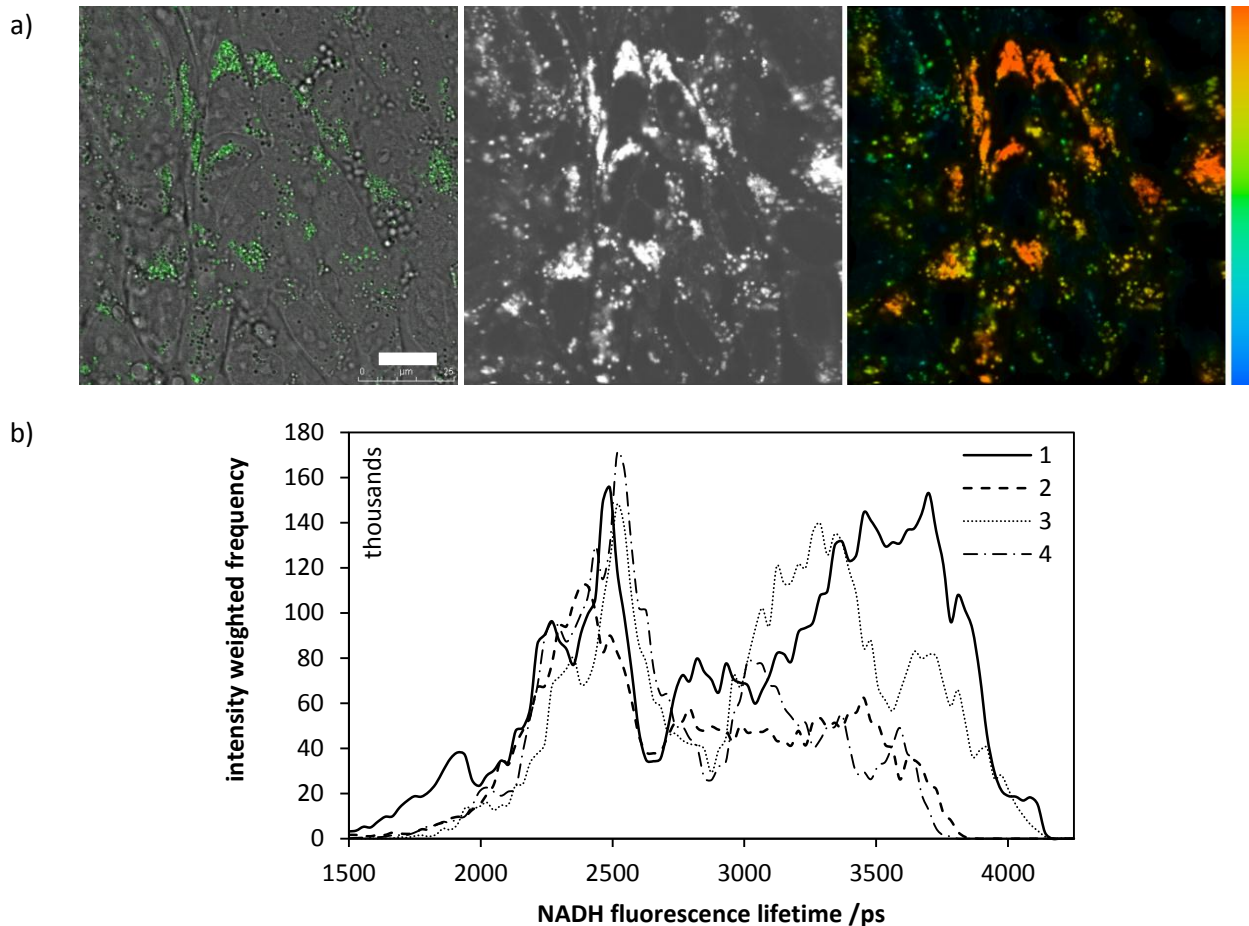


Figure 9.21: 2P imaging of H7 hESCs. a) From left to right, combined brightfield and NADH fluorescence image, NADH fluorescence only and fluorescence lifetime image of NADH. Scale bar 20 μm . The lifetime colour map extends from 2.0 ns (blue) to 4.0 ns (red). NADH is visualised as green/blue in the FLIM map whereas the predominant granule emission is orange. b) Histogram of NADH fluorescence lifetime of separate hESC colonies demonstrating the ability to contrast the granules in lifetime, although it is more straightforward to apply an upper intensity threshold, in order to separate out NADH fluorescence.

The average fluorescence lifetime of the granules is $\sim 3 - 4$ ns and is long compared with that of NADH when fit to a single exponential model, suggesting that these regions do not contain NADH. Fitting the fluorescence to a single exponential model, rather than a double, provides contrast between NADH ($\sim 2 - 2.5$ ns) and granules ($\sim > 3$ ns) (figure 9.21b). The high intensity granules may

be removed more straightforwardly by applying an upper limit intensity threshold. The remaining pixels contain NADH signal and are binned to construct a fluorescence decay. The NADH free and protein bound fluorescence lifetimes and pre-exponential factors are $57.7 \pm 3.3 \%$ (a_1), 600 ± 17 ps (τ_1) and $42.3 \pm 3.3 \%$ (a_2), 3508 ± 74 ps (τ_2), respectively. Additionally, the FAD free and protein bound fluorescence lifetimes and pre-exponential factors are $50.2 \pm 2.2 \%$ (a_1), 507 ± 53 ps (τ_1) and $49.9 \pm 2.2 \%$ (a_2), 3017 ± 38 ps (τ_2), respectively.

The intracellular granules are observed to be lost during differentiation and are not present at day 4 of differentiation of hESCs into ESC-derived cardiomyocytes (hESCMs) by the differentiation programme outlined in §7.3. As cells differentiate into cardiomyocytes, an increased engagement of aerobic metabolic pathways is expected in hESCMs compared to undifferentiated hESCs.

Slow beating hESCM clusters are identified on day 8 of differentiation, mechanically isolated and transferred to 0.5 % gelatine-coated glass-bottomed 35 mm dishes, dissociated into single cells and allowed to plate. Cardiomyocyte-like cells are identified morphologically as large cells including sarcomeric striations (figure 9.22a). The fluorescence parameters of NADH and FAD of undifferentiated hESCs (d0) are compared to day 8 (d8) and older day 37 (d37) hESCMs (figure 9.22b-d).

With the exception of the protein-bound NADH and FAD fluorescence lifetime (τ_2), there is no statistically significant difference in the redox ratio and NADH and FAD fluorescence lifetime parameters between d0 and d8 cells. As cells differentiate further and mature into beating cardiomyocytes, their redox ratio, NADH and FAD fluorescence lifetime parameters change significantly ($P < 0.01$, unpaired t-tests). Due to the increased metabolic activity of cardiomyocytes, it is expected that redox ratio should decrease upon differentiation from hESCs; however, the redox ratio is calculated to increase, instead. It may be the case that the metabolic demands of ESCs in the pluripotent state and undergoing differentiation are higher than beating cardiomyocytes but this seems unlikely.

As cells differentiate from d0, to d8 and to d37, the protein-bound NADH fluorescence lifetime (τ_2) decreases from 3510 ± 70 ps to 2890 ± 70 ps. Additionally, the proportion of protein-bound (a_2) NADH also decreases from 42.3 ± 3.3 at d0 to 28.4 ± 1.4 at d8 and further to 23.3 ± 2.5 at d37. The NADH lifetimes and free FAD (a_2) and NADH (a_1) lifetime contributions are significantly different between undifferentiated (d0) and differentiating cells (d8, d37) ($P < 0.001$, unpaired t-test).

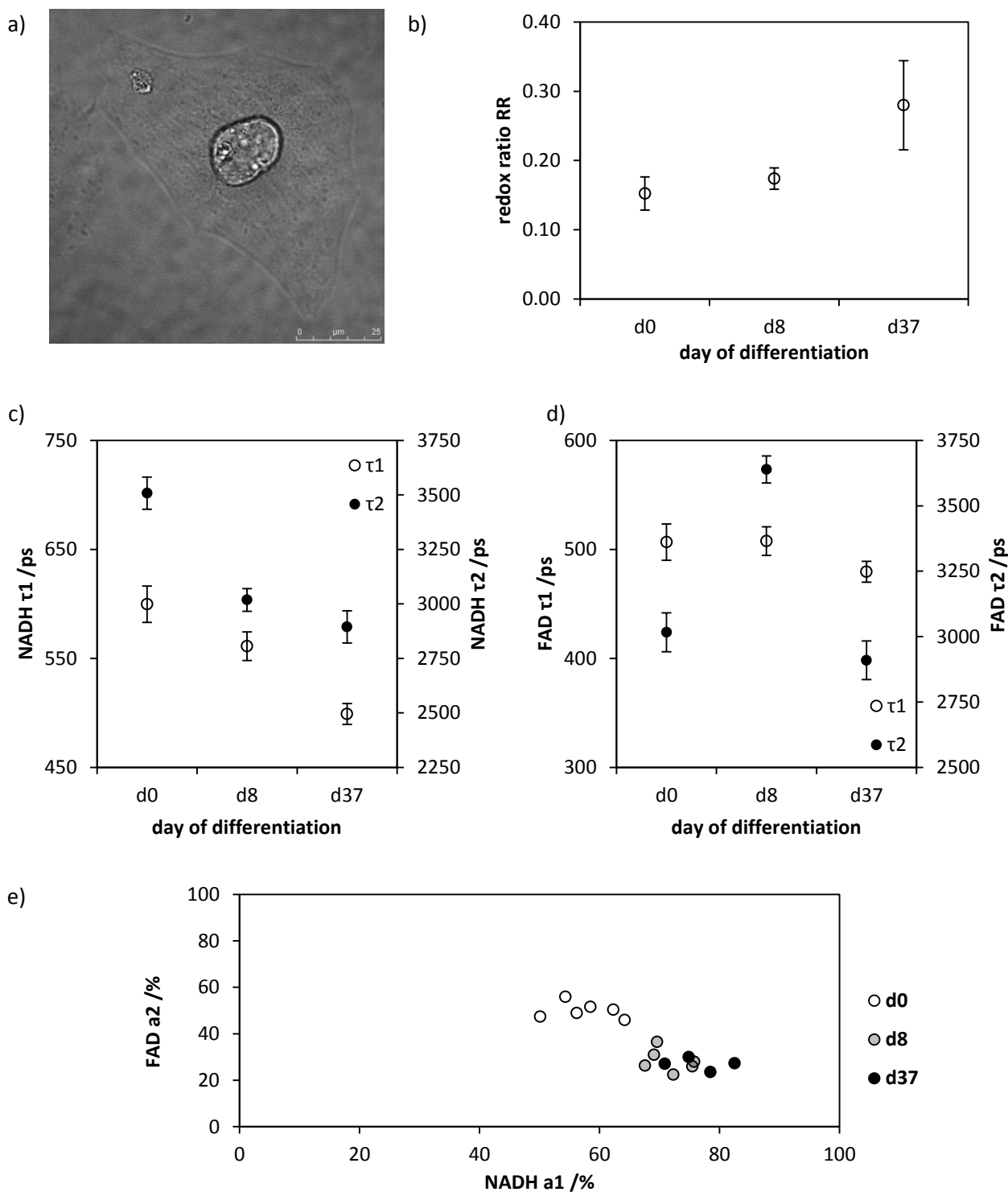


Figure 9.22: Imaging hESCs at different stages of differentiation; d0, undifferentiated cells; d8 and d37, 8 and 37 days of differentiation to hESCMs. a) Bright-field image of a d37 beating ESC-derived cardiomyocytes where sarcomeric striations can be clearly observed. b) Redox ratio, c) NADH and d) FAD fluorescence lifetimes and d) a plot of the relative amount of free NADH (a_1) and free FAD (a_2) of cells at each stage of differentiation. Error bars represent the standard error of the mean for $n = 6$ replicates.

Cells in hypoxic culture and cancerous cells often display a decrease in NADH lifetimes and contribution of protein-bound NADH. These cells favour glycolysis over oxidative phosphorylation [264]. It is not certain whether a similar decrease observed as hESCs differentiate to hESCMs is due to such a shift in metabolism.

9.10 Conclusions

Metabolic multidimensional fluorescence imaging of single cells has been demonstrated. By employing 2PE and TCSPC, the fluorescence intensity and lifetime parameters of the metabolic cofactors NADH and FAD were measured.

The BE cancer cell line was chosen to develop the method. During an investigation into the z-dependence of the fluorescence parameters, it was found that the redox ratio and FADH fluorescence lifetime exhibited significant variation between planes (1 μm and 5 μm) within the same cell. The parameters that are observed to be homogeneous throughout the cell are the free and bound NADH fluorescence lifetimes. The variation in the fluorescence parameters between planes is most likely due to intracellular variation. Furthermore, this variation is sufficiently high that the 2PE optical setup undersamples the cell. If the cell was homogeneous with respect to the redox ratio and the fluorescence parameters of NADH and FAD, undersampling would not be an issue. It was proposed that reducing the power of the objective would result in increasing the depth of focus and therefore improve sampling. However, this is estimated to result in approximately 40-fold reduction in signal that would be unable to be compensated by increases in excitation power owing to damage to the cells being imaged. In order to maximise sampling, 2P Imaging was performed at a set distance above the coverslip of 1 – 2 μm employing a 63 $\times$ oil-immersion objective. This resulted in a sampling efficiency of approximately 60% for cells of 5 μm thickness.

The fluorescence parameters of NADH and FAD were investigated in response to metabolically stimulating cells with the metabolic substrates glucose and pyruvate. Glucose was chosen as this is the primary substrate which feeds glycolysis. Pyruvate was chosen since its metabolic fate relies on whether a cell is respiring aerobically, in which case pyruvate is transported to the mitochondria, or anaerobically, in which case it remains in the cytosol. By considering how the intensity and lifetimes change it was inferred that glucose and pyruvate are metabolic substrates of BE cancer cells. Additionally, by spatially resolving the response, it was inferred that exogenous pyruvate is metabolised in the mitochondria and glucose is metabolised in the cytosol. Similarly, mitochondrial NADH intensity was measured to increase when cells are incubated with buffer containing 10 mM

glucose suggesting that glucose-derived pyruvate is metabolised in the mitochondria. This suggests that the Warburg effect (see §7.3) does not apply to these cancer cells. Indeed, not all cancer cells are observed to undergo altered metabolism; however, the metabolic alterations, if present in BE cells, may be too subtle to be elucidated by the measurements made here. But there is no basis for comparison and further studies into metabolic imaging for the detection of altered metabolism would require a non-transformed and transformed 'pair' of cancer cell lines to help elucidate and characterise any metabolic alterations. Such cell lines may include Hs578T (cancer) and Hs578Bst (normal) [281].

Measuring cell response continually in a fully time-resolved manner with the use of pharmacological agents permitted a more direct measure of how metabolic substrates are being metabolised by the cell. Pharmacological agents enable key points in the metabolic network to be inhibited. However, interpretation of the results is not as straightforward as initially anticipated. For example, a reduction in fluorescence intensity, read as a decrease in NADH, upon addition of pyruvate would seem to indicate a preference in metabolising pyruvate to lactate by lactate dehydrogenase concomitantly oxidising NADH to NAD⁺. Further investigation into BE cell's metabolism of pyruvate with the inhibitors oxamate and CIN showed that the drop in NADH is due to a mitochondrial fate, instead. This drop is most likely due to oxidation of TCA cycle-produced NADH by mitochondrial membrane complex I. To fully determine if this is the case, an inhibitor of complex I may be employed such as rotenone. This inhibits the transfer of electrons from Fe-S centres in complex I to ubiquinone. Without the ability to inhibit key points in the metabolic networks a model of metabolism would most likely be required.

It is difficult to interpret the results of changing fluorescence parameters and what these changes signify. A kinetic model of metabolism may aid such interpretations but this depends on how metabolic imaging will be employed. Of course, the availability of a model that considers the interconversion of NADH and FAD between their fluorescent and non-fluorescent forms, and perhaps the relative amounts of the cofactors bound to enzymes, would offer additional insight into these results. However, if the goal is only to provide a biomarker to determine the general state of a cell, e.g. cancerous/non-cancerous, differentiated/undifferentiated, then simply exploring a difference when measuring the fluorescence intensity or lifetime prior to a single cell proteomic measurement will prove adequate.

Such a biomarker is sought for heart failure and development by imaging ARCMs and hESCs undergoing differentiation. ARCMs were imaged using the same techniques that were developed with BE cells. The NADH and FAD intensity was measured to be an order of magnitude greater than

when imaging BE cells. It was found that long term imaging induced cells to spontaneously beat and prevented measurements. Attempts to prevent beating included removing calcium from the buffer, to deprive cells of metabolic substrate and to switch from 2PE to 1PE. These all failed to alleviate the problem and was only resolved by employing the drug thapsigargin, which disrupts Ca^{2+} signalling. Further tests have not been made on ARCMs following this and it is unclear how ARCM metabolism will be affected by thapsigargin.

Metabolic imaging was also performed on differentiating hESCs. Undifferentiated pluripotent hESCs were found to contain highly fluorescent granules of unknown origin, composition and function when imaging NADH, but not FAD. By applying an upper limit intensity threshold the fluorescence from the granules may be removed and the remaining fluorescence is assumed to originate from NADH. The granules are observed to be lost during differentiation. ESCs are differentiated into ESC-derived cardiomyocytes, which are observed to beat after day 8 of differentiation. The fluorescence parameters of NADH and FAD of undifferentiated hESCs (d0) are compared to day 8 (d8) and older day 37 (d37) hESCs. It is found that using the fluorescence parameters of intensity and fluorescence lifetime, cells from each stage of development could be contrasted. This result holds promise for using metabolic imaging to inform a proteomic measurement.

10 Conclusions

10.1 Microfluidic antibody capture with TIRF detection

A technology platform to undertake the analysis of protein copy number from single cells has been developed. The approach described is 'all-optical' whereby single cells are manipulated into separate analysis chambers using an optical trap; single cells are lysed by a shock wave caused by laser-induced microcavitation; and the protein released from a single cell is measured by total internal reflection microscopy as it is bound to micro-printed antibody spots within the device. The platform was tested using GFP transfected cells and the relative precision of the measurement method was determined to be 88%. Single cell measurements were also made on a breast cancer cell line to measure the relative levels of unlabelled human tumour suppressor protein p53 using a chip incorporating an antibody sandwich assay format. These results suggest that this is a viable method for measuring relative protein levels in single cells.

The technology is relatively scalable in terms of the ability to study multiple proteins in parallel. More importantly, it requires no genetic engineering of the samples and can therefore be applied to human tissue and clinical samples. One of the more important applications of this technology is likely to be the study of protein-protein interactions using a primary antibody against one protein and a secondary against its binding partner. In these cases it is important that the epitopes recognised by the antibodies differ from those recognised by the proteins. In the experiments reported in this work, in effect a population of p53 unbound to DNA or other proteins is being measured since the secondary epitope (aa 21-25) overlaps the Mdm2 binding pocket (aa 18-26) and this may prevent recognition by the secondary antibody and therefore prevent detection. Detection of proteins crucially depends on the availability of affinity reagents to capture target molecules. The quality of antibodies and in particular their specificity is paramount to providing good data. The current design and fabrication methods used in the chips leads to a 50% failure rate, largely due to the strict requirement upon antibody spot quality for single cell measurements. Microprinting proteins and antibodies is difficult and a one-size-fits-all is not a sensible approach therefore time-intensive optimisation of each antibody pair or system is required.

In the case of investigating the protein-protein interactions, it is important that the primary capture agent binds to a region of the protein which is decongested in terms of binding domains. A potentially attractive idea for capturing transcription factors is immobilising into spots the DNA sequence to which they natively bind. In the case of p53, each monomer that makes up the tetramer consists of a tetramerisation region, a DNA binding region and a transactivation region.

The vast majority of monoclonal antibodies bind to the transactivation region which also is the binding site for many of p53's partners and for post-translation modifications. Employing antibodies which bind to these regions results in improper protein counting but also limits investigation into protein binding partners. DNA microarrays are considered much more straightforward to produce, not least due to the maturity of the technology but because of the intrinsic molecular simplicity compared to protein.

To circumvent the problems of background protein free in solution being bound to antibody spots while cells are hydrodynamically trapped, single cell pulldown chambers are removed from the main channel and an optical trap is employed to transport cells. The demonstration devices used in this proof of principle study possess only 7 chambers but this could be increased as long as the cells can be loaded in an effective and efficient way. The optical trap may not be ideal for trapping large numbers of cells. However, for studies of rare cells such as circulating tumour cells (CTCs), where there are often only 1 - 10 CTCs per patient sample, the use of optical trapping can be a particularly good solution.

The use of optical traps was not initially intended since hydrodynamic trapping of cells can be carried out at higher throughput and in an automated fashion. The hydrodynamic traps presented in this work offer low trapping efficiency and so require thousands of cells in order to capture 10 or so. Trapping efficiency is low due to the requirements of the single cell chamber; it must contain a single cell, have a surface footprint large enough to accommodate one to several antibody spots and be designed such that loss of material by diffusion is minimised. This results in a design outlined in figure 10.1a. The increase in channel width has the consequence of spreading out the focussed flow width and reducing trapping efficiency. If the channels and chambers are the same width (figure 10.1b) the flow focussed width will not spread and a dramatic increase in trapping efficiency, approaching 100 %, is achieved.

However, an alternative strategy that defines a chamber volume and limits cross-talk by diffusion must be defined. This may be achieved with PDMS valves made by crossing two second-layer channels at right angles to the main first-layer channel in figure 10.1b. As pressure is applied to the second layer channels, the flexible interlayer PDMS membrane is deflected downward and defines a closed volume in the first layer channel.

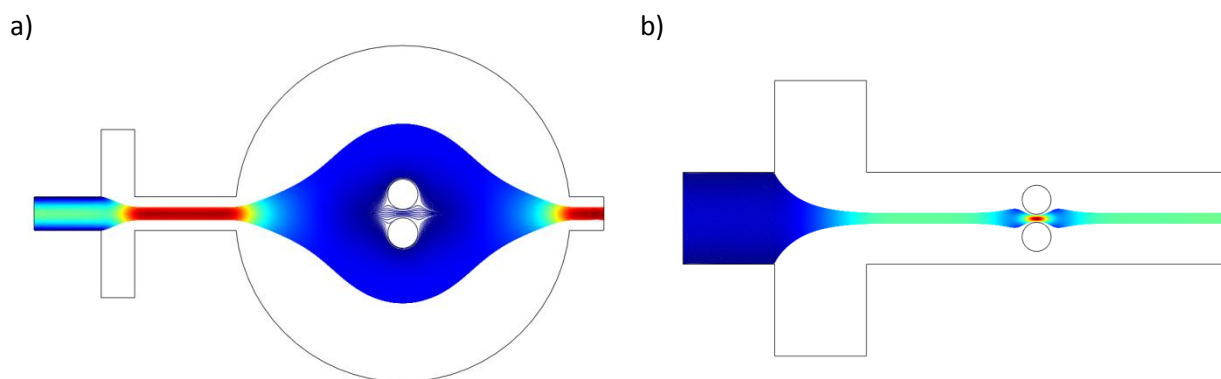


Figure 10.1: CFD simulations of two trapping strategies. a) Single-layer device where diffusion is limited by a high ratio of the chamber and channel widths. b) Two-layer device where width ratio is unity and chambers are produced by second-layer PDMS valves.

Results of any proteomic analysis on cells will be dependent on their physiological state immediately prior to whole-cell or part cell digestion. A measure of the proteome which reflects more closely the cultured state is desirable. Anchorage dependent cells require attachment upon a culture surface in order to proliferate. Reconciling this requirement with the need to flow material within a microfluidic device leads to the option to culture cells upon microcarrier beads (figure 10.2).

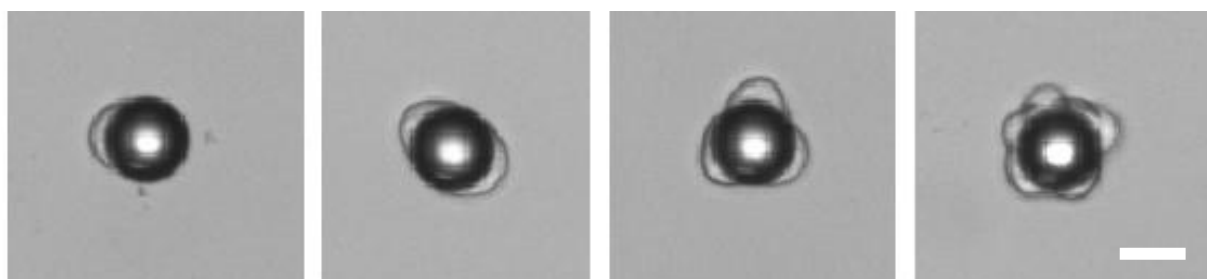


Figure 10.2: Examples of 1, 2, 3, and 5 BE cells attached to fibronectin coated polymethacrylate 30 μm beads. Scale bar 30 μm .

Polymethacrylate microcarrier beads may be functionalised with surface coatings such as collagen or fibronectin or with affinity capture agents such as antibodies for surface markers. CTCs are enriched from patient samples using the surface epithelial adhesion protein EpCAM. Incubation with antiEpCAM antibody functionalised beads would allow cells to be enriched and transported by the coordination of microcarrier beads and the optical trap. Furthermore, the use of microcarrier beads may lead to reduced cell loading times and photodamage by the optical traps. The greater difference in refractive index between beads and media than for cells allows stronger trapping and

beam power to be reduced. Furthermore, culturing cells on beads eliminates enzymatic detachment when harvesting cells and may eliminate the background protein level originating from compromised cells.

10.2 Autofluorescence determination of metabolic phenotype

Metabolism becomes altered in various diseases such as cancer and heart failure. Metabolic imaging of the autofluorescent species NADH and FAD has been performed in order to monitor or determine the metabolic phenotype of different cell types. By measuring parameters of fluorescence such as intensity and lifetime it is possible to contrast cell types and cell types under different metabolic conditions non-destructively and without the need for exogenous fluorophores.

The cell types investigated are BE cells, human embryonic stem cells and adult rat cardiomyocytes. Each tissue type represents a system that may be categorised by its metabolism and how it is altered during the progression of disease or differentiation.

For BE cancer cells it was determined that glycolysis and mitochondrial metabolism was active. Glucose is processed in the cytoplasm by glycolysis and results suggest that glucose-derived pyruvate is transported to the mitochondria where it is processed. Pyruvate was found to be metabolised by the BE cells. By employing pharmacological inhibitors, the response from which this is determined was found to originate from the mitochondria. Inhibitors of entire pathways or just specific nodes may be used in series to pin point the exact response a cell has for metabolic substrates. However, many of these inhibitors are irreversible and since they act upon metabolic enzymes and mitochondrial molecular machinery, such as the ETC, are poisonous to cells. It therefore remains questionable how feasible it becomes to perturb a cell with many different exogenous species before possibly inducing apoptosis. Less straightforward but perhaps less damaging to cell viability is the modulation of oxygen concentration to inhibit mitochondrial respiration.

The tests made upon BE cells provide somewhat binary information on whether metabolic substrates are utilised or not. In the case of some cancers, the metabolism shifts from predominantly deriving energy from oxidative phosphorylation to predominantly aerobic glycolysis. This may or may not be applicable to BE cells but regardless of this, the current method does not provide information on the relative contributions of each pathway to energy production. One possible method of doing so would be to overload the cell with metabolic substrate to maximally drive each pathway. By sequentially inhibiting each pathway the relative contribution of each

pathway may be determined by the magnitude of the sequential response. However, due to the complexity and interconnected nature of the metabolic networks it is not a straightforward task.

Embryonic stem cells are cells capable of differentiating into any somatic cell type. The differentiated cell will have distinct energy requirements and is exemplified by cardiomyocytes. ESCs are relatively metabolically inactive and rely on anaerobic metabolism whereas the cardiomyocytes are energetically more demanding. Consequently, mitochondrial metabolism becomes upregulated in order to meet such demands. Human ESCs were differentiated into beating cardiomyocytes using a directed protocol and imaged at 3 stages of development; undifferentiated, at day 8 and at day 37 of differentiation. It was found that NADH and FAD fluorescence parameters were significantly altered as development progressed. The redox ratio provided contrast between d8 and d37 whereas NADH fluorescence lifetime was measured to decrease from d0 to d37 and provided contrast between each stage of development. This demonstrates the ability of metabolic imaging to track the alteration of metabolism and its ability to serve as a biomarker.

In addition to cancer and stem cell differentiation, metabolism has been shown to undergo alteration in heart failure. Imaging of adult rat cardiomyocytes using two-photon excitation at 740 nm was problematic and induced cells to spontaneously beat during long-term imaging. Reducing excitation power or shifting to single-photon excitation at 360 nm did not alleviate the beating. Only by supplementing media with thapsigargin, a drug to deplete sarcoplasmic reticulum calcium ion stores, was spontaneous beating prevented during imaging. It is suspected that laser-induced calcium stimulation is responsible for this phenomenon. Unfortunately, cardiomyocyte metabolism in healthy and infarcted hearts could not be investigated further due to a breakdown of the laser system.

The utility of monitoring and being able to detect altered metabolism in cardiomyocytes lies in drug development. Many compounds are being screened in their efficacy in alleviating or eliminating heart failure. If a drug is able to rescue cardiomyocytes or whole heart tissue from heart failure then this may be determined non-invasively by metabolic imaging.

10.3 Further work

Microscopy provides a non-destructive method to monitor cellular metabolic activity with high resolution. This is complementary to the single cell proteomic platform developed in this work. Bridging both techniques will be invaluable to the investigation into what these metabolic changes

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Publications

Journal articles

1. A first step towards practical single cell proteomics: a microfluidic antibody capture chip with TIRF detection

A. Salehi-Reyhani, J. Kaplinsky, E. Burgin, M. Novakova, A. J. de Mello, R. H. Templer, P. Parker, M. A. A. Neil, O. Ces, P. M. W. French, K. R. Willison and D. Klug

Lab on a Chip, 2011, DOI: 10.1039/C0LC00613K

Patents

1. Method and apparatus for determining an analyte parameter

K. Willison, D. Klug, O. Ces, E. Burgin, A. Salehi-Reyhani, J. Kaplinsky

Patent WO/2011/007138, January 01, 2011

Conference presentations

1. Towards Single Cell Proteomics: Integration of Single Cell Manipulation, Lysis and Readout

A. Salehi-Reyhani, J. Kaplinsky, E. Burgin, A. Margineanu, C. Dunsby, R. H. Templer, P. M. W. French, A. J. de Mello, K. R. Willison, P. J. Parker, M. A. A. Neil, O. Ces and D. R. Klug

Presented at the 6th BSPR/EBI Proteomics Meeting, Hinxton (UK), 2009

2. Towards Single Cell Proteomics: Integration of Single Cell Manipulation, Lysis & Readout

A. Salehi-Reyhani, J. Kaplinsky, E. Burgin, A. Margineanu, C. Dunsby, R. H. Templer, P. M. W. French, A. J. de Mello, K. R. Willison, P. J. Parker, M. A. A. Neil, O. Ces and D. R. Klug

(Poster) Presented at the Joint FEBS/EMBO Summer School, Spetsai (Greece), 2009

3. Estimation of Membrane Lateral Pressure in Living Cells by Means of Multidimensional Confocal Fluorescence Microscopy

A. Margineanu, A. Salehi-Reyhani, H. Manning, O. Ces, C. Dunsby, R. H. Templer, P. M. W. French and D. Klug

(Poster) Presented at BIOS Photonics West, San Jose (USA), 2009