

Single cell analysis and cell sorting using microfluidic devices
with application to circulating tumour cells

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Declaration

I certify that the work presented in this thesis is my own original work except where referenced otherwise.

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Abstract

This thesis describes the development of integrated microfluidic technology for single cell proteomic analysis, focusing on circulating tumour cells (CTCs).

While single cell proteomic analysis has wide applicability across biology and medicine, CTCs form an ideal first application. Circulating tumour cells are intimately involved in metastasis, the step in cancer overwhelmingly responsible for death, yet have proved hard to study. Single cell microfluidic technology is ideal first because the quantity of material available is inherently at the level of a few cells and second because cell to cell variation is of great interest.

Chapter 1 is an introduction to the field. In chapter 2 a microfluidic sandwich assay for quantification of protein at the single cell level is described. In chapter 3 the isolation of CTCs in a microfluidic device is described. This relies on taking the output of the CellSearch[®] system and inputting it to a microfluidic device. While CTCs were identified, the result showed that a more systematic approach is required for counting and integration with the single cell assay previously described.

Chapters 4 and 5 describe development of technology suitable for counting and isolation of CTCs integrated into a microfluidic device with single cell proteomic analysis, although the work done here makes use of fluorescently labelled beads and model cell lines rather than CTCs from patient samples.

Chapter 4 describes microfluidic cytometry that can be used to count and identify a labelled population of cells, such as stained CTCs. Chapter 5 describes the preliminary development of a sorting system suitable for isolation of CTCs integrated with the cytometer.

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Abbreviations

cDNA	complementary DNA
CCD	Charge coupled device
CTC	Circulating tumour cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immuno-assay
ESI	Electrospray ionisation
FACS	Flourescence Activated Cell Sorting
FISH	Flourescence in situ hybridisation
FITC	Fluorescein isothiocyanate
GFP	Green flourescent protein
GPTS	3-glycidoxypopyl-trimethoxysilane
IPTG	Isopropyl β -D-1-thiogalactopyrranoside
LED	Light emitting diode
MALDI	Matrix assisted laser desorption ionisation
mRNA	messenger RNA
PC	Personal computer
PCR	Polymerase chain reaction
PDMS	Poly(dimethyl-siloxane)
PEG	Poly(ethyleneglycol)
PMT	Photomultiplier tube
PTM	Post-translational modification
RNA	Ribonucleic acid
TIRF	Total internal reflection microscopy

Contents

1	Introduction	15
1.1	Proteomics	15
1.1.1	Historical development	15
1.1.2	State of the art proteomics	17
1.1.2.1	Diversity of the proteome	18
1.1.2.2	Protein dynamic range	21
1.1.2.3	Protein-protein interactions	22
1.1.2.4	Tools for proteomics	25
1.1.2.5	Mass spectrometry	26
1.1.2.6	Antibodies	28
1.2	Single cell studies	29
1.2.1	Noise, heterogenity and development	31
1.2.1.1	Results from gene expression experiments	33
1.2.1.2	Results from experiments with fluorescent fusion proteins	34
1.2.1.3	Further results from single cell resolution experiments	37
1.3	Microfluidics	40
1.3.1	Theoretical description of microfluidics	42
1.3.2	Microfluidic fabrication and materials	43
1.3.3	Digital microfluidics	44
1.3.4	Microfluidic studies on single cells	44
1.3.5	Microfluidic cell sorting	45
1.3.5.1	Conventional cell sorting and counting	45
1.3.5.2	Single cell studies using flow cytometry	45

1.3.5.3	Microfluidic cell sorting devices	46
1.3.5.4	Moving cells across streamlines	47
1.3.5.5	Switching fluid flows	48
1.3.5.6	sorting of droplets	48
1.3.5.7	Isolation of circulating tumour cells in microfluidic devices . . .	48
1.4	Cancer, disseminated and circulating tumour cells	50
1.4.1	Cancer biomarkers diagnosis, prognosis	52
1.4.2	Development of cancer therapies	53
1.4.3	The cancer stem cell hypothesis	54
1.4.4	Breast cancer	55
1.5	Summary of the thesis	57
2	Single Cell Proteomics	59
2.1	Introduction and strategy	59
2.2	Theory of protein diffusion and binding	60
2.2.1	The diffusion equation and boundary conditions	60
2.2.2	Solution of the diffusion equation	62
2.2.3	Numerical evaluation	63
2.3	Simulation results	65
2.4	Total Internal Reflection (TIRF) microscopy	68
2.5	Validation of antibodies	69
2.5.1	Development of antibody spotting	71
2.6	Construction and operation of the microfluidic device	72
2.6.1	The cell inlet	75
2.6.2	Optical trapping of cells	75
2.6.3	Laser lysis of single cells	76
2.7	Binding data	77
2.7.1	Bleaching data	77
2.8	Results	78
2.9	Signal processing	78
2.9.1	Mean intensity analysis	78

2.9.2	Single molecule counting	78
2.9.3	Bleaching data	80
2.10	Discussion	82
3	Isolation of Circulating Tumour Cells	84
3.1	Introduction	84
3.2	Isolation of Circulating Tumour Cells	85
3.2.1	The CellSearch system	85
3.2.2	The CellSearch protocol	86
3.3	Specificity of anti-EpCAM antibody staining	87
3.3.1	Staining using anti-EpCAM antibodies	89
3.3.2	Spiking experiments using anti-EpCAM antibodies	90
3.4	Cytokeratin expression	90
3.5	Development of ERBB2 staining	92
3.5.1	Background subtraction	92
3.6	Arraying cells	96
3.7	Results with clinical sample	98
3.8	Discussion and further work	101
4	Microfluidic cytometry	105
4.1	Hydrodynamic focusing	106
4.1.1	Hagen-Poiseuille flow	106
4.1.2	Theory of hydrodynamic focusing	109
4.2	Glass devices	111
4.3	Introduction of beads and cells into the microfluidic device	111
4.3.1	Pumping	114
4.4	Experimental results for focusing	114
4.5	Correction of the amplifier time constant	118
4.6	Cytometry of beads	121
4.6.1	Width distribution of fluorescent bursts	122
4.7	Cytometry of cells	125

4.8	Poisson statistics cells	127
4.9	Discussion and further work	128
5	Microfluidic Cell Sorting	131
5.1	Design and characterisation	131
5.2	Valving	132
5.2.1	The solenoid valve	132
5.2.2	The pressure reservoir	134
5.3	Pressure stability	136
5.3.1	Instability from stepper motors	137
5.4	From positive to negative pressure	138
5.5	Results of sorting	139
5.6	Work with tracers	140
5.6.1	Results	142
5.7	Discussion and conclusion	144
A	Octave / Matlab scripts	146
A.1	Building the matrix for the diffusion operator	146
A.2	Normalisation of eigenfunctions for cylindrical geometry	148
A.3	scalar product of functions in cylindrical geometry	148
B	Fabrication of microfluidic devices	150
B.1	Poly(dimethylsiloxane) devices	150
B.2	Glass devices	152
B.3	Characterisation of channels	153
C	Materials and methods	156
C.1	Jurkat cells	156
C.2	MDA MB 468 cells	156
C.3	BE cells	156
C.4	BT474 cells	157
C.5	Preparation of cells	157

C.6 Preparation of cover slips	157
D LabVIEW code	159

List of Figures

1.1	The structural complexity of proteins	18
1.2	Yeast two hybrid assay	23
1.3	Structure of an IgG antibody.	28
1.4	Schematic graphs showing forms of variation of protein concentration amongst a population of cells.	32
1.5	Schematic graph showing variation of proteins over time	33
2.1	Schematic of sandwich assay.	60
2.2	Geometry of a cylindrical chamber for numerical solution of the diffusion equation	64
2.3	Grid showing indexing of points used in numerical solution of the diffusion equation.	64
2.4	Contour plots of the first six eignmodes.	67
2.5	Western blot of p53 protein and two anti-p53 antibodies.	70
2.6	Western blot of lysate from the BE cell line and anti-p53 antibodies.	71
2.7	Illustration of how the morphology of spots changes with the number of spots printed.	73
2.8	Anti-p53 antibodies spotted on to a plain glass surface showing consistent mor- phology. Scale bar 100 μm	74
2.9	Layout of the microfluidic device for cell lysis with blow up of lysis chamber . .	74
2.10	Showing the cell loaded into the lysis niche. Scale bar 50 μm	75
2.11	TIRF images of labelled secondary antibody for p53 bound to a spot from single cells	79
2.12	TIRF images of labelled secondary antibody for p53 bound to a spot from lysis of three cells.	80

2.13	Mean spot intensity after 15 minutes incubation vs. number of cells lysed in a chamber	81
3.1	Diagram of nonspecific antibody binding	89
3.2	Western blot of cell lysate from three cell lines (BE, MDA MB 468 and Jurkat) with three pan anti-Cytokeratin antibodies.	91
3.3	Staining of ERBB2 positive BRT-474 breast cancer cell line with anti-ERBB2 affibody	93
3.4	Staining of ERBB2 negative Jurkat leukocyte cell line with anti-ERBB2 affibody	94
3.5	Staining of ERBB2 negative MDA-468 breast cancer cell line with anti-ERBB2 affibody	95
3.6	Composite of images bright field and fluorescence images of stained BRT 474 cells	96
3.7	Background sampled from the imaging of stained BRT 474 cells	97
3.8	Background from the imaging of stained BRT 474 cells as determined by a 50 pixel low pass Gaussian filter	98
3.9	Mean pixel intensity of averaged across $n = 74$ individual BRT 474 cells	99
3.10	Total intensity of $n = 74$ individual BRT 474 cells with local background subtraction	100
3.11	Areas of $n = 74$ individual BRT 474 cells	101
3.12	Jurkat cells injected into a microfluidic chamber, as described in the text. The top and bottom frames are separated in time by 30 seconds.	102
3.13	A thresholded difference between the images in figure 3.12 superimposed in red, illustrating the slow injection and deposition of cells.	103
3.14	CTC isolated from clinical sample	104
4.1	Design of microfluidic cytometer.	105
4.2	Co-ordinate system and dimensions for calculation of flow through a rectangular channel.	107
4.3	Contour plot of the velocity in the x - y plane of figure 4.2	108
4.4	Variation of the velocity across the width of the channel along a line through the centre of the channel.	108

4.5	Variation of the velocity across the height of the channel along a line through the centre of the channel. Note the parabolic form.	109
4.6	Notation used for calculation of hydrodynamic focusing.	110
4.7	First interface for introduction of particles to the analyte inlet.	112
4.8	Accumulation of particles in PDMS reservoir.	113
4.9	Modification of the inlet reservoir.	113
4.10	Images of hydrodynamic focusing with dye flow.	115
4.11	The measures used to analyse images of flow in the microfluidic.	116
4.12	Absorption of light for a flow of $0.25\mu\text{l}/\text{min}$ and a sheath flow of $12\mu\text{l}/\text{min}$ where distance across the channel is across the cross section indicated in figure 4.11.	117
4.13	Width of focused stream vs. length along the flow axis.	118
4.14	Variation of width of focused dye analyte with sheath flow	119
4.15	Variation of width of focused beads with sheath flow	119
4.16	Amplifier time constant.	120
4.17	Variation of peak width with flow rate	120
4.18	The PMT response for measurement of fluorescent beads in flow	121
4.19	The PMT response for measurement of fluorescent beads in flow, detail	122
4.20	The PMT response for measurement of fluorescent beads in flow, individual peak	123
4.21	Noise present in the PMT signal	124
4.22	Full width half maximum of peaks detected by PMT for beads in flow	125
4.23	Full width half maximum of peaks detected by PMT for beads in flow	126
4.24	The PMT response for measurement of cells in flow.	127
4.25	The PMT response for measurement of cells in flow, detail	128
4.26	The PMT response for measurement of cells in flow, individual peak	129
4.27	Fano factor for cells in flow	130
5.1	Initial design of microfluidic sorting stage.	131
5.2	Schematic diagram of the solenoid valve.	133
5.3	Circuit used to actuate cell switching	134
5.4	Schematic diagram of the pressure reservoir.	135

5.5	Schematic image of a stepper motor.	137
5.6	Refinement of sorter design.	139
5.7	Assembled microfluidic device mounted on the microscope.	140
5.8	Sorted beads colour coded and bright field.	141
5.9	The set up for switching of tracer beads.	143
5.10	Series of frames showing switching of tracer beads	144
B.1	Photograph of silicon master showing SU-8 photoresist channels for 6 designs. .	151
B.2	Comparison of glass and PDMS devices.	152
B.3	Inlet reservoir of glass device	153
B.4	Inlet reservoir of PDMS device	154
B.5	Glass channels before and after cleaning	155
D.1	Block diagram of LabVIEW code for cytometry and sorting.	160
D.2	Block diagram of LabVIEW code for subVI controlling opening with a time delay.	161

Chapter 1

Introduction

Section 1 puts the work in the context of the study of proteins and cells, highlights some historical developments leading to the technology used in the thesis and reviews some basic facts about proteins. Section 2 reviews the recent interest in single cell studies. Section 3 introduces the specific problem of circulating tumour cells focusing on breast cancer. Section 4 reviews microfluidic technologies and their application to cell sorting, particularly for the isolation of circulating tumour cells. Section 5 gives a synopsis of the thesis.

1.1 Proteomics

1.1.1 Historical development

The study of proteins dates back to the beginnings of modern chemistry. Early purified proteins included albumin from blood, casein from milk and collagen from tendons. Following Lavoisier's chemical revolution the central problem in protein analysis was their elemental composition, pursued most notably by Justus Liebig in his 1842 work *Animal Chemistry*. [1]

From Lavoisier through to Pasteur, fermentation was a central problem in biology. With Eduard Buchner's discovery of cell free fermentation by enzymes in 1897 it became clear that not all chemical transformations in living things required organised cells. Biochemistry in its modern sense was born, and proteins in the form of enzymes was at its heart.

By the end of the nineteenth century hydrolysis showed that proteins were composed of amino acids, and that composition differed from protein to protein. Techniques such as differential volatility of esters and colour reactions allowed identification of all naturally occurring

amino acids except methionine. [2] In 1902 Emil Fischer and Franz Hofmeister were able to independently and simultaneously propose a polypeptide structure for proteins.

Svedburg's invention of the ultracentrifuge showed that purified proteins were indeed large molecules with monodisperse weight [3]. Bensley, Hoerr and Claude's differential centrifugation of cell lysates in a sucrose gradient led to separation organelles, allowing their biochemical analysis. [4]

The study of protein function was advanced by Warburg and Christian's use of UV spectrometry to monitor enzyme activity by turnover of NAD(P)H. Schoenheimer ^{15}N and ^2H labelling studies of glycine and L-leucine lead to the idea of a dynamic state of flux. The production of artificial unstable isotopes, first ^{11}C then, during the Manhattan project ^{14}C , gave a huge boost to deciphering of metabolic pathways. [5] Details of the citric acid and the Calvin-Benson cycles were rapidly filled in.

The refinement of paper chromatography by Marin and Synge in 1941 made a wide range of protein separations practical. This was followed after the Second World War by a range of further chromatographic techniques: ion exchange, size exclusion, affinity and ultimately HPLC.

The amino acid sequencing of insulin and its disulfide bond structure was completed by Fred Sanger's team in 1955. This proved that proteins had a definite primary structure, dovetailing with insights emerging from the structure of DNA.

The first insight into protein secondary structure came from Pauling and Corey's work on alpha helices and beta sheets, based on fibre diffraction experiments. The complete crystal structure of myoglobin [6] followed in 1958. The first protein structure was determined by NMR in 1985. [7] Between 1990 and 2010 the number of structures deposited in the PDB rose from 498 at the start of 1990 to 70,901 at the end of 2010.

With an outline understanding of protein molecules in place, a new challenge, of "proteomics" has opened up. This is the study of which proteins are present in the cell at what time. It includes the study of which proteins interact with one another and the post-translational modification state.

In 1943 Erwin Schrödinger's *What is life?* proposed that the genetic material must take the form of an 'aperiodic crystal'. [8] From the realisation of this concept in the structure of DNA in 1953 to the completion of the human genome in 2001, [9, 10] the most rapid progress was

made through the study of genes and nucleic acids.

The deciphering of the genetic code by 1966, [11] was followed by the cloning of mRNA into cDNA [12, 13], DNA sequencing [14] and the polymerase chain reaction (PCR) [15, 16]. These techniques opened up another route to protein sequencing. With advances in DNA sequencing this technology has far eclipsed the direct sequencing of proteins.

Genomics continues to make rapid advances. However, a proliferation of "omics" as in transcriptomics, proteomics, lipidomics and metabolomics, indicate a desire to replicate its successes in other fields. It is increasingly clear that making sense of the mass of genomic information will require integration with a fuller understanding of these other components of biological systems. A variety of terms, such as systems biology, post-genomic biology and chemical biology have been used to describe this task.

1.1.2 State of the art proteomics

The proteome is defined as the totality of an organism's proteins. Various sub-proteomes can be associated with organs, cells or organelles. Proteomics refers broadly to the study of protein levels, interactions, post-translational modification, structure and functions. Growing interest in proteomics is reflected in the founding of the Human Proteome Organisation (February 2001) and the launch of journals such as Proteomics (January 2001), Molecular and Cellular Proteomics (January 2002), Journal of Proteome Research (February 2002).

Quantitative characterisation of the proteome is one of the most challenging problems in analytical chemistry. Biological samples are complex mixtures containing many species of both protein and other molecules, present in quantities spanning up to 6 orders of magnitude. [17] Unlike the genome, the proteome varies over many scales.

In space, variation of the proteome is intricate between organelles, membrane location, the nucleus and throughout the cytoplasm. Intercellular proteomic variation is responsible for different tissue types in the body. Genetic variation between individuals, species, or mutated cancer cells is expressed as proteomic variation.

In time, post-translational modifications to the proteome occur on sub-second time scales. The typical time scale from signalling to translation is minutes to hours. Amongst many biological cycles are the cell cycle in dividing cells and the 24 hour circadian rhythm. The

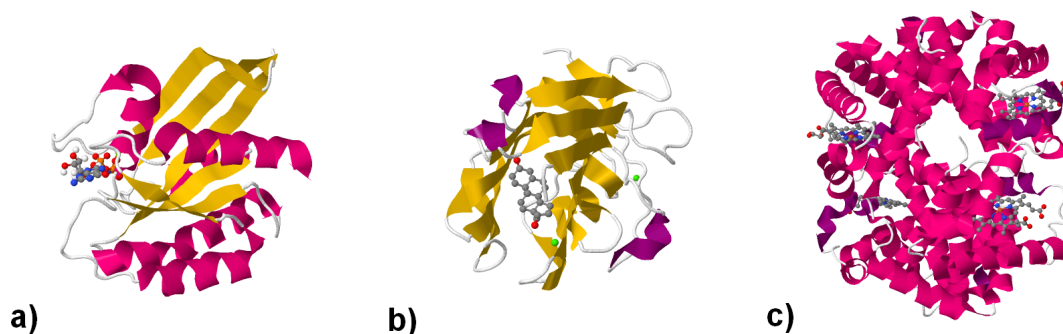


Figure 1.1: Illustration of the structural complexity of proteins using data x-ray crystallography. α helices are shown as cartoon helices and β sheets by cartoon arrows. Ligands are shown in ball and stick representation. a) H-Ras bound to a GTP analogue. [18] b) Sex-hormone binding globulin bound to dihydrotestosterone and two calcium ions. [19] c) A tetramer of hemoglobin chains each bound to a porphyrin ring. [20]

proteome varies over the life cycle of an organism in development and ageing. In humans this process spans decades. An important example relevant both between cells and over time is the distinction between stem cells and differentiated cells, which is a proteomic rather than a genetic one.

1.1.2.1 Diversity of the proteome

Even when a snapshot is taken of a particular proteome at a particular moment the analytical problem remains immense. Proteins are more structurally complex than DNA (see figure 1.1), and methods of sample preparation are more diverse.

Around 21,000 genes translated into protein have been identified in the human genome (as against around 4,300 in *E. coli* [21]). But this is a severe underestimate of the number of distinct protein species in the proteome.

The first layer of complexity is in isoforms arising from alternative splicing of mRNA, in which different combinations of exon are spliced together. Alternative splicing can become extremely complex. The gene *Dscam* in *Drosophila* produces 18,000 splice variants, which are used to produce unique cell surface markers. [22] More typically, human genes express several isoforms with some known to express 19 or 20. The number of known human isoforms is still expanding, but at present at least 75% of genes are known to express more than one mRNA splice form. [23]

Gene Ontology	Number of genes	Proportion of genes in database	Gene Ontology code
Protein binding	9221	50%	GO:0005515
Protein modification	1851	10%	GO:0006464
Membrane location	7646	42%	GO:0016020
Transcription factors and co-factors	1323	7%	GO:0000988, GO0030528

Table 1.1: Numbers of genes with selected annotations for location and function from the AmiGo database version 1.7 [27]

There is also a more exotic forms of splicing in so-called conjoined genes. Here exons from adjacent but nominally separate genes are spliced together. In humans 800 such genes have been identified. [24] In the *Drosophila* gene *mod(mdg4)* splicing takes place between exons transcribed from complimentary DNA strands. [25, 26]

Another mechanism that can produce protein diversity is alternative polyadenylation. The main consequence is direct and indirect regulation of protein production from an mRNA, affecting protein level. However, an alternative poly-adenylation site internal to an exon can result in new truncated products.

More diversity is produced by post-translational modification (PTM) of proteins. Unlike the primary structure of the polypeptide chain, these modifications are not genetically encoded. They consist of covalent bonds formed to (or sometimes broken in) the peptide chain after translation from mRNA. Many are reversible. They allow much more rapid responses than transcription and translation of new proteins. PTMs produce distinct chemical species which can be considered fully as distinct members of the proteome. Often the PTM is critical to protein function, so that the balance between modified and unmodified protein is of as much, or even greater, interest than total levels of the protein in itself.

The total number of human genes with at least some annotation in the Gene Ontology database is 18,410. [27] Since there are around 20,000 genes in the genome it is possible to begin building up an idea of the overall structure of the proteome, as shown in table 1.1.

It is also possible to break down proteins responsible for protein-protein modifications in more detail, as shown in table 1.2

The most important PTM is phosphorylation which, in metazoans, takes place at tyrosine, serine and threonine residues. The human genome contains 518 kinases, or 1.7% of genes,

Modification	Enzyme	Number of genes	Gene Ontology code
Addition of phosphate	Kinase	775	GO:0016301
Removal of phosphate	Phosphatase	256	GO:0016791
Addition of acetyl	Acetylase	74	GO:0006473
Removal of acetyl	Deacetylase	28	GO:0006476
Addition of ubiquitin	E3 ligase	298	GO:0016567
Removal of ubiquitin	Deubiquitinylation	298	GO:0016567
Glycosylation		145	GO:0006486
Deglycosylation		2	GO:0006517
Lipidation		56	GO:0006497
Protease		589	GO:0008233

Table 1.2: Numbers of genes annotated for selected protein–protein interactions from the AmiGo database version 1.7 [27]

which carry out phosphorylation. Around a 6,000 proteins are phosphorylated at a total of over 20,000 sites [28]

Acetylation was first studied in detail on histones. These are proteins around which DNA is wrapped to form chromatin. Lysine residues in histone tails are heavily modified by acetylation and methylation (while the serines are modified through phosphorylation). This, in turn, regulates chromatin structure effectively coding for gene expression, silencing, or cell division.

More recently it has become clear that acetylation rivals phosphorylation in significance. Reversible acetylation has been found on microtubules [29] and in mitochondria. SILAC mass spectrometry has been used to identify over 3,600 acetylation sites on 1750 proteins, showing that most enzymes in human metabolic pathways are acetylated. [30] Another mass spectrometry study showed different sets of proteins acetylated in leukocytes and liver cells, and a strong response of mitochondrial acetylation. [31]

Kinase inhibitors are already a well established drug class. Acetylation may form a similarly promising set of targets, with a histone deacetylase inhibitors already in the clinic for cancer and further drugs in clinical trials. As with phosphorylation, further understanding of drug action will require investigation of complex interaction networks. [32]

Glycosylation is the covalent attachment of sugars. [33] The most diverse form of glycosylation occurs on the extracellular domains of membrane proteins (it can also be found on cell surface lipids). Sequential attachment of sugar residues results in trees of branching sugar chains. Aside from diversity of sugar residues, branching chains have the potential for vastly more diversity than linear polymers.

Glycosylation is extensively involved in inter-cellular recognition, where the sugars are bound by proteins such as lectins. Recognition of sialic acid sugars on the cell surface has been found to play a role in metastasis. [34] Another class of glycosylation is the rapid and reversible attachment of O-linked β -N-acetyl glucosamine at tyrosine and serine residues residues form part of signalling networks throughout the nucleus and cytoplasm. [35]

Small protein domains can also be attached to another protein as PTMs. Most important is ubiquitinylation which regulates protein degradation. Polyubiquitination regulates protein degradation by the proteasome. But as with glycosylation ubiquitin can also form diverse branching tree structures, whose functions are not yet clear. [36]

In humans around a dozen ubiquitin-like proteins have been identified, including SUMO, NEDD8 and ISG15. Like ubiquitin, these modifications are reversible and can form poly-chains. [37]

Another form of PTM is cleavage of the polypeptide chain. While many proteinases act to degrade proteins, many others activate their substrate. An example is the maturation of proinsulin into insulin. A signalling peptide at the N-terminus of proinsulin is cleaved by sec11. The C chain is then cut out by pro-protein convertases 1 and 2 (PCKS1 and PCKS2) and two C terminal amino acids removed by carboxypeptidase E, proteinases that are responsible for the activation of a variety of hormones and signalling proteins. [38] Of course in nature multiple PTMs at multiple sites and protein-protein interactions are all at work simultaneously. It is this that ultimately defines the complexity of the proteomic problem.

1.1.2.2 Protein dynamic range

Protein levels are determined by the balance between synthesis and degradation. The process that regulates these levels is termed 'proteostasis'. [39] In eukaryotes, particularly, the proteostasis network is intricate.

The rate of protein synthesis is regulated by RNA transcription, maturation, translation and degradation. Transcription is regulated by binding of protein transcription factors to DNA and procession of RNA polymerase. Maturation of mRNA includes splicing of exons, capping, polyadenylation and export from the nucleus. The rate of translation depends primarily on the rate at which ribosomes initiate translation. This depends on the strength of the mRNA

binding sequence and mRNA secondary structure. mRNA with longer lifetimes will produce more proteins. This lifetime depends on the rate of mRNA degradation which is determined by proteins and RNA binding to the mRNA sequence and on the length of the poly-A tail. In addition, alternative sites of polyadenylation can leave alternate 3' untranslated regions, which can regulate stability and translation through binding of microRNAs.

The rate of protein degradation is determined by targeting to the proteosome or lysosome. Ubiquitylation is crucial to directing proteins to both of these pathways. [40] The degradation regulatory network is coupled to misfolding, aggregation and localisation of protein as well as to absolute levels.

The correlation between mRNA levels and protein levels has been of great interest, both because this should shed light on the logic of biochemical regulatory processes and because mRNA is more easily quantified. The first successes were in the yeast *S. cerevisiae*. Greenbaum et al. [41] merged data from a two experiments which quantified protein using 2D-PAGE [42, 43] and two experiments which quantified protein using mass spectrometry [44, 45].

They hypothesised that strong variation in transcription over the cell cycle is indicative of regulation of protein levels primarily by mRNA transcription. They indeed found a correlation $r = 0.89$ between mRNA and protein for the 100 most strongly varying genes, compared to $r = 0.2$ for the 100 least strongly varying. They also hypothesised that low ribosome occupancy of mRNA is indicative of regulation primarily by translation and found a correlation of $r = 0.78$ between mRNA and protein for the 100 most highly occupied mRNAs compared to $r = 0.30$ for the 100 least highly occupied.

While these results show that some genes may be regulated primarily at a single level, in general the picture may be more complex.

1.1.2.3 Protein-protein interactions

Beyond quantitative characterisation of the proteome, a second key challenge of proteomics is analysis of protein complexes and interactions, the full set of which is known as the interactome. For biology and medicine, interactions under biological conditions (the biological interactome) are of greater interest than interactions as such (the biophysical interactome).

Interactions can be transitory, as with an enzyme and its substrate, temporary, as with the

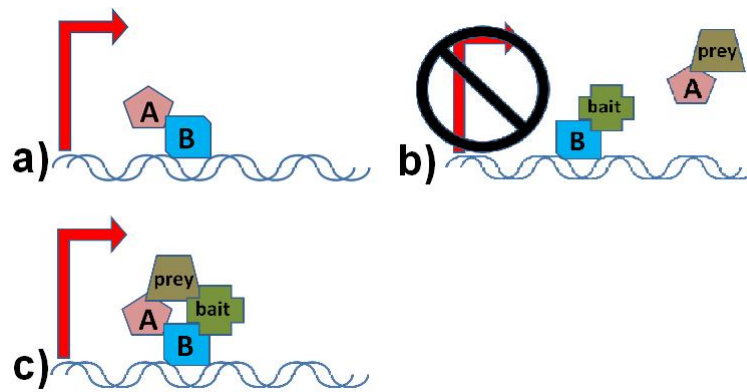


Figure 1.2: Yeast two hybrid assay. a) An intact transcription factor with domains A and B allows transcription to proceed. b) When the two transcription factor domains are separated and fused to 'bait' and 'prey' proteins which do not interact transcription is blocked. c) When the 'bait' and 'prey' proteins interact the transcription factor can be reconstituted allowing transcription to proceed.

dimerisation of receptors on ligand binding, or effectively permanent, as with the binding of the β and γ subunits of G proteins activated by G protein coupled receptors.

Currently available data on protein-protein interactions is limited. While a variety of techniques have been developed to investigate them, it is often unclear how reliable results are.

'Two hybrid' systems test interactions between two domains of interest by expressing them fused to two different halves of a transcription factor (see figure 1.2). The technique was developed in yeast [46], although it has more recently been extended to mammalian systems [47]. If a functional transcription factor is formed, resulting in production of a reporter protein, it is taken as an indication that the domains of interest bind. Because the interaction takes place under rather artificial circumstances it is unsurprising that high rates of false positives and false negatives for native biological interactions have been reported.

Yeast 2 hybrid data has been used to argue for a central estimate of 130,000 binary biophysical interactions in humans. [48]

Co-localisation of antibody staining, [49] FRET experiments [50] or links found through literature mining [51] can show that proteins may be associated or function in the same pathway, but are unable to establish protein-protein binding. Cross linking of proteins is more reliable in this respect. [52]

In silico structural prediction of protein-protein binding is especially difficult where it depends

on protein flexibility or weak interactions over relatively large areas.

Attempts to map protein-protein interaction networks across species with what data is available show little homology. A simulation of network evolution using the most widely accepted model showed that this model could not account for the observed lack of homology, and attributed it instead to paucity of good data [53]. Another example comes from the observation of scale free structures in empirical protein interaction networks.

Protein complexes can reach daunting complexity. One of the largest protein complexes in vertebrates is the nuclear pore complex. This is a ring structure of outer diameter ~ 125 nm and inner diameter ~ 35 nm that connects the nucleus to the cytoplasm forming a pore through the double membrane that separates the two. The structure contains around 30 different proteins (nucleoporins) arranged in multiple copies. The pore is built of repeated units with eight-fold symmetry. The whole structure has a mass of around 50 MDa and in addition to regulating transport interacts with processes on both sides of the nuclear membrane.

The spliceosome and the ribosome are important complexes consisting of both protein and RNA. There is increasing evidence that the active site of the of both are formed by the RNA component, and this is increasingly well established in the case of the ribosome. Nonetheless, these are amongst the most important protein complexes in the cell. The spliceosome contains or is associated with up to 300 proteins and is responsible for removing introns from pre-mRNA and splicing together exons to form mRNA. [54] The ribosome contains over 50 proteins and is responsible for translating messenger RNA into protein. [55]

It is important to characterise not just which proteins interacts, but which proteins interact at the same time. Trying to make sense of signalling networks without this information has been compared to 'trying to plan a journey with an incomplete railway network map lacking train time schedules.' [56]

One approach to conceptualising multiple interactions distinguishes between 'party' proteins which interact with many other proteins at one time and 'hub' proteins which interact with many other proteins, but the interactions are mutually exclusive happening one at a time. [57].

1.1.2.4 Tools for proteomics

The most fundamental difference between the tools for proteomics and those for genomics is the lack of a proteomic technology such as PCR that can exponentially amplify the number of molecules.

In other respects many of the tools for proteins and nucleic acids are similar. Recombinant DNA technology can be used to express proteins in prokaryotes such as *E. coli* or, especially where post translational modifications are required, eukaryotes such as Chinese Hamster Ovary (CHO) cells. Artificial modifications can be introduced to the protein sequence to study the effect of changes, to engineer functionality or to aid in purification (e.g. 6-His tags).

Proteins can be separated by techniques including column and gel chromatography, although the greater chemical diversity of proteins lead to a wider variety separation conditions than in the case of nucleic acids. Gel separations of proteins commonly take place along two dimensions (2D gel electrophoresis). A first separation along a pH gradient in an electric field focuses proteins to their isoelectric point. A second separation along the orthogonal direction then separates them according to their weight. 2D gels can separate up to thousands of proteins, including resolution of PTMs. With appropriate dyes they can quantify over 3-4 orders of magnitude. Their most severe limitation is in dealing with very hydrophobic proteins, such as membrane proteins, and in observing proteins present in small quantities. [58] The latter problem limits their applicability to single cell proteomics. The spots in a 2D gel can also be physically removed and the proteins recovered for functional assays or further identification and analysis.

Western blots transfer proteins separated using gel electrophoresis to a nitrocellulose membrane, allowing detection and quantification by autoradiography or labelled antibodies [59,60]. The name is from the related techniques for DNA and RNA blotting. The use of fluorescently labelled antibodies is now standard.

Protein molecules can be sequenced by sequential degradation of peptide units from the chain, although this technique has largely been superseded by techniques such as mass spectrometry.

1.1.2.5 Mass spectrometry

Mass spectrometry is a vast subject that cannot be covered in any detail in this introduction. [61] However, it is a core technology for proteomics, so it is important to provide an overview of the main approaches with their strengths and weaknesses.

For proteomic analysis mass spectrometers are typically coupled to another separation stage such as liquid chromatography. The real power of the mass spectrometer is the identification of fractions output from such a stage. However, it also plays a role in further separating and to some extent in quantifying the analyte.

The dynamics of an ion in an electromagnetic field are determined by its mass to charge ratio, m/z . This is the quantity that is measured in a mass spectrometer. To identify a protein the polypeptide chain must be fragmented. Multiple peaks will be measured corresponding to fragments with different masses and charges. Peak patterns are compared against databases to identify the parent proteins.

A mass spectrometer accelerates ions in an electric field. After this there are a number of means by which ions of differing m/z can be separated. A sector detector relies on the differential bending of particle tracks in a magnetic field such that a specific m/z value can be selected to enter a detector. A quadrupole mass filter will focus a particles with specific m/z down the central path allowing them to pass while other unfocused particles collide with the walls. In time of flight mass spectrometry linear acceleration by an electric field produces differing speeds for different m/z .

An alternative is to capture ions in a Penning trap and measure the cyclotron resonance frequencies. Here the ions are not separated in space or time but rather by frequency with which they oscillate in the trap. An alternative to a Penning trap using electric rather than magnetic confinement is the orbitrap, commercialised in 2004. [62]

In general mass spectrometer design can become very sophisticated. Two, three or even four mass spectrometer stages can be coupled together with additional fragmentation stages introduced between them.

Protein identification by mass spectrometry has been carried out on samples as small as the several hundred cells that make up individual mouse glomeruli and quantitative measurements have been made on samples of 2,000-4,000 cells from individual mouse pancreatic islets. [63]

Mizuno and others [64] applied ESI mass spec to single cells by poking the tip into the cell. Their refs 17-20 refer to single cell MALDI studies. Laser ablation has been applied to single cells with metabolites profiled by ESI. [65]

In higher organisms it is hard to see more than 10% of proteins present in a single experiment. [66]

The terms 'top down', 'bottom up' and 'shotgun' as used in proteomics are inspired by the related techniques in genomics. Typically in proteomics they are applied to studies using mass spectrometry. Top down proteomics refers to the isolation and identification of intact proteins. Bottom up proteomics digests proteins, for example with trypsin, before entry into a mass spectrometer. The original protein is then identified from the peptide fragments.

Shotgun proteomics is a bottom up technique in which proteins are digested, the fragments identified and the original proteins reconstructed using algorithmic methods. [67, 68]

The most important development in application of mass spectrometry to top down proteomics has been two techniques to inject large ions into the spectrometer. In matrix assisted laser desorption ionisation (MALDI) the protein is deposited on a plate in a chemical matrix. A laser pulse is used to evaporate the protein, injecting it into the spectrometer. In electrospray ionisation (ESI) a charged tip is used at the output of a chromatography stage which is coupled directly into the spectrometer, resulting in the injection of proteins as ions.

Here the breakthrough technique was MudPIT (Multidimensional Protein Identification Technology). This couples two-dimensional liquid chromatographic separation of peptide fragments with electrospray ionisation and tandem MS with collisional dissociation. [69]

The first chromatographic dimension is ion exchange and the second dimension reverse phase chromatography. Pulses of a salt gradient are interleaved with reverse phase gradients, so that each reverse phase gradient separates a new fraction from the ion exchange step. This can result in a lengthy separation. For example, a twelve step salt gradient each requiring a two hour reverse phase gradient would take 24 hours in total.

Quantification using mass spectrometry is less easy than identification. [66] Techniques such as Stable Isotope Labeling by Amino acid in Cell culture (SILAC), Isotope Coded Affinity Tags (ICAT) and Isotope Tags for Relative and Absolute Quantification (iTRAQ) rely on separating a sample into labeled and unlabeled streams which can then be compared an approach which

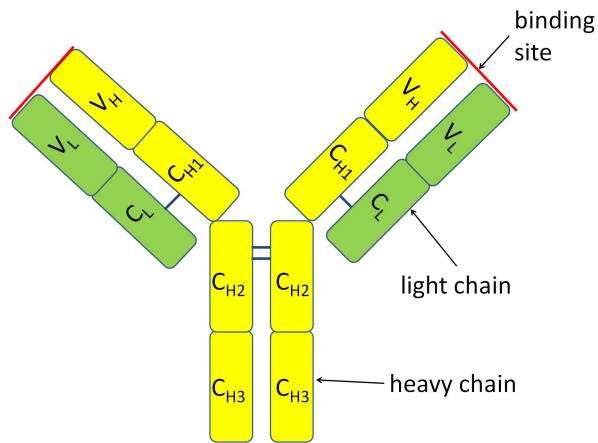


Figure 1.3: Structure of an IgG antibody.

is inherently unsuited to single cell experiments.

Another approach to quantification using mass spectroscopy is spectral counting. This is also of limited applicability with very small quantities of analyte.

1.1.2.6 Antibodies

The final important tool for proteomics discussed here is the use of antibodies. These are molecules produced by the adaptive immune system of jawed vertebrates. (Jawless vertebrates appear to have a similar but convergently evolved immune system.) By randomly creating a large 'library' this system has the potential to produce molecules of with high specificity.

The mammalian immune system produces several varieties of antibody, known as isotypes. The structure of an immunoglobulin G (IgG) antibody, the most commonly used isotype in biotechnology applications, is shown in figure1.3. It is made up of two identical heavy chains and two identical light light chains. The chains are held together by disulfide bonds.

The heavy chains consist of three constant immunoglobulin domains and one variable immunoglobulin domain. The light chains consist of one constant immunoglobulin domains and one variable immunoglobulin domain. Each immunoglobulin domain has a weight of around 50 kDa, and the total mass is around 150 kDa.

The variable domains of one heavy one light chain together form a binding site. Each antibody carries two identical copies of its binding site.

Antibodies against a specific antigen were first isolated by inoculating an animal with the

antigen, usually together with an adjuvant to stimulate the immune response, and isolating antibodies from serum. This produces polyclonal antibodies in which many different immune cells produce different antibodies against the same antigen.

Monoclonal antibodies are produced by fusing a single antibody producing cells with immortalised myeloma cancer cells. This produces a cell line that can be cultured to produce large quantities of an identical antibody.

Monoclonal antibodies were first developed using mouse and more recently using rabbits, in which it is often easier to get strong reactivity to human proteins. [70] Monoclonal antibodies can be sequenced, expressed recombinantly and modified.

Polyclonal antibodies are available from a wider range of species, commonly rabbits, goats, sheep and horses. The use of larger animals is beneficial as they are able to produce greater quantities.

There is considerable diversity across the immune systems of different species and non-mammalian antibodies have found applications. These include chickens, camelids and sharks.

Attempts have been made to systematically validate and document the performance of antibodies for particular applications [71]. However, the problem of inconsistency remains substantial with a need for testing of each new antibody in each new context.

Relative to the use of antibodies, mass spectrometry has the advantage that it is *unbiased*, meaning that any protein can be detected even if it is initially not known to be present. [72] Antibodies, by contrast, can only detect the presence of a protein that is specifically looked for.

Antibodies have a range of uses that mass spectrometry cannot reach, particularly when fluorescently labelled. They can be used for staining of cells or tissue, or in a sandwich type assay as in this thesis.

They have an advantage over 'bottom up' proteomic strategies commonly used in mass spectrometry in that they can recognise folded epitopes and can be used to recognise protein complexes.

1.2 Single cell studies

The study of cells has advanced in step with advances in microscopy. Robert Hooke first identified cells using a microscope in 1665. Joseph Jackson Lister's correction of chromatic

and spherical aberrations in the 1820s allowed the observation of cells in animal tissue, while Robert Brown identified the cell nucleus. [73]

Schwann and Schleiden confirmed that all living things were composed of cells. With the observation of cell division Virchow and Remak theorised that all cells come from cells. The same period led to the founding of cellular pathology based on microscopic observation of cell to cell variation.

The Abbe condenser (1872) and Köhler illumination introduced by the Carl Zeiss corporation at the end of the nineteenth century brought about the full development of classical bright field microscopy.

The contrast necessary to resolve subcellular detail was made possible by the use of dyes. This was pioneered by Paul Ehrlich drawing on dyes produced by the German chemical industry. The silver stain invented by Gogli and further developed by Cajal was used to investigate neurons. Further stains revealed details of the mitochondria, chromosomes and Golgi apparatus.

Tissue culture began in 1910, when Carrel and Burrows, building on the work of Ross Harrision, grew chicken heart cells and tumour tissue from chicken infected with the Rous sarcoma virus, and adult human cells outside a living body. This allowed cells to be studied and controlled outside the context of the body. In 1913 Frederick Gowland Hopkins characterised life as a property of the cell as a whole making it a central unit of study in biology.

The invention of phase contrast microscopy by Zernicke in the 1930s allowed confirmation that structures such as the Golgi apparatus could be seen in unstained, unfixed cells. With time lapse photography the movement of living cells could be captured in detail.

The development of electron microscopy allowed the study of cellular ultrastructure, that is structure too small to be seen by optical microscopy.

The step that allowed characterisation of cell to cell variation at a molecular level was the development of fluorescent labelling of antibodies. This combined specific binding with an extremely sensitive readout.

The introduction of dichroic mirrors in the 1960s and CCD cameras in the 1980s greatly enhanced fluorescent imaging [74].

The availability of new small molecule fluorescent probes [75], soon joined by green fluorescent protein (GFP) and its derivatives created a renaissance in microscopy. In 1994 GFP was

first expressed in *C. Elegans* [76] and then as a fusion protein in *Drosopholia* [77]. Fluorescent proteins under the control of known promoters can be used to study gene expression and fusion proteins to study localisation.

The toolbox of fluorescent probes has continued to expand [78], including the new technology of quantum dots [79, 80] and dye doped nanoparticles. Single molecules (with single or multiple fluorophores can now be routinely detected) so that with techniques such as fluorescent in situ hybridisation (FISH) discrete gene copy numbers can be counted.

Single cell studies have also progressed beyond microscopy. Dovichi coined the phrase 'chemical cytometry' meaning measurement of the chemical composition of a cell. His approach has been based on lysing single cells and analysing contents by capillary electrophoresis. [81] Flow cytometry has also been used to look at distributions across single cells [82]. As early as 1990 used to show bimodal expression in transfected Jurkats [83].

As discussed in more detail below, microfluidic techniques have opened up a new era in single cell studies. This involves predominantly microscopic, but also some non-microscopic techniques.

1.2.1 Noise, heterogeneity and development

A result of statistical mechanics is that for a process involving n copies of a molecule, the fraction of molecules involved in a fluctuation about equilibrium will generally be $\frac{1}{\sqrt{n}}$. This behaviour is rooted in the prevalence of ergodic dynamics combined with the central limit theorem, and is therefore widely if not universally applicable.

In macroscopic chemical reactions numbers are typically too large to observe fluctuations. On the scale of a cell, however, for many phenomena the number of molecules is brought into a range where fluctuations become significant. Thus for 10^4 molecules fluctuations will be approximately 1%, while for 10^1 molecules they will be about 33%.

Thermal fluctuations will arise in processes of interaction and binding, and these in turn will be related to fluctuations in processes regulating the number of molecules, such as synthesis, degradation and the apportioning of molecules in cell division.

Noise arising from these fluctuations sets limits to biological processes, such as the fidelity of signalling or the precision of circadian clocks.

Noise is also exploited by evolution, providing a general mechanism to create phenotypic diversity in a genetically identical population. In these instances stochastic fluctuations which exceed a threshold can send the cell down a new phenotypic pathway.

For single cell organisms this is important in generating diverse responses to an environmental stress, including evasion of host immune response. This raises the probability of at least a subpopulation surviving the stress without the need for genetic diversity. In metazoan organisms which require differentiated cell types, noise can play the role of symmetry breaking. It therefore plays a crucial role in development.

The mean protein concentration in a population of cells does not contain information about fluctuations or subpopulations. Figures 1.4 and 1.5 show schematically some ways in which protein concentration can vary across a population without this being reflected in the mean.

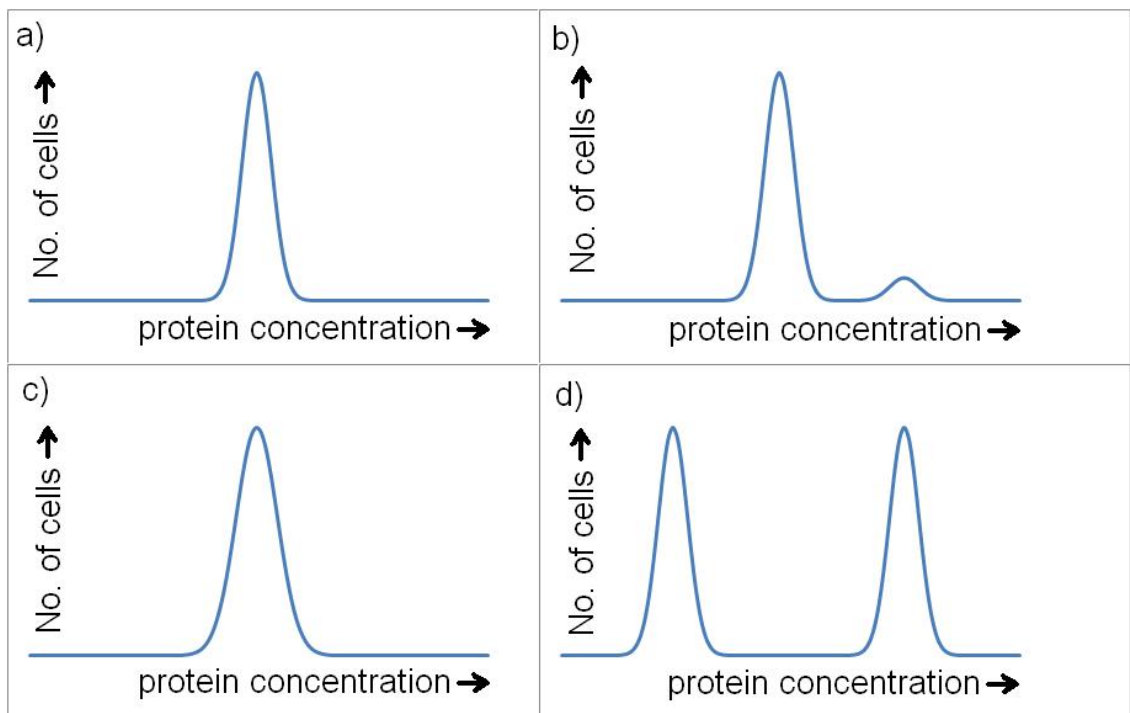


Figure 1.4: Schematic graphs showing forms of variation of protein concentration amongst a population of cells which cannot be distinguished by measurement of the mean, which is identical in all four distributions. a) shows a normal distribution of concentration about an average. b) shows a rare population cells with high concentration with the bulk of cells near to the mean. c) shows a wider normal distribution than in a). d) shows two evenly split populations, each well away from the mean.

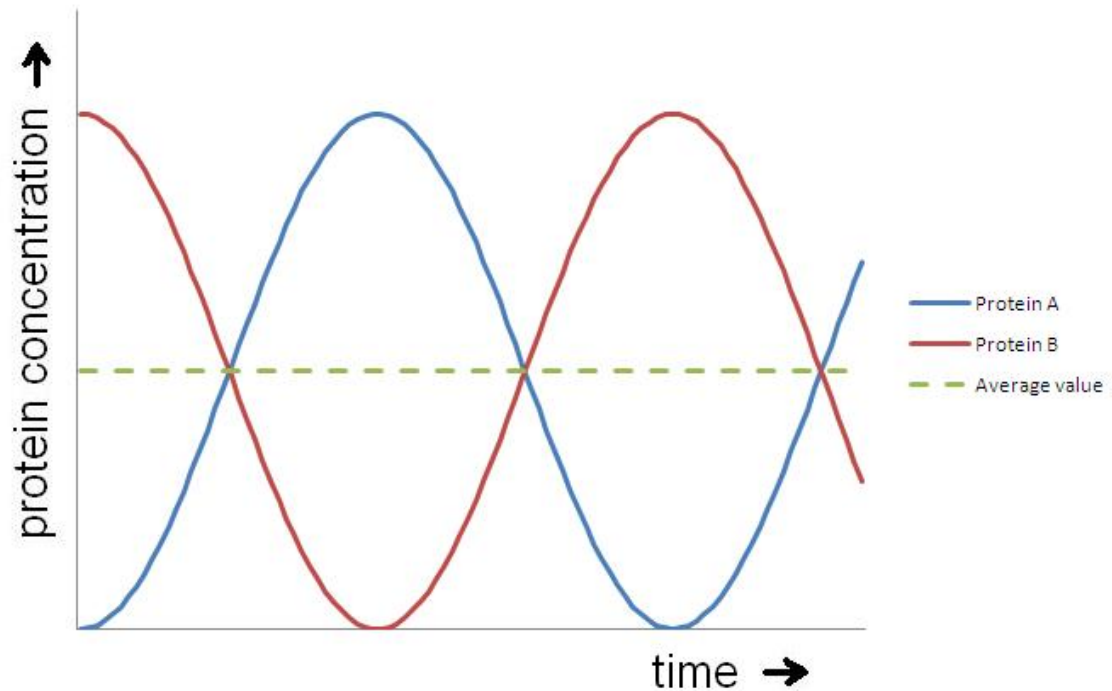


Figure 1.5: Schematic graph showing variation of protein over time averaged away over time.

1.2.1.1 Results from gene expression experiments

Experiments with RT-PCR on single cells show log-normal distributions of mRNA transcripts in a variety of important proteins. For example, this has been shown for transcripts of the transcription factors OCT4, NANOG and SOX2 in single human embryonic stem cells. [84] Another example comes from expression of cells from the pancreatic islets of Langerhans, including insulin genes *Ins1* and *Ins2* in the presence of high and low levels of glucose. [85]

The signal from microarray experiments on lysate from large numbers of cells measure the arithmetic mean of the population. For a log-normal distribution this is a relatively poor proxy for the distribution as a whole. For example, the log-normal distribution is often produced by multiplicative noise (rather than the production of normal distributions by additive noise) which suggests that the geometric mean is a more useful indicator.

The geometric mean is always below, or at most equal to, the arithmetic mean. More specifically, in the case of a log-normal distribution the arithmetic mean is raised above the geometric mean by a relatively few highly expressing individuals. This suggests that these few individuals are not a discrete population that can be sharply distinguished from the majority of

lower expressing cells.

1.2.1.2 Results from experiments with fluorescent fusion proteins

The first experiments to link the distribution of protein number to noise in translation and transcription were carried out in *Bacillus subtilis* by Ozbudak et al. [86] They engineered strains to express GFP under the control of an IPTG inducible promoter that allowed them vary the rate of transcription. They also engineered four levels of translational efficiency, ranging from 0.66 to 1.00 by introducing mutations into the mRNA ribosome binding site and initiation codon. They then used flow cytometry to measure the variance and mean of the cell-to-cell GFP distribution.

They characterise noise by the ratio of variance to mean ($\frac{\sigma^2}{\mu}$, or Fano factor). This measure is useful when comparing results to those expected from a Poisson process. The Poisson distribution applies to counting the number of times that a discrete event (such as production of a protein molecule) occurs in a set time. The condition that specifies a Poisson distribution is that the events occur with a constant probability through time and are therefore uncorrelated. The probability of an event should be low enough that an occurrence twice at the same instant is negligible.

The Poisson probability distribution for n events during a time interval t is then

$$P(n) = \frac{\lambda^n e^{-\lambda}}{n!} \quad (1.1)$$

where λ is the mean number of events expected in time t , that is $\lambda = \rho t$ where ρ is the constant probability of an event per unit time.

The significance of the Fano factor comes from the fact that the variance of a Poisson distribution is equal to its mean, $\sigma^2 = \mu = \lambda$. This means that for a Poisson distribution the Fano factor is equal to one, $\frac{\sigma^2}{\mu} = 1$. Fano factors greater than one indicate processes that are more bursty than Poisson statistics while Fano factors less than one indicate processes that are less bursty.

They find values of $\frac{\sigma^2}{\mu}$ in the range 20 to 40, that is much more 'bursty' than a Poisson process. They interpret the bursts as arising from transcription of single mRNA molecules.

Similar experiments using GFP fusion proteins in a eukaryote, the yeast *S. Cerevisiae*, were

carried out by Blake et al. using flow cytometry. [87]

Elowitz et al. carried out experiments using fluorescent proteins expressed in *E. coli* using fluorescence microscopy. Unlike flow cytometry experiments, this allowed them to follow individual cells – although they averaged over time to still generate a single point for each cell.

Elowitz et al. defined two types of noise in cell to cell variation, *extrinsic* and *intrinsic*. To be precise they speak about rates of gene expression, i.e. rates of mRNA transcription. By extrinsic noise they mean variation due to “concentrations, states, and locations of molecules such as regulatory proteins and polymerases” which vary from cell to cell. Intrinsic noise is then defined as variation that would occur in a hypothetical population of cells that are “identical not just genetically but also in the concentrations and states of their cellular components.” [88]

This definition begs the questions of what is to count as identical. Cells in literally identical microstates would evolve along identical pathways. Presumably they mean to refer to thermal processes rather than just stochasticity associated with quantum processes. Yet to talk about thermal fluctuations requires averaging over microstates corresponding to an identical macrostate. In textbook thermodynamics this is straightforward, as there is a clear cut distinction between micro and macro. In the case of cellular states the line between micro and macro is less obvious.

The meaning of intrinsic noise is none the less intuitively clear. Because transcription is dependent on events involving single molecules at a single DNA site, the thermal stochasticity associated with molecular dynamics and binding in otherwise similar cells will be amplified in to bursts of mRNA and then protein.

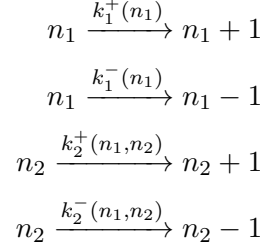
Fortunately Elowitz et al. provide a second, operational definition of noise as “the extent to which the activities of two identical copies of that gene, in the same intracellular environment, fail to correlate”. [88] They realise such a system by inserting two variants of GFP with different colours in to an *E. coli* genome.

In reporting noise they use the ratio of standard deviation to mean ($\frac{\sigma}{\mu}$, or co-efficient of variation) so their results are not directly comparable to those of Ozbudak et al. or Blake et al.

They found that intrinsic and extrinsic noise added in quadrature and were able to fit each to kinetic models. [89]

A useful general framework for analysis of these experiments has been provided by Paulsson.

[90] He considers a simple system. Define species 1 as mRNA and species 2 as the protein encoded by species 1. Let n_i be the concentration, σ_i^2 the variance of the concentration, k_i^+ the rate of production, k_i^- the rate of degradation and τ_i the lifetime of species i .



Near a stable equilibrium the noise in protein concentration is then given by

$$\frac{\sigma_2^2}{\langle n_2 \rangle^2} = \frac{1}{\langle n_2 \rangle H_{22}} + \frac{\sigma_1^2}{\langle n_1 \rangle^2} \frac{H_{21}^2}{H_{22}^2} \frac{H_{22}/\tau_2}{H_{11}/\tau_1 + H_{22}/\tau_2}. \quad (1.2)$$

Here the variables H_{ij} are the logarithmic gains

$$H_{ij} = \frac{\partial \ln(k_i^-/k_i^+)}{\partial \ln(n_j)} \quad (1.3)$$

the meaning being the (negative) differential fractional increase in concentration of species n_i for a differential fractional increase in species n_j . The definition includes a minus sign to make expansions about an equilibrium convenient: a 1% increase in n_i will produce an H_{ii} percent increase in k_i^- relative to k_i^+ .

A simple case models all steps as Poisson processes. Degradation of species n_i proportional to concentration $k_i^- = \alpha n_i$, and production of species n_i to be a constant independent of its own concentration $k_i^+ = \beta$. This is a good simple model for proteins and mRNA (although not for DNA, which is a template for its own synthesis).

It is easily seen that in this model $H_{11} = H_{22} = 1$. For the case of protein production proportional to mRNA concentration $H_{21} = -1$. In this case equation 1.2 simplifies to

$$\frac{\sigma_2^2}{\langle n_2 \rangle^2} = \frac{1}{\langle n_2 \rangle} + \frac{\sigma_1^2}{\langle n_1 \rangle^2} \frac{\tau_1}{\tau_1 + \tau_2}. \quad (1.4)$$

The first term on the right hand side of equation 1.2 (or equation 1.4) gives the expected gaussian fluctuations about equilibrium. The second term is additional noise arising from

fluctuations in mRNA levels. It can be seen that if protein lifetime is much longer than mRNA lifetime then the second extrinsic noise term falls to zero as fluctuations in mRNA levels are averaged away.

Equation 1.2 clearly shows the relationship between measurements of $\frac{\sigma^2}{\mu}$ and of $\frac{\sigma}{\mu}$. For intrinsic noise the Fano factor $\frac{\sigma^2}{\mu}$ is a constant independent of concentration. For extrinsic noise it is the co-efficient of variation $\frac{\sigma}{\mu}$ which is independent of concentration.

It can be seen that in fitting these models absolute quantitation of the number of molecules rather than relative signal strenghts are necessary.

The analysis of fluctuations based on similar models is taken further by Pakka et al. [91].

1.2.1.3 Further results from single cell resolution experiments

Cai et al. used isolation in 100 pL chambers to measure expression of β -galactosidase (β -gal) by *E. Coli* [92]. They achieved single molecule sensitivity by relying on an amplification of the signal through the enzymatic action of β -gal. (This strategy is therefor not suitable for application to proteins that lack enzymatic activity that can be detected by fluorecence of it's product.)

Previous studies assumed total fluorecence was a normally distributed continous variable and fitted σ and μ . Cai et al. measured not just the noise but the discrete distribution of of protein numbers, which they fitted to a gamma distribution

$$p(n) = \frac{n^{a-1}e^{n/b}}{b^a\Gamma(a)} \quad (1.5)$$

where a and b are parameters and Γ is the gamma function. Previous work by the group had shown by solving the master equation that for small protein numbers the parameters of the distribution can be intepreted as mRNA transcription burst size and translation rate.

Xie has shown that while mRNA numbers are correlated with protein numbers at the popu-lation level, at the single cell level they are uncorrelated. The explanation for this phenomenon is that the reduction of mRNA molecules in a cell is dominated by the process of cell division rather than by mRNA degradation. For this reason, the result is unlikely to extend to eukaryotic cells.

Guert at al. found that even relatively simple synthetic gene networks in *E. Coli* consisting

of three transcription factors modulated by two external small molecule regulators could not be understood by simple Boolean logic (simply chaining together arguments such as "*A* is high and represses *B*, therefore *B* is low..."). They concluded that nonlinearities, stochastic processes and the details of more subtle unknown interactions must play a role. [93]

If qualitative data is in some cases not good enough to understand such simple systems then a more complete understanding of regulatory networks in more complex cases, including eukaryotic organisms, will surely require more quantitative data.

Elowitz and Leibler constructed a small synthetic gene network, $A \rightarrow B \rightarrow C \rightarrow A$, which produced oscillations of GFP in *E. coli*. Because of one of the repressors in the network was LacI they were able to initially synchronise the cells using a pulse of IPTG. However, in order to track oscillations beyond the first maximum required single cell tracking, the period being measured as 160 ± 40 min ($n=63$). [94]

In deriving the gamma distribution they write that it "is conceivable that the two measurements are related, analogous to the ergodic principle in statistical physics." Such an analogy, however, requires a steady state. As discussed above, the data does indeed give a good fit for *E. coli*. But it is limited in how far the approach could be extended to a more complex case such as oscillations.

Several groups have studied oscillations over time in NF- κ B using fluorescent fusion proteins. Tay et al. studied activation of NF- κ B by TNF α . [95] Turner et al. observed oscillations in NF- κ B nuclear localisation with a period of around 100 minutes in response to TNF α stimulation. [96] Bartfeld et al. have used a fusion of GFP to NF- κ B subunit p65 to study oscillations in nuclear localisation in response to bacterial infection. [97]

Similar oscillations in nuclear localisation have been studied in fusions of p53 [98] and ERK [99].

Sprinzak et al. use GFP fusion proteins to examine the way in which the protein delta activates notch on neighbouring cells (trans-activation) while inhibiting notch in the same cell (cis-inhibition). [100]

Skotheim et al. studied the cell cycle in *S. Cerevisiae* by putting GFP under the control of the promoter for a cyclin gene, CLN2. They showed cell cycle entry is regulated by positive feedback rather than a linear cascade. [101]

Spencer et al. studied cell-to-cell variability in response to the signalling protein TRAIL in a human cell line. They were able to show that non-genetic variability was inherited by daughter cells on cell division. Unlike the other results reviewed here, their interesting results did not depend directly on measuring changes in intensity of a fusion protein. However, they did still use fluorescent reporter proteins to determine pairs of daughter cells and also to time the onset of apoptosis in response to signalling. [102]

Sigal et al. created twenty fluorescent protein fusions in a human cell line and measured variability over time. They found that amongst different proteins co-efficients of variation ($\frac{\sigma}{\mu}$) ranged from 0.12 to 0.28. The mixing time for protein concentration auto-correlation to fall to half ranged from 0.8 to 2.5 generations at about 20 hours per generation. By constructing cells lines with two fluorescent protein fusions they found that the concentrations of proteins in the same pathway showed higher correlations than proteins in unrelated pathways. [103]

Cohen et al. studied the response of to an anti-cancer drug at the single-cell level. They prepared a library of over 1,000 cell lines, each with a distinct fusion protein, and tracked the response in concentration and location of each in turn to create a wider picture of cell response. Amongst other results they found co-efficients of variation ranging from 0.1 to 0.6, although they attributed 20% of this to cell cycle variation. They found that on addition of the drug this variation increased on average by 30%, with 24 proteins showing very different and distinct cell-to-cell variation. [104]

New techniques for genetic engineering promise to allow easier construction of multiple fusion proteins in human cell lines. However, at present even the construction of fusion proteins for two specified proteins in one human cell line is challenging. [105]

Acar et al. use flow cytometry to study the distribution of expression of fluorescent protein under the control of the Gal transcription machinery in *S. Cerevisiae*. By studying strains in which each member of the Gal network is reduced from 2 to 1 they find that regulation of bistability is undercontrol of network motif that is dosage independent. [106]

These studies are only beginning to investigate the role of heterogeneity. Every difference between cells could be considered a form of heterogeneity. This includes both molecular composition (nucleic acids, proteins, lipids, metabolites together with all their modifications) as well as arrangement of molecules within the cell (localisation to different compartments, areas and

binding into complexes.) Heterogeneity between individual cells takes on a greater significance in cancer. This is because whereas in normal evolution the unit of selection is the individual organism, in evolution of cancer the cell becomes the unit of selection.

Many new tools will be needed to investigate heterogeneity, particularly in biological samples from organisms and patients, which cannot be engineered to express fusion proteins. Even where engineering is possible, it is still not an ideal solution.

Immortalised cell lines have a biology which is severely altered as compared to healthy - or even cancerous - cells. The introduction of a fluorescent reporter will further disrupt the biology, and construction of a fusion protein will alter the behaviour of any protein of interest to an unpredictable degree. Moore and Murphy [107] argue that if a fluorescent fusion construct with a protein rescues the knockout phenotype for the protein, that is evidence that the fusion construct is functional. However, they go on to note that this does not ensure that localisation or quantity of a protein is similar to the wild-type. Most obviously, a fusion construct may be present in excess or at new locations.

1.3 Microfluidics

At the same time that the importance of single cell studies has become clear, the tools to undertake these studies have emerged. The most important of these is the field of microfluidics. This is loosely defined as the use of fluid flows in which at least one dimension is confined on the scale of between 1 and 100 microns.

While microfluidics is a good fit for single cell analysis, its development has been motivated by issues common to bio-analytical chemistry and analytical chemistry. These include the reduction of power and reagent consumption, increasing the speed of analysis and reducing quantities of analyte used.

Whitesides, a pioneer of microfluidics, has identified four broad origins of microfluidic technologies [108]

1. Microanalytic technologies such as gas phase chromatography, HPLC and CE.
2. Post cold war biodefense, which motivated much initial funding for academic research.
3. The needs of molecular biology which, from the 1980s, demanded higher throughput and

sensitivity analyses.

4. Microelectronics, which provided much initial inspiration and continues to provide tools such as lithography.

The broader advantages of microfluidics are also realised in the related field of nanofluidics. This has also found biological applications, but mainly for the study of bio-macromolecules rather than at the cellular level. An important example is sequencing of DNA by nanopores. There are also many overlaps between nanotechnology and biological microfluidics, such as the delivery of molecules to cells using carbon nanotubes.

However, the importance of the micro scale is that it co-incides with the size of cells. A number of distinct phenomena arise at microfluidic scales that are useful both for the understanding of biology at the micro level [109] and for technological microfluidic design. This opens many new opportunities but also poses the challenge of validating new microfluidic assays, making them clearly comparable to existing biological data derived using older techniques. [110]

The advantages of miniaturised analysis systems were first laid out in the 1950s by Golay, which led to the adoption of first capillary gas chromatography and then capillary electrophoresis. Early devices used metal and then nylon tubing. [111] Fused silica capillaries became available as a spin-off from fibre-optics and soon became standard. However, such capillaries could not easily be built into *systems*, with branches, junctions and valves.

Another early device was a gas chromatograph micromachined in silicon in 1979, where the minaturisation was motivated in part by the needs of space exploration. [112]

A "miniaturized-Total Analysis System (μ -TAS)" was proposed by Manz, Graber and Widmer in 1990 [113], again drawing attention to the scaling advantages for chemical separations and analysis. An early glass device was constructed by Harrison et al. [114] Growing interest through the 1990 resulted in the founding of the journal *Lab on a Chip* in 2001.

Microfluidic devices make use of wide variety of physical forces and effects [115, 116] including pressure, elasticity, electricity, diffusion, optics and magnetism. [117]

1.3.1 Theoretical description of microfluidics

The flow of fluids in microfluidic devices can be accurately described by the Navier-Stokes equation which can be written in it's basic form as

$$\rho \left(\frac{\partial \mathbf{v}}{\partial t} + (\mathbf{v} \cdot \nabla) \mathbf{v} \right) = -\nabla p + \eta \nabla^2 \mathbf{v} + \rho \mathbf{g} + \mathbf{F} \quad (1.6)$$

where ρ is the density, $\mathbf{v}$ is the velocity of the fluid, p is the pressure and η is the viscosity, $\mathbf{g}$ is the acceleration due to gravity and $\mathbf{F}$ is any other body force such as the electrical force on a charged fluid.

This may most simply be understood as an application of Newton's second law to a parcel of fluid. The left hand side is the rate of change of momentum which has a contribution both from change in the flow with time and from the movement of the parcel within the flow. The right hand side is the total force applied to the fluid parcel.

The equation 1.6 can be extended in various ways. For example, compressible fluids (in a microfluidic context that means gases), non-Newtonian fluids which have velocity dependent viscosity and liquid crystals which have anisotropic properties all require additional terms on the right hand side. These will not be needed in this thesis, so are not included here.

Instead the condition for an incompressible flow, vanishing of the divergence of the velocity, is used

$$\nabla \cdot \mathbf{v} = 0. \quad (1.7)$$

The Navier-Stokes equation also requires specification of boundary conditions. At solid surfaces the velocity goes to zero, as determined by adhesions resulting from molecular interactions between the fluid and the wall. (In fact this results in a boundary condition such that the velocity would be extrapolated to zero a small distance inside the solid surface, known as the *slip length*. This can be significant in low pressure gas flows but in liquids the slip length is typically several molecular diameters. The phenomenon subject of active research in nanofluidics but in microfluidics can usually be neglected in favour of the simpler "no slip" condition.) At a free surface the boundary condition is that the stress is zero.

Exact solutions of the Navier-Stokes equations are rare, and even accurate approximate solutions are often impossible. Much of the difficulty can be traced to the nonlinearity introduced

by the term $(\mathbf{v} \cdot \nabla)\mathbf{v}$ in equation 1.6. Physically, this term allows for *turbulent* flow. Non-turbulent flow is known as *laminar* flow, and for these flows the Navier-Stokes equations are much more tractable.

Osbourne Reynolds discovered a criterion separating turbulent from non-turbulent flow. He characterised flows by a dimensionless ratio now known as the Reynolds number, Re . This is defined by

$$Re = \frac{\rho v l}{\eta} \quad (1.8)$$

where ρ is the density of the fluid, v is a characteristic flow velocity, l is a characteristic length scale and η is the dynamic viscosity. This measures the ratio of inertial to viscous effects. At high Reynolds numbers inertia dominates, resulting in turbulent flow. At low Reynolds numbers viscosity dominates resulting in laminar flow. The number at which the transition takes place depends on the exact geometry. For typical microfluidic channels it takes place at around 2000.

The characteristic length scales l of microfluidic devices are in the range of $100\ \mu\text{m}$ or less. This generally ensures laminar flows, simplifying analysis.

1.3.2 Microfluidic fabrication and materials

The most popular technique for microfluidic fabrication is soft lithography for creation of polydimethylsiloxane (PDMS) devices. [118] This is the technique used in this thesis, and the protocol is described in more detail in chapter 2. Briefly, lithography is used to create a negative master image of the desired channel pattern. Monomer is poured over the master, and polymerised in a curing step.

The advantage of soft lithography is that it is relatively quick, easy and cheap to create new devices, especially to a fixed design. As a material for bioanalysis, PDMS has the advantage that it is permeable to gases such as CO_2 important to cell survival.

An alternatives to soft lithography include injection molding or hot embossing and of polymethylmethacrylate (PMMA). [119] Here a master is used to impress a channel pattern on a thermosetting polymer.

For synthetic chemistry and other applications requiring chemical resistance glass is the preferred material. A common technique for producing channels in glass is hydrogen flouride etching.

Laser ablation using femtosecond pulses can be used to fabricate channels. This technique generally needs to be followed by either chemical etching (possibly using a photosensitive glass [120]) or under conditions where water can be used to wash debris from the channel during fabrication. [121] Careful control of focussing can allow for specific shaping of channel cross sections. [122]

A third technique that has been applied to glass is micromachining. This has also been applied to silicon.

Glass, and to a lesser extent PDMS are good choices for integration with optical techniques. The right combination of optics and microfluidics is often responsible for the best devices. [123]

1.3.3 Digital microfluidics

An important recent development has been the use of droplets, [124] sometimes known as 'digital microfluidics'. [125] Droplets can be contained either in a carrier fluid, for example aqueous droplets in oil, or in a gaseous atmosphere, such as air. Droplets have the advantage that diffusion and mixing are contained. As the term digital suggests, the discrete presence or absence of a droplet can overcome the accumulation of small errors that is otherwise inevitable in continuous flow systems.

Manipulation of droplets has both advantages and drawbacks compared to continuous flow systems. The technology to break a continuous flow into droplets is well developed. Notably it is also possible to controllably move merge droplets back into a continuous phase.

Digital microfluidic devices have been used for analysis of cells. [126]

1.3.4 Microfluidic studies on single cells

Microfluidic systems for single cell studies are reviewed by Zare and Kim [127]. The Quake group developed microfluidic devices for isolation of mRNA, reverse transcription to cDNA and amplification by PCR from single cells lysed on chip. This has been used to study bacteria, most strains of which cannot be cultured.

Some of the work described previously has been carried out in a microfluidic context, such as study mRNA transcripts in human progenitor stem cells, the presence of reactive oxygen species in cancer stem cells and, more recently, NF- κ B.

1.3.5 Microfluidic cell sorting

1.3.5.1 Conventional cell sorting and counting

The most widespread established technology for counting and sorting cells is fluorescence activated cell sorting (FACS).

The counting step is called flow cytometry. Here cells are fluorescently labelled, entrained in a sheath flow which centers them and directed through the focus of a laser beam. Detectors measure fluorescence as well as side scatter and forward scatter of the excitation light.

Sorting is achieved by breaking the flow into charged droplets where each cell is encapsulated in a separate drop. A voltage is applied across plates as drops fall between them, deflecting the drops on to different paths for collection.

1.3.5.2 Single cell studies using flow cytometry

Flow cytometry is relatively rarely thought of in the context of single cell proteomics. None the less, it is perhaps the most developed single cell proteomic technology. In so far as flow cytometers makes use of fluid sheath flows to align a stream of individual cells through the focus of an optical system with micron precision they can be considered microfluidic, if not 'lab on a chip', systems.

While the most advanced commercial flow cytometers can analyse 17 or more fluorescent markers at one time. [128] One study has measured as many as 26 surface markers on cells from umbilical cord blood. [129]

In addition to fluorescence side scatter and forward scatter of light are routinely measured to give information on cell size and morphology.

However, detection of many markers requires highly co-ordinated optics and dye chemistry as well as algorithms for automated analysis of high dimensional data [130] It is more routine for flow cytometry to measure one or a few markers. While flow cytometry is a powerful technique for measuring surface markers it is more limited in its ability to quantify cytoplasmic proteins.

Flow cytometry has also been developed in the direction of imaging cytometry, in which an image of each cell is captured. [131]

1.3.5.3 Microfluidic cell sorting devices

Microfluidic cell sorting is desirable as a development of conventional cell sorting. It could potentially bring the well known benefits of microfluidic devices to cell sorting: low consumption of reagents and power, speed, disposability and portability. Such of applications are about making existing measurements more convenient. While they might potentially open access to data on a new scale they do not open up the possibility of basically new measurements.

A second motivation for microfluidic cell sorting is integration with single cell analysis stages of the sort discussed in the previous section. Here the emphasis is on providing cell handling and manipulation. Advantages such portability, reduced cost and speed are less significant than the possibility of accurately, reliably and delicately manipulating single cells. It is desirable, for example, to minimise the stresses experienced by cells in order to increase viability and reduce perturbations which might affect the measured cellular state.

Whereas traditional cell sorting works well with large numbers of cells, microfluidic cell sorting can be applied either to high throughput applications or to lower throughput applications dealing with rare cells.

Conceptually a cell sorter has two components. First some sort of *measurement* to discriminate between cells. This may be quantitative, as in flowcytometry. For sorting it must also be the basis of a binary (or ternary or higher discrete) decision on which stream to allocate the cell. Second a *separation* stage must physically segregate cells into physically distinct compartments.

In practice the two stages may be coupled together. For example, early attempts at microfluidic cell sorting made use of AC dielectrophoresis. [132, 133] This relies on a contrast in dielectric constant to apply a differential force to cells. These devices both measure dielectric constant and use dielectric constant to achieve separation. A more recent AC dielectrophoretic device separated 10 μm latex particles from white blood cells, although not cells from cells. [134]

Another example that relies on contrast in dielectric constant (although at optical rather than AC frequencies) are devices that set up a three dimensional optical trapping lattice through which cells are left to drift. Those most strongly trapped are retained.

The combination of measurement and separation has some elegance, but it is inherently limited to special applications. For a widely applicable cell sorting system it is desirable to not to rely for separation on any specific physical characteristic of a cell population. The

general strategy of coupling together physically distinct detection and separation stages through computer control is less elegant but more flexible, allowing universal application.

One of the earliest microfluidic cell sorters used a switching of electrokinetic flow at a simple T junction in a PDMS device to separate *E. coli* expressing GFP from non-fluorescent cells. The device achieved a 30-fold enrichment from 1:100 to 1:2. Recovery, however, was poor. The throughput achieved was 20 cells per second. [135]

The same group later argued that electrokinetic switching creates problems with buffer incompatibilities, voltage changes due to ion depletion, pressure imbalances and evaporation. They therefore developed a system with similar geometry but making use of a multilayer PDMS system. Flow was created through peristaltic pumping and switching was achieved by closing off one branch of the T. The best performance from this device was able to sort 44 cells per second with a recovery rate of 39% and an enrichment factor of 83. [136]

More recent devices generally make use of hydrodynamic focusing. This is discussed further in chapter 4.

1.3.5.4 Moving cells across streamlines

Optical forces have been used in active devices. Unlike the passive optical sorting mentioned above, this strategy can be coupled with optical detection. Wang et al. sorted HeLa cells expressing GFP from non-fluorescent HeLa cells. They worked at rates of 20–100 cells per second. They achieved enrichment from about 1:80 up to 6:1, with recovery rates approaching 90%. [137].

Applegate et al. have built a 'optical conveyor belt' using a diode bar laser. [138, 139]

The 'hydrodynamic metamaterials' are in some sense an analogue of optical lattice sorting making use of a period array of physical posts rather than optical fields. They effectively 'diffract' particles larger than a certain size (rather than particles with a certain refractive index as with optical sorting) physically deflecting them at an angle across streamlines. [140]

Affinity patches fall into this category.

1.3.5.5 Switching fluid flows

In contrast to the sorting of cells which requires dragging cells across fluid stream lines, in this strategy the cell remains at rest relative to the fluid while the fluid flow itself is diverted. The advantage is that forces do not have to be applied specifically to the cell. The disadvantage is that there is typically greater perturbation to the flow.

Sugino et al. suggest that because active sorters so far seem restricted to rates of around 1-100 cells s^{-1} the best strategy for achieving high throughput will be parallelisation, and present a device with 8 parallel streams making use of thermoreversible gelation for valving. [141]

A device built by Franke et al. makes use of acoustic streaming. [142] (This phenomenon is unusual in microfluidics in that inertial effects are significant.)

Here an ultrasonic sound wave is absorbed by the fluid, resulting in a transfer of momentum. As is usual for acoustic streaming in microfluidics, the wave was a surface acoustic wave generated by interdigitated electrodes deposited on the surface of the device.

One of the most effective sorters to date makes use of a piezoelectric lead zirconate titanate (PZT) actuator. [143] It achieves rates of sorting up to 1000 stained mammalian cells per second, and enrichment factor of 230, from 1:123 up to 1.86:1, which they claim as the highest achieved in a μ -FACS device.

1.3.5.6 sorting of droplets

A third strategy for sorting is to distribute the cells into droplets. While this is an attractive strategy, the inevitable complexity of 2 phase flows and of getting cells into droplets can make it difficult. [144–146]

1.3.5.7 Isolation of circulating tumour cells in microfluidic devices

A 7.5 mL blood sample contains around 4×10^{10} erythrocytes (or red blood cells), 3×10^7 leukocytes (or white blood cells) and 2×10^9 platelets. At typical levels of between 1 and 100 cells per sample, CTCs constitute around 1 in a million cells in the leukocyte fraction.

Several approaches have been taken to the isolation of CTCs. One is the use of density centrifugation. A second is sorting by size and flexibility. A third is the use of antibodies.

The first strategy has been combined with high throughput microscopy.

Nagrath et al. built the first microfluidic chip to isolate CTCs. A chamber of pillars was fabricated in silicon. This was then functionalised with anti-EpCAM antibodies. Whole blood was flowed through the device and CTCs were immobilised by the antibodies. Capture yields were in the range of 60% when tested with spiked cells. The flow rate of whole blood was in the range millilitres per hour. [147]

This device has been used to profile the DNA of CTCs for EGFR mutations. [148]

Similar devices have used herringbone structures to improve mixing, resulting in improved recovery and flow rates. [149]

Two microfluidic chips have been built using capture by EpCAM antibodies. The first [147] and the second [150]

A pillar device coated with the lectin concanavalin A has been used to separate cell lines spiked into mouse blood. [151]

The second strategy has also been implemented in microfluidic formats. In both cases clinical validation has not yet been carried out.

Aptamers have been used to enrich cancer cells, but only in proof of principle [152]

A device for separation of CTCs from whole human blood based on size and mechanical flexibility has been used to separate spiked cell lines from blood, but has not yet been applied to clinical samples. [153] An array of holes was fabricated using nickel electroforming, which effectively trapped large cancer cell lines (size $>8\ \mu\text{m}$). Recovery rates greater than 90

A filter based system has the potential to eliminate the need for fluorescent staining, but this work has not yet fully achieved that goal.

A system which integrates magnetic bead pulldown onto a chip by incorporating a mixer. [154] Spiked cells only, but does PCR.

It has been found that a cancer cell line and white blood cells will electroporate in a microfluidic at a lower voltage ($300\text{-}400\ \text{Vcm}^{-1}$ and $400\text{-}500\ \text{Vcm}^{-1}$ respectively) than red blood cells ($1100\text{-}1200\ \text{Vcm}^{-1}$), suggesting that blood could be purged of CTCs by flow through an electric field in a microfluidic [155]

1.4 Cancer, disseminated and circulating tumour cells

Cancer encompasses a wide range of diseases, the common feature of which is an uncontrolled proliferation of cells. Where growth is unlimited, invasive of surrounding tissue and spreads to distant sites (metastasis) the disease is said to be a *malignant neoplasm*. In contrast, where a growth lacks all three of these characteristics (i.e. it is self limited and contained) it is said to be a *benign neoplasm* rather than a cancer.

In the US cancer was responsible for 23.2% of deaths in 2007, second only to heart disease. In the population younger than 85 it was the leading cause of death. Amongst women the most common cancer sites were breast, lung, and colon while amongst men they were prostate, lung and colon. [156]

Each cancer is the product of a unique set of mutations. But despite the diversity, some common hallmarks are apparent. Hanahan and Weinberg identified 1. self-sufficiency in growth signals, 2. insensitivity to growth-inhibitory signals, 3. evasion of apoptosis, 4. unlimited replicative potential, 5. sustained angiogenesis and 6. invasion of surrounding tissue and metastasis [157]. Mantovani has since suggested a seventh hallmark: cancer-related inflammation [158].

The detailed ways in which these hallmarks are a product of perturbation at a molecular level is not yet understood. The importance of DNA mutations is clear. The somatic mutation theory first put forward by Boveri in 1914 is now well established, as is the need for multiple mutations. [159] Understanding of a common chromosomal rearrangement in chronic myelogenous leukemia was the basis for development of the successful drug Imatinib.

Whole genome cancer sequencing is just beginning to reveal patterns across the disease. [160] Although cancer proteomics is somewhat less developed, some important pathways have been studied in detail. An example is the transcription factor p53 which co-ordinates response to DNA damage and is the most commonly mutated oncogene. [161]

However, metastasis remains poorly understood. From a clinical point of view metastasis is the most significant process, because it is responsible for most cancer deaths [162]. The importance of metastasis may be seen from the American Joint Committee on Cancer system of breast cancer staging which runs from 1 to 4. Stage 4 is defined primarily through the presence of metastasis. Lazebnik has suggested that given its significance the first five of of Hanahan

and Weinberg's hallmarks are not 'in the same league' as the sixth, metastasis. [163]

In order for a tumour to metastasise its cells must go through a number of steps. First the cells must migrate away from the primary tumour. This involves both becoming mobile and cutting through the extracellular matrix that binds cells in place.

Second, cells must enter into either blood or lymphatic circulation. This is known as intravasation. The lymph nodes of the lymphatic system act to filter and collect cells that enter circulation in this way. A section of the lymph node closest to the primary tumour is therefore often used as a diagnostic tool.

Thirdly, cells must exit from circulation at a distant site, known as extravasation. This again requires the ability to pass through the basement membrane.

Fourthly, cells must establish growth at a distant site. Cells which fail to do this may lie in a dormant state or form micrometastases. To successfully grow ultimately requires recruitment of surrounding tissue, as with the formation of blood vessels (angiogenesis) to feed the growing tumour.

Each of these stages will be best enabled by a different complement of proteins. The relation between protein expression and the mutational spectrum of a cancer at each stage in its development is unclear but it is likely that new mutations are required for a cancer to progress.

Despite its clinical significance, the process of metastasis is also the least well understood stage in the natural history of cancer. Even basic facts remain mysterious.

Intravasation has been observed in mice from a tumour even before it becomes morphologically invasive, and disseminated tumour cells have been detected in women with noninvasive breast ductal carcinoma in situ. [164]

The rival theories of linear progression, in which metastasis follows after development of the primary tumour, and parallel progression, in which metastasis begins much earlier and secondary tumours evolve in parallel with the primary, make contrasting empirical predictions. Yet Cristoph Klein finds that with contradictory evidence pointing in both directions it is not yet possible to settle which is correct. [165]

The 'seed and soil' hypothesis, states that the probability of metastasis depends on the tissue type of both the primary ('seed') and the secondary ('soil') tumour. This was first

demonstrated by Paget's 1889 study of breast cancers which showed that breast cancer preferentially metastasised to the lung, brain and liver. [166]

An example of further evidence for the importance of the 'seed' comes from experiments by Podsypanina which showed that engineered mouse mammary cells will distribute themselves to the lung but will remain dormant until induction of MYC and Kras^{D12}. [167]

An example of further evidence for the importance of the 'soil' comes from tumour self seeding, in which circulating tumour cells re-enter the primary tumour, offers evidence that the not just the tissue but also the microenvironment of the 'soil' is important. [168]

It is possible to access tissue from both primary and secondary tumours. This data has been used, for example to demonstrate the clonal selection theory of cancer. However, distinct differences between primary and secondary tumours have been identified. [169]

The profile of circulating tumour cells is therefore of interest for a number of reasons. They should shed light on when the differences between primary and secondary tumours appear. It would also be of great interest if profiles could be identified specific to the circulating phase of cancer natural history.

Perhaps most interesting is the possibility of studying heterogeneity of CTCs. It is clear that only a small fraction of CTCs ultimately go on to form secondary tumours - or else metastasis would proceed much more rapidly at multiple sites. However, at present it is unclear whether there is anything distinct about the fraction of CTCs responsible for metastasis, whether at the genetic or at the proteomic level.

CTCs that form metastasis may be statistically uniform, and secondary tumour formation a matter of chance. But any differences that can be identified will be of immense therapeutic interest.

1.4.1 Cancer biomarkers diagnosis, prognosis

The discovery of reliable biomarkers to aid diagnosis and prognosis is a major challenge for cancer medicine. At present the most reliable diagnostic results rely on biopsy of the solid tumour. Similarly, detection of minimal residual disease, which can result in relapse, relies on detection of disseminated tumour cells in the bone marrow.

For the patient a 'liquid biopsy' in the form of a blood test would be far preferable to fine

needle biopsy of the tumour or sampling of the bone marrow. While some serum biomarkers, such as prostate specific antigen, are available they are of limited reliability.

The number of CTCs detected by the CellSearch[®] system in cases of metastatic breast cancer has been shown to predict both progression free survival and overall survival. Detection of more than 5 vs. less than 5 CTCs in a on 7.5mL blood sample maximised the Cox hazard ratio. The median time to progression for patients with more than 5 CTCs was 2.1 months vs. 7.0 months for patients with fewer than 5 CTCs. The corresponding figures for overall survival was 8.2 months vs. >18 months. [170]

These results from cell counting are encouraging. But molecular profiling of CTCs could provide even more information which at present is only available from analysis of tumour cells, including the spectrum of mutations and protein expression.

1.4.2 Development of cancer therapies

A neoplasm can be surgically removed or attacked by localised radiotherapy. Chemotherapy can be used before surgery to shrink the tumour (neo-adjuvant therapy) or after surgery to destroy any remaining cancer cells and prevent recurrence (adjuvant therapy). Metastasis limits the effectiveness of surgery and radiotherapy. If the cancer has become widespread then some form of chemotherapy is the only option.

Most chemotherapies have relied on cytotoxins killing cancer cells before the therapy kills the patient. This results in extremely severe side effects. The most significant advance would be to find toxins that *specifically* target cancers. Widely used drugs such as cis-platin achieve this to some extent through targeting dividing cells. This is responsible for such characteristic side effects as hair loss and infertility.

Molecular profiling of tumours opens the possibility of more specific targeting of chemotherapy drugs. If a drug can be found that targets a pathological pathway which is of critical importance to the cancer but of limited significance in normal cells this may result in drugs with less severe side effects. This, in turn may allow the patient to tolerate much higher doses, resulting in more effective therapies.

Even as a first step, development of such drugs will require accurate molecular profiling. By definition, a highly specific drug will show little efficacy except in a population which exhibits

the targeted mutation. Clinical trials on unstratified patient populations will be unable to show significant improvement.

As more such drugs are developed, stratification by molecular profiling will become more important in assigning treatments.

Even more beneficial would be treatments to block the process of metastasis. Here understanding of CTCs will likely be critical. Molecular profiling of CTCs may provide targets for new or existing drugs. Understanding the way in which the CTC burden changes through the course of the disease will provide an opportunity to maximise the effectiveness of therapy.

1.4.3 The cancer stem cell hypothesis

An important question that requires analysis of CTCs heterogeneity is clarification of the cancer stem cell hypothesis.

This states that only a small subset of cancer cells are responsible for growth, renewal and metastasis of the cancer. Its exact meaning is far from understood. Rosen and Jordan grapple with 'the increasing complexity of the cancer stem cell paradigm', [171] while Maenhaut and colleagues attempt to clarify whether is 'a reality, a myth, a fuzzy concept or a misnomer?' [172] The term 'tumour initiating cell' is sometimes preferred as this does not prejudge the relationship of supposed 'cancer stem cells' with normal stem cells.

The hypothesis was first put forward by John Dike in 1994, in the context of experiments injecting leukaemia cells into mice. [173] Here interpretation was made easier by the good understanding of the developmental hierarchy in haematopoietic cells. It has since been extended to solid tumours, on the basis of similar experiments implanting breast [174], brain [175], colon, [176] pancreatic, [177] liver, [178] melanoma, [179] lung [180] and ovarian [181] cancer cells into mice.

The hypothesis remains controversial. The CD133 marker widely used for isolation of putative cancer stem cells is widely expressed in normal and cancerous tissue, not just in stem cells, and CD133⁺ cells have been found to be capable of tumour initiation. [182, 183] The mice used are nonobese diabetic-severe combined immunodeficiency (NOD-SCID), but the proportion of cells acting as 'stem cells' is dependent on the exact animal model used, as well as the protocol for handling and isolation of the cancer cells. [184, 185] In addition, for solid

tumours it is increasingly clear that the microenvironment rather than just the 'stemness' of a cell plays a significant role. [186, 187] This suggests that an analysis of isolated cancer cells will not in itself be sufficient to gain full understanding. On the other hand, it also suggests that 'cancer stemness' is in part a proteomic state, dependent on gene expression (as in 'normal stemness') not just a product of cancer mutations.

It has been suggested that it is unreasonable to expect that different tumours should behave in similar ways, and that the proportion of cancer stem cells may vary dramatically from patient to patient and over the course of time, including in response to treatment. This again highlights the need for technologies capable of gathering more data.

Technologies capable of analysing cellular heterogeneity can make a major contribution to resolving the identity and role of tumour initiating cells. In particular, techniques that can analyse human patient samples will be an essential complement to animal data. From a clinical point of view a technique that was able to provide a personalised understanding of these factors in each patient would be of great benefit.

1.4.4 Breast cancer

A major challenge for the development of cancer chemotherapy is the diversity of the disease at the molecular level. The efficacy of a given drug depends on the molecular rather than the anatomical characteristics of a tumour. There is therefore a desire to increasingly shift in pathology to the molecular level.

The goal is the stratification of patients by using molecular information to enable prescription of the most effective drug and enable the development of new molecularly targeted drugs. At present, as important is avoiding giving drugs that are ineffective with their associated iatrogenic morbidity (side effects) and cost.

An example of the way that molecular profiling has affected clinical practice comes from breast cancer. The diversity of breast cancer can be seen even in the histological examination of tissue sections. [188] Classifications devised on the basis of traditional pathology are now being supplemented by molecular characterisations. These overlap with, but do not perfectly co-incide with older classifications.

A broad division, originally based on microarray analysis of expression of 427 genes, [189, 190]

separates breast cancers into five categories. These have been found to be robust to further anatomical and molecular studies, and to be of relevance for clinical outcome. [191, 192]

As with cancer in general, breast cancer may be treated with both drugs that broadly target proliferating cells and drugs that more specifically target the cancer.

Examples of drugs that are commonly used to target proliferating cells in breast cancer include cyclophosphamide (a DNA cross linker), methotrexate (an inhibitor of dihydrofolate reductase) and 5-fluorouracil (an inhibitor of thymidylate synthase), anthracyclines (a DNA intercalater) and taxane (a disruptor of microtubules). The severe side effects of targeting DNA with drugs such as cyclophosphamide can include secondary cancers.

An example of more specific targeting comes from estrogen receptor (ER) positive breast cancers which can be treated with drugs that specifically target the ER pathway. Aromatase inhibitors (such as anastrozole) which block the production of estrogen and antagonists of the estrogen receptor in breast (such as tamoxifen) can both be used.

Breast cancer is now profiled for expression of the estrogen receptor (ER), progesterone receptor (PR) and the Her2 receptor. Overexpression of Her2 can be treated either with herceptin (as described in more detail in chapter 3) or with small molecule inhibitors of the kinase domain.

Epidermal Growth Factor Receptor (EGFR, also known as Her1) shares around 50% homology with HER2, is a related tyrosine kinase receptor. The extracellular domain is targeted by cetuximab (brand name Erbitux). An inhibitor of the kinase domain gefitinib (brand name Iressa) has also been developed.

The family of human epidermal growth factor receptors contains four members: epidermal growth factor receptor (EGFR), receptor tyrosine-protein kinases erbB-2, erbB-2 and erbB-4 (ERBB2, ERBB3 and ERBB4). They are known by a number of synonyms, most commonly HER1, HER2, HER3 and HER4. In particular, ERBB2 is often known as HER2.

While ERBB2 belongs to the family of epidermal growth factor receptors, its actual ligand is unknown. However, it is known to be a receptor tyrosine kinases (RTKs), and like other RTKs ligand binding causes them to dimerise, autophosphorylate and then phosphorylate their target. Deactivation takes place by internalisation of the membrane containing the dimerised receptor as vesicle, and ultimately by targeting to the lysosome for degradation.

In breast cancer the most important member of the family is ERBB2, which heterodimerises on activation and triggers a growth signal. It activates two well known growth pathways: MAP kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K). The action on the PI3K pathway is via phosphorylation of src. This in turn phosphorylates phosphatase and tensin analogue (PTEN), which deactivates PI3K signalling.

The clinical significance of ERBB2 was first realised in 1987. ERBB2 tumours are more aggressive, and result in poorer outcomes. However, a number of drugs targeting ERBB2 have been developed.

Trastuzumab (brand name Herceptin) is a breast cancer drug developed by Genentech and approved by the FDA in 1998. It is monoclonal antibody that achieves its therapeutic effect by binding the extra-cellular domain of ERBB2 – a target for which it was specifically designed [193]. The antigen binding region was developed from a murine antibody. This was then fused to a human variable region framework and IgG1 constant region. This process of 'humanisation' reduces immunogenicity of the drug. [194]

ERBB2 is amplified and overexpressed in 25–30% of breast cancers, in which cases it increases aggressiveness. It is also found to be overexpressed and often amplified in many other cancers. [195] However, up to now trastuzumab has only been found to be effective in the case of breast cancer.

Resistance to drugs can also be understood at the molecular level. Deletion of PTEN is an important mechanism for evolution of resistance to trastuzumab. Obviously, if PTEN is not present then it will not be reactivated when ERBB2 is inhibited. Another mechanism for resistance to trastuzumab is expression of MUC4. This is a cell surface protein that inhibits the internalisation of ERBB2.

1.5 Summary of the thesis

Chapter 2 shows the development of a technique to carry out proteomic measurements at a single cell level. The technique is based on a miniaturised antibody pull down. The work includes calculations for design of microfluidic devices, construction of microfluidic chambers, manipulations of single cells and measurements of p53 protein levels in single cells.

An ideal application of the single cell proteomic measurements shown in chapter 2 is to

circulating tumour cells. The remainder of the thesis deals with the development of technology to isolate and make measurements on circulating tumour cells.

Chapter 3 shows the separation of circulating tumour cells using the CellSearch system and their isolation in a microfluidic device. This involves adaptation of the CellSearch protocol, development of antibody staining for small cell samples, and quantitative microscopy of stained cells in a microfluidic device.

While chapter 3 shows that it is possible to observe circulating tumour cells in a microfluidic, it is harder to count them systematically or to manipulate them. Chapters 4 and 5 address these problems.

Counting of circulating tumour cells produces valuable clinical data. Counting in a microfluidic format carries the possibility of quicker, cheaper and more accurate results. Chapter 4 shows the development of a microfluidic cytometer suitable for counting a small number of stained cells in a sample of several thousand cells, i.e. samples similar to those produced and stained in chapter 3.

Chapter 5 shows initial development of a microfluidic sorter capable of isolating circulating tumour cells in a configuration suitable for single cell proteomic measurements. Sorting with beads is shown and an initial analysis of instabilities that limit its application to biological samples.

Chapter 2

Single Cell Proteomics

2.1 Introduction and strategy

The principle used to make single cell proteomic measurements in this work is to miniaturise an antibody sandwich assay. A microfluidic format is used to isolate a single cell in a chamber. A primary antibody against the protein of interest to be measured is spotted on to the floor of the chamber. A laser pulse is used to lyse the cell, releasing proteins in to the chamber where they bind to the primary antibody. A fluorescently labelled secondary antibody is introduced. This binds to a second epitope on the protein of interest forming a sandwich bound to the spot on the floor of the chamber, as shown in figure 2.1. Finally, fluorescence from bound secondary antibody is detected using total internal reflection (TIRF) microscopy.

The use of TIRF to excite the fluorescent labels means that only those labels bound to the surface will significantly contribute to the signal. This allows the assay to be run without the need to wash away excess secondary antibodies.

While cytosolic proteins are easily detected at a single cell level, the report here of p53 detection is the first example of measuring cytosolic protein at a single cell level without fixation of the cell. Compared to fixation of cells it offers a better opportunity for quantification. It also allows greater control of selection of cells. The technology for fixing single cells is generally optimised for use with large samples of cells, with the possibility for single cell data collection *post hoc*.

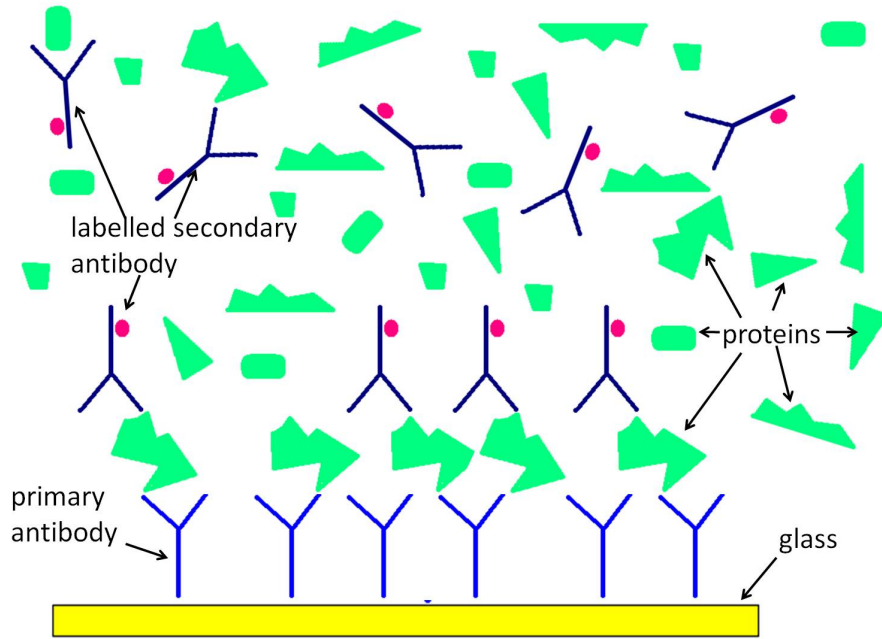


Figure 2.1: Schematic of sandwich assay. The primary antibody specifically binds one protein from a mixture. Only those labelled secondaries bound to the glass are detected by TIRF.

2.2 Theory of protein diffusion and binding

The success of any miniaturisation strategy depends on an understanding of scaling. In scaling down an antibody sandwich assay to microfluidic dimensions device turbulent mixing is eliminated, and mass transport is dominated by diffusion. The dimensions of the chamber in which the cell is lysed must be sufficiently small that the protein from a single cell can diffuse to an antibody spot in a reasonable amount of time (or else some form of active mixing must be introduced). This understanding is important for design of a device and also for interpretation of results, to understand time scales for equilibration.

2.2.1 The diffusion equation and boundary conditions

A protein of concentration ϕ will move according to Fick's law

$$\mathbf{j} = -D \nabla \phi \quad (2.1)$$

where $\mathbf{j}$ is the current density and D is the diffusion constant, which is specific to the protein and solute. Taking the divergence of both sides and using the conservation of matter $\text{div } \mathbf{j} = -\frac{\partial \phi}{\partial t}$

gives the diffusion equation

$$\frac{\partial \phi}{\partial t} = D \nabla^2 \phi. \quad (2.2)$$

Solution of this equation requires specification of boundary conditions. At a wall the current in the direction of the normal to the wall must be zero - protein cannot flow into or out of the wall. From equation 2.1 this implies that the derivative of ϕ along the normal to the wall is zero:

$$\frac{\partial \phi}{\partial n} = 0. \quad (2.3)$$

This known as a Neumann boundary condition. At an antibody patch, presuming that the protein binds at a rate proportional to protein concentration, the boundary condition will set the current into the wall proportional to the concentration. This is written in general terms as

$$\alpha \phi + \beta \frac{\partial \phi}{\partial n} = 0, \quad (2.4)$$

known as Robin boundary conditions. (This encompasses Neumann conditions as the special case $\alpha = 0$ and Dirichelet conditions which set $\phi = 0$ at the wall as the special case $\beta = 0$.) Importantly, with these boundary conditions $-\nabla^2$ will be a self adjoint operator. [196]

To evaluate $\frac{\alpha}{\beta}$ at an antibody patch, consider the total flow of molecules in to a patch of area A in time t . According to equation 2.1 this will be $AtD \frac{\partial \phi}{\partial n}$. This must equal the number of molecules that can diffuse into the patch. In time t molecules diffuse a mean distance $\sqrt{tD}$. Over an area A , neglecting edge effects, molecules in a volume $A\sqrt{tD}$ can diffuse to the patch giving a total number of molecules $A\sqrt{tD}\phi$. Therefore

$$AtD \frac{\partial \phi}{\partial n} = \gamma \delta A \sqrt{tD} \phi \quad (2.5)$$

where γ is a geometrical factor. For a plane patch where approximately half the molecules diffuse toward the patch and half diffuse away from it, γ will be approximately 0.5. δ is the probability of a protein binding to an antibody on contact.

Chosing units (this work will use square microns and seconds) and setting $A = t = 1$ the

boundary condition at the patch is therefore given by equation 2.4 with

$$\alpha = -\gamma\delta\sqrt{D}, \quad \beta = D, \quad \frac{\alpha}{\beta} = -\frac{\gamma\delta}{\sqrt{D}}. \quad (2.6)$$

2.2.2 Solution of the diffusion equation

Equation 2.2 can be solved by separation of variables in which ϕ is written as a product of a function S only of spatial variables and function T only of time. (The general solution to equation 2.2 cannot be written in this way, but it can be written as a linear combination of such functions.) From substituting

$$\phi(\mathbf{r}, t) = S(\mathbf{r})T(t) \quad (2.7)$$

in to equation 2.2 the standard separation follows,

$$\frac{1}{T} \frac{dT}{dt} = \frac{D}{S} \nabla^2 S = -\lambda \quad (2.8)$$

where λ is a constant that must be independent of space and time.

The equation for T is easily solved,

$$T(t) = T_0 e^{-\lambda t}. \quad (2.9)$$

The three dimensional eigenvalue equation for S is trickier:

$$-\nabla^2 S = \frac{\lambda}{D} S. \quad (2.10)$$

Because the lysis chamber is finite this equation will have a sequence of solutions with discrete eigenvalues μ_i which will give discrete values for $\lambda_i = \mu_i D$. The boundary conditions guarantee that $-\nabla^2$ is self adjoint so that the eigenvalues will be real and the corresponding eigenfunctions S_i form a complete orthogonal set.

The general solution for ϕ will then be given by

$$\phi(\mathbf{r}, t) = \sum_{i=1}^{\infty} \nu_i S_i(\mathbf{r}) e^{-\lambda_i t} \quad (2.11)$$

where S_i is the eigenfunction corresponding to eigenvalue $\frac{\lambda_i}{D}$ and ν_i is a constant weight that can be calculated from the protein concentration at time 0,

$$\nu_i = \int \phi(\mathbf{r}, 0) S_i(\mathbf{r}) d^3\mathbf{r}. \quad (2.12)$$

2.2.3 Numerical evaluation

For simplicity, speed of computation and economy of computer memory I chose a geometry that maximises symmetry, namely a cylindrical chamber with a circular antibody patch at the centre of the floor. This geometry is shown in figure 2.2. With a cylindrically symmetric initial concentration distribution there is then no angular variation, and the problem becomes two dimensional, dependent only on radius r and height z . For this chamber geometry angular variation of the initial concentration makes no difference to the total bound protein quantity vs. time in any case.

The Laplacian is written in cylindrical co-ordinates as

$$\nabla^2 = \frac{\partial^2}{\partial r^2} + \frac{1}{r} \frac{\partial}{\partial r} + \frac{1}{r^2} \frac{\partial^2}{\partial \theta^2} + \frac{\partial^2}{\partial z^2}. \quad (2.13)$$

Setting angular variation to zero by neglecting the third term leaves

$$\nabla^2 = \frac{\partial^2}{\partial r^2} + \frac{1}{r} \frac{\partial}{\partial r} + \frac{\partial^2}{\partial z^2} \quad (2.14)$$

where the influence of the cylindrical geometry is felt through the second term which distinguishes the operator from the standard two dimensional Laplacian. First and second differences are used to approximate the first and second derivatives on a grid with points indexed as shown in figure 2.3:

$$\begin{aligned} \frac{\partial^2 \phi_i}{\partial r^2} &\approx \frac{\phi_{i+1} - 2\phi_i + \phi_{i-1}}{\Delta r^2} \\ \frac{\partial^2 \phi_i}{\partial z^2} &\approx \frac{\phi_{i+m} - 2\phi_i + \phi_{i-m}}{\Delta z^2} \\ \frac{\partial \phi_i}{\partial r} &\approx \frac{\phi_{i+1} - \phi_{i-1}}{2\Delta r}. \end{aligned} \quad (2.15)$$

With appropriate accounting for boundaries these expressions for derivatives can be used with equation 2.14 for ∇^2 to approximate equation 2.10 as a matrix eigenvalue equation.

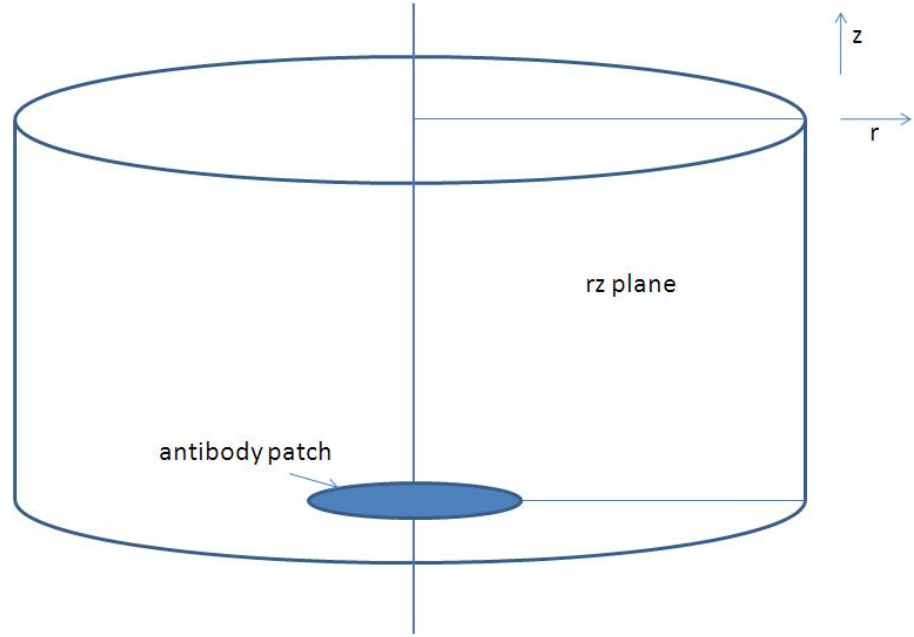


Figure 2.2: Geometry of a cylindrical chamber for numerical solution of the diffusion equation. A solution is obtained on the rz plane shown. By assuming no angular variation in the concentration this is extended to the whole cylinder.

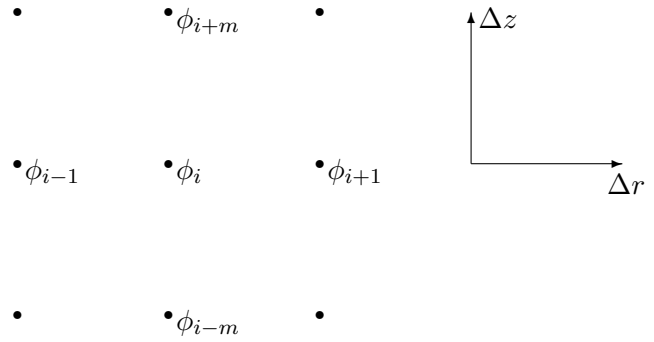


Figure 2.3: Grid showing indexing of points used in numerical solution of the diffusion equation. The r co-ordinate is divided in to m steps and the z co-ordinate is divided in to n steps. The center of the antibody patch is point 0 while the top right hand corner point has index nm .

At a wall a term needed in equations 2.15 to approximate a derivative normal to the wall will not be available, as it will correspond to a point beyond the wall. If ϕ_0 is the value at the wall, ϕ_{-1} is the unavailable value beyond the wall and ϕ'_0 is the normal derivative of ϕ_0 at the wall then rearrange

$$\phi'_0 \approx \frac{(\phi_1 - \phi_{-1})}{2} \quad (2.16)$$

to give

$$\phi_{-1} = \phi_1 - 2\phi'_0. \quad (2.17)$$

Applying boundary conditions allow this expression to be evaluated. Away from the antibody patch the normal derivative vanishes giving

$$\phi'_0 = 0 \Rightarrow \phi_{-1} = \phi_1 \Rightarrow \phi''_0 = 2\phi_1 - 2\phi_0. \quad (2.18)$$

This expression for ϕ''_0 at a wall is written entirely in terms of points inside the chamber and enforces the boundary condition. At the antibody patch the boundary conditions give

$$\alpha\phi_0 + \beta\phi'_0 = 0 \Rightarrow \phi'_0 = -\frac{\alpha}{\beta}\phi_0 \Rightarrow \phi_{-1} = \phi_1 + 2\frac{\alpha}{\beta}\phi_0 \Rightarrow \phi''_0 = 2\phi_1 - (2 - \frac{\alpha}{\beta})\phi_0. \quad (2.19)$$

Equation 2.6 then results in

$$\phi''_0 = 2\phi_1 - \left(2 + \frac{\gamma\delta}{\sqrt{D}}\right)\phi_0. \quad (2.20)$$

At the axis $r = 0$ symmetry and smoothness (continuous second derivatives) imply the same Neumann boundary condition $\frac{\partial\phi}{\partial r} = 0$ as at a wall.

The resulting matrix eigenvalue equation is solved using the Matlab / Octave built in function `eigs`, which uses the ARPACK implementation of the implicitly restarted Arnoldi algorithm. (Octave is an open source version of Matlab. When compared for this problem, running on different machines the two give numerically identical answers.)

Scripts used to build the matrix diffusion operator, to normalise and to calculate eigenfunction overlaps with appropriate weighting to account for the cylindrical geometry are given in appendix A.

2.3 Simulation results

The equation was solved for a chamber with height $50 \mu\text{m}$ and radius $170 \mu\text{m}$ with an $n = 50 \times m = 170$ grid and a uniform initial protein concentration. These dimensions were chosen to match preliminary experiments carried out in the group using circular chambers of this size. These experiments used green fluorescent protein to detect bound protein without the need for

a labeled secondary antibody. For this reason the diffusion co-efficient for GFP was used. [197]

Some sample results are shown in the following table:

	$D/\mu m^2 s^{-1}$	δ	spot radius / μm	time constant/s	percent of protein
mode 1	87	1	65	287.2	97.5
mode 2	87	1	65	16.1	2.1
mode 3	87	1	65	6.2	0.2
mode 1	87	0.5	65	430.9	98.9
mode 2	87	0.5	65	18.1	1.0
mode 3	87	0.5	65	6.4	0.1
mode 1	87	0.1	65	1583.6	99.9
mode 2	87	0.1	65	21.2	0.08
mode 3	87	0.1	65	6.6	0.03
mode 1	87	1	40	668.9	99.3
mode 2	87	1	40	18.7	0.5
mode 3	87	1	40	6.2	0.1
mode 1	87	1	100	120.2	94.0
mode 2	87	1	100	14.6	5.2
mode 3	87	1	100	5.9	0.0004

Higher modes (described by eigenfunctions S_i of successively higher index i) account for less and less protein. As can be seen, only the first three modes are of any significance. We are justified in assuming that more than 95 per cent of the protein is pulled down with a single exponential time constant, where the time constants shown cover the range we appear to see experimentally. The results may justify considering an initial spike of fast binding protein consisting of that protein not in mode 1.

Examining the contour plots of concentration for the eigenmodes shown in figure 2.4 an interpretation is readily arrived at. (For the range of parameters shown in the table, modes are qualitatively similar.) These plots show the concentration of protein established in the chamber.

Mode 1, which is established as the long term concentration pattern in the chamber has a lower concentration near the patch and a higher concentration at the far walls. Binding to the patch maintains the concentration gradient which drives a diffusion current toward the patch.

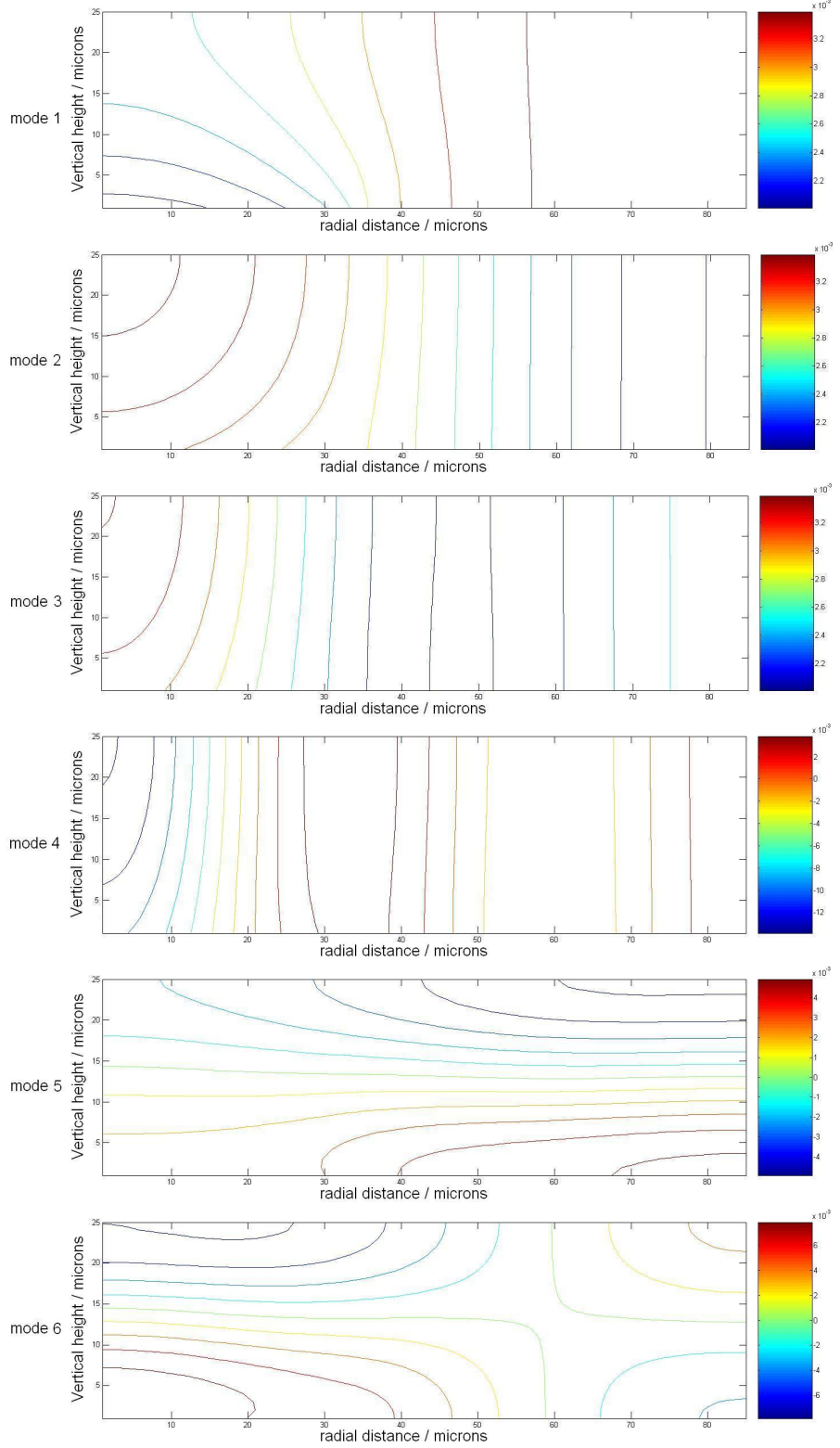


Figure 2.4: Contour plots of the first six eignmodes, from mode 1 at the top through to mode 6 at the bottom. In each case an $r-z$ plane is shown with the cylindrical axis of the chamber corresponding to the left hand edge and the antibody patch in the lower left corner (c.f. figure 2.2).

Modes 2 and 3 show maxima near the patch, decay away more quickly than mode 1. This corresponds to protein near the patch rapidly binding. The result is a depletion near the patch as described under mode 1. This may result in a spike of fast binding protein at the start of an experiment.

Higher modes consist either of rapidly varying inhomogeneities which rapidly smooth out (modes 4 and 5), more fast binding protein of the sort seen in modes 2 and 3, or both (mode 6).

The actual concentration is a weighted combination of these modes. As noted above, in practice, only the first three modes are significant.

2.4 Total Internal Reflection (TIRF) microscopy

Total Internal Reflection Microscopy is a method to selectively excite only fluorophores close to a surface. In this experimental set up it is used to excite fluorophores bound to the spot without a background signal from unbound labelled secondary antibodies in solution.

In the TIRF system used here an oil immersion objective mounted on a TE2000 inverted microscope (Nikon) is used both to deliver the excitation beam and to collect the emitted light. These are then separated using a filter cube designed for FITC imaging (Nikon).

The excitation beam is focused to a point at the back focal plane of the objective, off the optical axis. This is therefore focused by the objective to fill the front focal plane with the rays making a constant angle. The rays pass between the oil and the cover slip without encountering a change of refractive index. At the boundary between the cover slip and aqueous sample the refractive index changes. If the angle at which the rays are focused is greater than the critical angle they experience total internal reflection back into the glass cover slip.

However, an evanescent wave extends past the interface by around a third of a wavelength. (More precisely the amplitude decays exponentially with a characteristic length $\frac{2\pi}{\lambda} \sqrt{(n_1 \sin \theta_i)^2 - n_2^2}$ where λ is the wavelength, n_1 and n_2 the indices of refraction of the two media, and θ_i is the angle of incidence.) For an excitation wavelength of 488 nm used here, that amounts to around 160 nm. The power is proportional to the square of the amplitude and so decays exponentially over around half that distance, 80 nm.

The antibody-protein-antibody sandwich will be less than about 30 nm in height. Allowing

for some surface roughness and tilt in the cover slip up to about 0.5 degrees it will be within the excitation region across the field of view. The total chamber height is 50 μm , so more than 99.5% of the background will be excluded from excitation.

Focus is retained on the spot by use of a perfect focus microscope stage. This optically monitors and adjusts the z -position of the microscope stage using a dedicated LED to maintain a constant distance between the coverslip and the objective. This allows the collection of TIRF images over long periods of time, potentially up to several days. It also allows the stage to retain focus while moving from one spot to another and back again.

Flourescence is collected by an electron multiplied CCD camera (IXON DU-897E, Andor Technologies, Ireland) with 512x512 pixels and 16 bit dynamic range. It is possible to extract either a total signal intensity or to count single molecules.

NIS Elements software (National Instruments, USA) is used to control the TIRF beam, the mechanised microscope stage and a CCD camera.

2.5 Validation of antibodies

Key to a successful antibody sandwich assay is the quality of the antibodies. Both the affinity and, even more so, the specificity are important for an accurate assay. Lower affinities are tolerable to some extent because both antibodies are present in excess, but higher affinities will give improved results. The sandwich assay format has the benefit of requiring two binding events before detection, increasing overall specificity to the product of the specificities of the two antibodies. However, it also requires that the two antibodies bind to different epitopes, so as not to interfere with each other.

An assay was carried out in which the distance that antibodies ran on a polyacrylamide gel was compared in the presence and absence of recombinant p53. A pair of antibodies were tested, labelled Ab1* and Ab2 on the Western blot shown in figure 2.5. Only Ab1* was flourescently labelled so that only bands of Ab1* or a complex containing Ab1* are visible.

Lane two shows a single band containing the free antibody Ab1*. (The additional signal from the stack, present in all lanes, is material that has not moved at all.)

Lane one shows that in the presence of p53, the Ab1* antibody formed a complex. This shows up as an additional band at higher mass than the free Ab1*. The free Ab1* is still present

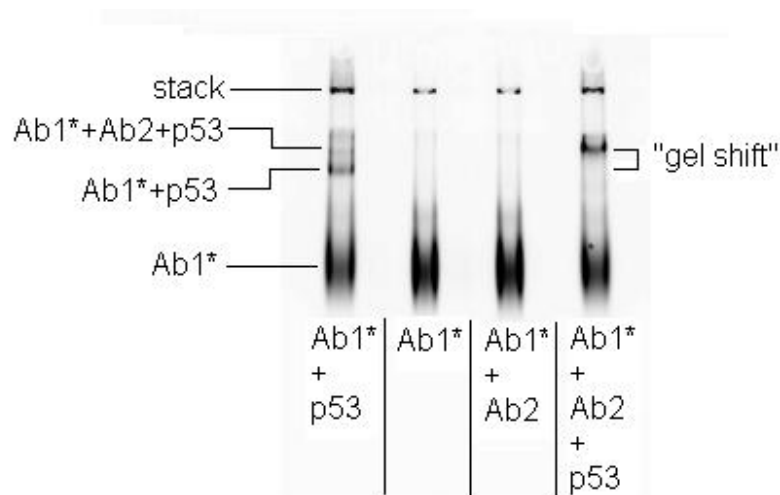


Figure 2.5: Fluorescent image of western blot showing p53 protein and two anti-p53 antibodies. Ab1* is a monoclonal mouse anti-p53 antibody from SantaCruz labelled with a dye for excitation at 488 nm. Ab2 is an unlabelled mouse monoclonal anti-p53 antibody from AssayDesigns.

as the antibody is present in excess.

Lane four shows that the presence of Ab1*+Ab2+p53 results in a heavier complex than Ab1*+p53. This shows up as a the 'gel shift' in the figure. It shows that Ab1* and Ab2 simultaneously bind p53.

Lane three shows there is no direct interaction between Ab1* and Ab2 in the absence of p53. (Any such interaction, were it present, could produce a false gel shift.)

After showing that the antibodies bind to pure protein there was an attempt to validate the anti-p53 antibodies using whole cell lysate from the BE cell line. This would test the specificity of the antibodies.

The result is shown in figure 2.6, where no p53 band is seen (lane one is repeated from figure 2.5 for comparison). In view of other positive results this was interpreted as a result of protein degradation in the BE cell lysate sample. While results from degraded samples should be interpreted with caution, it is nonetheless encouraging that no other bands were observed in this blot which is consistent with low non-specific binding by the antibodies.

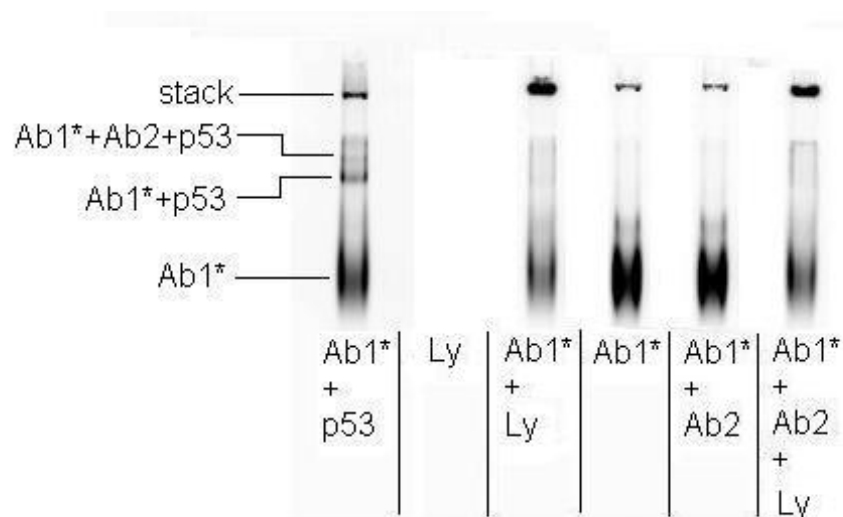


Figure 2.6: Fluorescent image of western blot of lysate from the BE cell line and anti-p53 antibodies. No p53 band is observed. Antibodies are as in figure 2.5. Ly is the BE cell lysate.

2.5.1 Development of antibody spotting

Antibody spots are produced by an Omnigrid microarrayer (Genomic Solutions, Holliston, MA, USA). This is a robotic system for producing microarrays of nucleic acid or protein. It functions by dipping a split pin into a microwell containing a solution of the molecule to be arrayed, in this case antibody. The slit in the pin then fills by capillary action. The pin is then touched pin down at an array of locations on a standard glass cover slip forming antibody microspots.

Preliminary experiments in the group had shown that the spotting conditions, particularly the surface chemistry and preparation of the cover slip are critical to getting good antibody spots (personal communication). These experiments had mainly been carried out using just a primary antibody to detect binding of Green Fluorescent Protein.

For development of a sandwich assay against p53 I therefore carried out experiments spotting down anti-p53 antibodies on a variety of surfaces. p53 from cell lysate and from recombinant sources was added to the spots, followed by addition of labelled secondary anti-p53.

Microfluidic confinement is necessary only where the quantity of of protein to be bound is small, as with single cell experiments. For the larger quantities used in these preliminary experiments it is not necessary. Experiments could therefore be performed in an open droplet rather than a microfluidic format. It was possible simply to use a wax pen to draw on the cover slip around the spots. The wax then confined the fluid droplet to the spots. Samples of up to

200 μ l could then be pipetted on to and removed from the spots

This allowed for easier manipulation and more rapid turnover of experiments.

Experiments were also carried out spotting down p53 protein and cell lysate to confirm that surface bound protein could directly pull down labelled secondary anti-p53 antibody and that this could be detected with TIRF microscopy.

Experiments were carried out on three cover slip surfaces: plain glass, a coating of polyethylene glycol (PEG), and a coating of 3-glycidoxypyl-trimethoxysilane (GPTS).

The first result was that to achieve good spot morphology it proved necessary to dip the pin in to the antibody reservoir, then *preprint* a series of spots on a first cover slip before printing the functional spots on the cover slips for experimental use. Spot morphologies are shown figure 2.7. In this case the spots were printed on a PEG coated glass cover slip, but the results for other surfaces were similar.

The preprint is show in the top panel of figure 2.7. These consists of the first spots after the pin has been dipped in to reservoir. It can be seen that the spots are large and merge into each other as excess material is removed from the pin.

After a preprint of 50 spots it was possible to print functional antibody spots, as shown in the middle panel of figure 2.7. After printing several hundred functional spots the spot morphology degrades, with too little material printed. Examples of this degraded morphology is shown in the lower panel of figure 2.7.

For experiments printing runs consisted of a 50 spot preprint followed by seven spots on each of six cover slips (for a total of 42 spots) before refilling the pin. Thus the pin was refilled well before spot morphology degraded.

The best TIRF signals for binding of p53 from cell lysate were obtained on plain glass surfaces, largely because of high non-specific binding away from the spot on PEG and GPTS surfaces. Figure 2.8 shows the morphology of spots printed on to plain glass prior to the introduction of fluid.

2.6 Construction and operation of the microfluidic device

The layout of the microfluidic device is shown in figure 2.9. The antibody spots were aligned under the lysis chambers by use of a custom jig designed by Beatrice Gauthé. This allowed

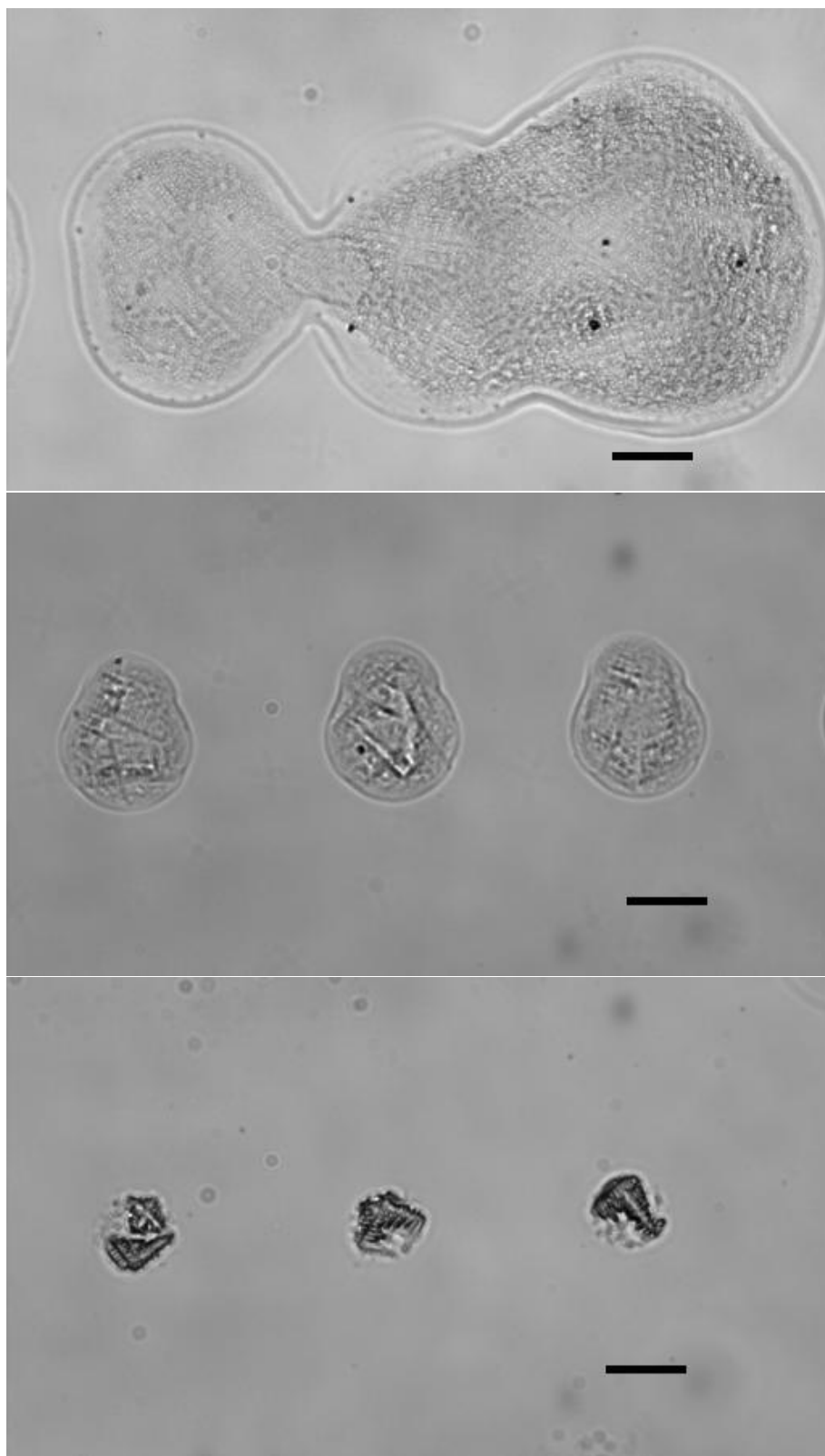


Figure 2.7: Illustration of how the morphology of spots changes with the number of spots printed. In this instance spots are printed on a surface functionalised with PEG. In each panel the order of spot printing is right to left. The top panel shows the initial spots from a series. The middle panel the mid-range and the lower panel the last spots. Scale bars $100\ \mu\text{m}$.

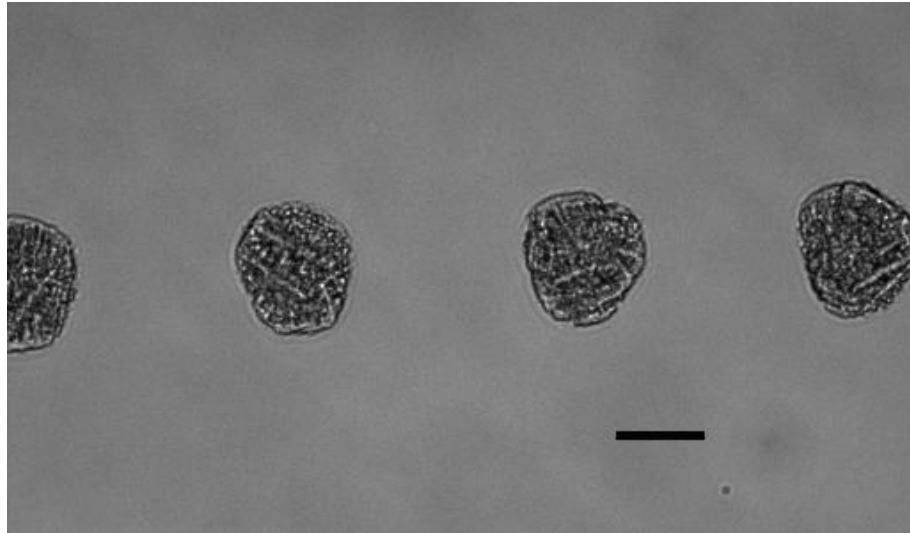


Figure 2.8: Anti-p53 antibodies spotted on to a plain glass surface showing consistent morphology. Scale bar 100 μm .

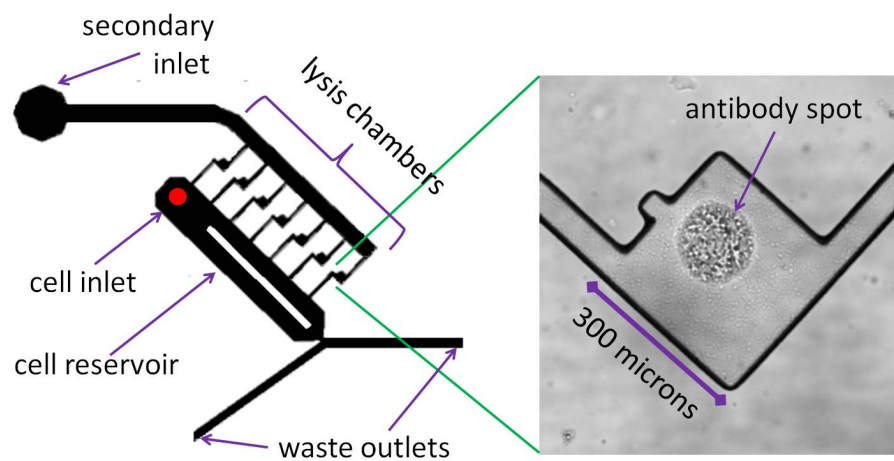


Figure 2.9: Layout of the microfluidic device for cell lysis. The red spot indicates the inlet for the cells. The blow up on the right shows a bright field image of the lysis chamber containing the antibody spot.

spots to be viewed on a microscope as the PDMS layer of the microfluidic device was sealed to the cover slip.

Cells are introduced through the cell inlet. They are arrayed in to the cell reservoir.

Cells were then be selected and transported to a lysis chamber using an optical trap. [198] Cells were then lysed using a pulsed laser beam and the binding recorded using TIRF microscopy. Each of these steps is described in a subsection below.

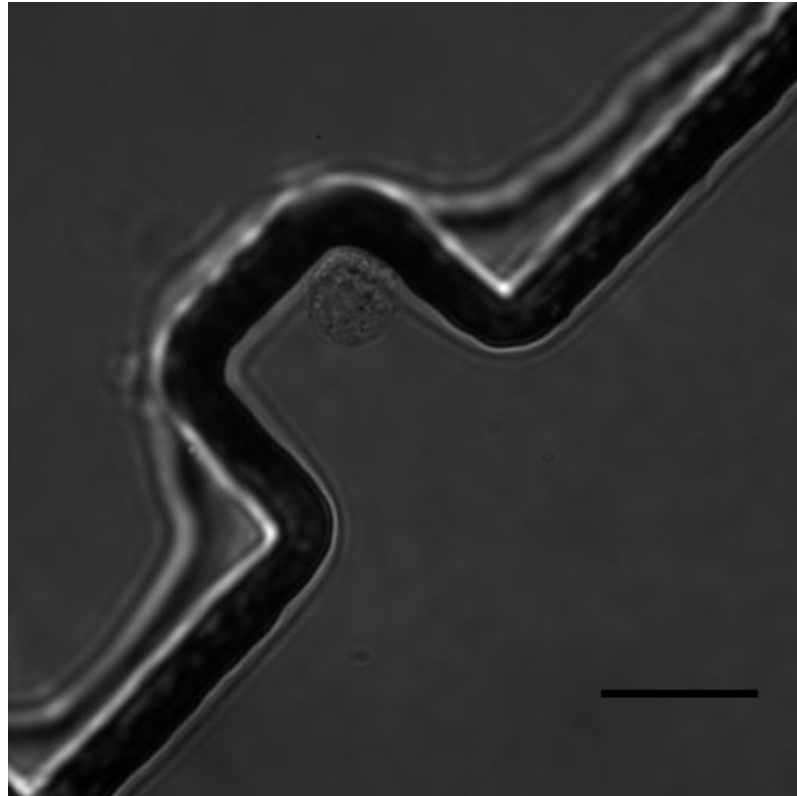


Figure 2.10: Showing the cell loaded into the lysis niche. Scale bar $50\mu\text{m}$.

2.6.1 The cell inlet

The fluid inlets and outlets were constructed using capillaries as described in appendix B. The cell inlet was constructed using PVC sleeving through which a capillary could pass into the device. This allowed cells loaded into the tip of the capillary to be directed deposited into the microfluidic chamber. Vacuum grease was used to seal the top of the sleeving. The arraying of cells is described in more detail in chapter 3.

2.6.2 Optical trapping of cells

Cells arrayed in the chamber were transported to the lysis chamber using an optical trap. For this purpose a 1074 nm continuous wave laser was focused at the back aperture of the microscope objective. The laser was focused at a constant point in the microscope field of view. Trapped cells could be moved through the microfluidic by actuation of the motorised microscope stage.

They were moved and lodged into the niche in the lysis chamber, as shown in figure 2.10.

Consistent trapping of cells proved to be a bottleneck in throughput of cells to be lysed,

although once a cell was trapped it was usually relatively easy to transport all the way into the lysis chamber. It is possible that the difficulty with trapping arises for cells that have settled too close to the surface, either because of an optical effect or because they have in some way bound or stuck to the surface.

Increasing the power of the trap above 1 mW clearly increased the force with which cells were held in the trap, but was of marginal help in trapping new cells. More drastically, 'nudging' the microfluidic sometimes dislodged stuck cells but the effects are unpredictable and uncontrollable.

The optical trapping system is good enough to capture cells for lysis if there is a large supply of cells any number of which can be discarded before lysis, as with the data presented in this chapter. However, difficulties with optical trapping were the main reason why the CTCs isolated in a microfluidic, as described in the next chapter, were not lysed.

It is possible that a coating may make cells less likely to stick to the cover slip, although this is not necessarily straightforward to implement without damaging the antibody spots. On the other hand, it is possible that simply greater experience with the optical trapping system will lead to greater reproducibility and improved results.

2.6.3 Laser lysis of single cells

Cell lysis was achieved by use of laser induced cavitation. A 6 ns laser pulse at a wavelength of 1064 nm produced by a Q-switched Nd:YAG laser (Surelite SL I-10, Continuum, USA) is focused roughly 10 μm above the cell where it creates a small bubble of highly ionised hot plasma. The actual lysis is essentially mechanical, caused by the shock wave produced by the collapsing bubble. The plasma reaches its maximum extent after about 20 ns and the collapsing pressure wave becomes subsonic after around 100 ns. [199]

This has the advantage that it is quick – essentially instantaneous time scale that are relevant for changing protein concentrations. Because the pulse and plasma is outside the cell it should not result in destruction of protein by ionisation. The pulse power is small enough that the temperature rise is insignificant.

Ideally mechanical lysis can be tuned to be strong enough to disrupt cellular structure and organelles while leaving covalently bonded proteins or bound protein complexes intact. In practice it is not yet clear that this 'tuning' has been achieved. The method can certainly be

used to disrupt cells, as judged by bright field imaging. As discussed below, this allows the detection of cytosolic proteins that are not detected in the presence of unlysed control cells.

The laser is aligned on the same optical path as the optical trap. Pulse energy is controlled by a quarter wave plate followed by a polarising beam split. Rotating the wave plate adjusts the polarisation and therefore the fraction of the pulse transmitted by the beam splitter to the objective. The pulse energy was set to $14.1 \pm 0.3 \mu\text{J}$.

At present it often takes several laser bubbles to fully disrupt a cell. While this might be turned to advantage. It is certainly possible to partially disrupt a cell by nicking the membrane, which may have various applications. However, it is not possible to consistently lyse a cell with a single bubble. It is not clear the extent to which the cytosolic matrix is truly disrupted. To what extent, for example, are structures such as microtubules intact? Are smaller protein complexes left intact? Do proteins really escape degradation by ionisation? Perhaps more importantly, to what extent can these things be made reproducible? The results described below are encouraging but are far from showing a full understanding of cell lysis for single cell proteomics.

2.7 Binding data

In this mode of data collection the intensity of the TIRF beam is lowered in order to reduce photobleaching of fluorophores.

2.7.1 Bleaching data

After binding is essentially complete, data is collected in a second bleaching mode. Here the power of the TIRF beam was increased. Images were then recorded at short intervals, in the case of the data here every minute. Successive images can be differenced by subtracting one from the other. Differencing will eliminate background fluorescence that is constant between frames while showing single molecules that have been bleached between the first and second frames.

This data proved to be more useful for quantification of binding. In future work bleaching data should usually be sufficient.

2.8 Results

Images of the antibody spots after 20 minutes for the first two single cell pull downs are shown in figure 2.11.

An image of the antibody spot after 20 minutes for the three cells is shown in figure 2.12.

2.9 Signal processing

2.9.1 Mean intensity analysis

In this analysis the mean signal on the spot at maximum binding is taken as proportional to the number of proteins bound. In order to account for non-specific binding the mean signal in a region away from the spot was subtracted for each data set. It is likely that non-specific binding on and off the spot are in reality different, but this was used as approximation.

A second experiment was run in which multiple cells were loaded into chambers on the same chip. The results are shown in figure 2.13, analysed by mean intensity analysis. The straight line fit is an initial indicator that the technique could be linear in the single cell regime.

2.9.2 Single molecule counting

Measuring protein number by mean intensity has three drawbacks. First, it is hard to remove background in a consistent way. Third, the signal intensity depends on the varying intensity of the excitation beam, both between measurements and over the field of view. Third, there is a need for calibration in order to obtain a single molecule count. TIRF microscopy is sensitive enough to detect single fluorophores bound to the antibody patch. A single molecule counting approach has the potential to overcome all these problems.

A first attempt at single molecule could be made by eye. This involved magnification of a portion of the bound spot images and count molecules by eye. The result could then be scaled up to give a result for the whole image. These results were consistent with the result shown in figure 2.13. They suggest that around 1500 molecules per cell per spot are observed. However the method is labour intensive and, more significantly, rather subjective in distinguishing single molecules from background noise.

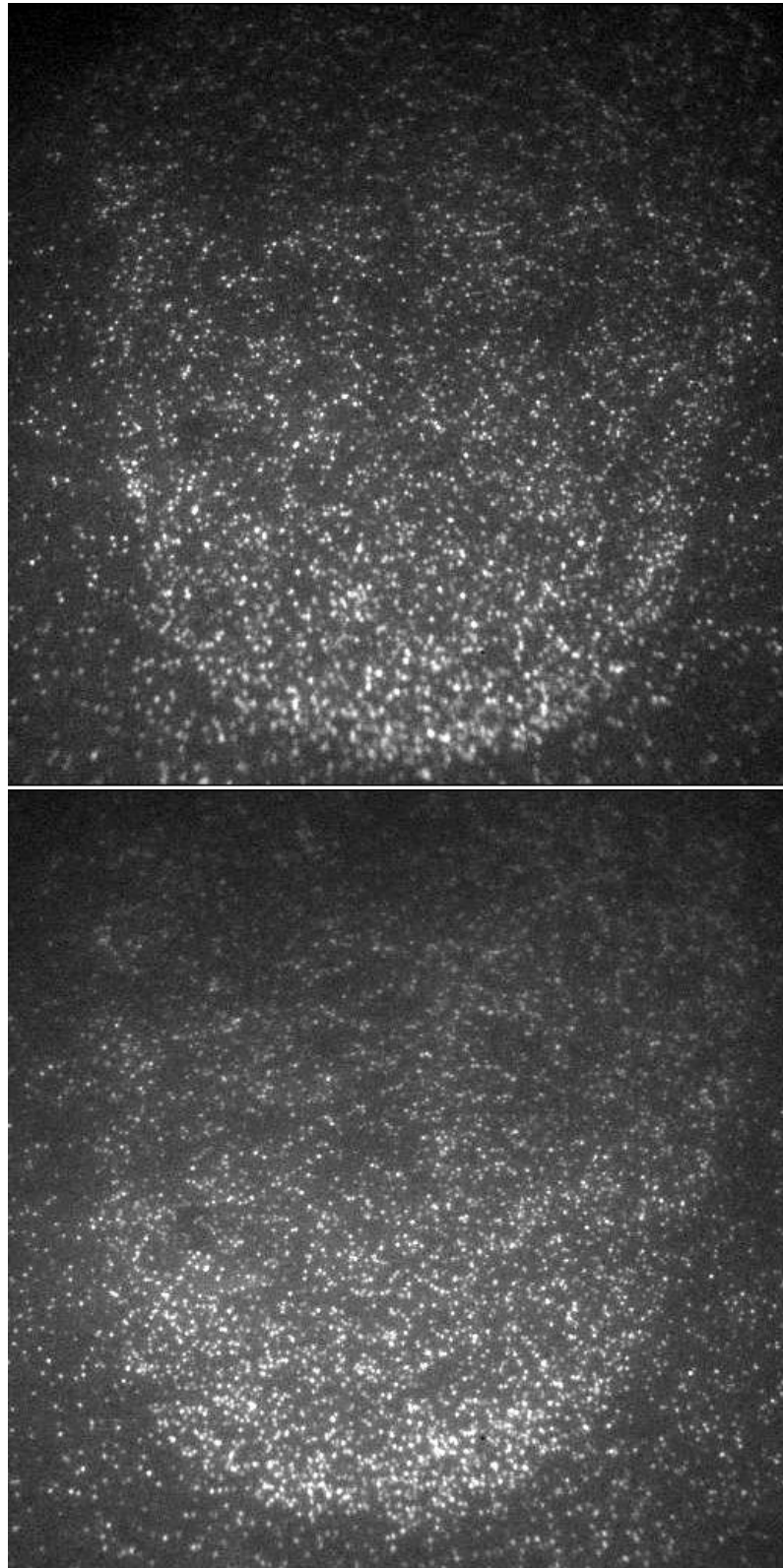


Figure 2.11: TIRF images of labelled secondary antibody for p53 bound to a spot from single cells after 15 minute incubation. Laser power was 1.5 mW and exposure time 1 s. (For clarity the dynamic range has been enhanced, with the maximum at 120 out of 255).

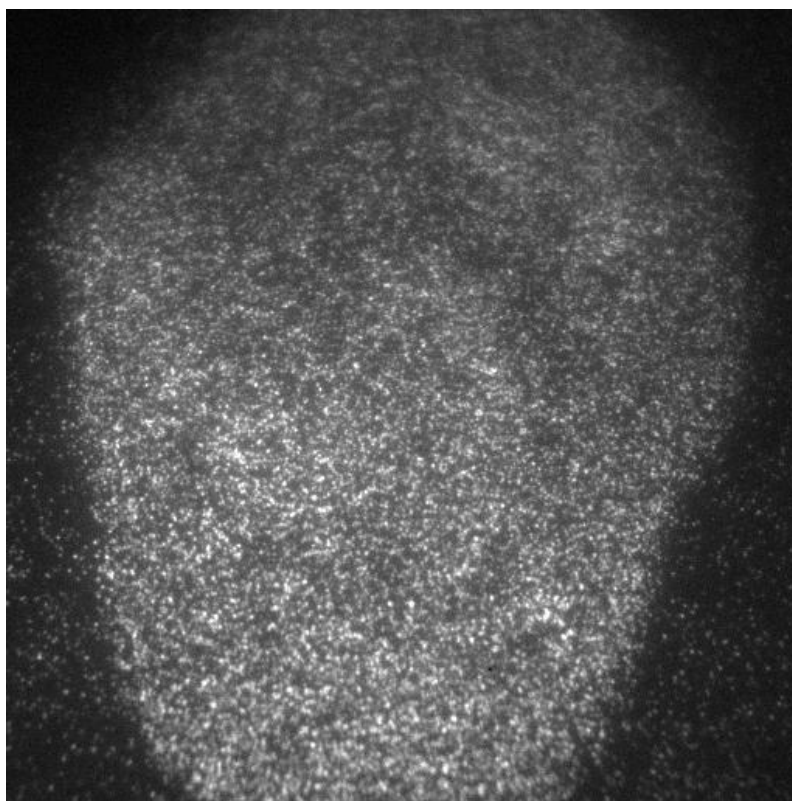


Figure 2.12: TIRF images of labelled secondary antibody for p53 bound to a spot from lysis of three cells after 15 minute incubation. Laser power was 1.5 mW and exposure time 1 s. (For clarity the dynamic range has been enhanced, with the maximum at 120 out of 255).

2.9.3 Bleaching data

In order to identify single molecules more clearly and to eliminate as much of the autofluorescent background as possible, analysis was carried out on differenced frames under bleaching conditions, as mentioned above.

Frames were differenced using ImageJ and imported in to Octave for processing. The differenced images were thresholded so that pixels above the threshold were assigned a value 1 and pixels below the threshold were assigned a value 0. Connected groups of positive pixels in the thresholded image were then counted, to give a count of single molecules.

A thresholding algorithm based on Otsu's maximum entropy method [200] was found to consistently produce thresholds that were too high (i.e. it eliminated real signal) when judged against a manual molecule count.

Instead, a threshold based on the mean signal in the image plus a multiple of the standard deviation was used. Because differenced frames were worked with, the large majority of pixels

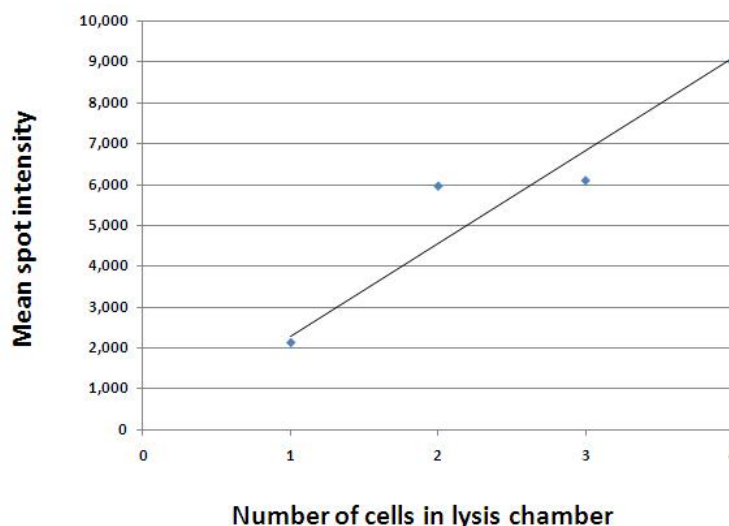


Figure 2.13: Mean spot intensity after 15 minutes incubation vs. number of cells lysed in a chamber. Each point represents a single experiment. The straight line fit is constrained to go through the zero point, giving $R^2 = 0.8925$. A free fit allowing a background not constrained through the origin (not shown) gives $R^2 = 0.9033$.

contributed only noise, which was approximately normal. Unfortunately the single molecules appeared to produce a signal only around 2 standard deviations from the mean, making them hard to objectively distinguish.

A filtering step was introduced to attempt to make use of the connected structure of molecules as opposed to noise. This filter involved discarding connected objects smaller than a fixed size. The results are shown in table 2.1.

Table 2.1: The number of connected objects detected after thresholding an imaging and filtering all objects smaller than the size shown. (Zero size corresponds to no filtering).

Threshold	Pixel size filtered out							
	0	1	2	3	4	5	6	7
$\mu + \sigma$	9446	1418	574	350	203	145	103	67
$\mu + 2\sigma$	2664	385	192	123	82	47	27	18
$\mu + 3\sigma$	774	154	81	45	30	16	12	5
$\mu + 4\sigma$	141	43	26	12	5	2	1	1
$\mu + 5\sigma$	75	27	16	5	3	2	1	0

It is likely that this spot contains around 1500 molecules. This confirms the difficulty of distinguishing spots from noise with this method.

Both data collection and data analysis could be improved. For example, data collection

could be improved by use of better dyes, higher power and greater magnification. Analysis could be improved by fitting gaussian peak shapes to single molecules.

The present data demonstrates that the method of pulling down protein from single cells is viable.

2.10 Discussion

The numbers of single proteins counted is well below numbers extrapolated from previous measurements on larger number of cells by ELISA techniques. These have ranged between 2×10^4 and 5.9×10^5 . [201, 202]

There are a number of possible explanations for this result. It is possible that the cell line used here, in the experimental conditions used, contained only a relatively small number of p53 molecules.

It is possible that the epitopes bound by the antibodies are already bound by other protein interaction partners, and that the experiment only measures 'free' p53. It is also possible that the epitope may be modified by post-translational modifications, preventing binding, as it is likely that the antibodies were raised against unmodified peptide fragments.

The epitope of the primary antibody is poorly defined as 'the C terminus' according to the manufacturer. The total length of p53 is 393 amino acids. The final well defined C terminal domain is made up of residues 319–360. This domain is involved in tetramerisation, in which state the p53 tetramer binds DNA as a transcription factor. It is possible that an un-tetramised fragment was used to generate the antibody and that tetramers may not bind.

The final 33 C-terminal amino acids are subject to a variety of post-translational modifications shown in table 2.2

The epitope of the labelled secondary antibody according to the manufacturer is amino acids 15–25, at the N terminus. p53 has interactions with at least 50 other proteins [203] in which this domain is heavily involved. When the p53 tetramer is bound to the DNA the N terminal reaches out along the helix to interact with other transcription factors. This domain also binds the protein mdm2 which regulates p53 levels by signalling for degradation on binding.

The 15–25 epitope also contains two post-translational modification sites, underlining its importance in protein interactions. Serine 15 is phosphorylated by PRPK and ATM. Threonine

Table 2.2: Post-translational modifications of the p53 C-terminus [203]

Residue number	Amino acid	Modification
370	Lysine	N6-methylation
372	Lysine	N6-methylation
373	Lysine	N6-acetylation
381	Lysine	N6-acetylation
382	Lysine	N6-acetylation
386	Lysine	SUMOlation
392	Serine	Phosphorylation

18 is phosphorylated by VRK1.

It would take considerable work to test the hypothesis that one or both antibodies bind specifically to a particular state of post-translationally modified protein. However, if it were shown that this is the case it would potentially open up a powerful tool. Quantification of p53 in a particular state of modification is of just as much interest as quantification of total p53. Indeed, given the lack of tools available for such quantification it may even be of greater interest.

Similarly, if binding to the secondary epitope is being blocked by p53 interaction partners such as mdm2 then it is an encouraging sign for the prospects of detecting protein complexes with the format. In this instance labelled secondary antibodies against mdm2 could be used in combination with primary antibodies against p53 to potentially quantify p53-mdm2 binding.

Full understanding of the biological and physical significance of the bound fraction remains for future work.

Chapter 3

Isolation of Circulating Tumour Cells

3.1 Introduction

Analysis of circulating tumour cells (CTCs) is an ideal application of the single cell analysis system described in the previous chapter. Firstly, these cells are of great biological and clinical interest. Secondly, only quantities of material at the level of a few cells are available. Thirdly, it is of interest to measure heterogeneity in the CTC population.

An important goal of this thesis is therefore the isolation of CTCs in a microfluidic device in a format suitable for integration with single cell lysis. A first step is described in this chapter, with work on improvements in this technique described in the remaining chapters.

As will be discussed below, the technique for isolation of CTCs used here relies on staining with fluorescently labelled antibodies against the EpCAM membrane protein. Independently of its use for CTC identification, staining of membrane proteins is a useful technique to quantify protein expression. This is complementary to the work described in chapter 2, as it measures membrane bound but not cytosolic protein. Work on quantifying the membrane protein ERBB2 (also known as Her2) in this way is also included in this chapter.

3.2 Isolation of Circulating Tumour Cells

3.2.1 The CellSearch system

The only technique for counting circulating tumour cells currently approved for clinical use by the US Food and Drug Administration is the CellSearch system produced by Veridex (a subsidiary of Johnson & Johnson). [204] I decided that a modification of this system would provide a useful starting point for isolation of CTCs in a microfluidic format such that single cell analysis described in the previous chapter could be carried out.

The strategy used by the CellSearch system is based on immunomagnetic separation. Magnetic nanobeads coated with an anti-EpCAM antibody are used to isolate circulating tumour cells from a 7.5 mL blood sample. A unit known as the CellTracks machine takes as input a blood sample which has been separated by centrifugation. It optically detects the white blood cell layer from the stratified sample in the centrifuge tube and removes the other layers by suction. It then adds the immunomagnetic particles and carries out a pull down, depositing isolated cells onto a cartridge where they are fixed and stained.

The fixed cell fraction contains the recovered CTCs, which in a typical sample will number between 0 and 5 but may range up to hundreds. It also typically contains between hundreds and thousands of white blood cells. Final identification of CTCs takes place by fluorescence microscopy. The cartridge is scanned by an automated fluorescence microscopy and images are presented to the human operator for a final decision. The cells are stained with

1. A labelled mouse anti-cytokeratin (CK) 8 and 18/19 monoclonal antibody. CK8 and CK18/19 are present only in epithelial cells. CTCs must be scored CK⁺.
2. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI). This dye stains the nucleus and is indicative of an intact cell. CTCs must be scored DAPI⁺.
3. A labelled mouse anti-CD45 monoclonal antibody. CD45 is a surface marker present on white blood cells. CTCs must be scored CD45⁻.

The course of treatment for a patient can depend on the presence or absence of a single positive cell. In so far as the judgement of whether a cell is positively stained is made by eye rather than by quantified measurement and objective algorithms, the CellSearch count is partly subjective. With weak staining whether a cell is positive or negative can be ambiguous.

The strategy in this work is to interrupt the CellSearch protocol before the cells are deposited, fixed and stained. Instead, the fraction containing CTCs and white blood cell contaminants can be put into a microfluidic device for analysis. Staining and counting of CTCs could potentially be automated to give an objective assessment. The next chapter of this thesis pursues this possibility.

In this chapter I show that staining, and manual identification of CTCs can take place in a microfluidic device integrated with a single cell analysis stage. This enables the possibility of more versatile and quantitative proteomic CTC profiling. This chapter details the identification of CTCs from CellSearch output in a microfluidic device by staining with a labelled anti-EpCAM antibody. It also describes staining of cells in a microfluidic device with anti-ERBB2 affibodies, and the possibility of quantifying ERBB2. Finally, it describes a first experiment in which a clinical sample was taken from a patient with metastatic breast cancer and probable CTCs were identified in a microfluidic device.

3.2.2 The CellSearch protocol

The standard protocol for isolating CTCs with the CellSearch system, as specified by Veridex is as follows:

1. Add 7.5 mL of blood to a 15 mL conical tube
2. Add 6.5 mL of proprietary dilution buffer
3. Seal the tube with cap and mix by inverting five times
4. Centifuge at 800g for 10 minutes
5. Immunomagnetic binding using the CellTracks machines
6. Deposit immunomagnetically isolated cells into a cartridge
7. Fix and stain cells
8. Pull magnetically labelled cells on to cartridge base
9. Identify fluorescently labelled cells and count CTCs

This protocol is used for patients with metastatic breast, colon or prostate cancer, all of which express EpCAM. This thesis is concerned exclusively with breast cancer.

The change in protocol in this work is at step 6. Instead of depositing cells into a cartridge for staining they are deposited into an eppendorf tube, in about 1 mL of buffer. As described below, these cells containing both CTCs and contaminating white blood cells are stained and washed using conventional protocols then run through a microfluidic device in which CTCs are identified.

3.3 Specificity of anti-EpCAM antibody staining

EpCAM is a 40 kDa glycosylated membrane protein normally expressed in epithelial tissue. It was first discovered in colon cancer, [205] but is expressed in many cancers including most adenocarcinomas, some squamous cell carcinomas, retinoblastomas and hepatocellular carcinomas.

EpCAM was the first human tumour-associated antigen against which a monoclonal antibody was found. It was also the target of the first monoclonal antibody cancer therapy and of the first to show clinical efficacy (edrecolomab). [206] It's detailed biology has only been investigated more recently.

In many breast cancers EpCAM is overexpressed by 100–1000 times. Silencing of EpCAM results in a reduction in proliferation, migration and invasion. [207] In normal cells it is thought to be expressed on the basolateral membrane, tightly bound to other cells, while in cancer it is expressed across the cell surface, potentially more exposed to antibody binding.

EpCAM signalling takes place by cleavage of a 26 amino acid cytoplasmic domain, EpICD. This activates the wnt signalling pathway, [208] which is known to play a role in both stem and cancer phenotypes, through activation of LIF/STAT3 and c-Myc leading to activation of the transcription factors oct3/4, Klf4 and sox2. Significantly, EpCAM is also expressed by stem and progenitor cells. [209]

EpCAM directly interacts with PI3K, suggesting cross talk with another important growth signalling pathway. Conversely, p53 which is important in blocking cancer growth, appears to regulate expression of EpCAM by binding to an intron in the DNA sequence, blocking transcription. [210]

Identification of CTCs by EpCAM expression can result in both false positive and false

negative results. The possibility of CTCs that do not express EpCAM is an active area of research, in both breast and in other cancers. However, EpCAM expressing CTCs have been shown to be of clinical relevance, and the case of false negative non-EpCAM expressing CTCs is beyond the scope of this work.

In the first stage of the CellSearch assay around 99% of the cells isolated by immunomagnetic pulldown are not CTCs. In the full CellSearch assay these contaminant cells are excluded by testing for the expression of an additional marker. The 'gold standard' for identification of EpCAM expressing cells as CTCs is the expression of cytokeratins 8 and 18, and this is used in the CellSearch assay. Cytokeratins 8 and 18 are cytoskeletal proteins expressed in epithelial cells. Because they are cytosolic, testing for cytokeratins using antibody binding requires fixation or lysis of a cell. In the conventional CellSearch assay this is done by fixation of cells.

The exact reason that contaminating cells are pulled down in immunomagnetic separation is not known. Several hypotheses are possible:

1. They may be a rare subpopulation of blood cells that expresses EpCAM.
2. They may show some non-specific binding to the anti-EpCAM antibodies, or other non-specific binding to the magnetic beads (for example, the beads may be endocytosed).
3. They may simply be mechanically caught up with EpCAM positive cells, and not show any binding to the immunomagnetic beads at all.

It is desirable to find a way of identifying CTCs from contaminants that does not depend on testing for cytosolic proteins such as cytokeratin. This would allow CTCs to be selected for lysis, and also open up the possibility of culturing or studying intact CTCs.

The simplest possibility is to stain cells with a labelled anti-EpCAM antibody. If the contaminants express EpCAM or otherwise bind to anti-EpCAM antibodies (hypotheses 1 or 2 above) then this strategy will not work. It would be necessary in to introduce in addition a stage such as anti-CD45 staining, and reject cells that score CD45⁺. This would introduce major extra complexity at the cytometry stage with the need for 2 colour rather than 1 colour fluorescent excitation and detection.

If, however, an anti-EpCAM antibody can be found that does not bind contaminating cells then this antibody can be fluorescently labelled and used to isolate CTCs.

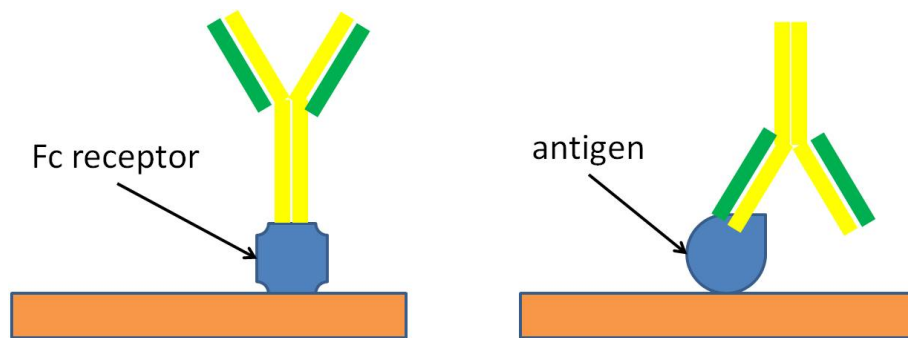


Figure 3.1: Binding of antibodies to Fc receptors takes place at the constant domain which is common to all antibodies (left) rather than specifically to a single antibody antigen (right)

The test described below shows that staining with a labelled anti-EpCAM antibody does appear to have specificity for epithelial cells. (The epitopes bound by the labelled and the immunomagnetic antibodies are not known. They may be the same or different.) This suggests that the contaminants do not arise from EpCAM expression on the contaminants. The successful use of anti-EpCAM staining is consistent with the successful use of anti-EpCAM coated pillars to isolate CTCs in microfluidic devices reported by other groups.

3.3.1 Staining using anti-EpCAM antibodies

To test the specificity of anti-EpCAM antibody binding, a stain was attempted against both an EpCAM expressing epithelial cell line (MDA MB 468) and against an EpCAM negative T cell line (Jurkats). Cells were washed and diluted to approximately 2×10^6 cells per ml. Anti-EpCAM antibody was added to a final concentration of $5 \mu\text{g}$ per ml, incubated for 30 minutes, and washed three times in PBS.

Nonspecific binding appeared to be a problem, as the T-cells showed significant fluorescent labelling. T-cells express Fc receptors that bind the constant region of antibodies. They can therefore show high affinity for antibody binding without expressing the specific antibody antigen (see figure 3.1).

It is likely that many of the contaminating cells from which it is desirable to separate CTCs express such receptors. Non-specific binding to such Fc receptors is a well known problem. One solution is to block binding by pre-incubation with an antibody of the same isotype as that used for labelling. However, incubation with mouse polyclonal isotype G (sigma) for 30 minutes prior to repeating the staining protocol did not eliminate labelling of T-cells.

While Fc receptors on T-cells in culture are available for binding, the receptors on T-cells from blood are thought to be occupied by antibodies. This is why, for example, the standard CellSearch protocol does not call for blocking of Fc receptors.

For this reason it was decided to attempt experiments spiking MDA MB 468 cells into blood and retrieve them using anti-EpCAM staining. If strong non-specific staining is present then it should be clear from the large number of cells stained. However, the gold standard proof that retrieved cells are really epithelial would be to show expression of CK 8/18 by single cell analysis.

3.3.2 Spiking experiments using anti-EpCAM antibodies

MDA 468 cells were trypsinised, counted, diluted and spiked into whole human blood taken from a healthy volunteer. Two concentrations of spiked cells were used. The first was at 10^3 cells spiked into a 7.5 mL sample. The second was at 10^6 cells spiked into a 7.5 mL sample.

The blood sample was pipetted from the sample collection tube into the conical tubes mentioned in step 1 of the protocol in section 3.2.2. At step 6 the isolated cells were deposited into an eppendorf tube. Cells were stained with anti-EpCAM at a final concentration of $5\mu\text{g}$ per ml, incubated for 30 minutes, and washed three times in PBS.

Cells were arrayed on a cover slip and observed in epifluorescence. These results showed retrieval of around 50% of spiked cells, indicating that anti-EpCAM had not large numbers of leukocytes. While the need for CK 8/18 analysis has been noted, this was encouraging enough to proceed with CTC experiments.

3.4 Cytokeratin expression

The decisive test of whether CTCs can be detected by EpCAM staining alone will require a pull down of cytokeratins 8 and 18 using the system described in the previous chapter. A first step in this direction is the identification of a suitable antibody pair for the sandwich assay. As noted in the previous chapter, it is particularly important to find antibodies with good specificity.

An attempt was made to validate pan anti-cytokeratin antibody against purified cytokeratin 8 protein. (Antibodies with specificity against cytokeratins 8 and 18 are commonly known as 'pan anti-cytokeratin'.) This was frustrated by the poor quality of the protein, commercially

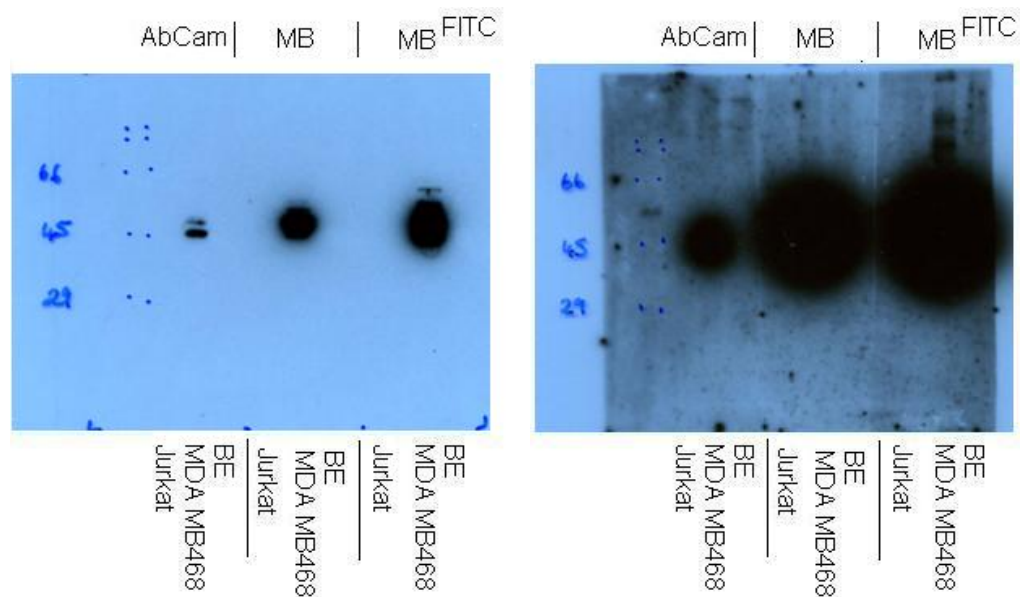


Figure 3.2: Western blot of cell lysate from three cell lines (BE, MDA MB 468 and Jurkat) with three pan anti-Cytokeratin antibodies, detected by fluorescently labelled anti-mouse antibody. The left hand figure is a short exposure showing a clean signal at the correct mass. The right hand figure is a long exposure showing a very low level of non-specific interactions.

sourced from Origene (USA).

However, tests against cell lysates gave good results. Figure 3.2 shows a Western blot against three different cell lines. BE is a colon cancer cell line that is not expected to express cytokeratins 8 and 18. MDA MB 468 is a breast cancer cell line that is expected to express cytokeratins 8 and 18, and is used as a model for CTCs. Jurkats are a leukocyte cell line that is not expected to express cytokeratins 8 and 18. They may be a good model for the contaminating cells in the CellSearch assay, that is likely to contain leukocytes.

The Western blot was taken with 10 μ g of total cell lysate from each cell line probed with pan anti-cytokeratin proteins from AbCam and Miltenyi Biotec. The Miltenyi Biotec antibody was tested both as an unlabelled antibody and labelled with a FITC tag, suitable for detection by TIRF.

The result shows that both antibodies have good specificity. It suggests spotting down the AbCam antibody and using the labelled Miltenyi Biotec antibody as a secondary. The outcome of this will depend on how well the primary functions when printed. This will have to be tested in further work.

3.5 Development of ERBB2 staining

As reviewed in chapter 1, it is desirable to stratify patients by ERBB2 tumour status.

At present stratification of patients as ERBB2 positive takes place by membrane staining of tumour tissue samples. Quantification of staining takes place by eye and cannot be readily be adapted to CTCs. Amplification of ERBB2 gene copy number has been measured in CTCs by FISH, but proteomic expression on ERBB2 has not been quantified.

It is not known in general whether, with respect to ERBB2 expression, CTCs share either genotype or phenotype with the primary tumour. If, for example, some CTCs were shown to express ERBB2 even when this is not present in the primary tumour and these cells are important for metastasis then the use of drugs such as trastuzumab may form an effective treatment.

It would therefore be useful to develop an assay for expression of ERBB2 at the single cell level that can be applied to CTCs isolated in a microfluidic device. Here an affibody was tested against three cell lines. BRT 474 is a ERBB2 positive breast cancer cell line, intended as a model for ERBB2 positive breast cancer CTCs. MDA MB 468 is a ERBB2 negative breast cancer cell line, intended as a model for ERBB2 negative breast cancer CTCs. Jurkat is ERBB2 negative leukocyte cell line, intended as a model for non-CTC contaminants in the CellSearch output.

3.5.1 Background subtraction

To quantify ERBB2 staining it is necessary to subtract a background.

Seventy five cells were selected by eye and masked out by hand, using the bright field image for guidance. Only cells that were well separated single cells were selected. The masks were used to define regions of interest on the fluorescent image over which the signal was averaged, to find mean intensity, and integrate, to find total signal from the cell.

The standard deviation of mean intensity from cell to cell before background subtraction was 5.6275 arbitrary units. After subtraction of a background determined from an empty region immediately adjacent to each cell the standard deviation was 4.8966 arbitrary units, a reduction of 13%. This suggests the standard deviation of the background should be $\sqrt{5.6275^2 - 4.8966^2} \approx 2.8$.

This is consistent with the standard deviation of the background as determined by using

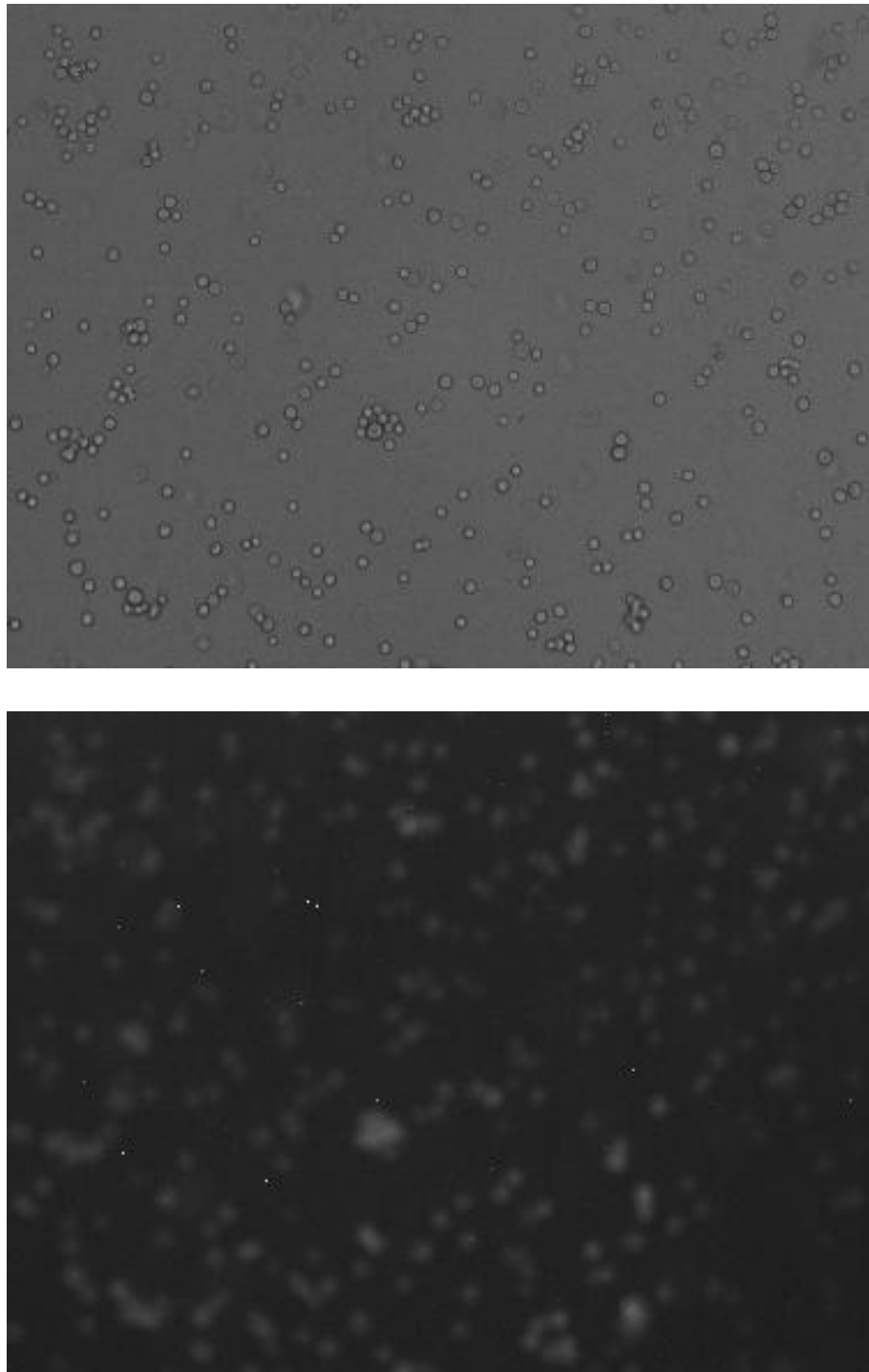


Figure 3.3: Staining of ERBB2 positive BRT-474 breast cancer cell line with anti-ERBB2 affibody. Top panel shows bright field image. Bottom panel shows wide field fluorescent image. Cells were stained in $1\mu\text{g}$ per mL of antibody at 4°C for 30 minutes and washed 5 times to remove excess dye. They were imaged using a Plan Fluor 10x/0.5NA objective. Fluorescence excitation used a mercury lamp and fluorescence collected using a Nikon TRITC filter cube.

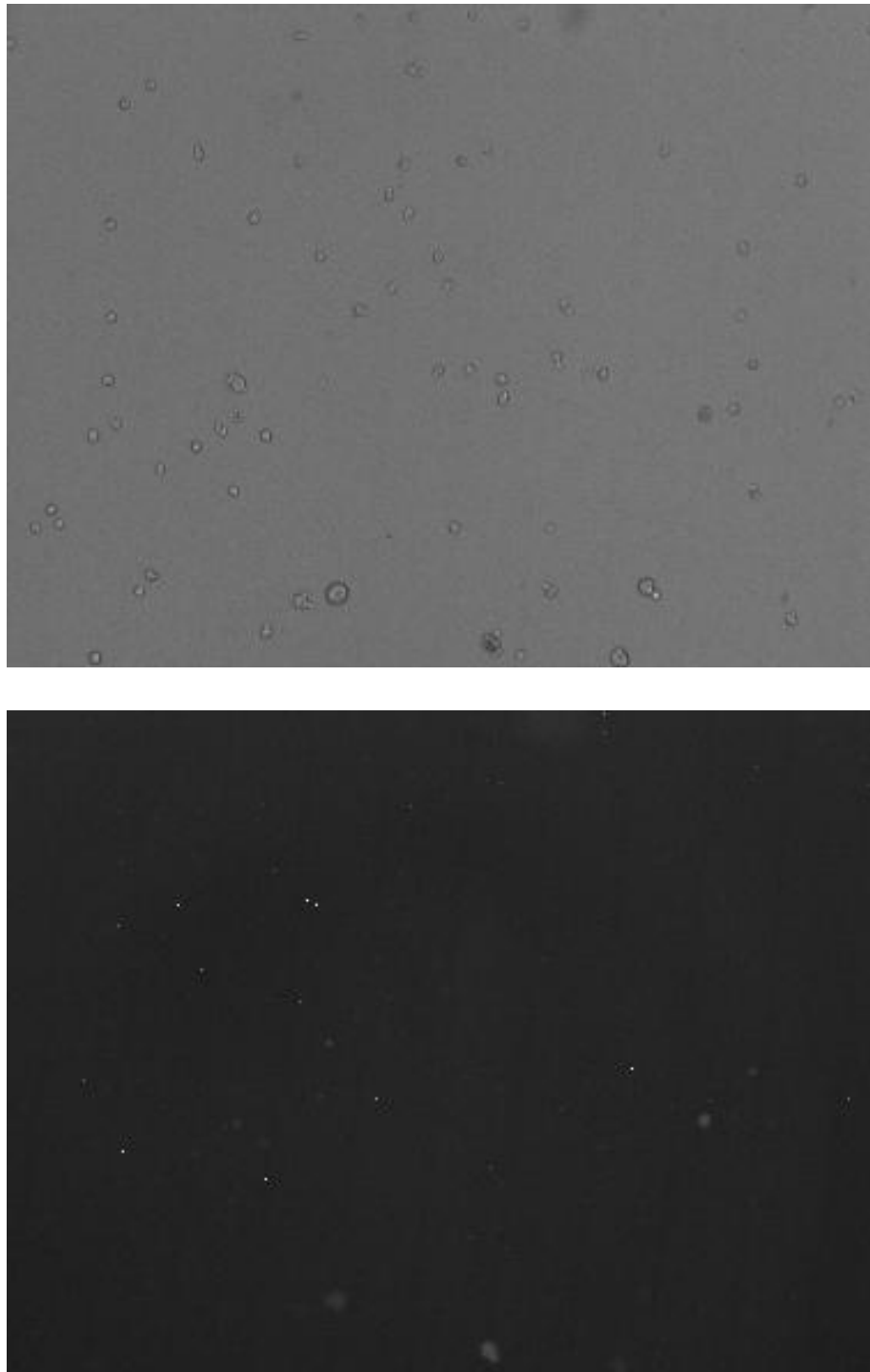


Figure 3.4: Staining of ERBB2 negative Jurkat leukocyte cell line with anti-ERBB2 affibody. Top panel shows bright field image. Bottom panel shows wide field fluorescent image. Cells were stained in $1\mu\text{g}$ per mL of antibody at 4°C for 30 minutes and washed 5 times to remove excess dye. They were imaged using a Plan Fluor 10x/0.5NA objective. Fluorescence excitation used a mercury lamp and fluorescence collected using a Nikon TRITC filter cube.

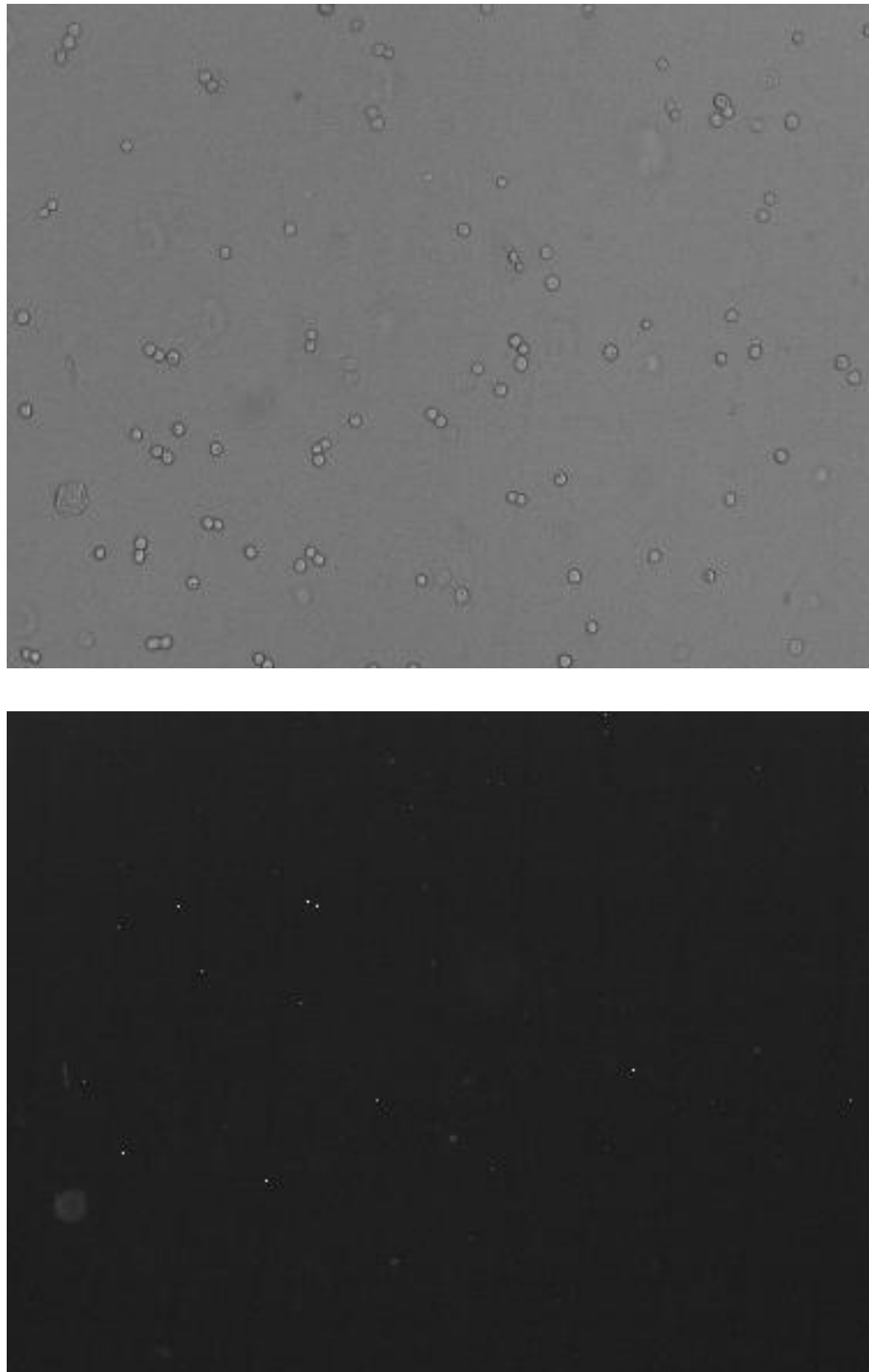


Figure 3.5: Staining of ERBB2 negative MDA-468 breast cancer cell line with anti-ERBB2 affibody. Top panel shows bright field image. Bottom panel shows wide field fluorescent image. Cells were stained in $1\mu\text{g}$ per mL of antibody at 4°C for 30 minutes and washed 5 times to remove excess dye. They were imaged using a Plan Fluor 10x/0.5NA objective. Fluorescence excitation used a mercury lamp and fluorescence collected using a Nikon TRITC filter cube.

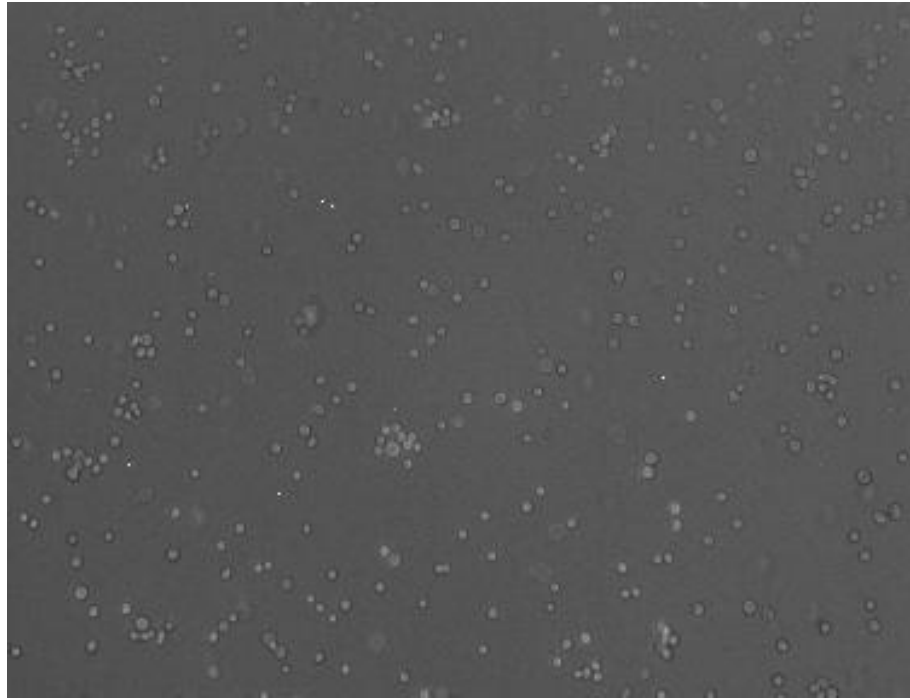


Figure 3.6: Composite of images bright field and fluorescence images of stained BRT 474 cells (images shown separately in figure 3.3) illustrating consistent staining of cells. Cells were stained in $1\mu\text{g}$ per mL of antibody at 4°C for 30 minutes and washed 5 times to remove excess dye. They were imaged using a Plan Fluor 10x/0.5NA objective. Fluorescence excitation used a mercury lamp and fluorescence collected using a Nikon TRITC filter cube.

a low pass spatial filter. A filter of $11.25\mu\text{m}$ gives a standard deviation of 2.749 arbitrary units. The average area of a cell is $76.0\mu\text{m}^2$, giving an average diameter of $9.9\mu\text{m}$. The local background subtraction has therefore accounted for variation at spatial scales greater than the cell.

Cells showed significant variation in area in bright field imaging (and therefore presumably in volume), as shown in figure 3.11 which gives rise to greater variation in integrated intensity than in mean intensity. This is seen in the high integrated intensity tail of figure 3.10.

3.6 Arraying cells

For simple studies on fluorescence imaging of cells in a microfluidic device, cells were directly injected into a large at low flow rates.

Initial experiments were carried out using BE cells, a colon carcinoma cell line, just because this cell line is a routinely used in our laboratory. However, this is an adherent cell line and the

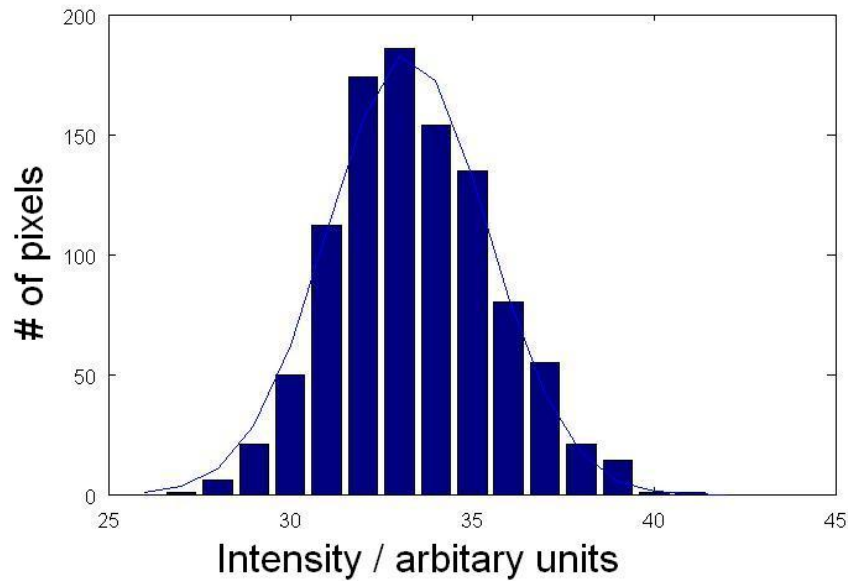


Figure 3.7: Background sampled from the imaging of stained BRT 474 cells (shown in 3.3) Plan Fluor 10x/0.5NA objective, fluorescence excitation used a mercury lamp and fluorescence collected using a Nikon TRITC filter cube. Bars show histogrammed data ($n = 1011$ pixels, $\mu = 33.4$, $\sigma = 2.4$). Line shows a best fit normal distribution ($\mu = 33.2$, $\sigma = 1.8$).

cells proved to be very 'sticky', resulting in clumping rather than an even flow of cells.

A capillary could be evenly filled with dispersed cells by placing its end into an eppendorf tube containing a cell suspension and filling the capillary using a syringe pump. However, on attempting to eject the cells by reversing the direction of pumping the clumping process could be observed taking place inside the capillary under a microscope. Cells would move until they touched another cell, at which point they would stick to each other. This would lead to the formation of giant clumps of cells.

It is possible that the clumping was due to a change in phenotype such as expression of surface proteins. The cells may have become more 'sticky' over time in the capillary. This seems unlikely because when testing for clumping the cells were left in the capillary for less than a minute before pumping out. The effect persisted whether cells were suspended in cell medium or PBS, and whether they were taken directly from cell culture into the capillary or whether they were left for several hours. It therefore seems more likely that the effect was due to a hydrodynamic difference between the flow loading and unloading the capillary.

The problem was overcome by the use of the Jurkat cell line. This is a non-adherent cell

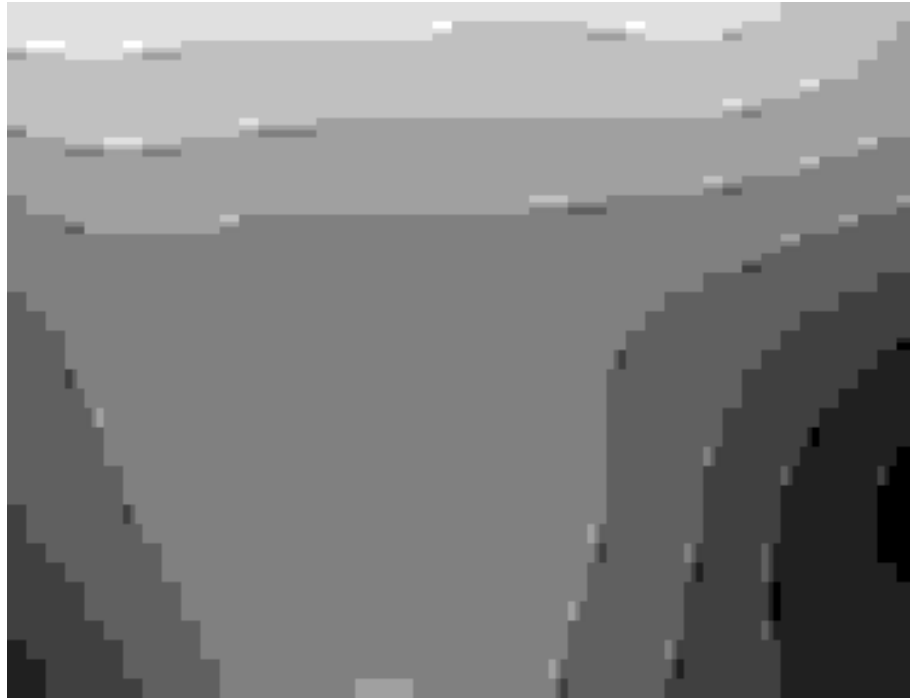


Figure 3.8: Background from the imaging of stained BRT 474 cells (as shown in 3.3) Plan Fluor 10x/0.5NA objective, fluorescence excitation used a mercury lamp and fluorescence collected using a Nikon TRITC filter cube, as determined by a 50 pixel low pass Gaussian filter. The greyscale runs from 33 to 41.

line, presumably much more similar to the non-CTC component of the CellSearch output.

The differenced image shows motion in around 143 cells.

3.7 Results with clinical sample

Samples were taken from two metastatic breast cancer patients and run through the standard CellSearch protocol by Rachel Payne at Hammersmith hospital. The CellSearch system encountered an error in processing one of the two samples. The other sample was successfully processed and outputted into 1 mL of buffer in the bottom of the 15 mL conical tube. This sample came from a patient whose primary tumour was an invasive ductal carcinoma grade 3.

The sample was then transported by bicycle to the South Kensington campus. The conical tube contained a few spots of what appeared to be whole blood around the rim, which had possibly been trapped by the sealing of the cap. During transport one or more of these spots appears to have contaminated the separated sample, accounting for a relatively high number of red blood cells contaminants. These red blood cells were easily recognised under microscopic

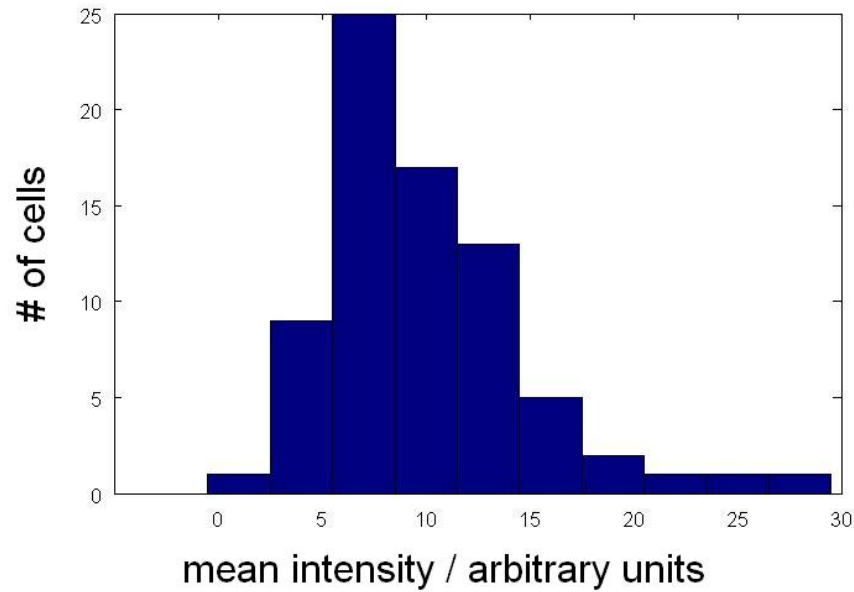


Figure 3.9: Mean pixel intensity of averaged across $n = 74$ individual BRT 474 cells with local background subtraction. Plan Fluor 10x/0.5NA objective, fluorescence excitation used a mercury lamp and fluorescence collected using a Nikon TRITC filter cube.

examination by their size and more particularly by their highly distinctive dimpled discoid morphology.

The contamination would be avoided in the standard CellSearch protocol because the AutoTracker is able to suck the buffer containing the CTCs from the bottom of the tube for final deposition in to a cassette with minimal mechanical disturbance. For the microfluidic analysis protocol the decision was made to transport the sample in the conical tube in order to minimise the number of transfers, which is an important consideration when dealing with so few cells of interest.

Red blood cell contamination was inconvenient, and in principle should be easily avoided in future experiments. However, it was still possible to carry forward the purpose of the initial experiment identifying the presence of CTCs.

In future protocols contamination should be easily avoidable. Options include carefully transferring the CellSearch output from the 15 mL conical tube before transport; cleaning contamination from the top of the conical tube; transporting the sample with much greater mechanical stability; or carrying out fluorescent staining steps before transport between campuses.

Cells were stained for both EpCAM and for ERBB2 and washed twice in PBS. The sample

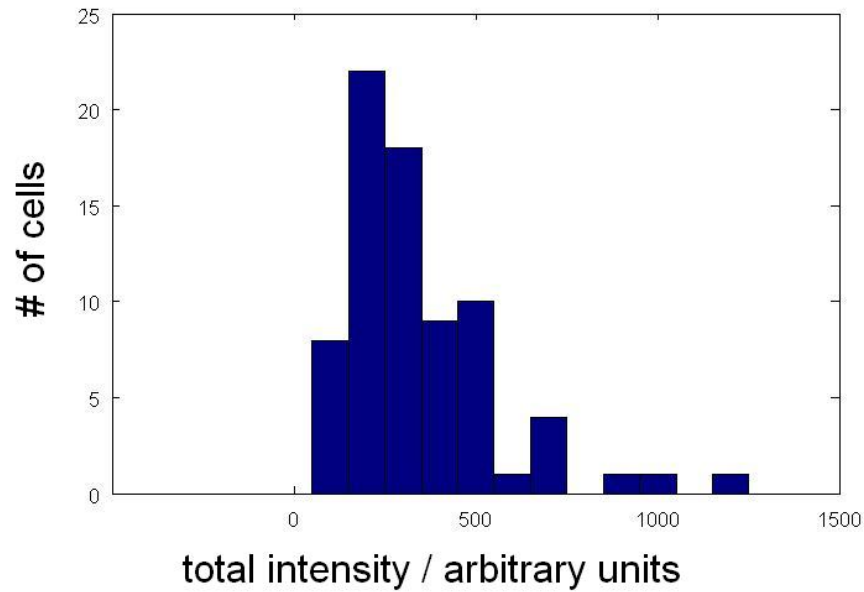


Figure 3.10: Total intensity of $n = 74$ individual BRT 474 cells with local background subtraction. Plan Fluor 10x/0.5NA objective, fluorescence excitation used a mercury lamp and fluorescence collected using a Nikon TRITC filter cube

was injected into a microfluidic device which had been assembled containing p53 antibody spots, as shown in figure.

The original intention was to reduce the sample volume to as close to $1\mu\text{l}$ as possible before injection into the microfluidic. In practice the volume could not be reduced below around $10\mu\text{l}$ without losing significant numbers of cells. About $2\mu\text{l}$, around one fifth of the volume, was injected into the microfluidic device.

Thousands of cells were observed. The chamber contained at least 3 stained cells which clearly stood out from background when viewed in epifluorescence. An example is shown in figure 3.14. These were presumed to be CTCs, and an attempt was made to capture them by optical trapping and transport them to a lysis chamber. However, this proved impossible, due in part to the unreliability of the trapping system and in part due to the large number of contaminating cells.

The procedures described in this section were carried out under ethical approval in NHS Research Ethics Committee reference number RREC 0795, amendment 6, December 2006.

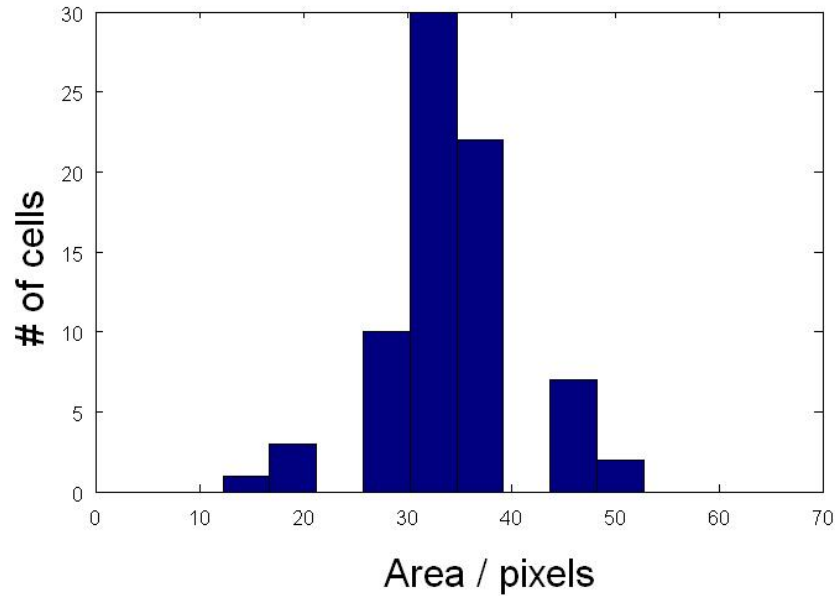


Figure 3.11: Areas of $n = 74$ individual BRT 474 cells. Plan Fluor 10x/0.5NA objective, fluorescence excitation used a mercury lamp and fluorescence collected using a Nikon TRITC filter cube

3.8 Discussion and further work

To systematically and accurately quantify the effectiveness of EpCAM staining it is necessary to develop a form of microfluidic cytometry to process a larger volume of fluid without losing material.

The experience also shows the difficulty of selecting stained cells from a randomly arrayed set of cells. Some greater automation would be desirable, for both identifying and isolating CTCs. This is especially the case for objective quantification of CTCs in a blood sample in a test suitable for diagnosis or prognosis.

A first possible approach would be to automate the scanning of randomly arrayed cells by a CCD camera. Data could be analysed either from fluorescence imaging alone or, more likely, both fluorescence and bright field imaging together, and using image recognition software to record the position of stained cells. This would allow for counting of CTCs. Further manipulation of selected cells, for example movement in a lysis chamber by laser trapping could then be either automated or carried out manually.

A refinement of this approach would be to array cells in mechanical traps. This would have

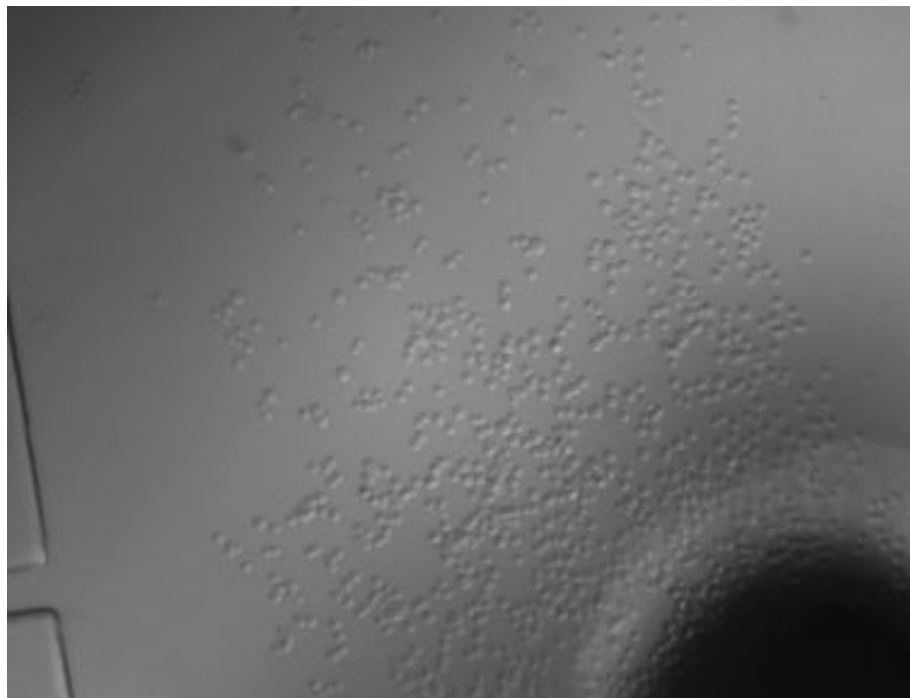
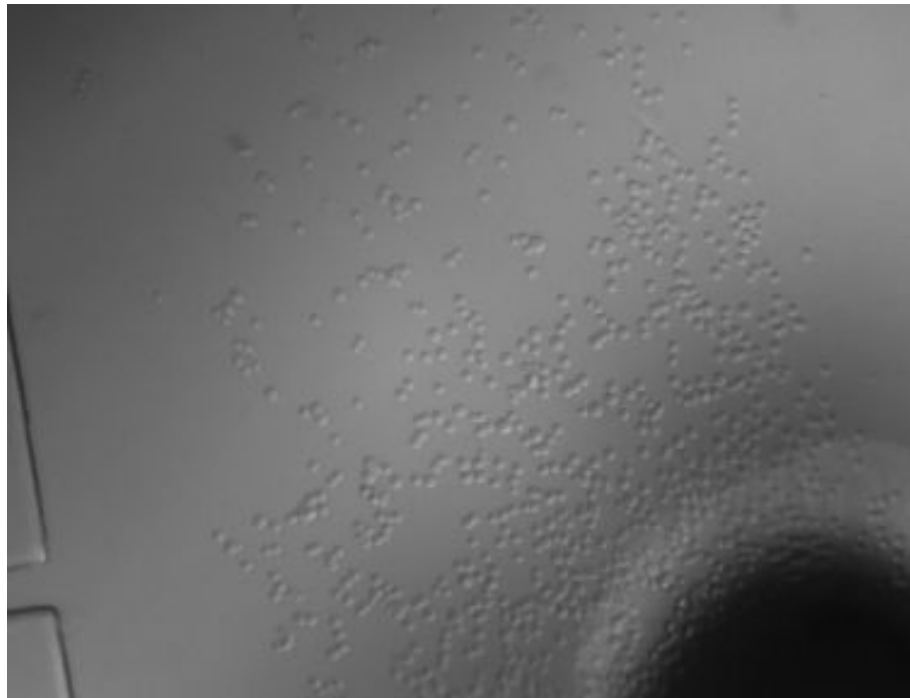


Figure 3.12: Jurkat cells injected into a microfluidic chamber, as described in the text. The top and bottom frames are separated in time by 30 seconds.

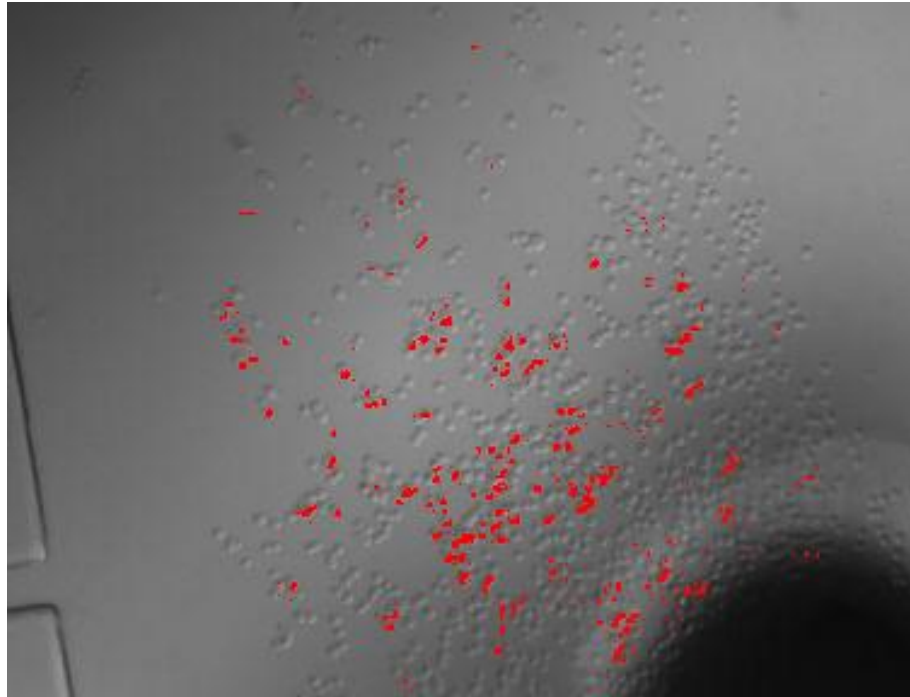


Figure 3.13: A thresholded difference between the images in figure 3.12 superimposed in red, illustrating the slow injection and deposition of cells.

the advantage of (perhaps approximately) locating cells in defined locations. Additionally it would allow for analysis of larger samples at higher flow rates. The random arraying of cells as shown in figure 3.12 relies on cells settling out of flow. This necessitates low flow rates. More significantly, even at low flow rates a fraction of cells will reach the chamber exit before settling. The only way to ensure 100% capture is to stop the flow before the first of these cells exit the chamber. This limits the size of the sample that can be run to a few microlitres. An array of mechanical traps should be able to expand this sample size by orders of magnitude.

A second possible approach would be to develop a microfluidic cytometry and sorting approach to measure and isolate stained cells. In some respects this is a more ambitious approach. In the longer term it may have the potential to work with whole blood samples, dispensing with the CellSearch pre-enrichment step, far more easily than mechanically trapping and scanning an entire blood sample.

If a single cell in a trap with surrounding space requires a $100\text{ }\mu\text{m}$ square, then 10,000 cells can be arrayed on a 1cm square. Such a chamber size approaches the practical limit for conventional microfluidic devices, but would be suitable for arraying the output of a CellSearch

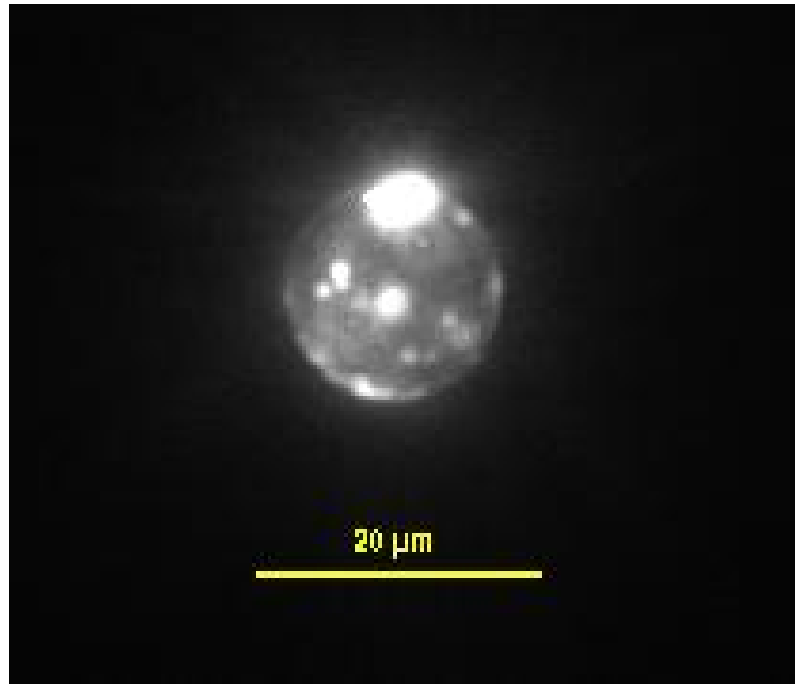


Figure 3.14: CTC isolated from clinical sample. Labelled anti-EpCAM antibody imaged using a x60 NA 1.49 oil immersion objective, wide field fluorescence excited by a mercury lamp and a TRITC filter.

pre-enrichment step. By contrast, even the white blood cell count in a 7.5 ml blood sample would be at least 2 orders of magnitude larger.

Some form of microfluidic sorting is therefore considerably more powerful than simple arraying, even with mechanical traps. A cytometry and sorting device which is integrated into standard microfluidic format could also find a variety of other uses, as it is easy to adopt a modular approach to design. While features such as the introduction of loops into the fluid path can have undesirable effects, for the most part it is possible to literally adopt a 'cut and paste' approach to combining microfluidic modules when using computer aided design to layout a photomask.

This is the motivation for the following chapters of this thesis.

Chapter 4

Microfluidic cytometry

This chapter details the attempt to automate the counting and measurement of fluorescence from cells. The first step is to hydrodynamically focus cells into a controlled stream. The second step is to pass this stream through the focus of a laser beam. This excites fluorescence of labels on the cells which is detected by a photomultiplier tube. The data is then processed to detect and count cells.

Much of the development was done using beads rather than cells, as these are generally much easier to handle. References in this chapter and the next to ‘particles’ refer to both beads and cells.

The basic layout of the microfluidic devices discussed in this chapter are shown in figure 4.1.

These were fabricated using the same soft lithography protocol as described appendix B. Refinements to the design and construction are described in later sections.

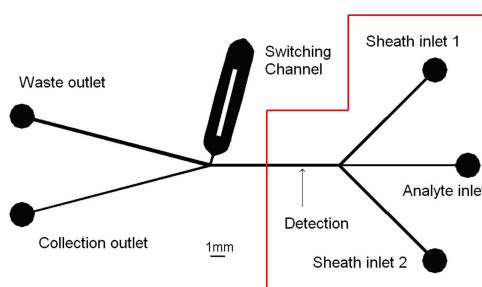


Figure 4.1: Initial design of microfluidic cytometer. The section relevant to this chapter is contained in the red box to the right, consisting of the analyte inlet, the sheath flows and detection. The left hand section is designed for switching, as detailed in the next chapter.

4.1 Hydrodynamic focusing

The particles are introduced into the inlet labelled 'analyte inlet' in figure 4.1. They flow right to left. Sheath flows are introduced into the 'sheath inlets'. When they meet the low Reynolds number means that the streams do not mix. Instead the analyte flow is focused down to a narrow stream between the two sheath flows. The effect is seen in figure 4.10.

As mentioned in chapter 1, hydrodynamic focusing takes place in conventional flow cytometer. In that context it is easy to achieve focusing in two dimensions, centering the focused flow along the third dimension. This two dimensional hydrodynamic focusing is harder to achieve in microfluidic devices. Various techniques to produce two dimensional focusing have been devised, but they require complex fabrication. [211–213] Here a simpler one dimensional focusing device is developed. As discussed in the conclusion to this chapter, achievement of complete two dimensional focusing would be a valuable improvement. However, the work was carried out with a view to achieving good enough performance for integration into a full sorting and analysis system. In that sense it is a valuable first step.

Hydrodynamic focusing (of fluid analyte – not particles) in a microfluidic device was first carried out by Knight et al. [214]. In that system the input of the analyte and the sheath flows were held at constant pressure. In the system described here the input of the analyte and sheath flows are connected to a constant current operated from syringe pumps.

4.1.1 Hagen-Poiseuille flow

The study of pressure driven flows through small channels was pioneered in the nineteenth century by Jean Louis Marie Poiseuille, who was interested in the flow through capillary blood vessels and, independently by Gotthilf Hagen. They found that the flow rate was proportional to the pressure drop and inversely proportional to the fourth power of the channel radius. This dependence holds for all channels, regardless of cross section, so long as flow remains laminar (i.e. the Reynolds numbers remains below about 200.) However, the coefficient and the detailed velocity distribution depend on the exact geometry.

The microfluidic devices described in this work have channels of approximately rectangular cross section. A flow through a channel of height h , width w and length L as shown in figure 4.2 can be calculated from the Navier-Stokes equations.

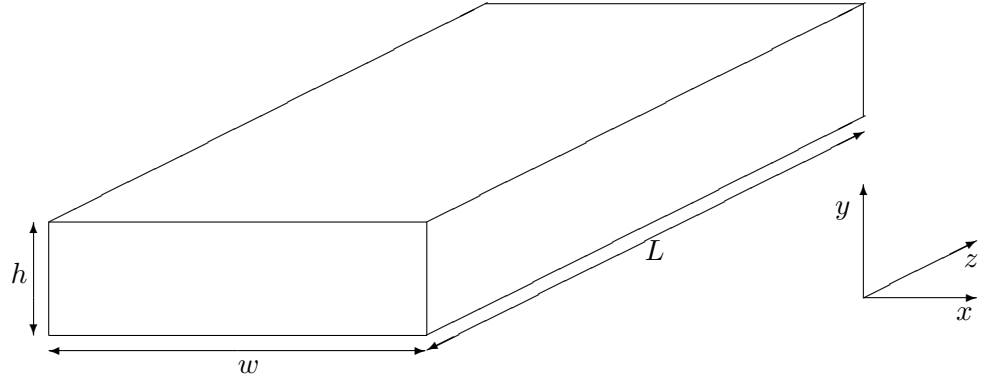


Figure 4.2: Co-ordinate system (x, y, z) and dimensions (height h , width w and length L) for calculation of flow through a rectangular channel. The pressure gradient and velocity are in the z direction.

For flow under a constant pressure drop in the the z -direction, the only non-zero component of the velocity is in the same direction and is given by

$$v_z(x, y) = \frac{4h^2 \Delta p}{\pi^3 \eta L} \sum_{\text{odd } n} \frac{1}{n^3} \left(1 - \frac{\cosh\left(\frac{n\pi x}{h}\right)}{\cosh\left(\frac{n\pi w}{2h}\right)} \right) \sin\left(\frac{n\pi y}{h}\right) \quad (4.1)$$

where Δp is the pressure drop, η is the viscosity, the origin of the x co-ordinate is the centre of the channel and the origin of the y co-ordinate is the base of the channel. (Gravity is balanced by a vertical pressure gradient, and in any case is not significant here.)

This is an exact solution of the full non-linear Navier-Stokes equations. The linear approximation for laminar flow that is often useful in microfluidic work is not necessary here. The limit to applicability of the solution comes at high Reynolds number where the smallest irregularities in pressure or surface profiles will generate turbulent flow. The height of the channels used in this thesis are nominally $50 \mu\text{m}$. Experimentally measure profiles shown in appendix B show heights closer to $60 \mu\text{m}$. At a flow through of $10 \mu\text{L min}^{-1}$, higher than any of the rates used here, this gives a Reynolds number of less than 20. Since this is well below the transition to turbulence at around 2000, the assumption that the flow will be linear is therefore well justified.

A contour of the velocity distribution is plotted in figure 4.3. Plots of velocity along horizontal and vertical line profiles through the centre of the channel are shown in figures 4.4

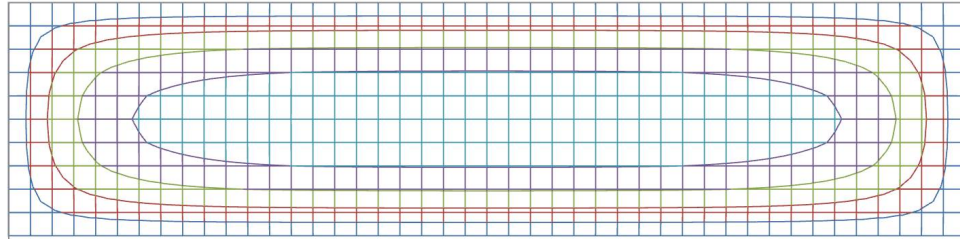


Figure 4.3: Contour plot of the velocity in the $x-y$ plane of figure 4.2 calculated from equation 4.1. Flow is in the z direction, perpendicular to the page. Flow is highest in the centre of the channel and falls off toward the edges.

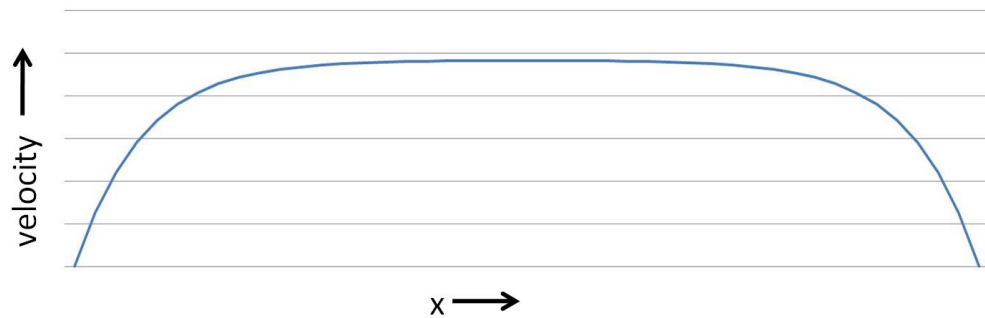


Figure 4.4: Variation of the velocity across the width of the channel along a line through the centre of the channel.

and 4.5. The no slip boundary conditions ensure that flow is much faster near the centre of the channels than near the walls. The profile across the shallow dimension of the channel, has an approximately parabolic shape, similar to that found in a circular pipe. For an infinitely wide channel this profile becomes exactly parabolic. In the wide dimension the flow profile is much flatter, and in an infinitely wide channel becomes constant.

It can be seen that the corners of rectangular channels are regions of particular stagnation, in a way not found in circular geometries. Experimentally it is observed that beads or cells that fall out of flow into the corners are far harder to wash out using high flow rates than particles that fall out of flow along the center line of channels. Clearing such particles generally requires a flow combined with sonication.

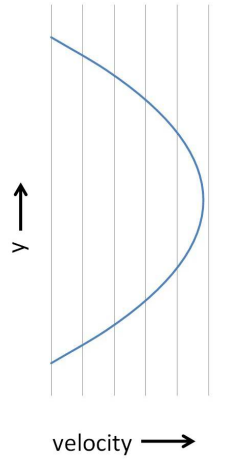


Figure 4.5: Variation of the velocity across the height of the channel along a line through the centre of the channel. Note the parabolic form.

4.1.2 Theory of hydrodynamic focusing

Focusing flows in this work are generated using syringe pumps which generate a constant flow rate. The flow into the analyte channel is set at Q_a and that into each sheath flow is set at Q_s . The total outlet flow must be $Q_o = Q_a + 2Q_s$. The width of the focused stream consisting of the current Q_a will be w_{focus} while the total flow fills the channel width $w_{channel}$. This notation is illustrated in figure 4.6.

Lee et al. [215] have performed the calculation of w_{focus} . They start from the statement that current is equal to cross sectional area multiplied by the mean velocity of fluid through that area:

$$\begin{aligned} hw_{focus}v_{focus} &= Q_a \\ hw_{channel}v_{channel} &= Q_a + 2Q_s \end{aligned} \quad (4.2)$$

where v_{focus} and $v_{channel}$ are the mean velocities in the focused flow and the total channel flow respectively. From this it follows that w_{focus} can be written

$$\frac{w_{focus}}{w_{channel}} = \frac{Q_a}{\gamma(Q_a + 2Q_s)} \quad (4.3)$$

where γ is given by the ratio of mean velocity in the focused flow to the total channel flow

$$\gamma = \frac{v_{focus}}{v_{channel}}. \quad (4.4)$$

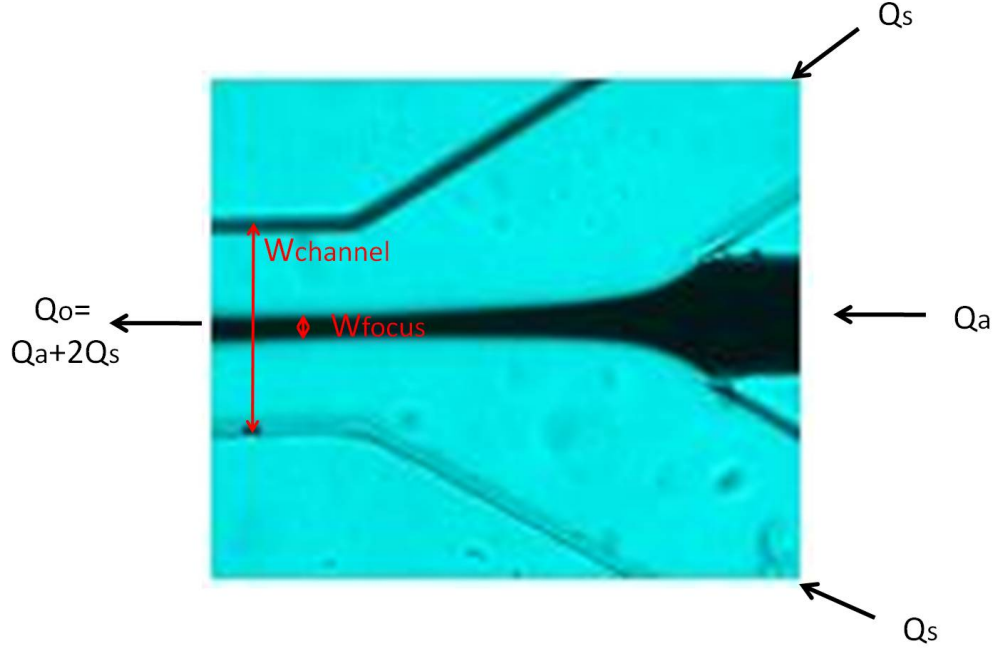


Figure 4.6: Notation used for calculation of hydrodynamic focusing. The figure shows a photograph of a dye stream entering into a PDMS device built according to the design of figure 4.1 through the central channel from the right. The dye current in is denoted Q_a . The dye stream is focused down by sheath flows from the top right and bottom right. The current entering in each of these sheath flows is denoted Q_s . A laminar flow produces no significant mixing between the sheath flow and the dye. The total current flows out to the left. This current is denoted Q_o . The width of the outlet channel is denoted $w_{channel}$. The width of the focused dye stream within the channel is denoted w_{focus} .

This can be evaluated analytically by integration of velocity profile equation 4.1 to yield the relation

$$\gamma = \frac{1 - (192h/\pi^5 w_{focus}) \sum_{n=0}^{\infty} \frac{1}{(2n+1)^5} \frac{\sinh[(2n+1)\pi w_{focus}/2h]}{\cosh[(2n+1)\pi w_{channel}/2h]}}{1 - (192h/\pi^5 w_{channel}) \sum_{n=0}^{\infty} \frac{\tanh[(2n+1)\pi w_{channel}/2h]}{(2n+1)^5}}. \quad (4.5)$$

Experimentally it is the flows Q_a and Q_s that are controlled. However, the dependence of γ on w_{focus} and $w_{channel}$ means that equation 4.5 cannot be rearranged to give an explicit expression for the width in terms of these variables.

Instead, the ratio $\beta = Q_a/Q_s$ can be expressed in terms of $w_{focus}/w_{channel}$:

$$\beta = \frac{Q_a}{Q_s} = \frac{2\gamma \frac{w_{focus}}{w_{channel}}}{1 - \gamma \frac{w_{focus}}{w_{channel}}}. \quad (4.6)$$

This expression was numerically evaluated using Octave and plotted to give a curve of β against $w_{focus}/w_{channel}$ for comparison to data. This is done later in the chapter when

discussing results in figures 4.14 and 4.15.

Further analysis along the lines developed here can be found in [216] and in [217, 218], where the effects of diffusion are also considered.

4.2 Glass devices

PDMS devices showed persistent experimental problems including leaking and accumulation of debris that blocked channels. Another key problem was poor flow of particles into the device. They would, instead, tend to drop out of flow. It was therefore decided to explore the alternative of all glass devices.

The channels in these devices followed a similar design to those shown in figure 4.1. Fabrication is described in appendix B

As can be seen from data in the appendix, if glass devices were to be pursued it would be necessary to create smoother channels. This might be achieved by, for example, etching laser ablated channels to remove roughness, by using etching to create the channels in the first place or another technique such as sand blasting.

However, for this work it was possible to overcome the problems shown by PDMS devices by more careful fabrication and design, so glass devices proved unnecessary. Further development of the PDMS designs are detailed below.

4.3 Introduction of beads and cells into the microfluidic device

In the initial design particles were introduced into the microfluidic device by first loading them into a Hamilton syringe. The syringe was then interfaced to a connecting teflon tubing with 1/16" outer diameter using an adaptor and nut. The other end of the connecting tubing was then interfaced to 1/16" outer diameter tubing sealed into the microfluidic device with epoxy. This arrangement is illustrated in figure 4.7

The syringe was placed on a syringe pump and particles pumped through the connecting tube into the microfluidic device. This arrangement had several shortcomings.

A first problem was that particles would settle in the syringe, falling out of suspension. This

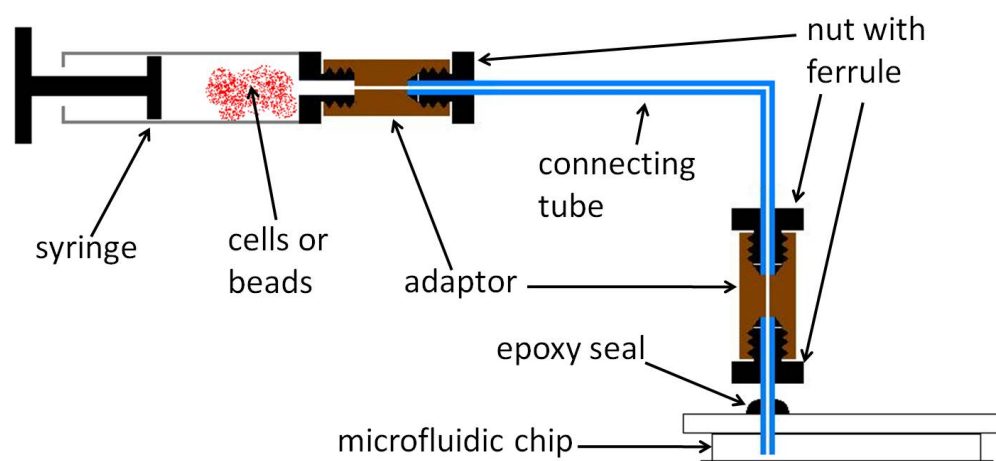


Figure 4.7: First interface for introduction of particles to the analyte inlet.

limited the amount of time for which they could be held in the syringe before pumping, or the time for which particles could be pumped to around 10 minutes.

A more serious problem for small samples of a few thousand particles, for which the device is ultimately designed, is the length of the connecting tube which was at least 20 cm long. Particles that are loaded into the syringe have to enter and traverse the entire length of the syringe before entering into the microfluidic. This might have been reduced by an elaborate mounting of the pump directly above the microfluidic, but this would be inconvenient to construct and to operate.

This design also requires a relatively large reservoir in the microfluidic chip. This resulted in particles dropping out of flow. This is illustrated in figure 4.8. The hole punched to accommodate the teflon tubing at the inlet was around 2 mm diameter, and could only be reduced to 1.6 mm before a smaller diameter tubing would be needed. This large hole significantly slowed the flow in the region between the end of the tubing and the entrance to the microfluidic channel.

Two changes were made to create a device shown schematically on the right hand side of figure 4.8. First, the inlet hole was scaled down to accommodate a capillary. The best technique for punching a hole was found to be use of a hollow needle, sawn off and sharpened with a file.

This produced an improvement, but the large circular reservoir in the PDMS design still produced considerable settling. The next step was therefore the elimination of the analyte reservoir. A new photomask and master were made up according the design shown in figure 4.9. The inlet hole now has a diameter comparable to that of the channel. The needle could

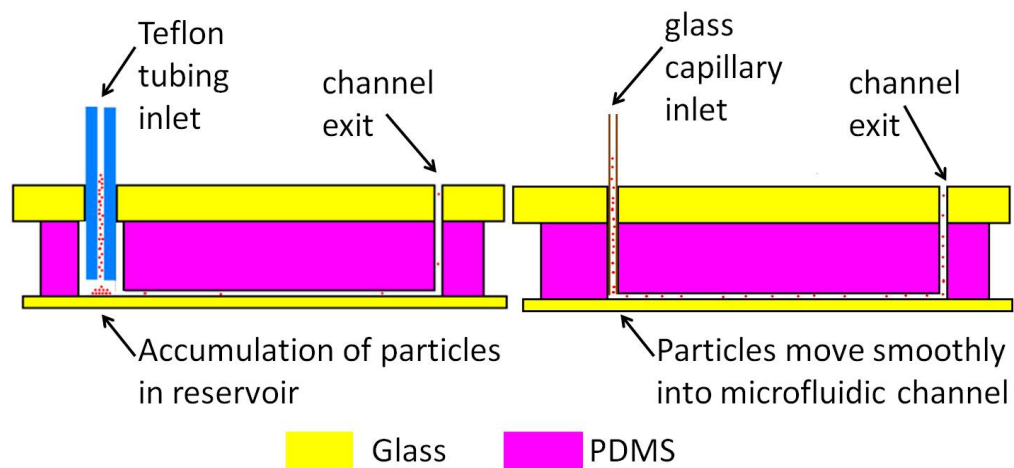


Figure 4.8: Accumulation of particles in PDMS reservoir.

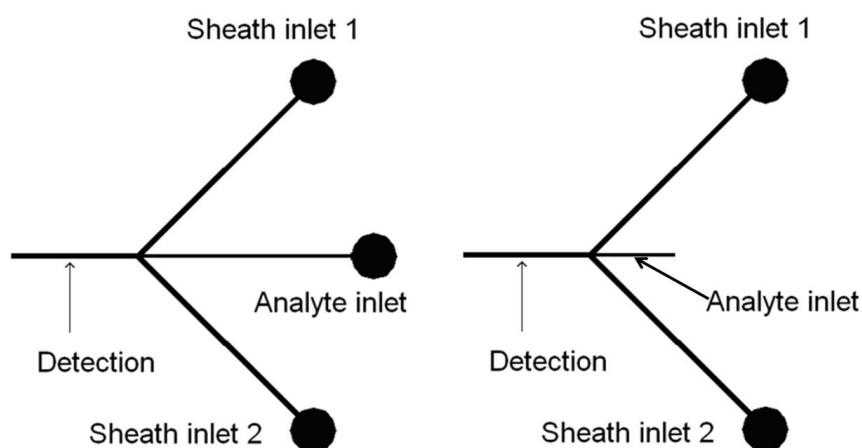


Figure 4.9: Modification of the inlet reservoir. The inlet can be punched anywhere in the inlet channel, typically about a millimeter upstream of the focusing junction.

easily be aligned into the channel by gently sliding it over the surface of the PDMS until it found the channel groove.

This produced holes that could nicely accommodate a capillary of outer diameter $360\text{ }\mu\text{m}$. However, simply inserting the capillary into the hole would not produce a leak tight joint. That would require sealing the capillary in place with, for example, epoxy. Instead a pipette tip (VWR 89041-382, UK) was inserted into the hole and sealed with epoxy. This acted as a sheath which allowed the capillary to be reversibly inserted. A second pipette tip sealed to the capillary then pushed into the first capillary forming a leak tight joint.

This allowed a capillary to be loaded with particles and the tip to then be directly inserted in to the entrance of a microfluidic channel.

4.3.1 Pumping

Pumping was carried out using KDS200 syringe pumps (KDS scientific, KDS Scientific (Holliston, MA, USA). Each of these pumps is capable of holding two syringes. A first pump was used to drive two Hamilton syringes (Sigma), one for each sheath flow. A second pump was used to drive a single Hamilton syringe (Sigma) for introducing analyte.

The syringes had luer lock tips which were used to interface them with PTFE tubing (Anachem, Luton, UK) using finger tight fittings from Upchurch Scientific (Anachem, Luton, UK).

4.4 Experimental results for focusing

Images of a stream of dye focused in a microfluidic channel can be seen in figure 4.10. These show the position of dye by absorption of light from bright field illumination. These images were taken with the device mounted on a TE2000 (Nikon, Japan) inverted microscope using a x10, 0.3NA objective (Nikon, Japan). The images were captured using a CCD camera (C7780-20, Hamamatsu, Japan).

The dye used was red food colouring (Tesco), the active pigments being azorubine (E122) and sunset yellow FCF (E110). Increased contrast was gained by using a FITC filter cube (Nikon, Japan). The filter cube transmits only wavelengths between 515 and 555 nm, which are strongly absorbed by the dye. Bright field illumination by white light then produces good contrast with the dye showing no transmitted light.

Images such as those in figure 4.10 were analysed by taking a series of cross sections across the channel. Figure 4.11 shows the lines indicated by 'cross section' and 'flow axis'.

A representative example is shown in figure 4.12. Here the central peak indicates absorption by the focused dye, and its width gives the width of focused dye.

Profiles such as those in figure 4.12 are taken at each point down the flow axis, showing how the focused width varies with length. The result of such a measurement is shown in figure 4.13. For each set of flow conditions, the minimum width in this profile was identified.

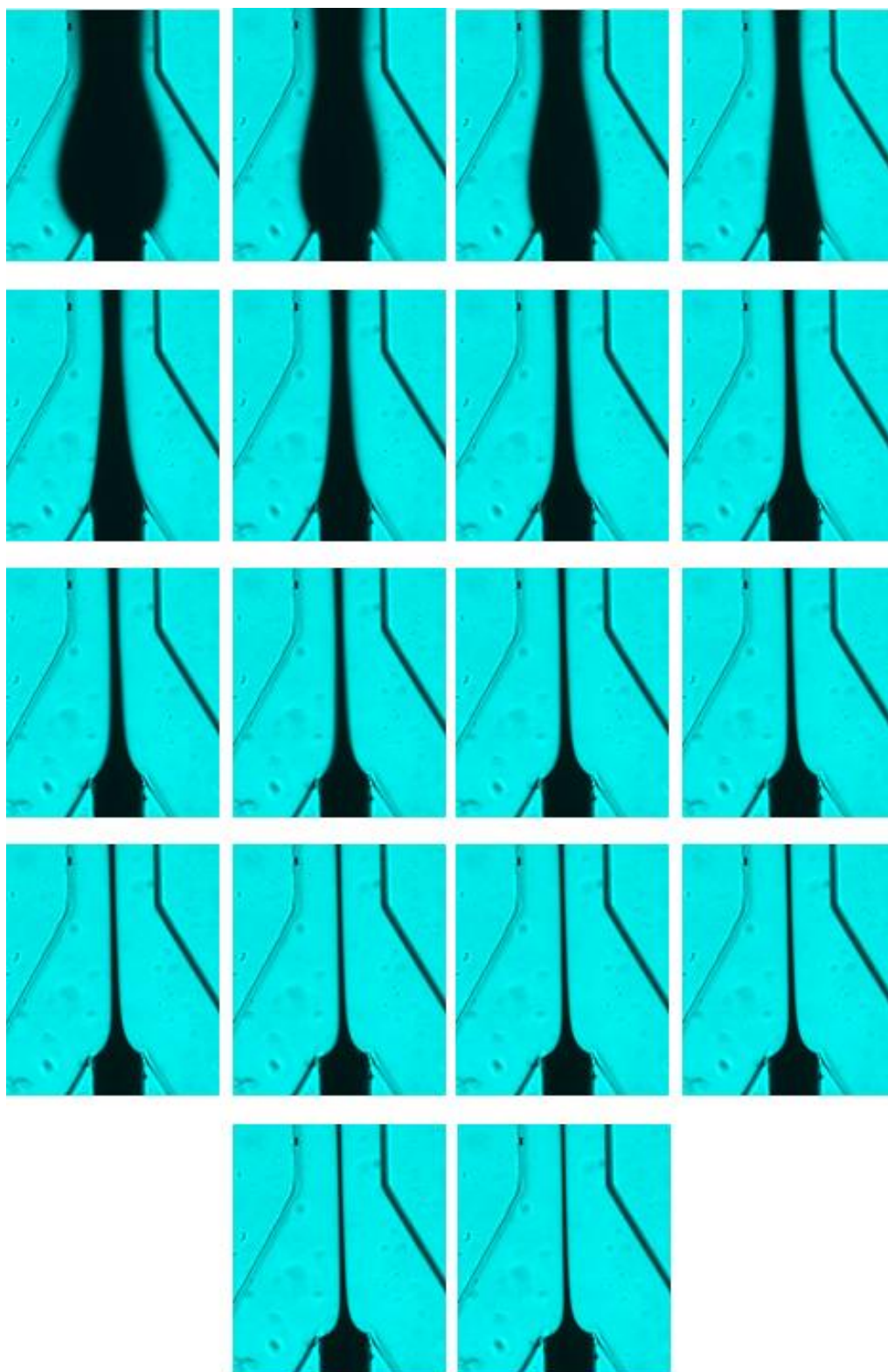


Figure 4.10: Images of hydrodynamic focusing with dye flow at $0.5 \mu\text{l} / \text{min}$ and sheath flows of 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 25, 30, 35, 40, 45 and to $50 \mu\text{l} / \text{min}$, left to right and top to bottom.

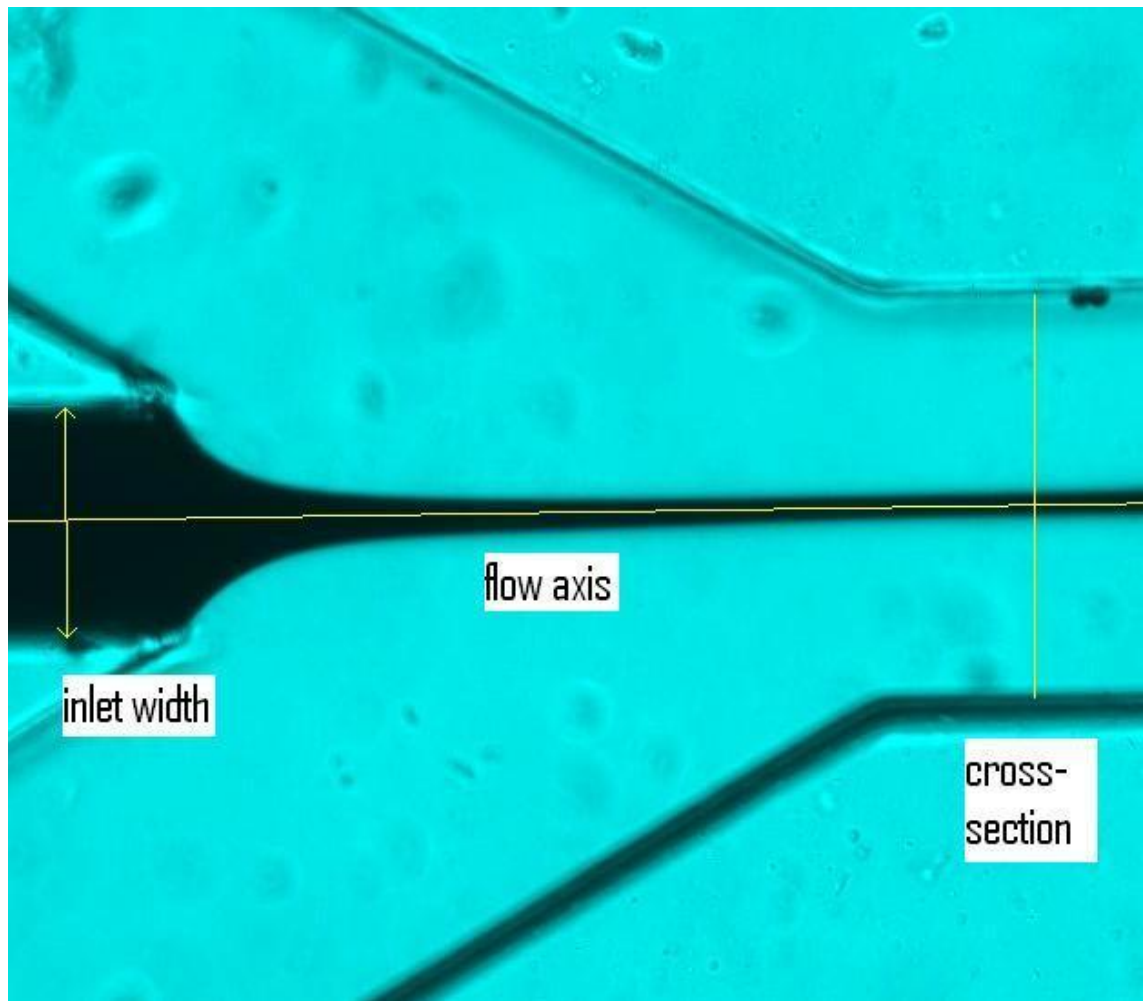


Figure 4.11: The measures used to analyse images of flow in the microfluidic.

This data is used to produce a curve showing the variation of focused width with flow condition. Figure 4.14 shows a comparison of theory with measurements. Agreement is qualitatively correct and is reasonable considering that the theory contains no fitable parameters. Widths are consistently higher than theory predicts. Because the excess width increased with both total flow rate and with the ratio β I hypothesised that this was due to diffusion currents.

I therefore repeated the measurements using $0.1\ \mu\text{m}$ fluorescent beads as flow tracers. Since their radius is around 100 times as large the diffusion co-efficient should be around 100 times smaller. The distance travelled will be around 10 times less for the same time.

The reduced impact of diffusion could be seen in the uniform cross section of the focused flow.

The data for focused width versus β shown in figure 4.15. It can be seen that the deviation

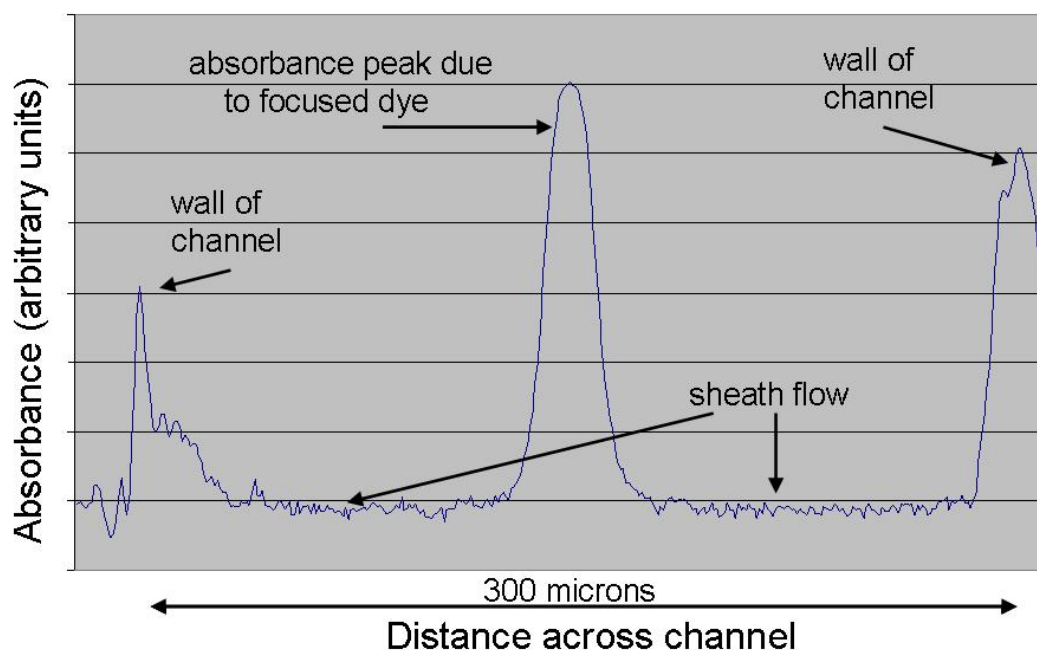


Figure 4.12: Absorption of light for a flow of $0.25\mu\text{l}/\text{min}$ and a sheath flow of $12\mu\text{l}/\text{min}$ where distance across the channel is across the cross section indicated in figure 4.11.

from the theoretical curve is not significantly decreased from the case of dye absorption. This suggests that although the effect of diffusion can be seen in the curve of figure 4.13, diffusion is not responsible for the deviation from theory.

The results from figures 4.14 and 4.15 were clearly qualitatively replicated when both $10\mu\text{m}$ beads and cells were used in the place of dye. It is expected that particles will follow flow lines in these circumstances. In the results below focusing is sufficient to put all fluorescent particles through the laser focus.

The channel width of $200\mu\text{m}$ is fairly wide relative to a single cell. However, a wider channel enables higher sheath flow rates which, in turn, produces smoother flow. Furthermore, narrowing the channel would not address the primary limitation on focussing quality, namely lack of focusing in the y direction. The above results show that the $200\mu\text{m}$ is adequate for counting cells.

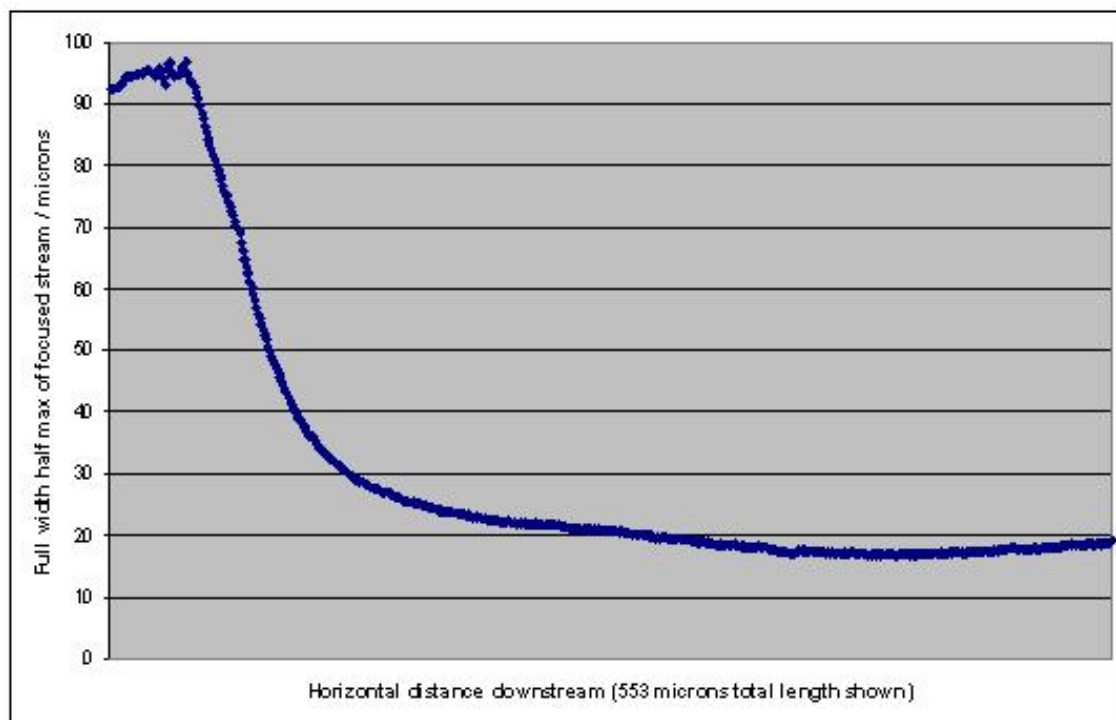


Figure 4.13: Width of focused stream defined as the full width half maximum of the peak in absorbance (as seen in figure 4.12) vs. length along flow axis (defined in figure 4.11) for a flow of $0.25\mu\text{l}/\text{min}$ and a sheath flow of $12\mu\text{l}/\text{min}$.

4.5 Correction of the amplifier time constant

The signal from the PMT was amplified using a circuit previously constructed by Chris Barnett. The amplified signal was then recorded by a PC.

Initial results were encouraging, showing a detection of peaks in the PMT output corresponding to the passage of fluorescently labelled cells. The peak shape showed a sharp rise followed by a slow decay. Averaging of many peaks, centered on the signal maximum, revealed this as an exponential decay, as shown in figure 4.16. The fitted time constant of 19 ms was traced to capacitance in the amplifier circuit. While such capacitance can be useful for averaging of noise, for the present purposes a faster response was required. A resistance was wired in parallel with the amplifier to reduce the time constant.

After correction the limit to the rate at which cells could be detected was set by the rate at which LabVIEW can process data. For the standard edition of LabVIEW used in this work, this is 1 ms. This is demonstrated in figure 4.17, which shows how the average peak width decreases with higher flow rates.

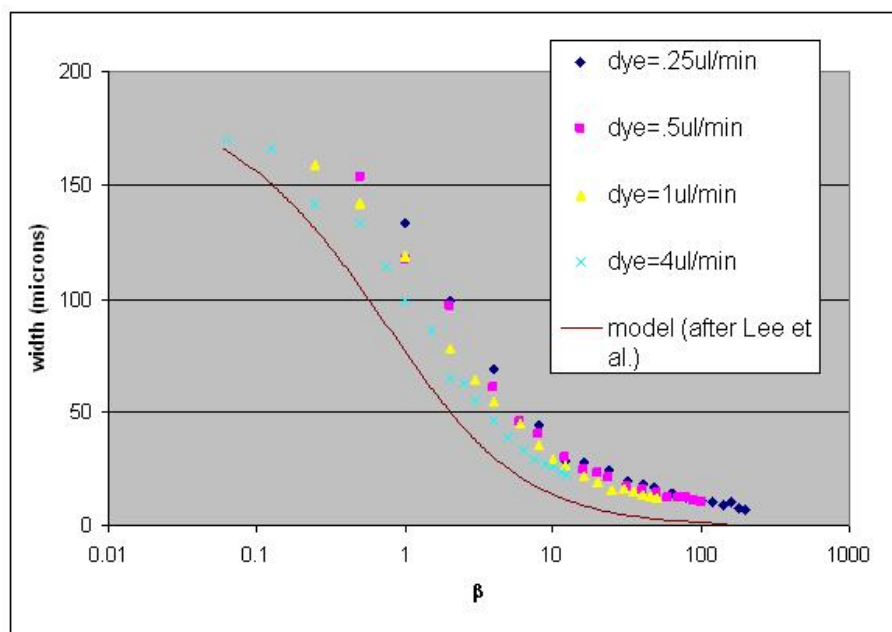


Figure 4.14: Variation of width of focused dye analyte stream (vertical axis) with ratio of sheath flow rate to analyte flow rate (horizontal axis)

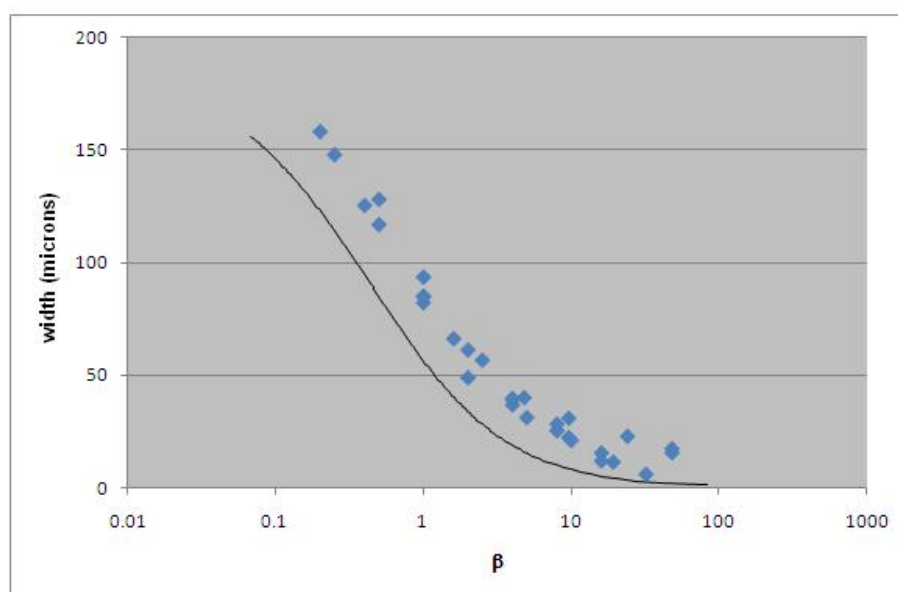


Figure 4.15: Variation of width of focused bead flow (vertical axis) with ratio of sheath flow rate to analyte flow rate (horizontal axis)

Average peak width should be inversely proportional to flow velocity which, in turn is proportional to total flow rate. This behaviour is shown in the linear portion of figure 4.17. At higher flow rates the average peak width is less than one millisecond, and it can be seen that

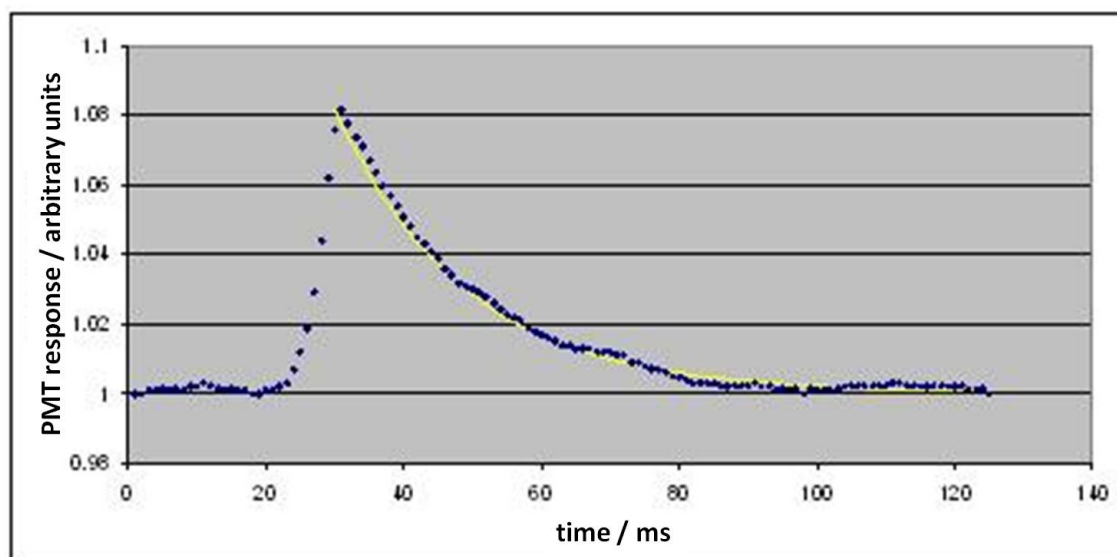


Figure 4.16: Amplifier time constant. The blue squares show the average signal from 224 cells detected in flow. The yellow curve shows an exponential fit to the decay, with time constant 19 ms.

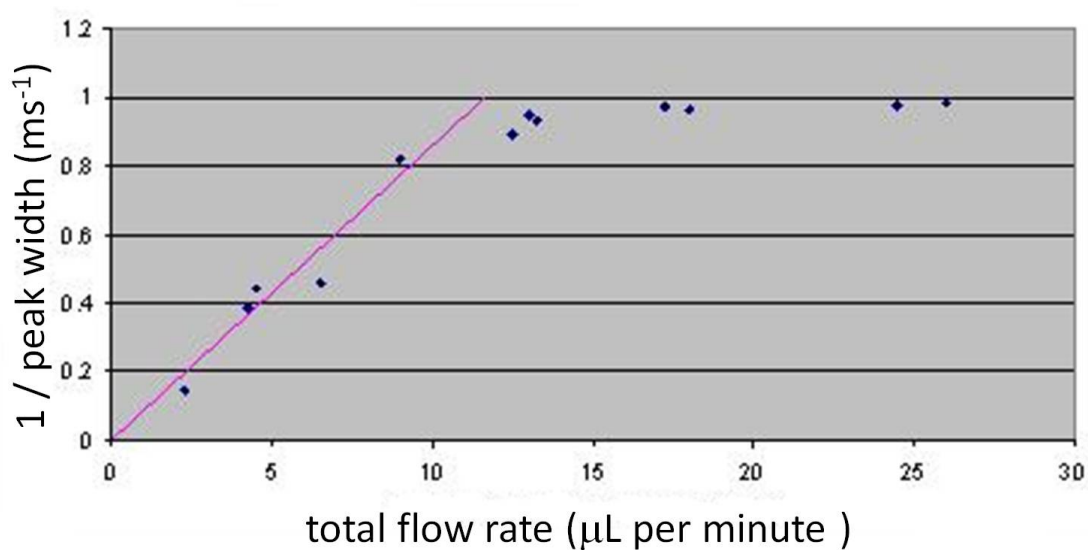


Figure 4.17: Variation of inverse peak width with flow rate. At low flow rates inverse peak width is proportional to total flow rates. At high flow rate widths are too short to measure, so the linear variation breaks off.

the inverse peak width saturates. Widths shorter than one millisecond cannot be measured.

LabVIEW Realtime software, capable of faster processing is available and could easily be implemented. However, as will be seen in the next chapter, 1,000 cells per second is considerably faster than cells can be switched. For a sorting system this is therefore not yet the rate limiting

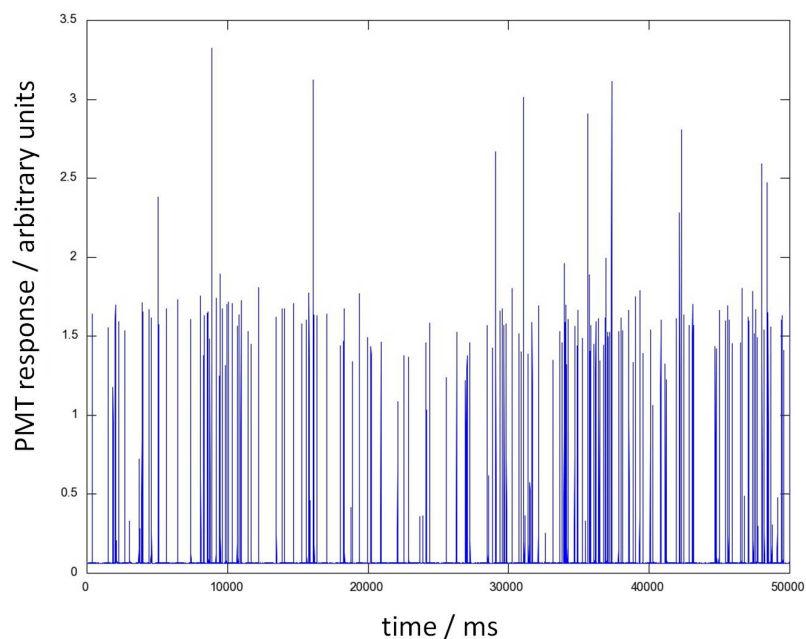


Figure 4.18: The PMT response for measurement of fluorescent beads in flow excited at 530 nm. Each peak corresponds to a bead passing through the laser focus. The flow rate is $0.5\mu\text{L}$ per minute of bead solution at a concentration of 2000 beads per μL and the sheath flow rate is $10\mu\text{L}$ per minute. The sampling rate of the PMT signal is 800 Hz . The threshold is 0.18 , resulting in 1035 peaks.

step.

4.6 Cytometry of beads

It was then possible to carry out cytometry of fluorescent beads. Representative results are shown in figures 4.18–4.21. The signal from the PMT was simply thresholded with a single ‘peak’ called in every interval between which the signal rose above and then fell below threshold.

Dye loaded beads gave a very strong signal. Filters were placed in the optical path to reduce the power in the beam from 5 mW to 0.2 mW . To avoid saturation of the signal the voltage applied to the photomultiplier was reduced from 1 kV to 700 V . (It would have been more optimal to apply another filter to the excitation, but in these circumstances reducing the voltage did not create difficulties.) It can be seen from figure 4.21 that under these conditions the noise is around 0.002 units while the signal can be seen from figure 4.19 to be greater than 0.1 unit, giving a signal to noise ratio of 50 .

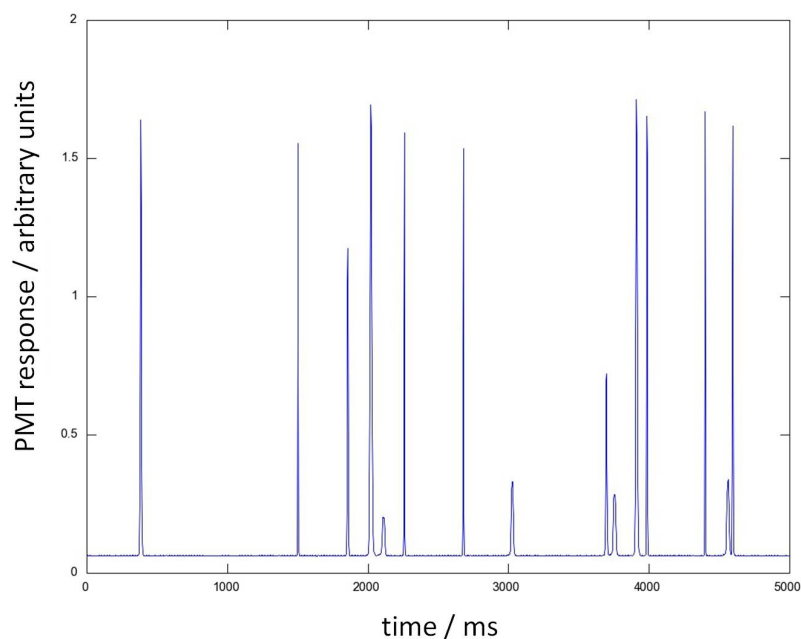


Figure 4.19: The peaks shown here are a close up of the first 5s of figure 4.18, showing that peaks are well distinguished at baseline. The PMT response for measurement of fluorescent beads in flow excited at 530 nm. Each peak corresponds to a bead passing through the laser focus. The flow rate is $0.5\mu\text{L}$ per minute of bead solution at a concentration of 2000 beads per μL and the sheath flow rate is $10\mu\text{L}$ per minute. The sampling rate of the PMT signal is 800 Hz. The threshold is 0.18, resulting in 1035 peaks.

The relatively high threshold of 0.18 was adopted not due to noise but rather so that peaks that were close to one another could more accurately be resolved.

4.6.1 Width distribution of fluorescent bursts

It can be seen in figure 4.19 that the widths of the peaks from beads show a significant spread. The peak shape will be determined by a convolution of the illumination intensity and the dye distribution. But assuming that this is constant for all beads the width should be inversely proportional to velocity.

This can be modelled by assuming that the spread arises from lack of hydrodynamic focusing in the vertical y direction.

Start by assuming that beads have a uniform distribution in y . Because the rate at which particles arrive at the laser focus is proportional to their velocity, the probability that a given bead observed at the laser focus has a height y is then not uniformly distributed. A parabolic

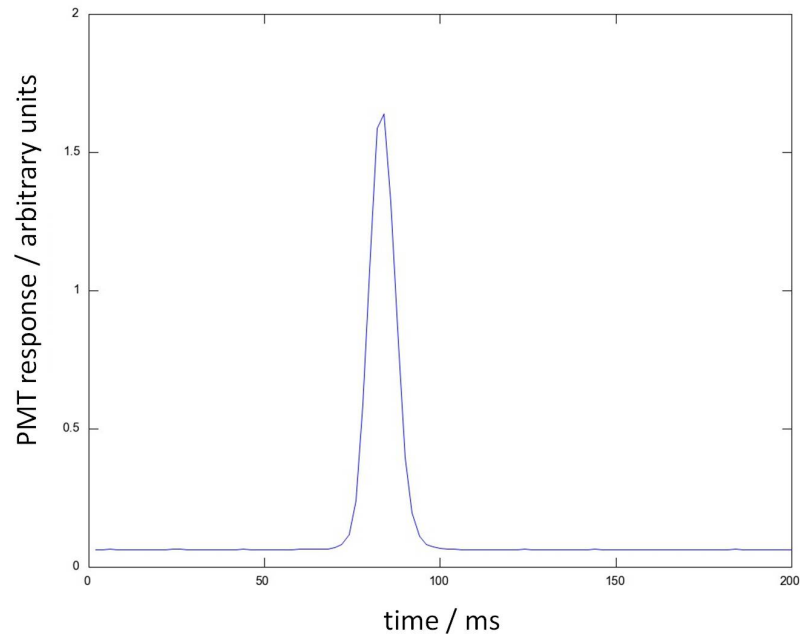


Figure 4.20: The PMT response for measurement of beads in flow. The peak shown here is a close up of the first peak in figure 4.19, showing a symmetric shape. The PMT response for measurement of fluorescent beads in flow excited at 530 nm. Each peak corresponds to a bead passing through the laser focus. The flow rate is $0.5\mu\text{L}$ per minute of bead solution at a concentration of 2000 beads per μL and the sheath flow rate is $10\mu\text{L}$ per minute. The sampling rate of the PMT signal is 800 Hz. The threshold is 0.18, resulting in 1035 peaks.

velocity distribution in y is an accurate approximation

$$v = 1 - y^2 \quad (4.7)$$

where the velocity v has been non-dimensionalised using the maximum velocity attained at the centre of the channel, v_{max} and y has been non-dimensionalised using half the channel height. The probability density function for an observed bead to be at height y is then just proportional to this velocity

$$P(y) = \frac{3}{2}(1 - y^2) \quad (4.8)$$

where the factor $\frac{3}{2}$ normalises the distribution to correspond to a single bead. The width, measured in time, of a fluorescent burst is proportional to the length of the laser focus and the inverse of the velocity:

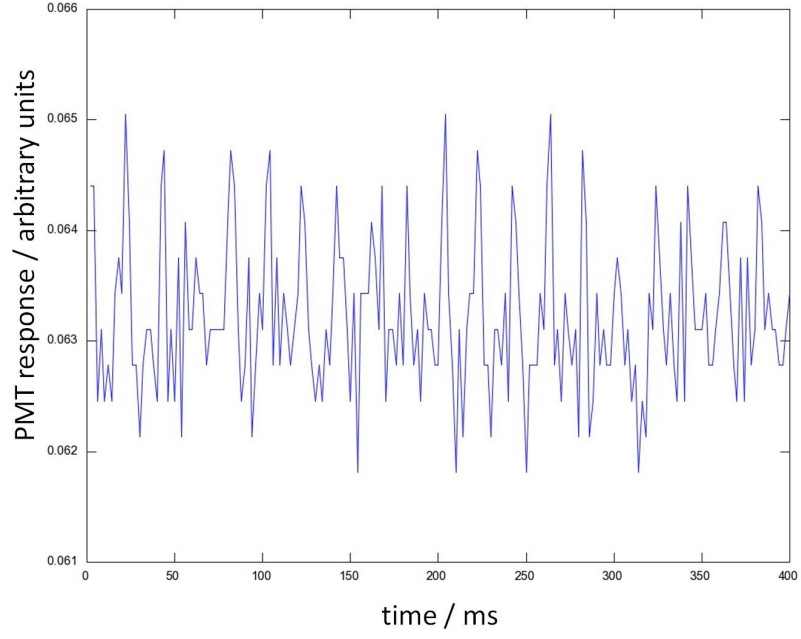


Figure 4.21: Noise present in the PMT signal sampled at 800 Hz in the presence of laser excitation but absence of beads. This is the control baseline noise for the conditions shown in figures 4.18–4.20.

$$w = \frac{1}{v} = \frac{1}{1 - y^2}. \quad (4.9)$$

Here w has been non-dimensionalised using v_{max}/ℓ , where ℓ is the length of the laser focus.

The probability distribution of w is then given by

$$P(w) = \left| \frac{dy}{dw} \right| P(y) \quad (4.10)$$

so that using

$$\left| \frac{dy}{dw} \right| = \frac{1}{2} \frac{1}{w^2} \left(1 - \frac{1}{w} \right)^{-\frac{1}{2}} \quad (4.11)$$

and

$$P(y) = \frac{3}{2} (1 - y^2) = \frac{3}{2} \frac{1}{w} \quad (4.12)$$

gives

$$P(w) = \frac{3}{4} \frac{1}{w^3} \left(1 - \frac{1}{w} \right)^{-\frac{1}{2}}. \quad (4.13)$$

For the comparison to experimental data the total number of observed particles is fixed

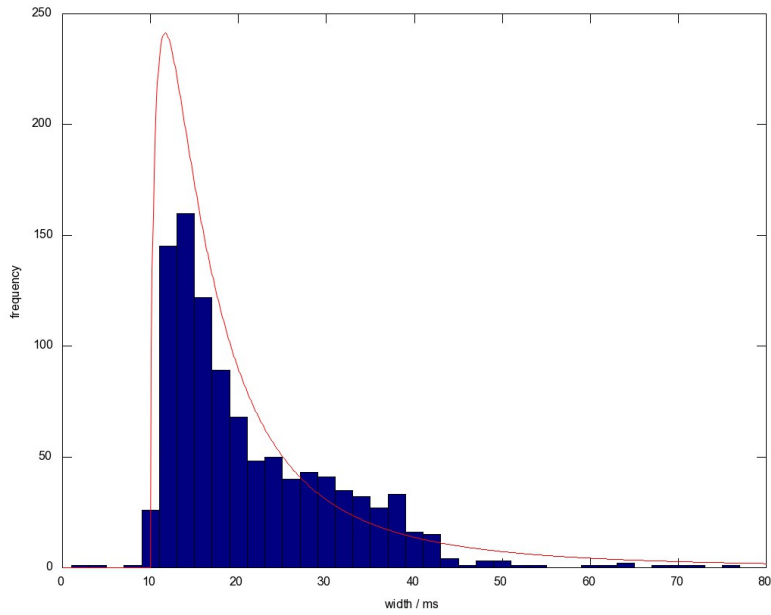


Figure 4.22: Full width half maximum of peaks detected by PMT for beads in flow. The threshold is 0.08, resulting in 1014 peaks and a fitted parameter of 5.00. The PMT response for measurement of fluorescent beads in flow excited at 530 nm. Each peak corresponds to a bead passing through the laser focus. The flow rate is $0.5\mu\text{L}$ per minute of bead solution at a concentration of 2000 beads per μL and the sheath flow rate is $10\mu\text{L}$ per minute. The sampling rate of the PMT signal is 800 Hz.

through normalisation of the probability distribution to observed number of particles, n . A single free parameter v_{max}/ℓ , which corresponds simply to scaling of the w axis, is fitted to binned experimental data.

The result of fitting equation 4.13 to a run of $n = 1014$ peaks is shown in figure 4.23. The qualitative fit is reasonable (a perfect fit would pass through the mid-point of the top of each histogram bin). The low number of points in the high w tail is likely due to a data set that is too small for collection of enough rare events.

4.7 Cytometry of cells

Measurements similar to those on beads were then performed on cells. BE cells, an adherent colon cancer cell line, were cultured, stained and introduced into the microfluidic device for counting.

BE cells were detached with trypsin which was neutralised with cell media. They were then

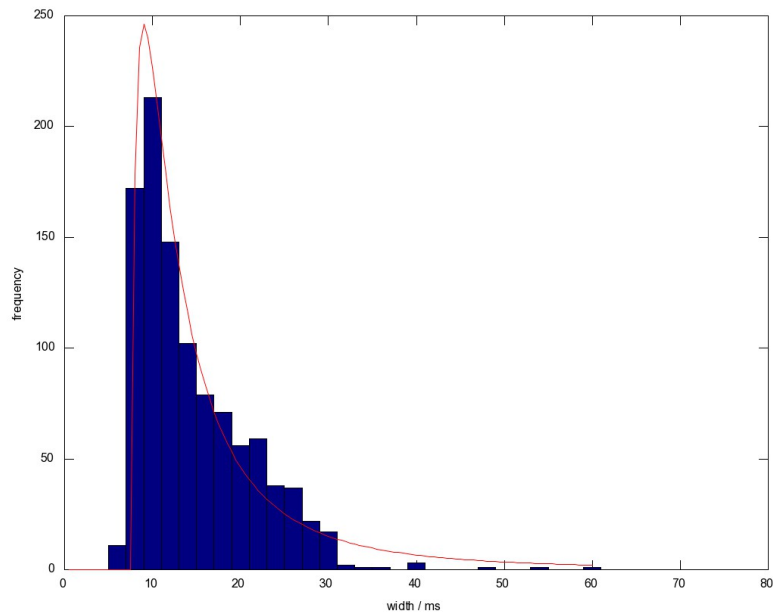


Figure 4.23: Full width half maximum of peaks detected by PMT for beads in flow. The threshold is 0.18, resulting in 1035 peaks and a fitted parameter of 7.70. The PMT response for measurement of fluorescent beads in flow excited at 530 nm. Each peak corresponds to a bead passing through the laser focus. The flow rate is $0.5\mu\text{L}$ per minute of bead solution at a concentration of 2000 beads per μL and the sheath flow rate is $10\mu\text{L}$ per minute. The sampling rate of the PMT signal is 800 Hz.

resuspended in PBS at a concentration of 1.5×10^6 cells / ml. They were then stained with Wheat Germ Agglutinin 555 (Invitrogen, UK) for 10 minutes at 37°C . Cells were then washed three times by centrifuging for 5 minutes at 600g and resuspending in PBS. This removed excess dye from the background solution.

Cells were resuspended at a concentration of 500 cells per microlitre. Around 2 microlitres were loaded into a capillary and inserted into the analyte inlet of the microfluidic device. They were detected in the same way as for beads.

A typical PMT trace showing peaks with detected cells can be seen figures 4.24–4.26. As is expected, the signal to noise ratio is considerably lower than for beads. The threshold was manually selected at 0.03 above the mean value of the signal in the absence of signal. The standard deviation of signal in the absence of signal is 0.005. This gives a signal to noise ratio of 6 for detection of the weakest cell signals.

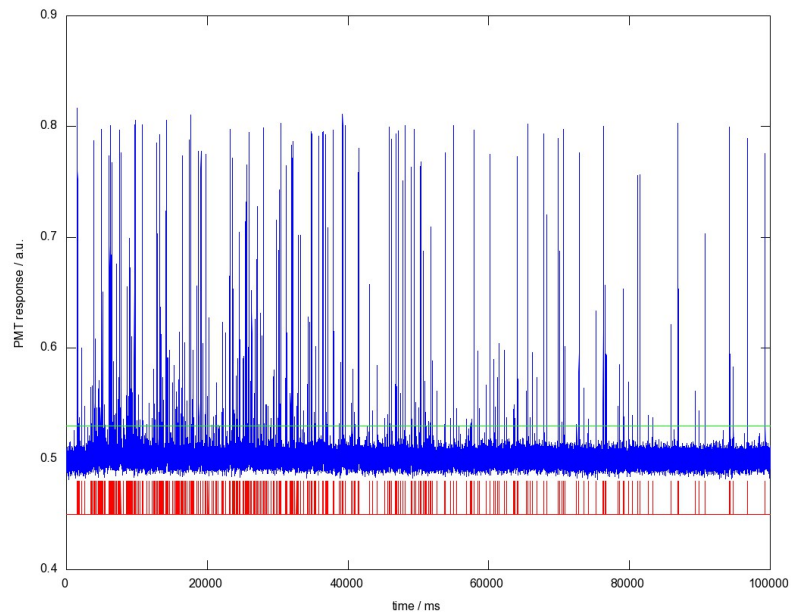


Figure 4.24: The PMT response for measurement of stained cells in flow through the focus of the laser beam (blue). The threshold of 0.53 is shown (green). Each peak detected above threshold is as a peak below the signal (red). The flow rate is $0.5\mu\text{L}$ per minute of bead solution at a concentration of 2000 beads per μL and the sheath flow rate is $10\mu\text{L}$ per minute. The PMT was sampled at a frequency of 800 Hz.

4.8 Poisson statistics cells

If particles are flowing through the device at a stationary random rate, independent of one another, then they should follow Poisson statistics. This results in number of testable features: the number of beads arriving in a set time interval should follow a Poisson distribution, the intervals between beads should follow an exponential distribution, and the ratio of the variance to the mean (the Fano factor) of the number of beads arriving in a fixed interval should be equal to one.

It is clear from figure 4.24 that the rate at which cells are detected falls over time. This is shown more quantitatively in figure 4.27. Here three quantities are plotted.

The mean arrival time over 250 milliseconds was calculated and this is plotted as a running average in the blue curve. The variance in arrival time over 250 milliseconds was also calculated and plotted in the red curve. The ratio of these two quantities is known as the Fano factor and is plotted as the green curve.

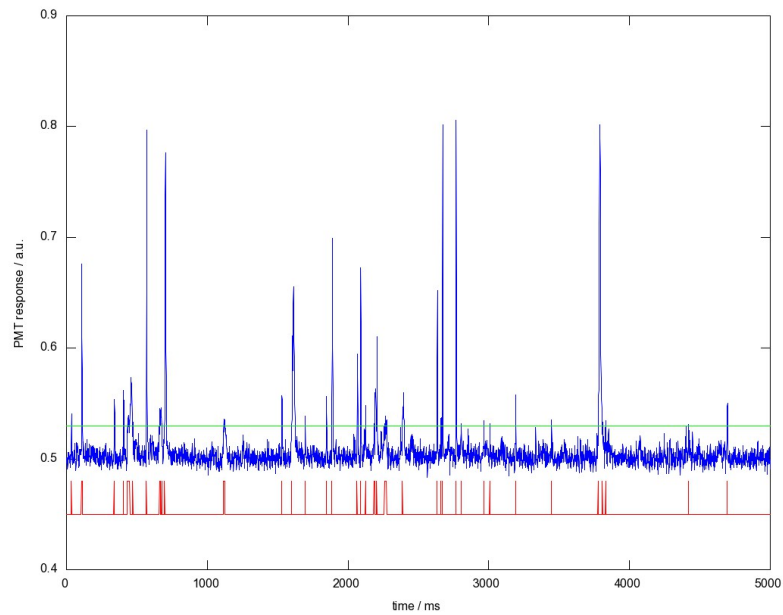


Figure 4.25: Close up of 5 seconds of figure 4.24. The PMT response for measurement of stained cells in flow through the focus of the laser beam (blue). The threshold of 0.53 is shown (green). Each peak detected above threshold is as a peak below the signal (red). The flow rate is $0.5\mu\text{L}$ per minute of bead solution at a concentration of 2000 beads per μL and the sheath flow rate is $10\mu\text{L}$ per minute. The PMT was sampled at a frequency of 800 Hz.

For a Poisson process the Fano factor is equal to one. The fact that the Fano factor in figure 4.27 oscillates around 1 shows that detection of cells is ‘locally Poissonian’, i.e. while there is an overall slow down in the number of cells detected events are not ‘clumping’ and there are not unusually long breaks between cells.

The total count of cells in this run was 409, giving a recovery rate of approximately 40%. Analyte was introduced at a rate of 1 microlitre a minute with few cells appearing after three minutes (as is apparent from figure 4.27). Most cells that were detected were therefore detected in the initial volume in which they were suspended. The remaining cells were most likely lost in the capillary used to introduce them into the microfluidic by sticking to the walls.

4.9 Discussion and further work

The system has demonstrated an ability to perform flow cytometry in a microfluidic format.

The two main limitations are non-uniform velocity distributions and the loss of sample.

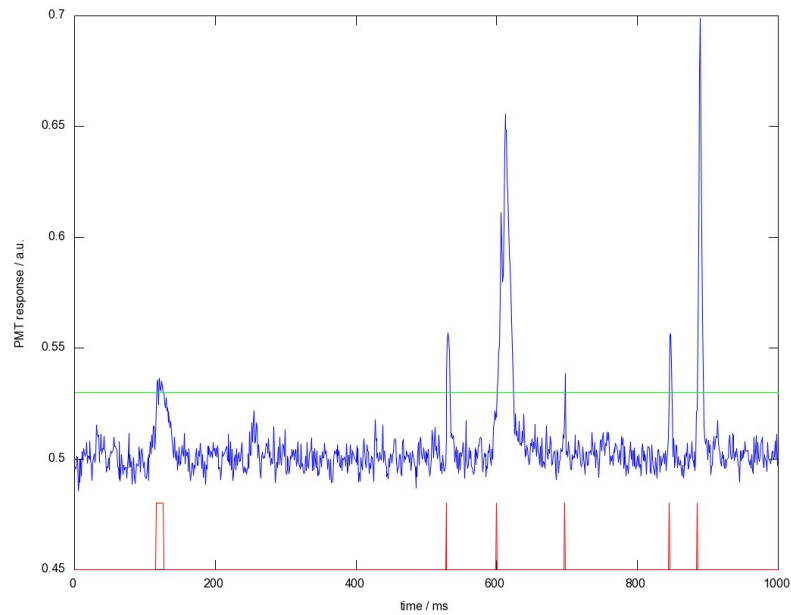


Figure 4.26: Close up of the first second of figure 4.25. The PMT response for measurement of stained cells in flow through the focus of the laser beam (blue). The threshold of 0.53 is shown (green). Each peak detected above threshold is as a peak below the signal (red). The flow rate is $0.5\mu\text{L}$ per minute of bead solution at a concentration of 2000 beads per μL and the sheath flow rate is $10\mu\text{L}$ per minute. The PMT was sampled at a frequency of 800 Hz.

The problem of non-uniform velocity distributions is a particular problem when it is desirable to quantitate the amount of staining rather than just to threshold above a low minimum. Even for thresholding, a uniform distribution will give more consistent results. This is desirable for cytometry of clinical samples stained with EpCAM.

Achieving uniform signals may require a sheath flow in both dimensions perpendicular to the flow. As has been mentioned, such devices have been made, but require more complex fabrication methods than are used here. [211–213]

The second limitation, loss of cells due to sticking to the walls is a particular problem for a device that aims to analyse small numbers of cells. Losses of hundreds of cells will often be acceptable for applications in which tens of thousands or more cells are analysed. For clinical samples consisting of a thousand cells, and many fewer cells of interest, it is a more severe limitation.

The sticking of cells to the capillary wall which was responsible for the low recovery rate was confirmed by placing a capillary containing cells under a microscope and observing the flow.

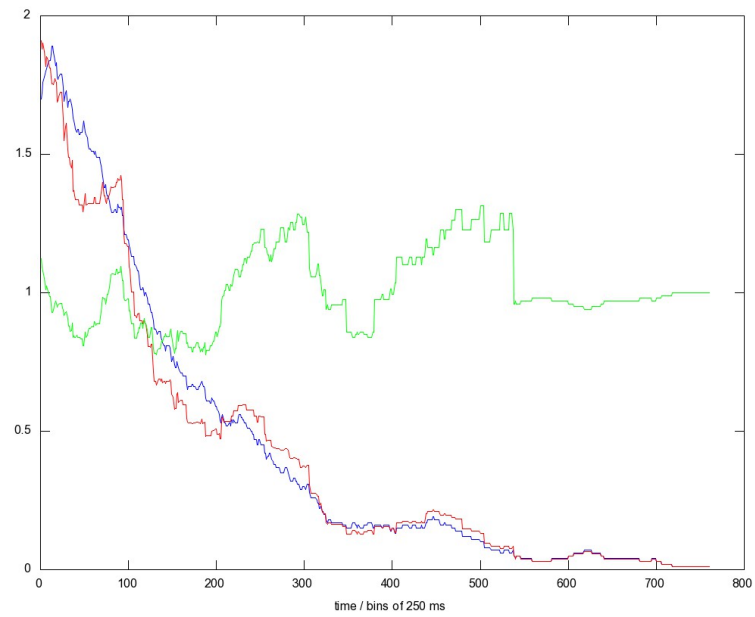


Figure 4.27: Mean interarrival time during 250 ms periods (blue curve, vertical axis milliseconds), variance of interarrival time during 250 ms periods (red curve, vertical axis square milliseconds), Fano factor for 250 ms periods (green curve, vertical axis milliseconds)

It is possible that sticking could be much reduced by preblocking or coating the inside of the capillary. However, sticking of BE cells used for these cytometry experiments is likely related to the specific adherent cell line used.

Optimisation of blocking should probably be carried out in samples that more closely approximate clinical samples.

Chapter 5

Microfluidic Cell Sorting

5.1 Design and characterisation

The initial plan for sorting was based the idea of switching fluid flows based on pressure changes. The layout of the chip is shown in 5.1 based on the design of Bang et al. [219]. The right hand portion of the figure shows the focusing stage discussed in the previous chapter. The left hand portion, boxed in red, shows the sorting stage discussed here. The final assembled device is shown later in figure 5.7.

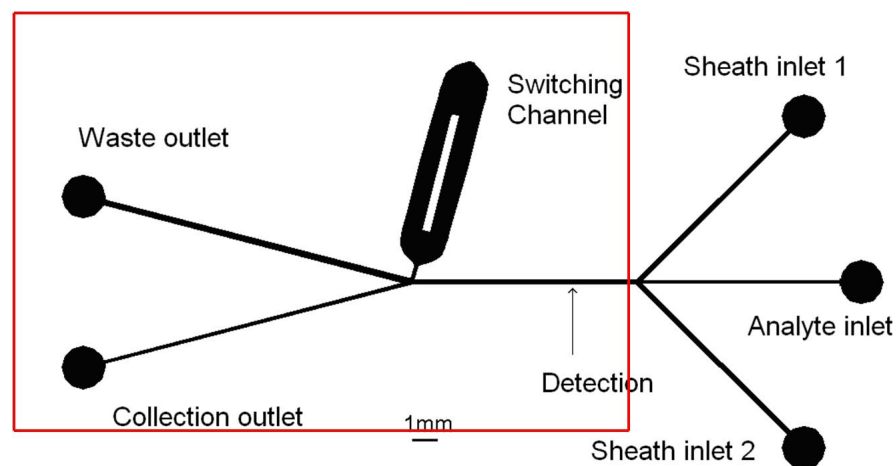


Figure 5.1: Initial design of microfluidic sorting stage. The device is as shown in figure 4.1. In this chapter the section in the red box to the left are used for sorting. The purple spot shows where the microfluidic is interfaced with a solenoid valve.

The principle of operation is that cells flowing past the detection stage reach a junction with

two possible exits, either the waste outlet or the collection outlet. The flow is biased so that in the absence of switching cells flow to the waste outlet. Switching is triggered by detection of a fluorescently labelled cell. On switching the labelled cell is diverted from the waste outlet to the collection outlet.

In the initial design, switching works by opening a valve which pressurises the switching channel. A parcel of fluid flows out of the switching channel diverting a cell from the waste outlet to the collection outlet. This mode of operation was later modified, as discussed in 5.4.

In the initial design pressure to the switching channel was provided by a gas cylinder. The pressure was found to be too high, and was replaced by a gravity siphon. While this provided greater control over pressure, flows were found to be unstable. It was found that use of negative pressure to suck particles into the switching channel, and collect them there, was more stable.

These developments and initial results of sorting are reported in this chapter. It concludes with a characterisation of instability in the switching flow which could form the basis for further development of the device.

5.2 Valving

5.2.1 The solenoid valve

A solenoid valve was purchased from Lee Products (Gerrards Cross, UK; catalogue number INKA4024212H). The maximum switching frequency of the valve is specified as 1.2 kHz, limited by heating effects. The response time is therefore under one millisecond.

The primary design application of the valve is for use dispensing ink in ink jet printers, but the specification makes it useful for microfluidic work. The valve was chosen to operate in a 'normally closed' state, with a view to brief opening to transmit a pulse of pressure into the microfluidic. When the solenoid energised the iron plunger is pulled into the coil, opening the valve. When the solenoid is switched off a spring closes the valve.

One end of the valve was sealed to teflon tubing leading to a pressure reservoir by means of a permanent epoxy bond. This reservoir is described in the next subsection. The other end of the valve carried a bayonet connection allowing reversible connection to teflon tubing. The connection was used to form a connection to tubing which was permanently epoxied into the

microfluidic device, entering the device at the end of the switching channel as shown in figure 5.1. So far as was detectable, for pressures at which microfluidics operate this seal was entirely leak proof.

A schematic of the valve is shown in figure 5.2 and a schematic of the connection between the valve and the microfluidic in figure 5.4.

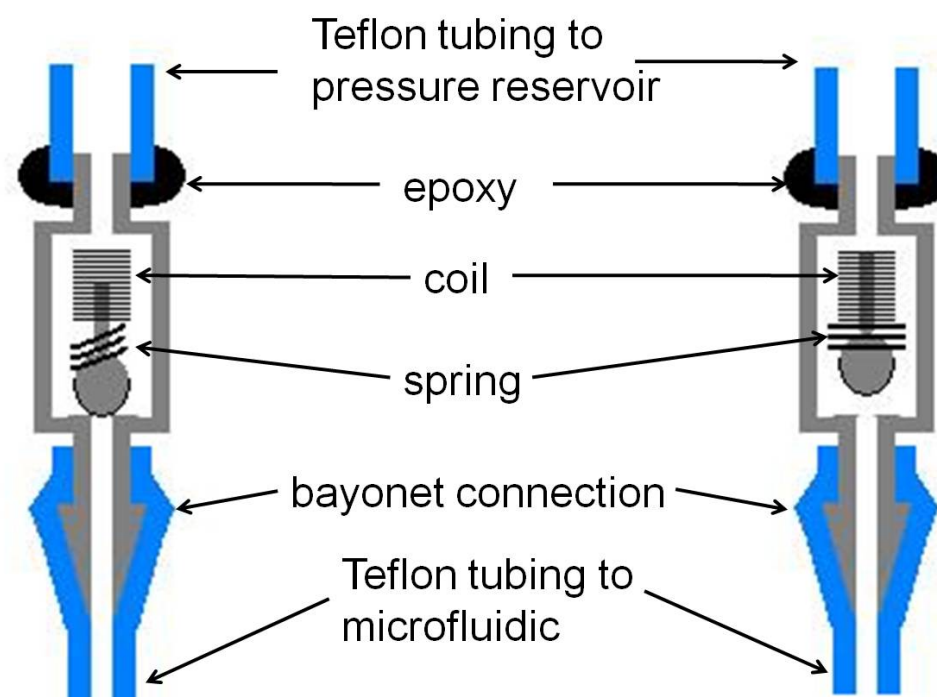


Figure 5.2: Schematic diagram of the solenoid valve. The left hand figure shows the normally closed case. The right hand figure shows the open case when the solenoid is energised.

In summary, when the valve was open the pressure from the pressure reservoir made contact with the switching channel.

To open the solenoid valve requires a voltage spike of 30V followed by a hold at a constant voltage of 7.4V to keep it open. LabVIEW was used to provide the a logical input to the circuit shown in figure 5.3 which then gave a valve opening signal across the valve.

LabVIEW VIs were written allowing the switch to be manually toggled open/closed, or to be manually trigger opening for a set time period, measured in milliseconds. These VIs were useful for troubleshooting, development and setting up of the system, for example in elimination of air bubbles.

For fluorescence activated sorting valve actuation was incorporated into the larger VI shown

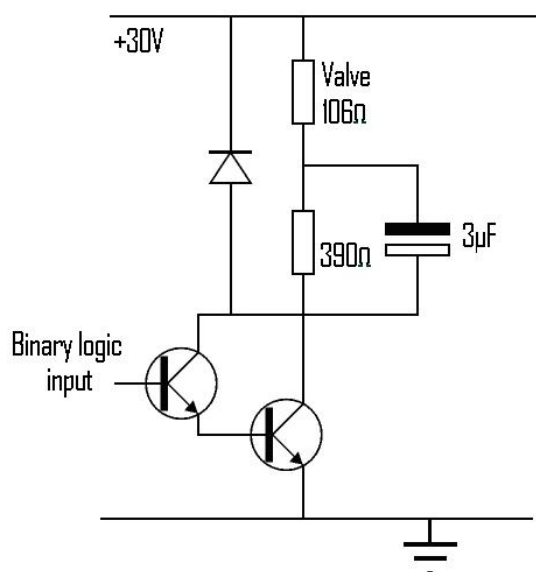


Figure 5.3: Circuit used to actuate cell switching

in Appendix B.

5.2.2 The pressure reservoir

A pressure reservoir was constructed from a 250 mL glass jar with a plastic screw cap (VWR). Two hole were drilled into the plastic cap. The first was used to connect the reservoir to the valve. The reservoir was partially filled with distilled water and the tubing connected to the valve was passed through the first hole so that the end of the tubing was below water level.

Initially, the second hole was used to connect the reservoir to a pressure source, again through teflon tubing. The pressure source used was a nitrogen cylinder with a 2 bar regulator (BOC gases, UK). Tubing from the pressure source was passed through the second hole in the reservoir cap. Both pieces of tubing were then sealed in place using epoxy.

A manual valve and pressure gauge were inserted in the line between the cylinder and the reservoir. This gauge had a linear scale running up to 0.8 bar. Pressures required for operation of the device are in the range 0.01 to 0.05 bar. The precision and reproducibility of pressure produced by the gauge / valve combination proved too low for this operating range.

It would have been possible to acquire a valve and gauge suitable for operation at pressures an order of magnitude lower. However, these are more specialist, expensive, devices.

Instead it was noted that 0.01 bar corresponds about 10 cm of water pressure (more precisely

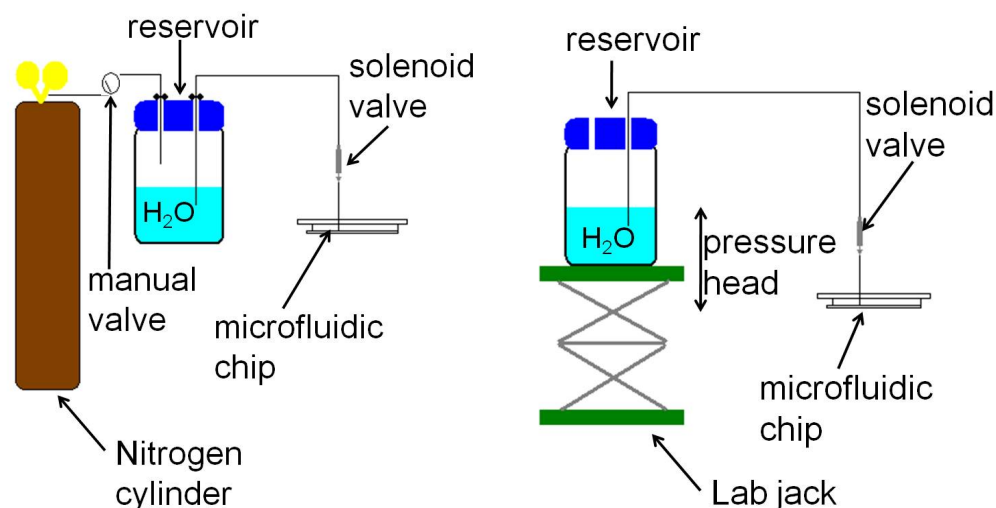


Figure 5.4: Schematic diagram of the pressure reservoir attached to two pressure sources. On the left is the initial arrangement driven by a nitrogen cylinder. On the right is the final arrangement driven by a gravitational pressure head.

10.197 cm). This is a convenient length scale over which to build a gravity driven pressure siphon. The pressure head of water could then easily be controlled to millimeter ($=0.1$ mbar) precision using a lab jack. This arrangement also simplified the plumbing in that it eliminated the need for a gas source and the need for a gas tight reservoir.

The initial and final arrangements are shown in figure 5.4.

With the successful implementation of the gravitational pressure head for the switching reservoir, the possibility of using a gravitational feed for the main inlet and sheath flows was considered. This has the potential advantage of eliminating the oscillations produced by stepper motors as described in the previous chapter. Some initial designs in this direction were attempted.

Unfortunately, the pressures required to drive these flows are higher, and would require in the range of three meters of pressure. This could be brought down somewhat by reducing the hydrodynamic resistance of the inlets, for example by using wider tubing instead of capillary. However even this would still result in impractically high pressure heads. In addition it is convenient to run these flows in constant current mode rather than constant pressure, as this gives control over the focusing width by adjusting either the sheath and inlet flows independently. Finally, in practice a reservoir providing gravitational head is not oscillation free. The motivation for running the focusing using gravity is therefore diminished.

5.3 Pressure stability

The major difficulty faced by the design turned out to be instability of the flow. Minor instability is acceptable, or even imperceptible, in cytometry measurement this is not the case when switching using rapid pressure changes is introduced in to the system.

The first instability arose when the focused stream reached the junction between the waste outlet and the collection outlet. Correct operation requires that in the absence of switching the focused stream be biased to flow down the waste outlet. However, this bias must be small enough that the focused flow may easily be diverted to the collection outlet. A second, more severe instability arose during and after switching.

Two attempts were made to achieve a stable balance overcoming the first instability.

The first was made use of differential fluid resistance between the two outlets. In these devices the width of the waste outlet was 200 μm while the width of the collection outlet was 100 μm . The flow should therefore be expected to divide in a ratio approximately 2:1 in favour of the waste outlet (with a precise calculation of the resistances the bias would be expected to be even higher).

However, in practice it proved impossible to establish a stable division between the outlets. This proved to be the case not just with variation from device to device, which would be expected to some extent for a technique relying on differential resistance, but also from run to run with the same device.

The second attempt to overcome the instability at the waste / collection junction made use of an additional pump to pump the two sheath inlets at different flow rates. In this way the position of the focused stream in the channel could be controlled. This technique succeeded in introducing a bias into the flow but again did not produce results that were stable in time.

It may have been possible to overcome some of this instability by reducing the distance between the focusing and the switching junction. However, it was desirable to maintain sufficient distance between these two stages that the actuation of switching did not interfere with focusing and detection.

Instead effort focused on reducing an important source of instability arising from the stepper motors in the pumps.

5.3.1 Instability from stepper motors

The KDS scientific syringe pumps are powered by stepper motors. A schematic diagram of a stepper motor is shown in figure 5.5. These motors operate by periodically reversing the current and therefor the magnetic field generated by the stator coils. The the rotor then rotates so as to keep the poles of its permanent magnets continuously aligned with their opposite poles (north with south and south with north) in the stator coils.

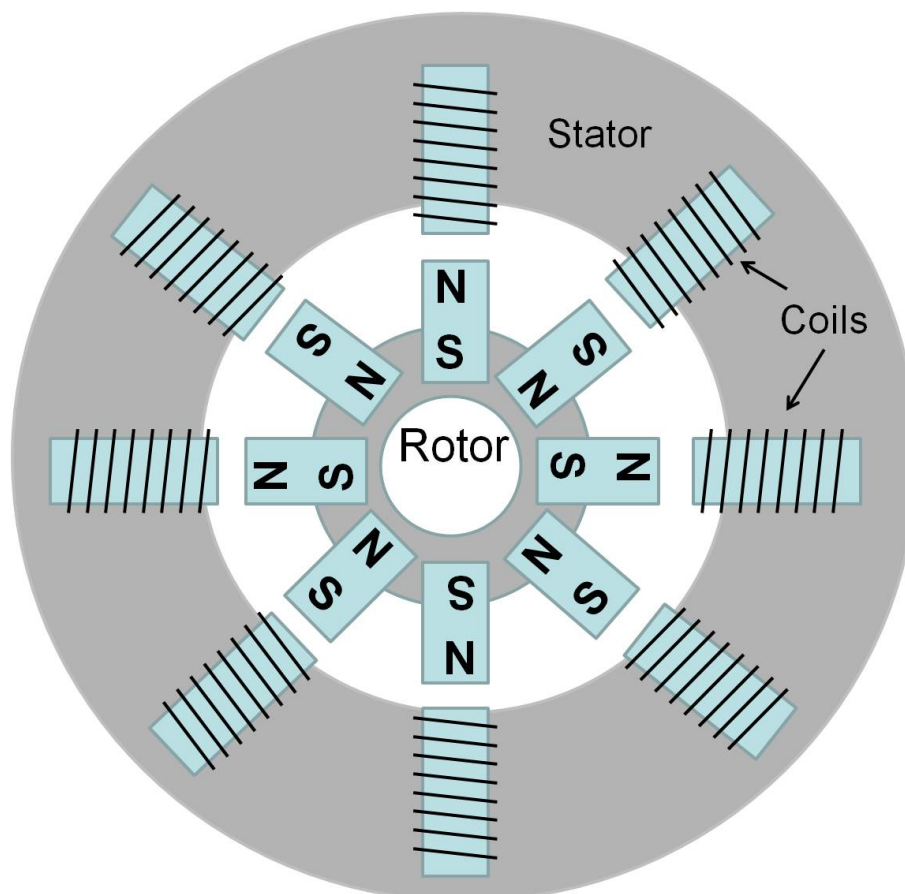


Figure 5.5: Schematic image of a stepper motor showing the rotor (which rotates) with permanent magnets inside the stator (which is static) containing electromagnetic coils whose magnetic polarity can be changed by reversing the current.

At low flow rates the discrete steps of the motor can be seen as the rotor jumps from one position to the next. The field reversals are not sufficiently rapid to produce motion. CCD images of dye in a focused stream were integrated. The total integrated signal over time showed oscillations in the dye flow. A fourier transform showed a peak that varied with the motor frequencies, suggesting these were an important source of the oscillations.

The solution is a motor in which the currents in the coils can be changed in much smaller steps, known as microstepping. A custom microstepping pump system which had been built for another application was adapted. This made use of Lynx microcontrollers to break down each motor step into 16,000 substeps allowing very smooth operation even at low flow rates.

The system required some basic renovation. It was found to be performing poorly, with even greater jolting than the KDS pump. This was presumably due to some damage during transport and storage. On rewiring the microstepping drivers, one combination of driver and pump was found that showed smooth microstepping. As the driver software for the custom system was only available in compiled form the pump was driven using a LabVIEW VI on a dedicated PC.

One pump was not sufficient to drive both sheath flows and an analyte inlet. While it is possible to drive more than one inlet from a single syringe pump the current will split according to the hydrodynamic resistance of each arm. This is generally not controllable, depending on minor details of fabrication that vary from device to device and on the details of joints which are remade with each run. It is also hard to measure. It is therefore far better to run each inlet from a separate syringe.

It was decided to use the one available microstepped pump to run the analyte inlet. This inlet is run at a significantly slower rate (a factor of between 5 and 10) than the sheath flows. It also showed up more strongly as a source of oscillations in the integrated image observations described above.

5.4 From positive to negative pressure

With the gravity pressure reservoir in place attempts were made to set the height at the appropriate pressure for switching, it was soon realised that the reservoir could be made to apply negative as well as positive pressure, simply by reducing the height of the reservoir.

Furthermore, because the switching channel is widened to create a relatively slow flow compared to that in the detection channel, particles which are sucked into the switching channel fall out of flow. Once they have settled they then tend to stay in place on subsequent switches. Particles of interest therefore accumulate in the switching channel. This mode of operation overcomes the need to make a separate arrangement or reservoir at the end of the collection

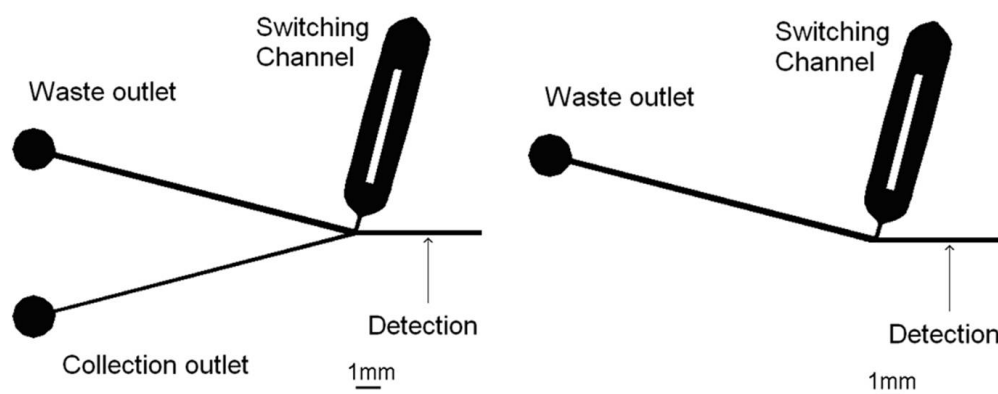


Figure 5.6: Refinement of of the sorter design. The left hand panel shows the original design. The right hand panel shows the elimination of the collection channel. Particles are instead collected in the switching channel.

channel.

It was therefore convenient to operate the switching channel as a collection reservoir. A new photomask and master were fabricated with the layout of the device modified to that shown in figure 5.6.

This new design also had the benefit of overcoming a second problem. In the initial design the focused stream had to be tuned to consistently flow down the waste outlet rather than the collection outlet when in ordinary flow without switching. Unfortunately this proved hard to achieve reproducibly. The splitting of flow between the outlets depends on the ratio of hydrodynamic resistances. It is possible that the difficulty in achieving reproducible flow down the waste outlet channel was due to small differences in the outlet capillaries themselves. However, with the elimination of a separate collection outlet there was no longer a need to resolve this problem.

Figure 5.7 shows an assembled sorting device mounted on an TE2000 microscope.

5.5 Results of sorting

With these results it was possible to achieve preliminary sorting results. The pressure (-5mm H₂O pressure head), flow rate (1/16 μ L per minute analyte, 1 μ L per minute per sheath flow), and switching time (10ms) were selected empirically by observing switching by eye and then using these parameters for automated switching at high throughput.

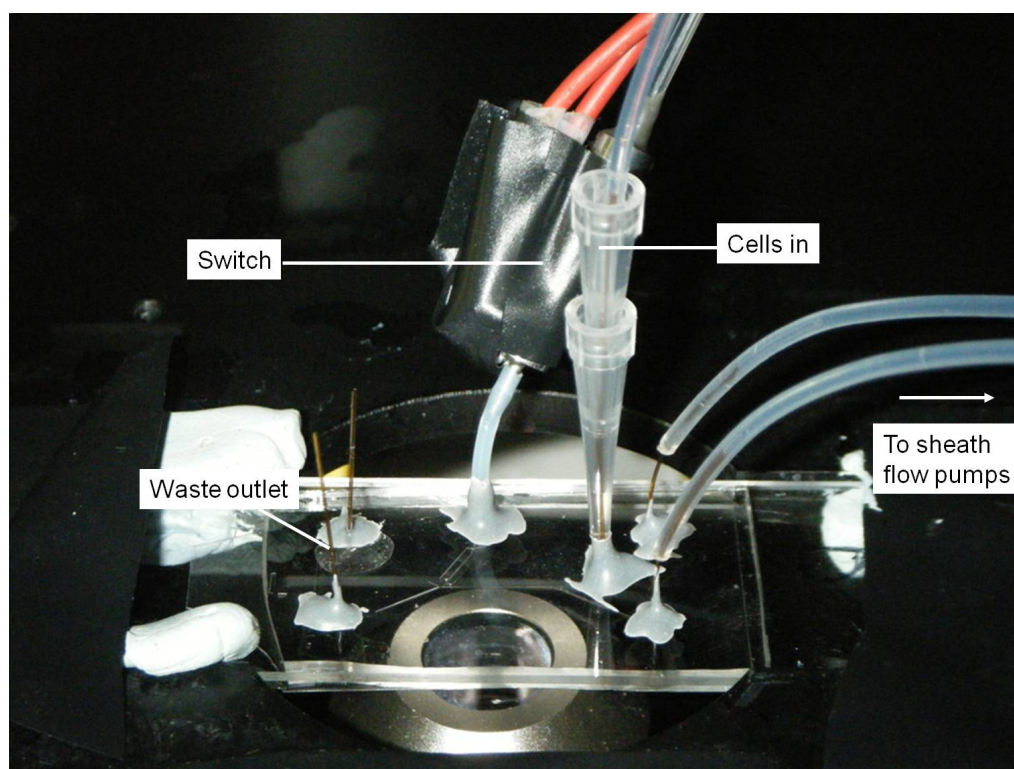


Figure 5.7: Assembled microfluidic device mounted on the microscope.

A mixture of 10% fluorescent beads was prepared in 90% unlabelled beads at a concentration of 1000 beads per microlitre. One microlitre of this solution was introduced into a capillary which was inserted into the microfluidic. Results in the collection chamber are shown in figure 5.8.

This shows 31 labelled beads (31% recovery rate) and 15 unlabelled beads (18 fold enrichment rate). However, trial and error proved inadequate to further optimise the pressure, flow rate and switching time to improve these results. I therefore needed data to investigate the switching process in more detail.

5.6 Work with tracers

To more systematically set parameters for consistent switching it is useful to make use of flow tracers. Here it is useful to distinguish between the different concept of streamline, streakline and pathline used in analysis of fluid flow. In the case of steady, time-independent, flow these concepts are identical. This is the case for the hydrodynamic focussing and cytometry

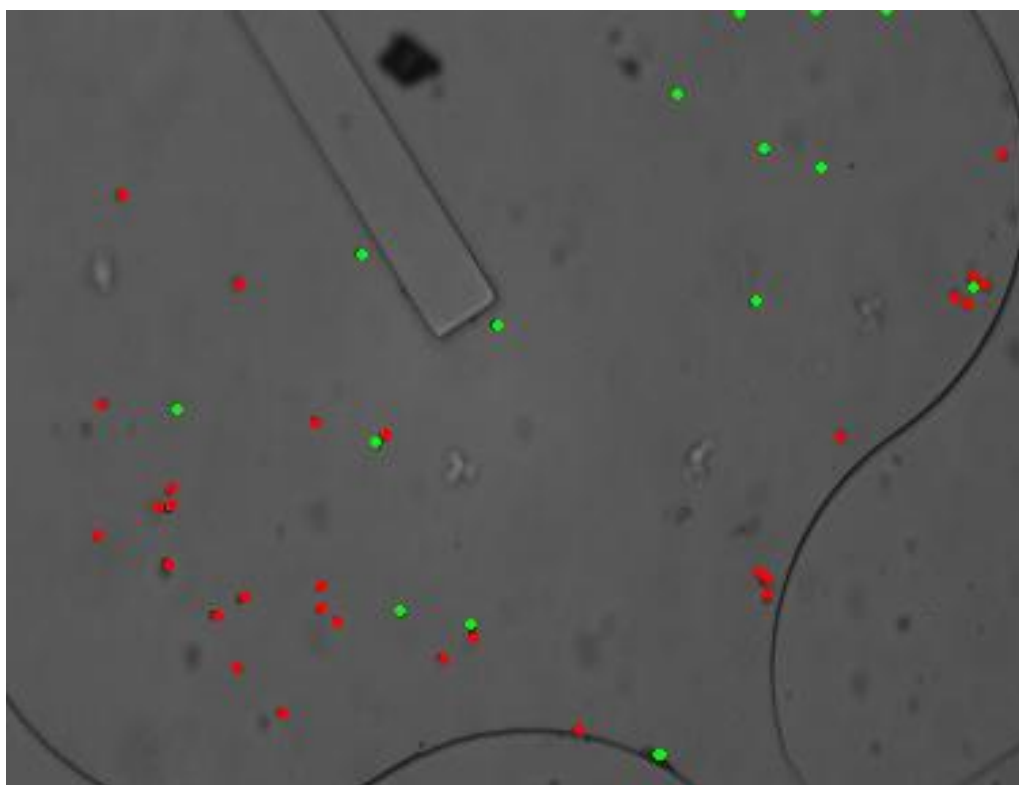
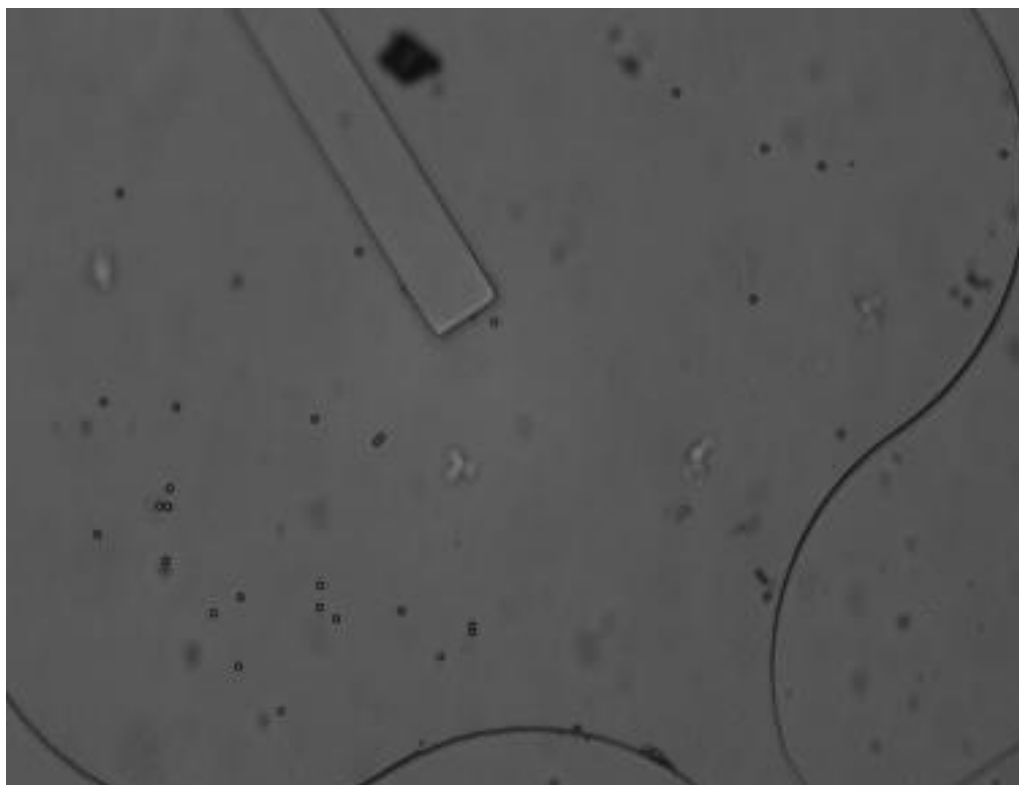


Figure 5.8: Sorted beads. The top panel shows bright field view. The bottom panel shows fluorescent beads colour coded red and non-fluorescent beads colour coded green.

experiments discussed earlier. In the analysis of switching the time dependence of the flow requires that they be distinguished:

- Streamlines are curves that, at a given time, are tangent to the velocity vector.
- Streaklines are curves defined, at a given time, by present location of all fluid parcels that have historically passed through a specified point. A tracer dye (neglecting diffusion) injected at a specified point will, at a later time, form a streakline.
- Pathlines are curves defined over time by a fluid parcel moving through the flow. A particle (a bead or cell) injected at a specified point will, over time, trace out a pathline.

Recording the trajectory of particles gives pathlines, and ultimately analysis of pathline this is what is of interest to answer the question of whether a particle can be successfully sorted. However, visualisation of streaklines gives greater insight into the structure of the flow during switching. This is because at a given time a pathline only provides information at a single point. A streakline gives information along an entire curve. Where a pathline effectively encodes information from a single tracer particle, recording streaklines over time shows the motion of a continuous distribution of tracers.

The attempts at experiments with tracers made use of dye. However, diffusion in this case was a large problem.

As an alternative, $0.1\ \mu\text{m}$ fluorescent beads (Molecular Probes, UK) were used as tracers. This overcame the problem of diffusion. In contrast to dye, which could easily be washed away, the particles could settle out of flow over time, gradually building up on the floor of the device. However, this was a far smaller problem than diffusion.

5.6.1 Results

A frame showing $0.1\ \mu\text{m}$ beads is shown in figure 5.9. Here the focused stream of beads in normal flow is shown at the bottom of the frame, flowing from right to left. The large chamber in the top part of the image is used to collect switched beads. When the valve is opened the the flow of beads is diverted into the large chamber. A streak of beads in the collection chamber is shown from a previous switching event.

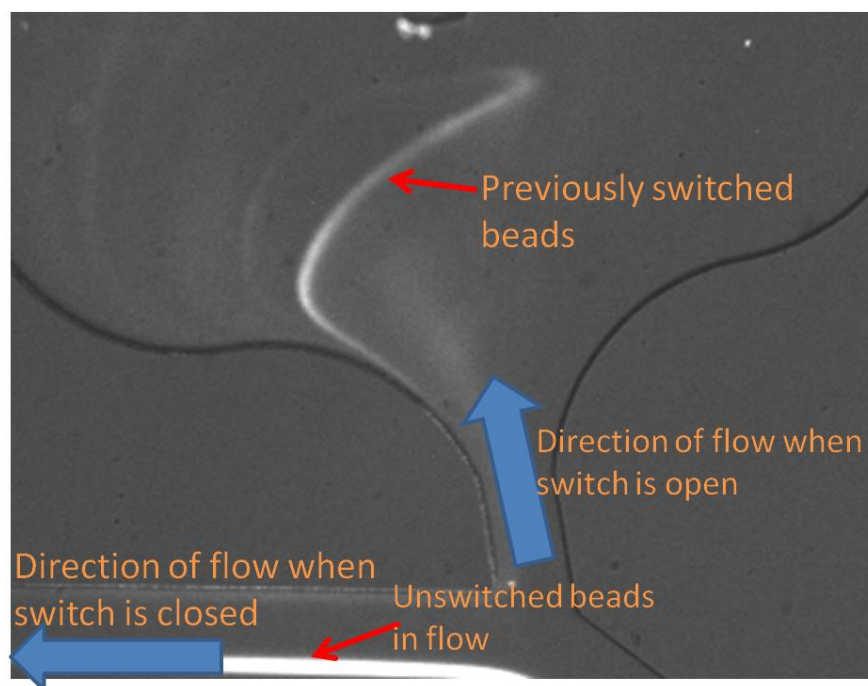


Figure 5.9: The set up for switching of tracer beads. Fluorescence is excited using a mercury lamp.

Figure 5.10 shows a sequence of frames illustrating the switching process. The valve in this instance was opened for 5ms. The sheath flow was $1\mu\text{L}$ per minute from each side while the flow of focused beads was at $0.0625\mu\text{L}$ per minute. Each frame is separated by 10ms, so that the whole sequence shown covers 240ms.

At the start of the process the collection chamber already contains a previously switched streak of beads. When the valve is opened (during the first frame) the old previously switched beads move further up in to the collection chamber while a new set of beads is sucked in from below. The switching here is fairly reproducible in that the new beads finish in a similar location to the old beads at the start of the process.

The most striking result is that the switching process lasts much longer than the valve opening time. This implies either substantial compressibility in the system or that the closing process of the valve is rather imperfect.

In other instances the switching process visualised in this way shows considerable instabilities. For instance, the focused flow does not consistently re-establish itself in the expected position after the switch is closed. A consistent equilibrium would be expected for such a low Reynolds number regime. Again, the explanation is probably either substantial compressibility in the

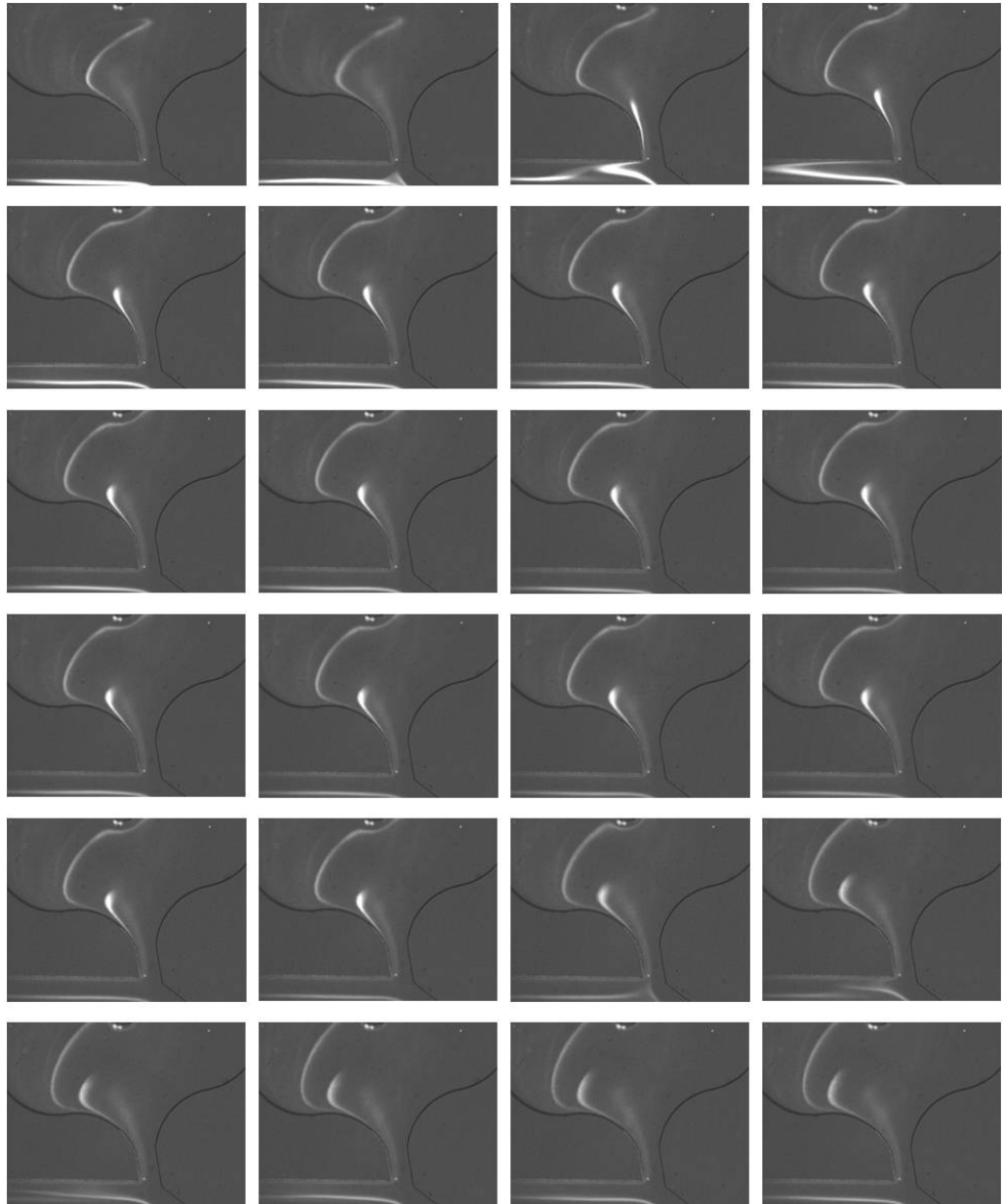


Figure 5.10: Series of frames showing switching in a flow of $0.1\mu\text{m}$ diameter fluorescent beads. Fluorescence is excited using a mercury lamp. The valve is opened to suck in flow for 5ms at the first frame. Frames are separated by 10ms for a total time shown here of 240ms.

system or that the closing process of the valve is rather imperfect.

5.7 Discussion and conclusion

This thesis has shown that it is possible to carry out single cell proteomic measurements and that it is possible to isolate circulating tumour cells in microfluidic devices.

It has also detailed work attempting to automate the identification and handling of CTCs so that they can be counted and sorted for introduction into a single cell analysis stage.

The work on microfluidic cytometry highlighted the difficulties of working with small quantities of cells. While microfluidics has the potential to overcome these problems more easily than larger systems such as conventional FACS, loss of sample still appeared as a problem to be overcome. This problem was in addition to the general problem of optimising a microfluidic flow cytometer, such as the need for two dimensional focusing.

The work with tracers on switching showed the presence of instability and delay in the switching process that make switching hard to optimise. These difficulties might be overcome with more rigid and reliable materials and components.

In January 2011 the journal *Lab on a Chip* identified the automated analysis of CTCs in microfluidic systems as a 'Grand Challenge'. [220] It is likely that a fully integrated system will require further advances in microfluidic cytometry and switching more generally. However, it is also probable that the techniques described in chapters 2 and 3 will be used to generate single cell analyses of CTCs considerably before the goal of automated analysis is met.

Appendix A

Octave / Matlab scripts

The following scripts have been run on both Octave and Matlab with identical results.

A.1 Building the matrix for the diffusion operator

```
% Script for diffusion operator
%
%set up an nxm grid:
%
n=50;
m=170;
l=n*m;
%
% Note: when plotting, use reshape(v(:,*),n,m)
%
% The matrix for the operator is built in the variable k
% A sparse matrix is used to save memory and increase speed
%
% Boundary conditions phi=0
%
k=sparse(diag(-4*ones(l,1)));
k=k+sparse(diag([ repmat([ones(n-1,1);0],m-1,1); ones(n-1,1)],1));
```

```

k=k+sparse(diag([ones(n-1,1); repmat([0; ones(n-1,1)], m-1, 1)], -1));
k=k+sparse(diag(ones(1-n,1), n));
k=k+sparse(diag(ones(1-n,1), -n));
%
%
% For Boundary conditions  $\phi'=0$  on top row add:
%
k=k+sparse(diag([ones(n,1); zeros(1-2*n,1)], n));
%
% For Boundary conditions  $\phi'=0$  on bottom row add:
%
k=k+sparse(diag([zeros(1-2*n,1); ones(n,1)], -n));
%
% For Boundary conditions  $\phi'=0$  on left col add:
%
k=k+sparse(diag([repmat([1; zeros(n-1,1)], m-1, 1); 1; zeros(n-2,1)], 1));
%
% For Boundary conditions  $\phi'=0$  on right col add:
%
k=k+sparse(diag([zeros(n-2,1); 1; repmat([zeros(n-1,1); 1], m-1, 1)], -1));
%
% For Boundary conditions  $\beta \phi' + \alpha \phi = 0$  along first z elements of left
% hand col use  $g = \beta/\alpha$  and add:
%
z=100;
g=-0.053606;
k=k+sparse(g*(diag([repmat([1; zeros(n-1,1)], z, 1); zeros(n*(m-z), 1)])));
%
%
% Add a  $1/r \phi'$  term for cylindrical geometry

```

```

%
a=zeros(n,1);
for p=1:(m-2)
a=[a; 1 ./ (p*ones(n,1))];
end
b=circshift(a,-n);
%
k=k+sparse(.5*(diag(a,n)));
k=k+sparse(-.5*(diag(b,-n)));
%

```

A.2 Normalisation of eigenfunctions for cylindrical geometry

```

function normalised=nor(a,n,m)
%
% This function normalises an eigenfunction in variable a
% on an nxm grid, where m indexes the radial direction
%
weight=repmat(linspace(1,m,m),n,1);
areshape=reshape(a,n,m);
norma=sqrt(sum(sum(weight.*areshape.*areshape)));
normalised=areshape/norma;
endfunction

```

A.3 scalar product of functions in cylindrical geometry

```

function tot=integrate(a)
%
% To calculates the integral of the product function f1 and f2
% defined on an nxm grid where m indexes the radial direction
% calculate a=f1.*f2 and then use this function to integrate

```

```
% the answer
%
[n, m]=size(a);
weight= repmat(linspace(1,m,m),n,1);
tot=sum(sum(weight.*a));
endfunction
```

Appendix B

Fabrication of microfluidic devices

B.1 Poly(dimethylsiloxane) devices

Polydimethylsiloxane PDMS chips were produced by casting a mixture of PDMS monomer with cross linker (Sylgard 184, VWR, UK) in a ratio of 9:1 over a lithographically fabricated master. PDMS devices for individual chips were then cut and peeled from the master.

Holes were cut using hollow needles through the PDMS to provide access to the channels from the top of the chip. A glass cover slip was bonded to the bottom surface of the chip forming a floor for the microfluidic channels. Holes were drilled in a glass microscope slide aligned with these access holes. The slide was bonded to the top surface of the PDMS chip to provide mechanical strength.

Capillaries and tubes were then passed through the access holes and epoxied into place.

Photomasks for lithographic fabrication of the master were designed using the Autocad (Autodesk Inc., USA) computer aided design program and sent for production to JD phototools Ltd (Oldham, UK). They exposed a polyester film coated with a silver halide emulsion to a light pattern corresponding to the desired design. The exposure was generated by flashes of laser light with the spatial patterning provided by a bank of digital micromirrors. After focusing through a lens system the pixel size of the system was $0.8\text{ }\mu\text{m}$. The pattern was developed, fixed, washed and dried by a carefully controlled version of the standard photographic process.

A variety of masks were ordered and produced, and selected masks were manually cut for use.

In order to produce the mask with a single outlet branch an existing mask was adapted. A

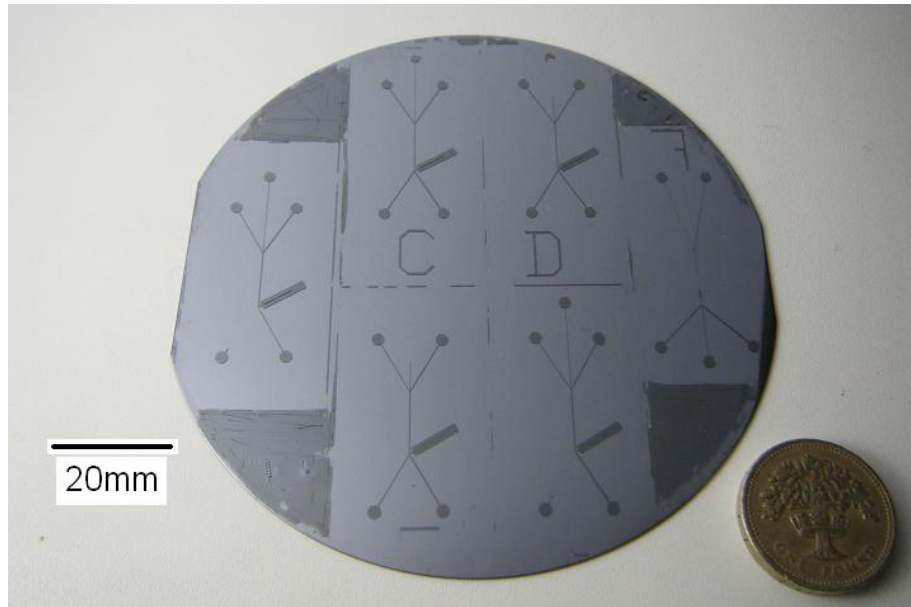


Figure B.1: Photograph of silicon master showing SU-8 photoresist channels for 6 designs.

peice of darkened film was taken an aligned under a microscope to block out the extra channel. Adhesive tape was used to secure the block in place.

Initial attempts at fabrication of masters followed a protocol that called for cleaning of the silicon wafer in piranha solution (70% sulfuric acid, 30% hydrogen peroxide) overnight to remove any organic material. However, it was found that this procedure was unnecessary. When unpacked in a clean room environmment the wafers as supplied by IDB technologies were adequately clean for our purposes. They were therefore used directly, without a cleaning step.

Piranha solution is both highly corrosive and potentially explosive. Eliminating its use has the advantage of removing the most hazardous step in the microfluidic fabrication protocol. In addtion, while the cleaning proceduce is not tremendously time consuming in itself, an overnight cleaning step reduces flexibility in fabrication schedules. In this context making direct use of wafers therefore adds appreciably to the rapidity of 'rapid prototyping'.

A master is shown in figure B.1.

In order to bond the the PDMS to glass both surfaces were cleaned by sonication for 5 minutes each in ethanol, water, NaOH (0.5M), then water, repeated three times. Surfaces were then dried with nitrogen. The structural support slide and, in the case of devices used for flow focusing studies, the cover slip were placed in a plasma oven (PDC-002, Harrick Plasma, USA) at full power for 18 seconds before being pressed together.

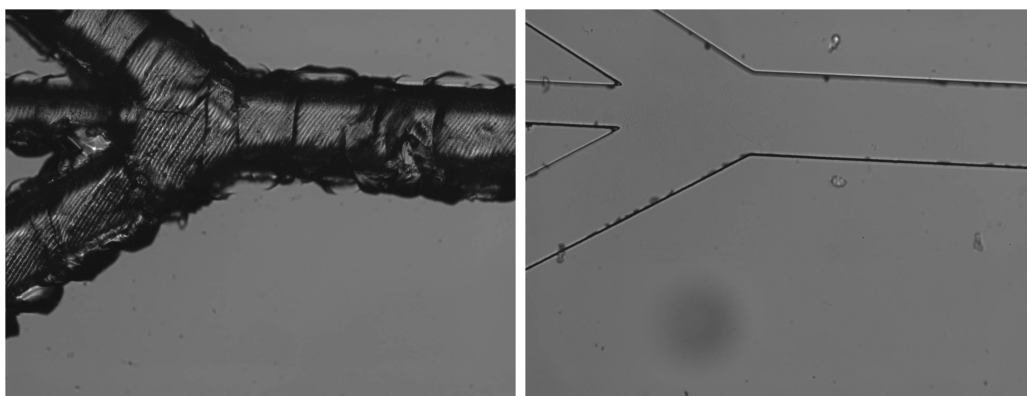


Figure B.2: Comparison of glass (left) and PDMS (right) devices.

The cover slip in studies with antibody patches was bonded simply by pressing without plasma bonding.

B.2 Glass devices

Glass devices were fabricated by laser ablation in a glass microscope slide. The work was carried out by an Oxford Laser micromaching system (Oxford, UK). CAD files were used to program the laser which scanned a raster pattern producing channels according to the desired design. After setting up actual process of etching each device took around 4 hours.

The devices that were produced with this method proved to be extremely rough as compared to PDMS, as is evident both in bright field images and in profiling shown in figures B.2 and B.3 to B.5. This roughness was partly due to the lines of raster scanning which are clearly visible and partly due to smaller scale irregularities.

It is possible that some of the roughness was due to debris that could be cleaned away. However, sonication for 1 hour in distilled H₂O and then for 1 hour in 0.5M NaOH followed by rinsing in distilled H₂O failed to produce any noticeable difference.

Despite the poor channel roughness, devices were constructed. Holes were drilled in the microscope slide bearing the channels at each inlet. The slide was then positioned on top of a cover slip and placed on a steel plate for easy positioning in an electric oven. The oven was heated to 600 °C for 2 two hours. This was sufficient to soften the glass causing the slide to fuse with the cover slip. The set up was then cooled by switching off the oven and leaving overnight. Attempting to cool to room temperature in less than about 8 hours resulted in

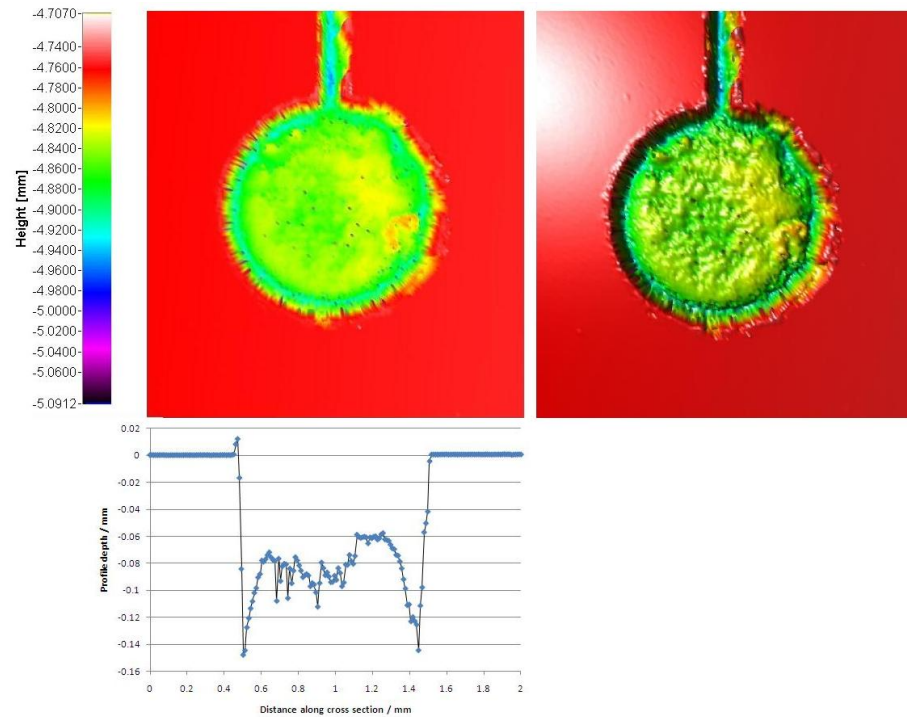


Figure B.3: Top left: Inlet reservoir of glass device after cleaning with colour scale giving depth. Top Right: Same reservoir rendered with 3D illumination. Below left: profile across the reservoir aligned with image above.

severe cracking.

B.3 Characterisation of channels

The PDMS and glass channels were optically profiled courtesy of Bio Nano consulting (London, UK). The results are shown in figures B.3 to B.5

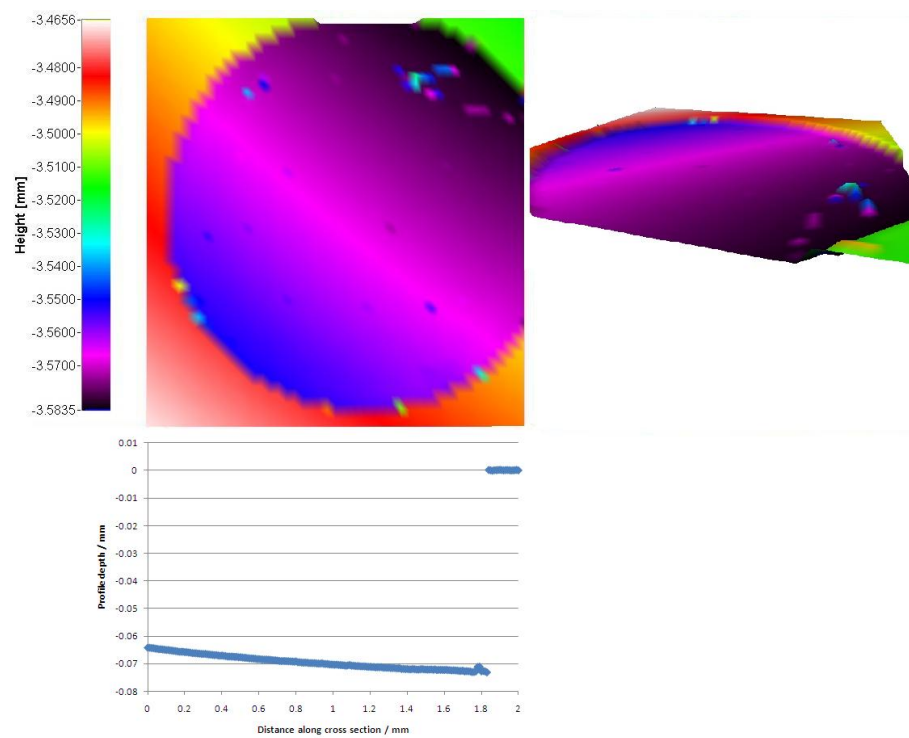


Figure B.4: Inlet reservoir of PDMS device after cleaning with colour scale giving depth. Top Right: Same reservoir rendered with 3D illumination. Below left: profile across the reservoir aligned with image above.

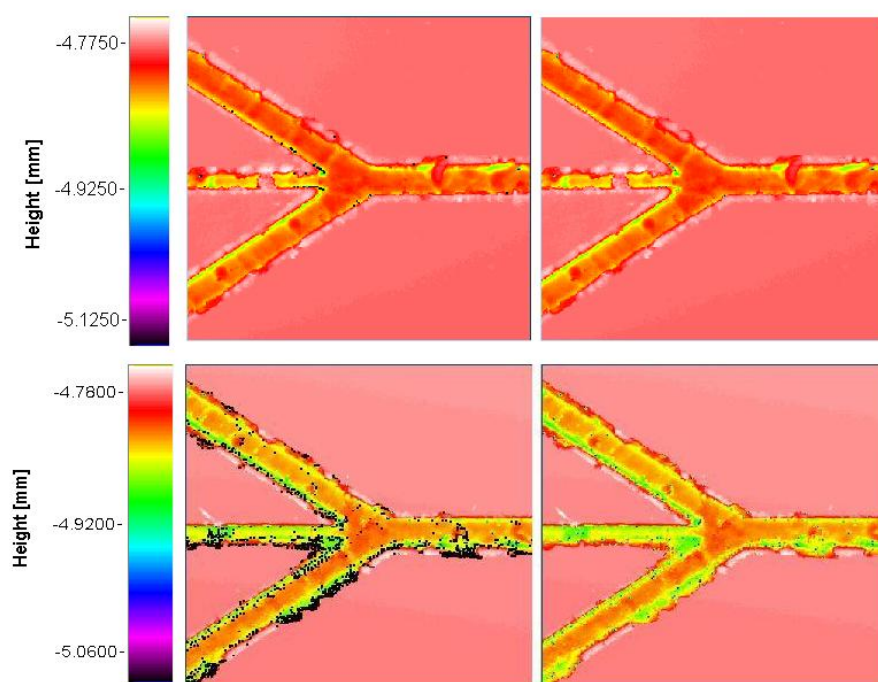


Figure B.5: Top row: Glass channels before cleaning by sonication. Bottom row: after cleaning by sonication. Left hand column: raw profiling data with black dots indicating a reading could not be taken. Right hand column: profiling data after smoothing.

Appendix C

Materials and methods

C.1 Jurkat cells

Jurkat cells were a gift from Professor Daniel Davies (Imperial College London, UK). They were cultured in 12mL of RPMI 1640 medium (Invitrogen) with 10% Fetal Calf Serum (Sigma) in 75 cm² culture flasks (VWR; UK) in incubator at 37°C and 5% CO₂. They are a non-adherent cell line and were split in to a new flask at a ratio of 1:9 cells to new medium every 2–3 days.

C.2 MDA MB 468 cells

MDA MB 468 cells were a gift from Professor Charles Coombes (Hammersmith Hospital, UK). They were cultured in 12mL of DMEM/F12 ham (Invitrogen) with 10% Fetal Calf Serum (Sigma) in 75 cm² culture flasks (VWR; UK) in incubator at 37°C and 5% CO₂. At 80% confluence (around every 4 days) they were washed with phosphate buffered saline solution (Sigma; UK), lifted with 1mL of trypsin (Sigma), split in to a new flask at a ratio 1:5 cell to new medium.

C.3 BE cells

BE cells were a gift from Professor Keith Willison (Institute of Cancer Research, UK). They were cultured in 12mL of DMEM (Invitrogen) with 10% Fetal Calf Serum (Sigma) in 75 cm² culture flasks (VWR; UK) in incubator at 37°C and 5% CO₂. At 80% confluence (around every

4 days) they were washed with phosphate buffered saline solution (Sigma; UK), lifted with 1mL of trypsin (Sigma), split in to a new flask at a ratio 1:5 cell to new medium.

C.4 BT474 cells

BT474 cells were a gift from Heather Rada (Institute of Cancer Research, UK). They were cultured in 12mL of DMEM/F12 ham (Invitrogen) with 10% Fetal Calf Serum (Sigma) in 75cm² culture flasks (VWR; UK) in incubator at 37°C and 5% CO₂. At 80% confluence (around every 4 days) they were washed with phosphate buffered saline solution (Sigma; UK), lifted with 1mL of trypsin (Sigma), split in to a new flask at a ratio 1:5 cell to new medium.

C.5 Preparation of cells

For the single cell lysis and staining protocols described in chapters 2, 3 and 4 adherent cells were trypsinised with 1mL of trypsin. This was neutralised with 4mL of medium. Cells were then washed three times by centrifugation at 400g for 5 minutes and removal of the supernatant followed by resuspension in 4% bovine serum albumin (Sigma; UK) in PBS at concentrations of 10⁵ per mL.

Preparation of cell lysate to test antibody staining described in chapters 2 and 3 was carried out by resuspending washed cells in 0.3mL of PBS with 0.1% Triton X-100 (Sigma; UK). Cells were placed on a roller mixer in a cold room for 30 minutes. They were placed in a Falcon tube (VWR; UK) and pulse spun at 1,500g at 4°C. Total contents was transferred to an Eppendorf tube which was spun at 1,000g at 4°C for 15 minutes. The supernatant was removed and the product resuspended in 100μL of PBS.

C.6 Preparation of cover slips

Cover slip surfaces for spotting antibodies were prepared as follows.

Batches of 6 cover slips were prepared in Hellendahl jars. Initial cleaning was carried out by sonication for 5 minutes in c, followed by a wash with 0.5M sodium hydroxide and sonication for 60 minutes. They were then rinsed four times in in MilliQ H₂O and twice in 96% ethanol.

For plain glass printing cover slides were dried with nitrogen gas and used directly.

For GPTS (3-glycidoxypropyl-trimethoxysilane) derivatisation cover slips were immersed for 1 hour in 2.5

For PEG (polyethylene glycol) derivatisation cover slips were immersed for 1 hour in 2.5% APTS (3-aminopropyl-trimethoxysilane) in 10mM acetic acid in 96% ethanol. They were then rinsed in 96% ethanol followed by sonication in 96% ethanol for 10 minutes and a final rinse in 96% ethanol. Cover slips were then baked at 110°C for 15 minutes and dried with nitrogen gas. A droplet of 25%w/v biotinylated PEG in 0.1M sodium bicarbonate was sandwiched between pairs of coverslips and incubated in the dark for 3 hours. Coverslips were then separated, rinsed in MilliQ H₂O, dried with nitrogen gas and used directly.

Appendix D

LabVIEW code

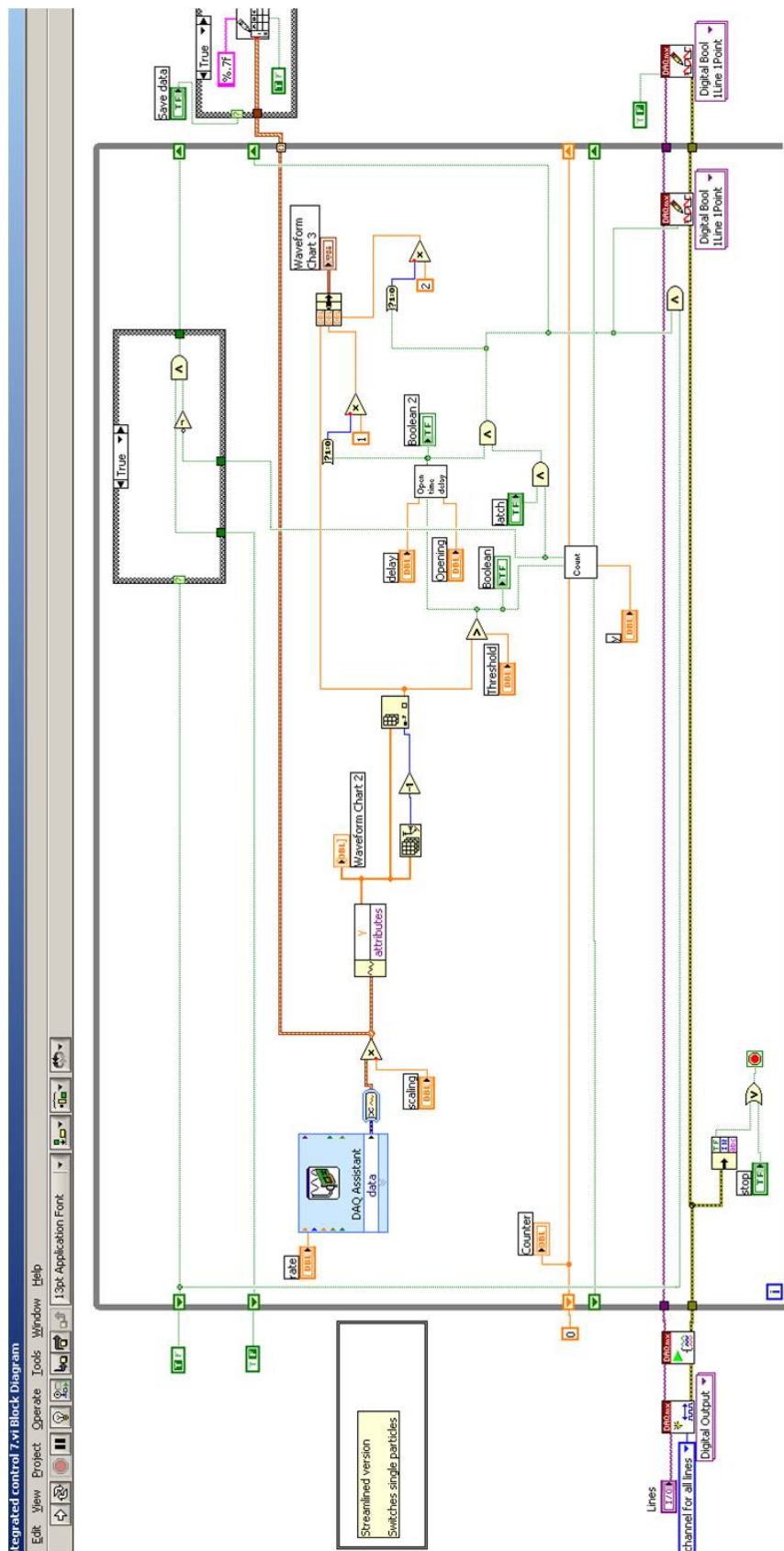


Figure D.1: Block diagram of LabVIEW code for cytometry and sorting.

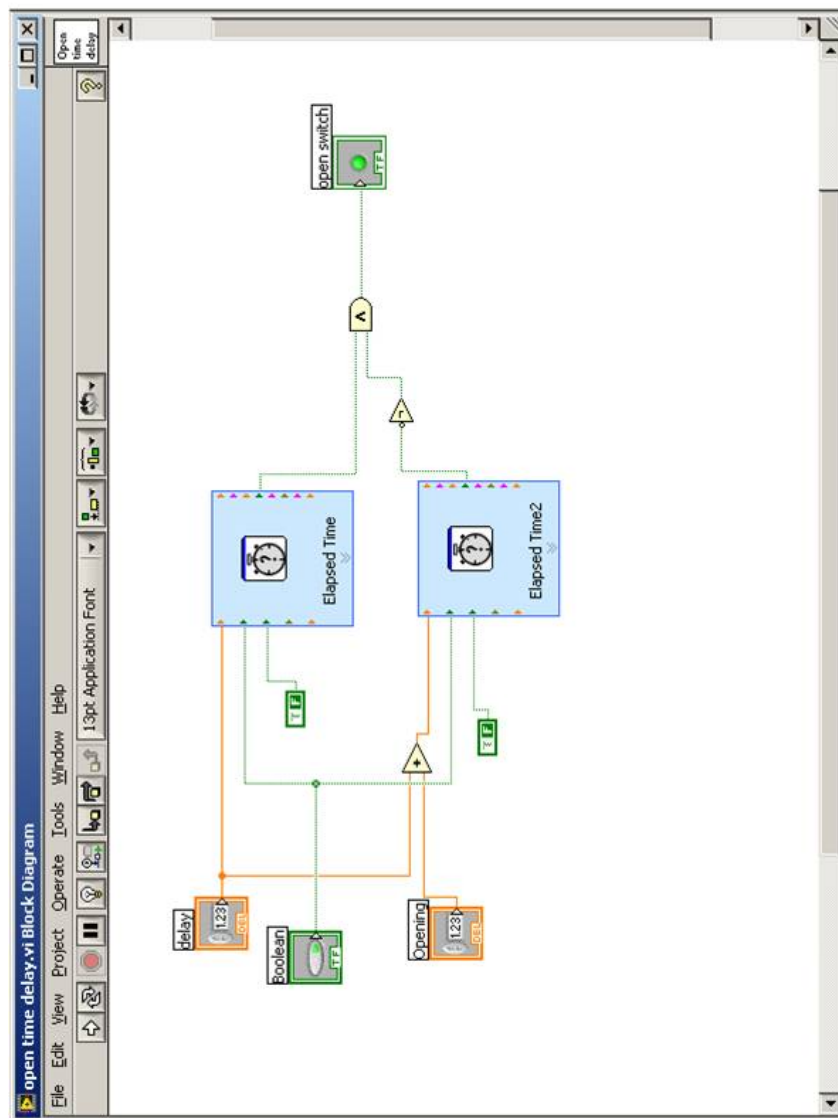


Figure D.2: Block diagram of LabVIEW code for subVI controlling opening with a time delay.

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