Diagnosing cancer one cell at a time with single molecule spectroscopy

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2017
Abstract

Mapping protein expression heterogeneity in cancer at single cell resolution is essential for the understanding of disease progression, emergent drug resistance, and metastasis, but is a great technical challenge. Longitudinal monitoring of heterogeneity pertaining to biomarker expression may provide the necessary medical cues for the administering of personalised therapeutics. Studying molecular heterogeneity in the ultra-rare circulating tumour cells [CTCs] found in the blood of cancer patients is a greater challenge still, but success may yield deep insight into the nature of the metastatic cascade, and also provide the technological means of a non-invasive ‘liquid biopsy’.

The MAC chip is a quantitative single molecule sensitive protein assay for the evaluation of protein copy number in single cells. In this thesis, we attempt the development of a new biomarker-targeting MAC chip assay for the breast cancer oncoprotein estrogen receptor alpha. We describe a series of improvements to the MAC chip assay architecture allowing multiplexed measurement of several proteins simultaneously, and improvements to analysis methods allowing for superior molecule counting. Building on the work of others in the field of circulating tumour cell isolation, we attempt the integration of the MAC chip analysis method into a multiple-stage device for the isolation and proteomic analysis of circulating tumour cells. Finally, using a multiplexed MAC chip device for the tumour suppressor protein p53 and its activated form phosphorylated at serine-15, we demonstrate for the first time that the MAC chip can be used to study protein expression heterogeneity in quasi-clinical samples. The patient-derived xenografts we use to perform this work are a key resource of clinically-relevant tumour material, and a model system directly analogous to primary patient biopsies, thus demonstrating the feasibility of translational single cell proteomics with the MAC chip system.
Declaration of originality

All the work presented in this thesis is my own, unless otherwise stated, and with the following exceptions:

-- James Alexander Squires developed the phospho-p53 MAC chip assay and performed a number of the xenograft experiments.

-- Beatrice Griffiths of the Institute of Cancer Research performed xenograft tumour disaggregation.

-- CTC microfluidic manufacturing and testing was performed in conjunction with Zainab Ahmed and Ksenia Katsanovskaja under my supervision.

-- CTC isolation with microtubes was performed by Celia Cheung and Ksenia Katsanovskaja.

-- The thresholding-based single molecule counting algorithm was written by Dr Ali Salehi-Reyhani.

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Publications based on this work

[1] ‘Multiplexed single cell protein expression analysis in solid tumours using a miniaturised microfluidic assay’


https://doi.org/10.1088/2057-1739/aa6aae
Acknowledgements

I would like to thank my supervisors Professor David Klug, Professor Keith Willison, Professor Charles Coombes and Professor Simak Ali for both the opportunity to undertake this PhD in the first place, and their support throughout its duration. I would like to express my sincere gratitude to Dr Marco Gerlinger for his scientific collaboration and extremely useful comments and advice on paper writing. I would like to thank Cancer Research UK for sponsoring this work, and all those who have donated or worked to raise the funds which have made this possible. Thank you to Sophie, Hugh, Jim, John, Mike Barclay and everyone else whose bacchanalian dedication has ensured that the pendulum of stress has always been swiftly forced back to its equilibrium point. Thank you to the patients whom participated in the clinical trials which enabled an important fraction of the work in this thesis to be brought into being. Thank you lastly to my family and all those others whom must remain unnamed for the sake of brevity, your friendship and support has been of inestimable value.
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>APTES</td>
<td>(3-aminopropyl)triethoxysilane</td>
</tr>
<tr>
<td>ASMI</td>
<td>Average single molecule intensity</td>
</tr>
<tr>
<td>BNO</td>
<td>Baseline noise offset</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating tumour cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM-CCD</td>
<td>Electron multiplying charged coupled device</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial cell adhesion molecule</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>MAC chip</td>
<td>Microfluidic affinity capture chip</td>
</tr>
<tr>
<td>MCF7</td>
<td>Human estrogen receptor-positive breast cancer cell line</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-specific binding</td>
</tr>
<tr>
<td>OH-TAM</td>
<td>Hydroxytamoxifen</td>
</tr>
<tr>
<td>PALM</td>
<td>Photoactivatable localisation microscopy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBSA</td>
<td>Bovine serum albumin in phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>pS15-p53</td>
<td>p53 phosphorylated at serine-15</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to noise ratio</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline sodium citrate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>STORM</td>
<td>Stochastic optical reconstruction microscopy</td>
</tr>
<tr>
<td>TAM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection fluorescence</td>
</tr>
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Chapter 1. Introduction

‘It is in the nature of the human being to seek a justification for his actions.’

– Aleksandr Solzhenitsyn

1.1. Why single cells?

It is important to spell out the rationale for studying single cells. The cell is the fundamental subunit of all life on Earth and indeed the tissues which work together to make the most complicated organisms can be comprised of billions, even trillions of cells. Against this vast canvas of biology, we must ask what the value is in searching for answers on the level of the individual cell, when it must seem obvious that so many processes are cooperative, and except in the simplest of organisms, involve many more than one cell.

The answer is twofold. Firstly, though cells from a distinct population [whether that be an organ, a cell line, or a clinical biopsy] may be genetically identical, phenotypically these cells can be very different, and will in virtually all cases display a spread, or distribution of characteristics, the ultimate shape of which may have profound consequences for the biological system. Secondly some samples may be so rare as to preclude a bulk analysis. There exist a few pathologically important rare cell types which ultimately must be analysed with single cell technologies – the canonical example being circulating tumour cells [CTCs], the purported agents
of metastatic spread in cancer, and a topic to which we will return below, and in depth in Chapter 4.

To focus on the former, cell-to-cell diversity is omnipresent in biological systems. Fundamentally this heterogeneity reflects the stochasticity inherent in the molecular mechanisms of gene expression and regulation, and the discrete differences in protein copy number this produces\(^1\). There is an emerging consensus that such heterogeneity does not occur arbitrarily, and that it is a potentially huge resource for new biological information that has only recently begun to be tapped\(^2\). Molecular stochasticity, or ‘noise’, appears to be an essential component of the cellular machinery that allows the cell to sense information pertaining to its internal states and external environment, and the process by which evolution and development are facilitated through a broadening of the range of phenotypes that can be produced from a given genotype\(^3\). This can produce highly advantageous outcomes such as the differentiation of critical blood components during haematopoiesis\(^4\), trichromatic vision in humans and other primates\(^5\), and the generation of ligand receptor diversity in the olfactory system\(^6\), but may also create conditions detrimental to human health. For example, inherent randomness in expression levels pertaining to physiologically-critical genes is a mechanism which enables some bacterial cells to survive antibiotic treatment when the rest of the clonal population does not, as the cells in question are preadapted to survival by a particularly advantageous variation in their molecular phenotype\(^7\).

Cellular variation is especially important in cancer. Genetic intratumour heterogeneity has been identified in many cancer types and presents a major diagnostic challenge\(^8\). Heterogeneity can be present at the macroscale, between large tumour subclones that have significantly expanded and also at the microscale level, between individual cancer cells\(^9\). Genetic heterogeneity can lead to phenotypic heterogeneity, for example of gene and protein expression levels or protein function\(^10\). In addition, phenotypic heterogeneity may also occur independently of underlying genetic variation, for example through microenvironmental influences or as a consequence of regulatory processes within cancer cells\(^11\). Overall, the current lack of insight into the extent of and the mechanisms that establish intratumour heterogeneity and of its clinical relevance is a central hurdle for the development of more effective therapies and biomarkers in oncology.

Many traditional biochemical techniques are unable to detect intrapopulation variability because by their very nature they require many cells for the measurements to be performed. Techniques such as western blotting are bulk – or ensemble – measurement techniques that produce a population averaged result that masks internal differences and returns the mean of some analyte parameter over hundreds to millions of cells. The mean is however in many cases a misleading metric because of the inherent property of the biological systems under interrogation to display subpopulations, outliers or otherwise non-normally distributed levels of protein expression [Figure 1.1]. Often these systems are perturbed through the addition of a drug,
and the net response is used to make inferences about the efficacy of the drug or the effect it has on the cells. Yet even in an isogenic cell population, the stochastic nature of gene expression and differing epigenetic characteristics mean that the response to external stimuli is not uniform, and the ensemble result necessarily fails to reflect the true response at the single cell level. This can have serious implications if the averaging obscures the presence of subpopulations that respond in different ways to the experimental conditions under consideration. It is a real and serious risk that a few outlying cells can bias the population average, or that the largest subpopulation obscures the behaviour of rare cells that are the ones that hold the greatest interest. A real-world example of this with pathological consequences is the emergence of chemotherapy resistance in tumours which have previously responded to a drug. If 99.9% of a tumour's cells are susceptible to a chemotherapeutic agent, then on average the patient's tumour is responding to treatment; however, it is the small minority of cells which are immune to the effects of the drug which will govern the future course of the disease, and the likelihood of the patient's survival. Unpicking the nature and causes of this variability could thus lead to therapeutic strategies that minimise drug resistance.

![Figure 1.1: Bulk measurements lead to misleading results.](image)

**A** The average level of expression in a multimodal distribution does not reflect the true expression level in either population. **B** Ensemble measurements will mask the distinct behaviour of a rare cell population.

Taking this into consideration, technologies to assess such phenotypes at the microscale level of single cancer cells are urgently needed. Single cell gene expression profiling has made major progress but technologies to evaluate protein expression and protein modification at the single cell level have been missing. Importantly, single cell technologies should ideally be able to work with very small quantities of tumour material, both to explore the heterogeneity at the highest levels of spatial resolution, and to make maximum use of often precious and small clinical tissue samples.

We are concerned in this thesis with single cell proteomics. The technical challenge of single cell proteomics is greater than that for its related -omics counterparts, due primarily to the lack of a controllable method of amplification of the protein signal, unlike RT-PCR in
single cell genomics and transcriptomics, but also the large dynamic range of expression levels. For example, in yeast protein copy number varies by a factor of $10^{6}$, while in human cells protein concentration varies by an even larger factor of $10^{7}$. Flow cytometry and immunofluorescence imaging are perhaps the two most commonly used methods for the study of population variability in proteomics, but both have notable drawbacks. Flow cytometry is a well-established technique which can look at the intercellular variability of protein expression on a large scale, but due to such considerations as the dead volume of the system and cell handling losses, it can still only be used with large initial samples, on the order of $10^{5}$ cells. It is thus unsuited for use with precious samples, such as CTCs or fine needle aspirates. Immunofluorescence experiments are simple to implement, and can in principal be performed on small to very large cell populations, but have practical limitations in terms of multiplexability [which is limited by spectral overlap of fluorophores], lengthy acquisition times, challenging data processing requirements, and the requirement that cells must be fixed prior to analysis, provided the cells are not expressing an endogenously produced fluorescent protein. While suitable analysis methods can allow expression levels to be compared between cells by quantifying relative fluorescence intensities, these techniques are only able to provide relative measurements of protein expression, and cannot tell us the true copy number of a protein within a cell. Due to intrinsic cellular autofluorescence, low-expressing cells may be easily confused with non-expressers. Consequently, there is an urgent need for robust single-cell technologies which are not subject to these limitations, and preferably which can attain proteomic information with single molecule resolution.

1.2. Microfluidics

Generally speaking, a microfluidic device is any one in which fluid flows in channels of sub-mm, and particularly sub-100µm dimensions. What we specifically mean when we talk of ‘microfluidics’ is a whole host of related miniaturised ‘Lab on a chip’ technologies which feature miniaturised fluidic channels designed for a specific experimental or analytical purpose. The field of microfluidics is a burgeoning field of research in 21st century science, and has found applicability in many diverse areas, from tissue engineering to organic chemistry. The reason for this proliferation is multifold, but hinges on the fact that microfluidic devices can be produced in-house to bespoke requirements, typically allow for drastic reductions in reagent usage and are relatively cheap to produce, which thus allows iterative improvement of promising prototype devices. To illustrate the potential reduction in reagent usage in miniaturisation of a system, consider that reducing the linear dimensions of a system by a factor of 1000 [e.g, from 1cm to 10µm] results in a reduction in volume of $10^{9}$, meaning an experiment that previously required millilitres of reagent can now be performed with only picolitres of the same substance. They have found particular applicability in the area of bioassays, and increasingly
in single cell analysis. Microfluidic single cell analysis was first developed for genomic applications, and these, along with transcriptomic applications are the most mature, but many new single cell proteomics analysis platforms have emerged in recent years based on microfluidic technologies, usually utilising antibody-based detection methods to discriminate different protein species in single cells. Notable methods include the single cell antibody barcode chip and single cell western blotting. An alternative to microfluidic methods in single cell proteomics is mass cytometry, where cellular proteins are targeted with transitional metal mass-tagged antibodies before being nebulised and analysed via mass spectrometry. The identity and abundance of the transition metals present can be traced back to the antibody labelling reagents, and thus quantify the proteins present in the cells. The variety of mass tags available leads to a high degree of multiplexing, although low abundance proteins are very challenging to detect.

1.3. The liquid biopsy

Perhaps the holy grail of current translational oncology, the so-called ‘liquid biopsy’ refers to attempts to develop minimally or non-invasive techniques to monitor the tumour burden, disease progression and therapy response of cancers through the routine sampling of patient blood. Efforts to develop various forms of liquid biopsy have resulted in a drive to create microfluidic technology capable of processing blood to extract CTCs and also to perform downstream genomic, transcriptomic or proteomic single cell analysis. It usually refers to either the assaying of circulating cell-free DNA [cfDNA] in blood plasma or the detection and analysis of circulating tumour cells [CTCs]. cfDNA was first described in 1948 and derives either from cellular necrosis or cell apoptosis, which results in spontaneous active shedding of DNA fragments into the bloodstream. The amount of cfDNA present correlates with tumour grade and prognosis, and has been investigated for early cancer diagnosis as well as a biomarker of mutagenesis and tumourigenesis in molecular epidemiology. CTCs are the direct agents of metastatic spread in cancer and occur when a primary, or indeed a secondary, lesion sheds whole cells into the blood, where a small fraction are able to survive and circulate, and eventually break out into new tissue at a distal site, seeding a metastatic deposit at the secondary location [Figure 1.2].
Figure 1.2: Circulating tumour cells. The primary sheds tumour cells into the bloodstream, and a small fraction with a more aggressive phenotype are able to survive and seed a new tumour in a distant organ.

CTCs as a component of whole blood are extremely scarce, usually only present at the level of a handful per 7.5ml blood sample [which may contain hundreds of thousands of leukocytes, and billions of red blood cells] $^{33,34,35}$. This presents an extreme technical challenge in terms of their isolation and analysis, but nevertheless it is one which must be surmounted, as the process of metastasis is responsible for approximately 90\% of cancer mortality, a statistic which cannot be reduced without a much deeper understanding of the metastatic cascade. CTC count is a well-established indicator for patient survival, and CTC enumeration has been used as a biomarker in over 200 trials involving patients with various cancers $^{36}$. It has been shown that a CTC count in excess of 5 per 7.5ml blood draw is indicative of considerably worse medium and long-term survival $^{37}$, but the potential information to be gained from analysing actual CTC phenotypes goes well beyond that accessible from simply counting the amount present in the bloodstream $^{38,39,40}$.

The onset of CTC dissemination is now thought to be an early event in the development of a tumour $^{41,42}$. The total number of disseminated cells and the comparatively low incidence of resultant secondary tumours, means that necessarily CTCs must vary in their malignant potential, and as such, identifying the molecular differences between CTCs is paramount to uncovering the roots of metastasis. Developing the technology to study this heterogeneity is therefore of paramount importance for the future of cancer research as well as fulfilling the promise of personalised medicine.
1.4. The MAC chip

The MAC chip is our research group’s own bespoke technology designed to address the demands of single cell proteomics. The workings of the MAC chip will be fully described in Chapter 2. Briefly, it was first introduced in 2011 as a microfluidic, label-free method of quantifying protein copy number in single cells, and has been used to quantify expression of the tumour suppressor p53 in a variety of human cancer cell lines. The MAC chip platform is an inherently single cell method. It uses optical cell handling in the form of laser trapping to manoeuvre cells inside the microfluidic channels of the device, and laser-induced lysis to initiate the analysis step. Readout is performed via single molecule-sensitive fluorescence imaging. The MAC chip assay may in theory be calibrated, allowing absolute quantification of protein in single cells. There is no lower limit to the number of cells needed to perform MAC chip analysis, and as such the MAC chip method is ideally suited to the analysis of rare clinical material, including CTCs.

1.5. Thesis overview

Taking the above into consideration the goal of this research is to apply the MAC chip technology to directly measure protein copy number in clinical or clinically-derived material, with a focus on circulating tumour cells isolated from the blood of cancer patients.

As p53 is not frequently used as a biomarker in many cancers, we aimed to develop a single cell-sensitive MAC chip assay for a diagnostic and prognostically relevant protein biomarker. The chosen target was estrogen receptor alpha, a hormone-activated transcription factor that plays a crucial role in female sexual development and one that is of critical importance in the treatment of breast cancers. The attempted development of this quantitative single cell ERα assay is the subject of Chapter 2.

The development of the ERα assay required a concomitant redevelopment of the image analysis and single molecule counting procedures used to extract quantitative information from MAC chip datasets. The development and testing of these new image processing methods is covered in Chapter 3.

Circulating tumour cells are a unique and challenging target for single cell analysis. Their scarcity and the complexity of the isolation task requires specific technologies to perform CTC isolation successfully before analysis of the resultant cells is even considered. Much work has been done on this aspect by other groups and many technical solutions have now been put forward. Nevertheless, these technologies are typically bespoke and must be reproduced in-house if they are to be used to study CTCs. Furthermore, the MAC chip itself places certain requirements on the properties of the sample that can be introduced to it and single cell analysis
successfully performed. Chapter 4 details the work done to reproduce an effective CTC isolation system in-house, as well as develop an efficient technological solution capable of coupling the output of the chosen CTC isolation method into a MAC chip for single cell analysis.

In Chapter 5 we focus on the establishment of a protocol for using the MAC chip to analyse heterogeneity of protein expression within solid tumours themselves. We will demonstrate the successful use of this protocol to study p53 levels and p53 phosphorylation in dissociated cells from mouse xenograft biopsies derived from colorectal cancer patients acquired through the ProspectC and ProspectR colorectal cancer trials at the Royal Marsden Hospital [London, UK]. These proof-of-principle experiments demonstrate conclusively that the MAC chip method is a viable technology for interrogating clinically-derived samples.
Chapter 2. A microfluidic affinity capture assay for estrogen receptor alpha

2.1. MAC chips

The MAC chip, a microfluidic platform for quantitative proteomic analysis of single cells, was first published in 2011 by Salehi-Reyhani et al. The MAC chip is a novel biosensor which consists of a miniaturised ELISA assay within a microfluidic environment, and which uses optical manipulation and lysis of cells, coupled with fluorescent TIRF readout to directly measure protein content in single cells. MAC chip assays are almost unique in the field of single cell analytics, as they have the potential to provide absolutely quantitative data on cellular protein copy number, because any MAC chip assay can be calibrated with a recombinant protein standard.

The first MAC chip developed was for the tumour suppressor protein p53, and at the time this project was commenced, all the published data from the MAC chip was for this assay.
To become a useful analytical platform, a broader repertoire of analytes was obviously required. As such, development was started on numerous other analytes [see for example Dr Gemma Milman’s PhD thesis, 2016], with protein targets chosen based on biomolecular significance. In this project, we were tasked with developing an assay for the human estrogen receptor alpha, a protein with immense importance for characterisation and treatment of breast cancer.

The MAC chip has already been demonstrated as an effective platform for the absolute and relative quantification of protein copy number in single cells derived from cell lines, but the MAC chip has several features which make it an intrinsically useful platform for the processing of clinical material [See Chapter 5]. These include: [1] a low requirement for primary cells in terms of their absolute number meaning analyses can be performed scarce or precious samples, or that very small biopsies could provide sufficient material for analysis, opening the possibility for less invasive methods; [2] no fixation is required to perform the assay meaning cells are viable till the point of analysis and thus functional and dynamic studies may be performed; and [3] the MAC chip workflow provides high selectivity for both viability and specific surface antigen or morphologically defined subpopulations of interest e.g. epithelial or mesenchymal cells, which may be of particular importance when working with processed primary samples that can contain a large proportion of dead material or that may be highly heterogeneous with respect to their cellular constitution. It is these properties and the desire to generate diagnostically or prognostically useful data with the MAC chip format that were the rationale behind developing assays for notable biomarkers, and this in turn made the estrogen receptor an obvious candidate.

2.1.1. Experimental overview

A schematic diagram of the MAC chip concept is shown in Figure 2.1. The design of the standard MAC chip consists of a large reservoir channel into which a suspension of single cells is loaded, perpendicular to which are ~50 smaller channels leading to individual analysis chambers. Cells are moved from the main channel into the analysis chambers via an optical trap. Optical trapping is a powerful technique that can create forces of an ideal magnitude to non-destructively manipulate cells and subcellular components, even allowing the direct mechanical interrogation of single biological macromolecules such as nucleic acids and proteins. It works because a laser beam tightly focussed though an objective lens creates a steep spatial light gradient, and dielectric particles such as cells placed in this gradient experience a force in the direction of greatest intensity, drawing the cell towards the focal point of the laser. The laser beam also gives rise to a scattering force which blows the cell along in the direction of beam propagation and is the result of forward momentum transfer from the photons to the object in the beam path. When the scattering and gradient force are in equilibrium the cell is trapped, and by moving the position of the trap relative to the cell’s environment, the cell can be moved
to a desired location, provided the viscous drag force on the cell as it moves does not exceed the trapping force of the laser. Use of the optical trap allows specific cells to be chosen for analysis based on criteria such as cell surface marker expression or viability.

Figure 2.1: The MAC chip. [A] Schematic overview of an OT2 MAC chip with 50 single cell analysis chambers. [B] Zoom of 300×300μm square analysis chambers and channels. [C] Schematic diagram of analysis chamber. A printed primary antibody spot which reacts with a protein analyte resides in a central ring in the chamber, while a fluorescent secondary antibody targeting a secondary epitope on the analyte floats in solution. A single cell is brought into the chamber via optical trap, and lysed with a concentrated pulse of high power laser beam,
allowing the cellular contents to diffuse freely in the chamber. With the cell lysed, the target proteins bind to the capture antibody spot on the solid phase and form complexes with the fluorescent secondary. Total internal reflection fluorescence illumination creates an exponentially decaying evanescent field allowing only the closest molecules to surface of the coverglass to be excited and thus detected in the experiment.

In fact, all cellular manipulation and analysis in the MAC chip platform is performed optically and is based around a standard inverted microscope. Cells are loaded into analysis chambers from the main sample chamber using an optical trap. Cellular lysis is achieved with a high energy laser pulse \([E=14\mu J; 6\text{ns pulse; } \lambda=1064\text{nm}]\) into the medium surrounding the cell which produces a cavitation bubble capable of rupturing the cell membrane and releasing its contents into the analysis volume. The assay readout is by total internal reflection [TIRF]-based optical single molecule imaging of the fluorescence at the antibody capture spot. Once the assay has reached chemical equilibrium, the bound protein is imaged using the readout TIRF laser \([\lambda=405, 488, 532 \text{ or } 647\text{nm}]\) and an Andor iXon DU-897E EMCCD camera [Andor Technologies, Ireland].

2.2. Estrogen receptor alpha

The human nuclear hormone receptor estrogen receptor alpha \([\text{ER}\alpha]\) is overexpressed in more than 60% of breast cancer cases. It is a prognostically useful biomarker: expression levels are negatively correlated with tumour grade and survival outcome\(^{48}\), and the hormone response phenotype of breast cancer is defined with respect to ER status\(^{49}\), which has important implications for the type of cancer therapy to be administered. When bound by its ligand estradiol \(\text{ER}\alpha\) acts as a transcription factor which transactivates a wealth of proliferative genes; its abnormal function can therefore be an essential precursor to cancer of the reproductive tissues, where estrogen receptor expression is greatest. \(\text{ER}\alpha\) function is targeted in ER-positive breast cancers by anti-estrogen therapies such as Tamoxifen whose metabolites act as antagonists to \(\text{ER}\alpha\)’s ligand binding pocket and disrupt the estrogen signalling cascade.

\(\text{ER}\alpha\), along with progesterone receptor \([\text{PR}]\) and the human epidermal growth factor receptor 2 \([\text{HER2}]\) forms a canonical trio of diagnostically and prognostically crucial biomarkers in breast cancer\(^{50,51}\). It has been known since the 1970s that positive expression of \(\text{ER}\alpha\) in primary breast tumours can enhance survival outcome and increase the likelihood of response to endocrine therapy with tamoxifen, as well as providing an independent parameter for predicting early recurrence in patients where the tumour has been surgically resected\(^{52}\).

The MAC chip is one of few technologies that may be suitable for the quantitative study of circulating tumour cells [See Chapter 4]. In breast cancer, there is already evidence to indicate that circulating tumour cells may have vastly different estrogen receptor profiles than the primary tumour that sheds them\(^{53}\), and the use of a MAC chip estrogen receptor assay would allow this effect to be quantitatively profiled.
2.3. Design and manufacture

MAC chips are designed and manufactured using well established photolithographic and soft lithographic techniques [Figure 2.2]. The layout of the microfluidic is first designed using appropriate software [AutoCAD, Autodesk Inc.]. The design is then converted into either a photographic film, or chromium-glass photomask, depending on the requirements of the final microfluidic. Generally, film masks are suitable for microfluidics where the minimum feature size is no less than 10μm. For microfluidics with higher spatial resolution requirements, and feature sizes down to the sub-micron level, a chrome on glass mask is necessary [for a more in-depth discussion of the determinants of resolution in the soft lithographic process, see Chapter 4]. The mask is designed to transmit UV light in the regions of the mask which contain the microfluidic design, and opaque elsewhere. The negative epoxy photoresist Su-8 [MicroChem] is deposited in a film of the desired thickness on a 100mm silicon wafer via spin coating, and the wafer is then exposed to intense UV light through the mask, usually with a mercury lamp. The UV light causes the Su-8 beneath the transparent portions of the mask to crosslink and solidify, and the uncrosslinked Su-8 can then be dissolved away with Su-8 developer [MicroChem], or propylene glycol monomethyl ether acetate. The wafer is then silanised with 1,1,2,2-perfluoroctyltrichlorosilane [Sigma Aldrich], which makes the surface of the mould hydrophobic and facilitates the subsequent lift-off of cured PDMS.

To create the microfluidic superstructure itself polydimethylsiloxane [PDMS] is mixed in a 10:1 ratio with curing agent [Sylgard 184 Silicone Elastomer Kit, Dow Corning], poured over the mould, and degassed inside a vacuum desiccator to remove air bubbles [approx. 15-30 minutes]. The PDMS is then allowed to cure at room temperature for 24 – 48 hours, until it is set solid. It is possible to cure PDMS quicker at elevated temperature, however this is not suitable for MAC chip microfluidics, as curing at higher temperature can cause the mould to swell slightly, and consequently the chambers of the MAC chip will no longer exactly align with the 500μm spacing of the spots deposited on the microarray slide. Once cured, the design is cut from the mould using a scalpel, and inlet/outlet holes are drilled through the PDMS at the required locations.
Figure 2.2: Essentials of microfluidic mould production. The photocurable polymer Su-8 is deposited onto a 100mm silicon wafer, then spin-coated at the appropriate RPM for a set period to create an even film of the desired thickness across the surface of the wafer. After spin-coating, the wafer is ‘baked’ to remove excess solvent and aligned with the photomask, then exposed to high intensity UV light, which causes the SU-8 beneath the transparent portions of the mask to cross-link and solidify in the desired pattern. After exposure, the wafer is baked again, then submerged in a developer, which removes all the un-crosslinked SU-8 that received no UV exposure, leaving only the wafer and the solidified mould of the microfluidic.

PDMS pieces are then washed to remove dirt and other contaminants. The PDMS is first submerged in detergent solution [1% Alconox, Alconox Inc], sonicated for at least 1 hr, then rinsed copiously in isopropanol and dried with nitrogen. To complete the MAC chip the PDMS superstructure must be aligned so that the analysis chambers of the chip align exactly with the antibody spots that have been microarrayed onto the coverslip. This is performed with the aid of a custom-built alignment rig, which allows the translation of the PDMS piece relative to the microarray slide in the x,y,z dimensions, as well as rotation in the x-y plane [Figure 2.3]. Once the chambers are aligned with the spots, the PDMS is lowered onto the coverslip and contact bonding occurs between the PDMS and the surface. Provided the PDMS surface and the slide are both clean, contact bonding is usually sufficient to maintain the integrity of the device during a MAC chip experiment. Contact bonding is also non-permanent, meaning the PDMS structures can be recovered post-experiment, and reused after cleaning. Some applications [See Chapter 4] require a more robust bond between the PDMS and the substrate, and in this case a process called plasma-bonding can be used to create a permanent bond between the surfaces.
2.3.1. Surface functionalization

The role of the glass supra-surface in microarray design is critical to maximising signal to noise ratios, and the best surface to use varies for different antibody pairs and their target antigen. Three distinct surfaces were tested in the anti-ERα and anti-p53 antibody experiments in this thesis: [1] ‘PEG-Neutravidin®’ slides, produced in-house to a custom protocol [below], and two commercially available coverslips designed for protein microarrays, [2] Schott Nexterion Type H, and [3] PolyAn 3D functionalized Neutravidin slides. PEG-neutravidin coverslips produce a very low background signal by inhibiting the non-specific binding of the fluorescent secondary antibody to the glass coverslip. The PolyAn Neutravidin coverslips are essentially a commercially available version of this in-house protocol, as they both result in a largely homogeneous neutravidin surface layer, whilst Nexterion Type H coverslips consist of a cross-linked, multi-component polymer layer activated with N-Hydroxysuccinimide (NHS) esters to provide covalent immobilization of amine groups.

PEG-neutravidin functionalisation was performed as follows: coverslips were first silanised with (3-Aminopropyl)triethoxysilane [APTES; Sigma-Aldrich, UK] and then functionalised with mPEG-SVA [M_w 5000] and biotin-PEG-SVA [Laysan Bio, USA]. Slides first underwent a strict cleaning preparation which consisted of sonication for 20 minutes in 1M potassium hydroxide [Sigma-Aldrich, UK], 5 minutes in deionised water, 15 minutes in acetone and finally another 20 minutes in KOH. Slides were washed ×3 in deionised water in between sonication steps and dried with nitrogen gas after the final step. Cleaned coverslips were then incubated at room temperature for 10 minutes in APTES solution [1 ml APTES + 5ml acetic acid + 100ml MeOH], sonicated for 1 minute, then left to incubate for a further 10 minutes. They were then rinsed 3× with MeOH and 3× with deionised water, then dried with nitrogen.
Functionalisation with mPEG/mPEG-biotin-SVA was performed as follows: 5 – 15 mg of PEG-biotin-SVA was mixed with 50 mg of mPEG in 400 µl of 1 mM sodium bicarbonate buffer; 80 µl of this was then pipetted onto an APTES coverslip, sandwiched with another then incubated in darkness at room temperature in a humidity chamber for 2 – 3 hours. The derivatised coverslips were then removed, rinsed copiously with deionised water and stored in the dark at room temperature until required.

Immediately prior to antibody printing coverglass surface composition was further modified with the addition of NeutrAvidin®. 80 µl of NeutrAvidin® at 0.1 mg/ml in Dulbecco’s phosphate buffered saline [PAA Laboratories GmbH, USA] was sandwiched between two PEGylated coverslips and incubated in the dark for 1 hour at room temperature. Coverslips were then again copiously washed with deionised water, dried with nitrogen and used immediately for microarray printing.

2.3.2. Microarray printing

Microarray technology has been used in biological experiments for several years. The first microarray biosensors were DNA-based54, however protein microarrays are now an established technology that be used to screen bulk samples [e.g. cell or tissue lysates] and characterise them for hundreds of thousands of proteins simultaneously55. The MAC chip takes the essence of a bulk analytical protein microarray, the immobilisation of an antibody to a target protein on a suitable capture surface via a microarray printing robot, and houses it within a set of microfluidic channels to provide the sensitivity required to measure protein from only a single cell.

The MAC chip antibody spots were printed onto functionalised coverslips using an Omniprint digital microarrayer. Antibodies were diluted from their stock concentration 1:1 with a print buffer, which, depending on the primary antibody typically resulted in a concentration of antibody of 0.5 – 1 mg/ml in the deposited antibody spot. The print buffers used were [at their final concentration in the antibody spot] 3XSSC 1.5M betaine, and 1X ArrayIt protein printing buffer [ArrayIt Corporation]. These were used as they have been shown to be among the best performing protein microarray buffers in the literature56, as well as in internal group evaluation experiments57.
2.3.3. Device filling and cell loading

MAC chip devices were filled immediately prior to cell experiments with a solution consisting of 4% bovine serum albumin in Dulbecco’s phosphate buffered saline, supplemented with the fluorescent secondary antibody at the stated concentration. Filling occurred by placing a small reservoir of the BSA + secondary antibody solution over the inlet/outlets of the device and degassing the device in a vacuum desiccator for 10-30 minutes. MCF7 cells were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% foetal bovine serum. Immediately prior to a cell experiment, cells were trypsinised, then resuspended in media and triturated to create a single cell suspension. The single cell suspension was introduced to the main channel of the device by drawing the cell solution through from a small reservoir over the device inlet with a microfluidic vacuum pump [LabSmith Inc]. From the main channel individual cells were manually moved into analysis chambers by optical trap.

2.4. Readout and data analysis

MAC chip readout is performed via single-molecule total internal reflection fluorescence [TIRF] microscopy. TIRF is widely used in single molecule microscopies due to its restriction of the excitation volume to an approximately 150nm thick region above the surface of the coverslip. TIRF excitation occurs via an evanescent field, the intensity of which decays exponentially with distance away from the coverslip surface, which dramatically reduces unwanted fluorescence background signal. The optical configuration of the MAC chip TIRF microscope is shown in Figure 2.5. Raw MAC chip data consists of a sequence of single molecule fluorescence images of the analyte complex on the printed antibody spot [Figure 2.6]. Images may be either time-course, showing fluorescence increase over time, or single time-points taken once the assay has reached equilibrium.
Data analysis is performed through a series of custom-developed single molecule counting procedures written in ImageJ. The analysis codes originally used when this project began were found to be inadequate for analysing much of the ER assay data which is the subject of this chapter, and these codes were gradually replaced with a new methodology over the course of the project. The development of these new codes is discussed in depth in Chapter 3, High dynamic range single molecule image analysis.

Figure 2.5: MAC chip optical setup. Modified from43. Vortran Stradus Versalase multiwavelength laser system is used to illuminate the sample with 405, 488, 532 or 647 nm light after passing through a motorised TIRF unit [MTU]. A 1070nm continuous wave laser is used for the optical trapping and manipulation of cells. A 1064nm pulsed laser is used for optical lysis of single cells in analysis chambers. λ/2 = half-wave plate, PBS = polarising beam splitter, M = mirror, FM = Flip Mirror, PM = Periscope Mirror, L1 and L2 = lenses in beam expander, FC = Filter cube.
2.5. Modifications of the MAC chip design

2.5.1. On-chip validation of viability of antibody spots: The Standard Candle MAC chip

The traditional design of the MAC chip does not contain a facility for positive controls. To rule out the failure of the antibody spots during experiments for the target analyte, the architecture of the MAC chip was altered to introduce additional control lanes which could be filled after an experiment with a control solution of analyte-positive lysate or recombinant protein to demonstrate that the printed antibody spot is capable of binding the analyte. In chips which are functioning correctly the antibody spot will ‘light up’ when a highly-concentrated control is introduced. The Standard Candle chip’s name is a nod to the field of cosmology, where supernovae of a particular spectral class with a known brightness are used as calibrators for cosmological distances [so-called ‘standard candles’]. Having said this, it should be borne in
mind that the SC chip is designed to be a qualitative binary control [spots are designated functioning or not functioning, depending on outcome], rather than a quantitative internal calibration. The reasons for this will be discussed below.

2.5.2. Volume reduction

Modelling work done by the group in 2013 raised the possibility of improving the signal response in the MAC chip by reducing the assay volume, as this acts to concentrate the analyte once has been released from cells, compared to larger chamber volumes. The 4.5nl chamber of the standard MAC chip was reduced to 0.74nl for the early MAC chip experiments in this chapter, to capitalise on this effect, and a range of intermediate miniaturised chambers were also developed with volumes of 1 and 1.25 nl.

Miniaturising the MAC chamber may impact the printed antibody spot, which is of defined size, typically varying for different assay systems between 100-150μm in diameter. Additional constraints are placed on the minimum height of the microfluidic by the size of the cells that need to be analysed: obviously the chamber height cannot be reduced below the diameter of the largest cells that must be analysed, and hence for a maximally miniaturised assay there is only a small range of x,y and z-dimensions that may be successfully used. Figure 2.8 shows the relationship between these dimensions for a range of analysis chamber volumes.

Figure 2.7: The standard candle MAC chip. The SC chip improves on the conventional MAC chip design by introducing a secondary inlet connected to five additional antibody capture chambers, allowing the introduction of a positive control solution during single cell experiments.
2.6. Assay development: antibody screening

Maximising assay sensitivity is critical to detecting analyte in single cells. A key [though not the only] determinant of this in the MAC chip system is the affinity of the component antibodies for their target protein. There are many antibodies commercially available for ERα detection and so to select the antibodies with the maximum likelihood of success in the MAC chip a series of parallel antibody screens were performed in order to identify antibody pairs that showed strong binding under test conditions. Table 2.1 summarises the ERα antibodies used in experiments. These screens were initially performed in an open-well format, where multiple primary antibodies were printed onto a slide and tested for their signal response to a solution of full-length human recombinant ERα [ThermoFisher Scientific] plus secondary fluorescent antibody at a known concentration. Printing multiple arrays of primary antibodies to the slide allowed for the parallel screening of multiple secondaries, or screening at multiple analyte concentrations. Typically for initial screening of primary antibodies the secondary antibody concentration was fixed [e.g. 1µg/ml] and the analyte was titrated over several orders of magnitude [e.g. 1×10³, 1×10⁵, 1×10⁷ molecules/ml]. Arrays were separated using a PDMS piece with well-structures cut to fit the outline of the array. Open chip experiments were usually performed in a 3-well format [Figure 2.9, upper panel].

Figure 2.8: Z-depth vs x-y lengths for a square analysis chamber over a range of target volumes. Geometric restrictions due to cell and antibody spot size have implications for multiplexed experiments, as smaller x-y lengths can accommodate fewer antibody spots.
Table 2.1: Summary table of antibodies screened for the ERα assay

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Class</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>E115 [ab32063]</td>
<td>Rabbit monoclonal</td>
<td>Abcam</td>
</tr>
<tr>
<td>ab205850</td>
<td>Rabbit monoclonal</td>
<td>Abcam</td>
</tr>
<tr>
<td>EPR703(2)/ab79413</td>
<td>Rabbit monoclonal</td>
<td>Abcam</td>
</tr>
<tr>
<td>AB1104</td>
<td>Mouse monoclonal</td>
<td>Abcam</td>
</tr>
<tr>
<td>C542/ab66102</td>
<td>Mouse monoclonal</td>
<td>Abcam</td>
</tr>
<tr>
<td>H151</td>
<td>Mouse monoclonal</td>
<td>Novus Biologicals</td>
</tr>
<tr>
<td>H4624</td>
<td>Mouse monoclonal</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>H184</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>HC20</td>
<td>Mouse polyclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>D12</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>F10</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>C311</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>2Q418</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>D-4</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>1F3</td>
<td>Mouse monoclonal</td>
<td>ThermoFisher Scientific</td>
</tr>
<tr>
<td>6F11</td>
<td>Mouse monoclonal</td>
<td>ThermoFisher Scientific</td>
</tr>
<tr>
<td>TE111.5D11</td>
<td>Mouse monoclonal</td>
<td>ThermoFisher Scientific</td>
</tr>
</tbody>
</table>

In the literature, the HC20 antibody is widely used in immunofluorescence experiments and others for the detection of ERα [>230 citations in the literature]. It was thus decided to use this antibody as the initial detection antibody for the screening process. HC20 was bought unlabelled and labelled in-house with Lightning Link DyLight-488 fluorescence labelling kit [Thermo Fisher Scientific].

Using this technique, several strongly binding candidate ERα antibodies were identified: D12, HC20, F10, and C311 [all Santa Cruz Biotechnology Inc]. These candidates then underwent a secondary open-chip screening round to identify the best secondary antibody and print buffer combination [Figure 2.10]. The candidates were printed in 3 arrays of 8×25 spots. Each candidate was printed 1:1 with both 6×SSC-3M betaine and 2×ArrayIt protein printing solution. Each of the 3 wells contained a different detection agent: either HC20 or D12 antibodies, both conjugated with Dylight-488, or Fluormone ES2, a small molecule fluorescent estrogen
analogue [ThermoFisher Scientific]. The results of this screen indicated that the HC20 antibody outperformed both D12 and Fluormone ES2 as detection agents, and produced maximum signal when paired with either D12, F10 or HC20 itself as the primary capture agent, and ArrayIt as the print buffer. D12 displayed binding equal to F10 within the margin of error, but D12 was chosen as the candidate as the spot morphology was superior. A double HC20-HC20 assay was rejected as the main assay candidate, even though it produced excellent signal, because this assay would only be able to detect dimerised protein.

Figure 2.10: Results of parallel antibody + print buffer screening. HC20=0.95ug/ml=3.7E6 per nl, d12=1ug/ml, fluormone1800nM [1e7/nl]. A=ArrayIt print buffer, B=3×SSC1.5M betaine. All primary antibodies had a final printed concentration of 1mg/ml.

2.7. The D12 + HC20 sandwich assay

The D12+HC20 candidate assay underwent testing to assert its suitability for the analysis of ERα from single cells. Firstly, the time to equilibrium of the system was measured [Figure 2.11], and subsequently a calibration curve was performed with recombinant protein [Figure 2.12] to quantify the response of the assay at multiple orders of analyte concentration. These were first performed in specialized microfluidics called Flow Chips [Figure 2.13]. The Flow Chip is a variant of the MAC chip which preserves the basic principle of a miniaturized ELISA assay, but is designed in such a configuration that allows the solution inside the MAC chip chamber to be replaced. The design consists of three or more long, thin, unconnected parallel channels, each with multiple circular chambers to house antibody spots, and inlet/outlets at
either end. Each lane of the device is connected to a syringe pump and a solution of the target analyte plus secondary antibody at a desired concentration is flowed in and allowed to bind to the primary antibody spot. With such a device, it is possible to test the effectiveness of the antibody sandwich pair over several orders of magnitude, and in doing so compute important parameters such as the assay’s dynamic range, limit of detection, and, by using solutions that contain only secondary antibody and no recombinant protein, the level of non-specific binding of the fluorescent secondary to both the primary antibody, and the PEG-neutravidin surface. The calibration curve is also the process through which absolute protein numbers can be obtained through MAC chip measurements. The chamber volume of the flow chips was 0.74 nl.

Figure 2.11: Time to equilibrium of the ERα D12+HC20 assay. Equilibrium is reached by 2 hours after analyte is introduced.
Figure 2.12: Calibration curve for the D12 + labelled-HC20 ER assay using 0.74nl chambers. Single molecule count above background means the average number of single molecules counted per frame minus the mean number counted with only labelled secondary antibody in the analysis chamber. The dashed line indicates an ideal assay that binds 100% of the analyte. Data points are the average of 10 spots ± 1 standard deviation. Secondary antibody concentration for all measurements was 0.125 µg ml⁻¹. The graph shows that ERα detection is linear with concentration over some 3 orders of magnitude.

Figure 2.13: The Flow Chip. This microfluidic consists of multiple [usually 3] separate, parallel lanes of antibody capture chambers. The lanes are connected to distinct inlets and controlled by separate pumps, such that the solution in the analysis chamber can be refreshed. This allows titrations of analyte to be performed in a microfluidic environment on a single device.

The calibration curve indicated that it should be possible to get a measurable signal with this assay for any cell that expresses over approximately 3500 molecules of ERα. To see if this was suitable for our purposes a back-of-the-envelope calculation was performed to estimate the number of ERα molecules per cell, based on quantities already measured in the literature.

2.7.1. Estimate of the expected value of ERα copy number in single MCF7 cells

To date there have been no direct measurements of the mean expected copy number of estrogen receptor per cell for any cell lines or primary tissue types, though a characteristic threshold
for determining the estrogen receptor positivity of clinical samples has been put forward by Welsh et al of 2 pg/µg total protein\textsuperscript{59}. Assuming that a typical cell has a mass of $10^{-9}$ g and that approximately 20% of the cell by weight is protein then a single cell on the cusp of estrogen receptor-positivity ought to contain $\sim 3600$ copies of ERζ. This is just below the linear detection region of the MAC chip assay, however it should still be possible to detect. More importantly highly expressing cells such as MCF-7 contain up to 150 times this lower threshold, putting their expected copy number at $\sim 0.5$ million proteins, safely within the linear portion of the assay’s dynamic range and leading to the conclusion that the clear majority of estrogen receptor positive cells will be able to be characterised by the assay.

2.7.2. D12 + HC20 assay: single cell experiments

Four separate repeat MAC chip experiments were performed with the model ERζ-positive cell line MCF7 under normal culture conditions [Figure 2.14 & Figure 2.15]. MAC chambers were filled with anything from 0-5 cells, with multiple cells in some chambers to maximise the chance of measuring a signal. In any fluorescence-based assay, photobleaching is an issue, as so as internal calibration for this, multiple antibody-spot chambers were left empty, and a compensation factor equal to the mean decrease in single molecule binding in the empty chambers after lysis, was added to all post-lysis measurements. Surprisingly, considering the prediction of the calculation above, only a small handful of chambers exhibited a significant increase in single molecule binding 2 hours after cell lysis. Additionally, most of these chambers where increased binding was detected, contained multiple cells, meaning the potential of the D12-HC20 system as a single cell ER measurement system was not promising.

Exposure to the drug tamoxifen [TAM] has been reported in the literature to increase estrogen receptor expression in MCF-7 cells by up to 5-fold\textsuperscript{60}. Accordingly, a subsequent experiment was performed where the MCF7 cells had been cultured in media containing 1μM TAM for 72 hours, however this too showed very little detectable ERζ [Figure 2.14C].

As this was counter to the expectation from the calibration curve, some controls were performed to ensure that the chip was functioning as expected. Firstly, the control lanes of the standard candle MAC chip were filled with a high concentration [10\textsuperscript{7} molecules/µl] recombinant ER solution to ensure that the spots were active inside the microfluidic, which proved to be the case [inset Figure 2.15D]. Secondly, immunofluorescence experiments were performed on fixed MCF7 cells to confirm that the cells were indeed expressing ERζ, as expected.
Figure 2.14: Result of D12-HC20 single cell experiments. [A] Plot of raw single molecule counts from 4 MAC chip repeats with the D12-HC20 assay with MCF7 cells. Empty MAC chambers are used to calculate a compensation factor to account for fluorophore bleaching. Histograms of the increase in single molecule binding after cell lysis indicate that only a handful of cells contain a measurable level of ERα for both untreated cells [A] and those treated with tamoxifen for 72 hours [C]. The change in single molecule count after lysis for empty chambers forms a normal distribution around zero.
Figure 2.15 [A-D]: Increase in SM binding with number of cells in chamber indicated. As is apparent from this plot, the majority of chambers in which we observed significant increases in single molecule binding actually contained more than one cell. [D-inset] Standard candle control lanes with $2 \times 10^7$ ERα molecules displayed an increase in binding of $\sim 10^5$ molecules.

2.8. Immunofluorescence of MCF7 cells

To confirm that the MCF7 cells were indeed expressing ERα, immunofluorescence staining was performed with the ab205850-Alexa488 antibody. The fixation and staining protocol was as follows, and adapted from Stadler et al. First cells were washed in ice cold PBS, then subsequently fixed in 4% paraformaldehyde in PBS for 15 minutes. The cells were then rinsed briefly in PBS and permeabilised with 0.1% Triton X-100 for 15 minutes at room temperature, then finally rinsed 3 times in PBS for five minutes each time. Antibody staining was performed.
overnight at 4°C at the manufacturer-recommended dilution [1-5μg/ml] in 4% foetal calf serum in PBS + 0.05% Triton X-100. A multiple panel of antibodies was used consisting of ab205850-488 for ERα and DO-1-647 for p53. After the incubation the cells were washed 3 times in PBS for 5 minutes, then exposed to the Hoechst nuclear stain at 1μg/ml for 10 minutes, then finally washed again 3 times in PBS prior to imaging. A representative immunofluorescence image is shown in Figure 2.16.

The distribution and cellular localisation of ERα is as one would expect for MCF7 cells, with both cytoplasmic and nuclear fractions. See, for example, Razandi et al 2013. This confirmed that ER was present in the cells, though it revealed nothing about the abundance, nor what fraction of ER in the cells was unable to be bound due to the blocking of epitopes by binding partners or molecular complexes.

Figure 2.16: Immunofluorescence of MCF7 cells confirms the presence of ERα. ERα is in green, blue is the Hoechst DNA stain highlighting the nucleus. Both nuclear and cytoplasmic fractions of ERα are present in the cells.
2.9. Repeat calibration curves

The positive result for ERα via immunofluorescence led to the calibration curve of Figure 2.12 being repeated, however instead of performing the experiment in a flowchip environment, the protein titration was performed in a series of standard MAC chips. That is, for a range of analyte of 6 orders of magnitude, from $10^2$- $10^7$ molecules/nl, 6 separate 50-chamber MAC chip devices were each filled directly with a solution of secondary antibody + protein at $10^2$, $10^3$ molecules/nl. This was done in order to make the correspondence between the assay environment during the calibration curve and that during single cell experiments as close as possible i.e. to account for any extra effects due to the different geometries in the flow chip vs the miniaturised MAC chip. The curves were performed for two secondary concentrations: 0.125μg/ml and 1μg/ml. The results of these ‘chip filling’ calibration curves is shown in Figure 2.17. Strikingly, these curves show a considerable discrepancy with those performed in the flow chip, and a considerably lower capture efficiency and worse limit of detection [Flowchip 0.125μg/ml secondary: 1145 counted molecules, or 3089 molecules in the chamber; 0.74nl MAC with 0.125μg/ml secondary: 558 counted molecules or 364,000 molecules in the chamber; 0.74ml MAC with 1μg/ml secondary: 940 counted molecules or 149,000 molecules in the chamber]. Both MAC chip calibration curves exhibited a much lower degree of non-specific binding compared to the flowchip, although NSB was worsened by the higher secondary concentration of 1μg/ml in the MAC chip.

![Figure 2.17: 0.74ml calibration curves repeated in standard MAC chips [red+blue curves] show a much lower capture efficiency than the original flowchip calibration curve [black curve].](image-url)
Why did the initial D12-HC20 standard curves performed in a flowchip differ so much to those performed later inside standard MAC chip geometries? A possible clue lies in the control lanes of the standard candle device. Like Flowchip lanes, the standard lanes of this device are attached to a pump at one end, and a reservoir of recombinant protein solution at the other. During a single cell experiment [until the control is introduced], this reservoir, and the control chambers themselves, contains only secondary antibody, and the pump is stopped so in theory the fluid in the channels should be static. The control chambers are otherwise identical to the single cell chambers. Yet the measured level of non-specific binding of the secondary antibody in these chambers is always markedly higher than in the cell chambers. These measurements are always taken at equilibrium [as measured in the non-control lanes] and suggest that, even though the pump has been stopped, the control lanes must have been exposed to more secondary antibody than the other chambers. As the volume of the chamber is so low, the degree of flow required to completely replenish the analyte and secondary in the chamber is minuscule: less than a nanolitre. This could easily be accounted for through an error in pumping, either through the intrinsic stability of the pump [the minimum step size of which is 167 nl] or through a systematic error caused by the presence of a bubble or cavity in the barrel of the syringe, for example. Another piece of evidence in support of this hypothesis is that the nonspecific binding observed in the D12-HC20 single cell experiments [36±14 molecules] was markedly lower than that recorded during the flowchip calibration curve [560±210 molecules]. Furthermore, plotting the on-spot single molecule for each spot in a flowchip lane [Figure 2.18] shows that spots nearest the source of the recombinant protein display more bound protein. The repeat 0.74 nl chamber D12-HC20 curve performed in standard MAC chips showed considerably lower capture efficiency than that performed in the flowchip.
Figure 2.18: Position dependent binding in the flowchip. Spots nearest to reservoir display more single molecule binding than those closest to the microfluidic pump. This is not attributable to spot size.

2.10. Parallel antibody screen 2

The above results suggested that a higher affinity antibody pair would be required to measure single cell ERα. A second round of parallel antibody screening was performed to attempt to identify an alternative sandwich system with even greater affinity than the D12-HC20 assay [Figure 2.19]. Two clear candidates were identified: mouse monoclonal F10 as the primary capture agent, coupled with ab205850 [Abcam], a monoclonal rabbit antibody purchased pre-conjugated with AlexaFluor-488.
Figure 2.19: Results of parallel antibody screening 2. The F10-ab205850 system considerably outperforms all other assays. Secondary concentration was fixed at 0.125μg/ml.

2.11. Antibody affinity measurements

After these screening stages, it was decided to have the affinity of the strongest candidate antibodies quantitatively measured in order that the constituents of the system could be understood relative to the MAC chip group’s other assays, and to rule out that the failure of the assay to measure single cell ERα reliably was down to a lack of affinity of a particular antibody. Consequently, the affinity of the lead anti-ERα antibodies from these screens to ERα recombinant protein was measured via surface plasmon resonance with a Biacore T200. This was performed by Dr James Noble at the National Physical Laboratory. The ERα antibodies this was performed for were D12, HC20, F10, and ab205850. The antibodies from the group’s p53 assay were measured as a reference. The results of these measurements are given below [Table 2.2 & Table 2.3].

Table 2.2: Measured affinities of the ERα antibodies to recombinant ERα.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>$k_\text{on} \text{[M}^{-1}\text{s}^{-1}]$</th>
<th>$k_\text{off} \text{[s}^{-1}]$</th>
<th>$K_D \text{[M]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D12</td>
<td>1.33×10^5±4.1×10^3</td>
<td>8.69×10^2±5.3×10^6</td>
<td>6.92×10^-9</td>
</tr>
<tr>
<td>F10</td>
<td>2.54×10^6±1.69×10^6</td>
<td>9.06×10^5±1.37×10^6</td>
<td>3.08×10^-10</td>
</tr>
<tr>
<td>ab205850-AF488</td>
<td>2.54±10^5±2.4×10^5</td>
<td>&lt;1×10^6</td>
<td>3.96×10^-12</td>
</tr>
<tr>
<td>HC20</td>
<td>1.20×10^6±2.17×10^4</td>
<td>9.96×10^5±3.43×10^6</td>
<td>8.18×10^-11</td>
</tr>
</tbody>
</table>
The order of binding tightness for the 4 anti-ERα antibodies is as follows: ab205850-488>HC20>F10>D12, and range from ~4pM to ~7nM. The results of the Biacore measurements are surprising, because they show that in fact all the ERα antibodies have very high affinities to their target. To emphasise this point, all sandwich assay systems involving any combination of the above antibodies have an avidity greater than that of the p53 assay which is routinely and reliably used for single cell p53 measurement. Using either F10 or HC20 as the capture antibody provides a capture agent with either 25 or 100× greater affinity for the analyte than the Enzo antibody has in the p53 system. Ab205850 provides almost 400× greater affinity for its target than DO-1 (488-labelled) in the p53 system.

The p53 measurements highlight an interesting phenomenon, namely that fluorescent labelling of antibodies seems to be responsible for a reduction in affinity to the antigen. As unlabelled HC20 was measured in these affinity tests, it possible therefore that the HC20 labelled with DyLight-488, used in the above single cell experiments, had a slightly lower affinity than the 81pM of the unlabelled version.

### Table 2.3: Affinities of the constituent antibodies of the p53 assay.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>kᵢ [M⁻¹s⁻¹]</th>
<th>kᵣ [s⁻¹]</th>
<th>Kₒ [M]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzo</td>
<td>2.93×10⁴</td>
<td>2.41×10⁴</td>
<td>8.21×10⁻⁹</td>
</tr>
<tr>
<td>DO-1</td>
<td>3.37×10⁴</td>
<td>1.78×10⁵</td>
<td>5.29×10⁻¹⁰</td>
</tr>
<tr>
<td>DO-1 488</td>
<td>2.37×10⁴</td>
<td>3.48×10⁵</td>
<td>1.47×10⁻⁹</td>
</tr>
<tr>
<td>DO-1 647</td>
<td>1.76×10⁴</td>
<td>1.09×10⁴</td>
<td>6.18×10⁻⁹</td>
</tr>
</tbody>
</table>

2.12. F10 + ab205850-Alexa-488 sandwich assay

A sandwich system comprising either F10 or HC20 as the primary antibody, and ab205850 as the detection antibody therefore seemed the likeliest route to a working single cell assay. Although F10 has a lower affinity than HC20, F10 was chosen over HC20 as HC20 is a mouse polyclonal antibody, whereas F10 is a monoclonal mouse antibody, and one would therefore hope to experience less variability in experimental outcome, particularly between antibody batches. D12 was discounted for pairing with ab205850, as both antibodies target the same epitope region of ERα, therefore limiting detection to multimeric forms of ER.

2.12.1. Single cell experiments: 150×150μm chambers

Single cell experiments were performed with the F10-ab205850 assay for both non-drugged MCF7 cells, and MCF7 cells drugged for 72hrs with 1μM hydroxytamoxifen [Figure 2.20]. The F10 capture antibody was printed with ArrayIt print buffer and the secondary concentration [ab205850] was fixed at 0.25μg/ml. For the undrugged cells, no increase in binding was observable after lysis, however for the tamoxifen-treated cells, binding was observed in a small minority [5] cells after lysis. No binding was observed for the basal population. The F10-
ab205850 assay displayed much higher cross reactivity between primary and secondary antibody than the D12-HC20 assay: 7100±1600 molecules compared to only 55±36 during single cell experiments for the D12-HC20 system.

![Figure 2.20: Results of MCF7 experiments for undrugged cells and those treated with 1μM OH-TAM, for the F10+ab205850 assay in 150x150x33μm chambers. Each graph is the combined data from 2 MAC chip repeats.](image)

### 2.13. Miniaturised vs standard chambers

During the course of the experiments with the 150x150x33μm MAC chambers it became apparent that the miniaturised assay chambers were presenting an issue during fabrication: namely that the smaller PDMS chambers were extremely difficult to align with the printed antibody spots, the size of which were only marginally smaller than the chambers themselves. Consequently, on many occasions the PDMS chamber would end up crushing, or partially sitting over the antibody spot itself, leading to detrimental effects on the protein capture efficiency [Figure 2.21].

As the volume of the analysis chamber is reduced, and specifically the x-y dimensions approach the diameter of the printed antibody spot, the process of alignment becomes very challenging, and frequently results in the partial, or full, crushing of the antibody spot on the microarrayed coverslip by the PDMS superstructure of the microfluidic. The full consequences of this are hard to establish empirically, but there are several possible effects on the assay. If substantial quantities of the high-affinity primary antibody become adsorbed to the PDMS or PEG-neutravidin surface near the antibody spot [and hence the field of view of imaging], then these antibodies could potentially form a sink for the target analyte in these areas and thus produce a concordant reduction in the number of bound analytes at the antibody spot itself.

It has also been observed that channels and chambers in reduced-volume chips fill at slower rates than conventional designs, and indeed the meniscus which should quickly wash away the excess of antibody from the antibody spot can stall in extreme cases. When this
occurs, it is plausible that the excess capture antibody is deposited on the walls and ceiling of the microfluidic, both in the downstream channel and in the antibody chamber itself.

A

Misaligned antibody spots

B

Crushed spots redistribute unpredictably around analysis chamber

C
Figure 2.21: Spot crushing in miniaturised chambers. A slight misalignment can cause antibody spots to be fouled or partially destroyed, though the exact effect this has on the primary adsorption layer at the PEG-neutravidin surface is not known. On contact, the gel-phase spot may interact with the PDMS chamber, with surface tension serving to redistribute the excess of antibody around the chamber, drawing or ‘wicking’ the spot to the sidewalls, where it is hypothesised it can re-adsorb and generate a sink for the protein analyte away from the primary antibody spot on the coverslip, thus lowering the detectable analyte and the apparent capture efficiency of the assay. [A] Schematic representation of a misalignment between miniaturised analysis chambers and comparably sized antibody spots. [B] Schematic representation of the redistribution of antibody around the analysis chamber on contact with the PDMS. [C] Real-life observation of [B].

2.14. F10 + ab205850-Alexa-488 single cell experiments: 1.2nl & 4.5nl chambers

To try to avoid this issue, single cell experiments were performed in slightly larger 1.2nl [190×190×33µm] and conventionally sized 4.5nl [300×300×50µm] MAC chambers. The results of these experiments [Figure 2.22 & Figure 2.23] showed that under these experimental conditions the assay could measure ERα in a small minority of cells; however, most cells tested in the assay did not produce a signal.

A successful calibration curve was able to be performed for the F10-ab205850 system in 300x300 chambers with 2µg/ml secondary [Figure 2.24]. This curve gives some insight into why we could not detect ERα in the majority of the cells that were tested. Defining the limit of detection according to Armbruster et al63, as before, we get an LOD of 9169 molecules bound to the spot or $4.4 \times 10^6$ molecules in the chamber.
Figure 2.22: Results of undrugged MCF7 experiments in 190×190×33 chambers, 0.25μg/ml HC20.

Figure 2.23: Results of undrugged MCF7 cells in 300×300×50 chambers with 2μg/ml ab305850.
2.15. HC20+ab205850 sandwich assay

The very highest affinity pair according to the Biacore measurements was HC20 with ab205850 as secondary. After the failure of the above assays to measure the basal ER levels, several single cell experiments were conducted for this pair. This was done somewhat reluctantly, because as stated before HC20 is a polyclonal antibody and is likely to exhibit considerable batch to batch variation. More urgently than this however was the fact that the manufacturer, Santa Cruz, had at this point announced that the HC20 antibody would soon be discontinued.

2.15.1. HC20+ab205850, 1.2nl chambers

Single cell experiments were performed in 190×190 as well as 300×300 chambers. The results of the 190-chamber experiments showed that once again the assay was unable to measure ERα in basal cells, though a larger fraction of cells were able to be measured in the case of 72hr exposure to 1μM tamoxifen [Figure 2.25]. A single cell test experiment was performed using 1×borate buffer [pH 8.5] with cells exposed to tamoxifen for 24 hours. Borate buffer was used to attempt to increase assay binding, as this was the same buffer as the Biacore antibody affinity measurements was performed in; using PBS might lower the reaction strength relative to the Biacore experiments due to e.g. ionic effects.
Figure 2.25: Single cell experiments for HC20-ab205850 in 190×190 [1.25nl] analysis chambers with 0.125ug/ml secondary. [A] 72hr treatment with tamoxifen showed a greater fraction of cells with measurable ERα content than any previous experiment. [B] Week-long treatment with tamoxifen with the same conditions as [A] produced no measurable signal. [C] 24hr tamoxifen treatment with the single cell experiment performed in borate buffer produced signal in >50% of cells tested. [D] Without tamoxifen the assay registered no binding after lysis for any cells tested.

2.15.2. HC20+ab205850, 4.5nl chambers

Experiments performed with the 300×300 chambers also showed poor ERα detectability in the bulk cell population [Figure 2.26], both with and without tamoxifen, even with secondary concentration increased to 0.5μg/ml. To test whether the failure to detect ERα was a result of the laser-based lysis being ineffective, a single cell experiment was performed using RIPA lysis buffer to lyse cells in the MAC chambers. This was performed by filling the main MAC chip channel with 10× RIPA buffer, then allowing this to diffuse into the chambers. Cells were fully lysed after approximately 1 hour, and 2 hours after this the chip was imaged. The results [Figure 2.26B], show no substantive increase in measured ERα, and provide evidence that the lysis method is not the issue, in agreement with previous publications investigating the efficacy of laser lysis.64
2.16. Effect of secondary antibody concentration

If the secondary antibody concentration is fixed, reducing the volume of the MAC chamber reduces the number of secondary antibodies available to bind in the assay by a factor approximately equal to the volume reduction factor [approximately because the number of antibodies can be partially replenished by the diffusion of more secondary from the channels which lead to the analysis chamber]. So, if we reduce the volume of our chamber from 4.5nl to 0.74nl [~6x], we have 6x less secondary molecules available to bind the analyte. For high secondary concentrations, the number of antibodies may remain in excess of the available analyte in the smaller chambers, but for lower concentrations this is quite possibly not the case. To counteract this effect, we could increase the concentration of secondary antibody, but increasing the secondary concentrations for a given chamber volume increases the level of non-specific binding, an effect we have sought to minimise, due to the challenges involved in analysing images with high degrees of non-specific binding [Chapter 3]. Nevertheless, high degrees of non-specific binding are now able to be coped with robustly with new image analysis methods, and so this should no longer be considered an assay limiting consideration.

As a final word, there has been a degree of nomenclatural ambiguity in the MAC chip group concerning ‘miniaturising the assay’ or ‘switching to smaller chambers’ as the panacea for problems of assay sensitivity. As the group’s own published results show however, miniaturising the assay should, for the avoidance of doubt, be thought of as increasing the concentration of both the analyte and the secondary.
2.17. Effect of analyte dimerisation

The effect of protein dimerisation cannot be ignored in the range of analyte concentration we are interested in measuring in the MAC chip. This is especially true of ERα which is capable of forming homodimers as well as heterodimers with its homologue ERß. A simple treatment of the reaction process can give us a good insight into the effect this process may have on-chip. Two monomeric proteins, M, which autoassociate to form a dimer, D, can be described as follows:

\[
M + M \overset{k_D}{\underset{}{\rightleftharpoons}} D
\]

At equilibrium the dissociation constant, \(k_D\) is the ratio of monomeric form to dimer:

\[
k_D = \frac{[M][M]}{[D]} = \frac{[M]^2}{[D]}
\]

The total amount of protein in the monomer-dimer system can be written:

\[
[M_t] = [M] + 2[D]
\]

\[
\therefore [D] = \frac{[M_t] - [M]}{2}
\]
Substituting this in to the expression for $k_D$:

$$k_D = \frac{2[M]^2}{[M_t] - [M]}$$

Rearranging this for a quadratic equation in $[M]$:

$$2[M]^2 + k_D[M] - k_D[M_t] = 0$$

Which has the solution:

$$[M] = \frac{-k_D + \sqrt{k_D^2 + 8[M_t]k_D}}{4}$$

As the equilibrium dissociation constant, $k_D$, of dimerisation has been measured experimentally for many of the proteins we are interested in, we can use this formula to calculate the concentration of monomers for a given value of the total concentration of the protein, $[M_t]$. For a protein such as p53 which can dimerise, and then these dimers can come together and form tetramers, provided the system is in equilibrium, we can perform the same analysis for $D + D \overset{k_D}{\underset{D_t}{\leftrightarrow}} T$, and linking the total concentration to the fraction of non-monomeric protein calculable from equation X.

$$[D] = \frac{-k_{D_{tet}} + \sqrt{k_{D_{tet}}^2 + 8[D_t]k_{D_{tet}}}}{4}$$

Where $[D]$ is the concentration of non-monomeric protein in the form of dimers, $D_t$ is the total concentration of non-monomeric protein and $k_{D_{tet}}$ is the equilibrium dissociation constant of tetramerisation. Figure 2.28 shows the degree of dimer- and tetramerisation for two MAC chip analytes, ERz and the tumour suppressor p53. The P53 dimerisation and tetramerisation equilibrium dissociation constants measured experimentally by Rajagopalan et al, and for ERz taken from. Measurement of the [unliganded] dimer dissociation rate by the same group gave a value of $t_{1/2} = 39\pm3$ minutes. This is an important result as the long duration of the dissociation underscores how important it is to wait for titrated protein to equilibrate before adding it to the MAC chip, when performing a calibration curve, else low concentrations of ERz may still be highly dimerised.
Figure 2.28: Intrinsic dimerisation and tetramerisation in the MAC chip. The higher the concentration, the more the fraction of available protein units decreases [dashed line], i.e. a solution that is completely dimerised reduces the available protein units to 50%.

Additionally, the presence of estradiol or other high affinity ligands stabilises the dimerised formation and therefore ERα from cells may be more likely to be in the dimerised form: in the presence of E₂ increases the dissociation rate almost quadruples to $151 \pm 9$ minutes, and in the presence of tamoxifen, increases further, to $184 \pm 4$ minutes. For a given concentration of ERα this will reduce the number density of distinct molecular complexes. An ER MAC chip experiment is allowed at least 2 hours to come to equilibrium post-lysis, however increasing this time beyond $\sim 3$ hours is very challenging due to time constraints on the available equipment, and thus taking measurements on a timescale that allows for complete dissociation of dimerised protein is almost impossible, which may considerably impact the amount of ERα measured. Furthermore, the dimer complex will exhibit a slower characteristic diffusion time than the monomeric form, which will affect the rate of mass transport to the capture spot in a sample where dimerisation is non-negligible.

As secondary antibody concentration is not in saturating conditions at high analyte copy numbers, the likelihood of two secondary antibodies binding both molecules in the dimerised complex is low, and hence we would expect to undercount the number of analyte molecules in our readout from the true value. Calibration of the MAC chip is therefore important so we know the degree of intrinsic dimer- and multimerization for a given readout, and can apply a correction factor if required.

2.18. Multiplexing the MAC chip

A key aim of this project was to develop strategies for making multiplexed protein measurements with the MAC chip. This can be performed in several ways. The first strategy is to adapt the MAC chamber such that it can accommodate multiple capture antibody spots; the second
strategy is to use a single printed spot but use multiple detection antibodies, each labelled with different wavelength fluorophores. This latter strategy has the potential to be used with either [a] a spot consisting of a single antibody and two secondary antibodies targeting protein binding partners or a single species and a post-translational modification, or [b] an antibody spot containing a mixture of antibodies for multiple target analytes.

The latter strategy is covered in more detail in Chapter 5; the former will be discussed now.

MAC chip variants were designed that allowed for two primary antibody spots to be printed inside a single analysis chamber [Figure 2.29, inset]. The dimensions of the chamber were fixed at $380 \times 190 \times 63$ μm so as to keep the volume at 4.5 nl, the same as for the $300 \times 300 \times 50$ μm standard MAC chip used for the p53 assay, and so that calibration curves generated for protein systems in this chip would still be valid. A microarray printing program was written that printed two rows of different antibody spots, offset by between 150-200 μm, with the exact offset depending on the morphologies of the antibody spots. A proof of principle experiment was performed multiplexing the F10-αb205750-Alexa488 anti-ERα assay outlined above with the group’s anti-p53 assay consisting of Enzo Life Sciences p53 capture antibody and DO-1-Alexa-647 anti-p53 secondary [Santa Cruz Biotech]. MCF-7 cells were lysed in the chambers as per a normal MAC chip experiment, and the results [Figure 2.29] show that it is possible to measure two distinct proteins simultaneously in the MAC chip with the multiple capture spot strategy. It should be noted that MCF7 cells are already known to have low expression levels of p53, but as the highest-expressing ER cell line the experiment had to be performed with these to stand a chance of measuring anything at all. This coupled with the already noted fact that the ERα assay is incapable of measuring the ER levels in the basal cell population, means that a strong multiplexed signal was only measured in a single assay chamber. Nevertheless, the method is viable and is a simple enhancement of the MAC chip methodology that should enable quantitative, single cell, multiplexed measurements to be performed easily for other antibody systems with superior detection capabilities. Care must be taken in these experiments to establish the optimum spot offset, as failure to do so leads to spot coalescence, which complicates imaging and subsequent analysis. Spot coalescence also precludes the use of single colour detection, which is theoretically possible using the spatial multiplexing mode.

Despite the overall lack of assay sensitivity, ERα and p53 are an interesting pair of proteins to perform multiplexed measurement on, as there is a great deal of crosstalk between the two regulatory networks; in MDM2 they even share the same E3 ubiquitin ligase. ERα can directly bind to the N-terminus of p53 and also form a tertiary complex with p53 and its regulator MDM2, with the evidence suggesting that ERα can prevent MDM2 from deactivating p53 and thus protecting it from being targeted for degradation and depleting in copy number. Overprotection of p53 from downregulation can lead to growth inhibition and an apoptotic
response and this may go some way to explaining the well-documented cytotoxicity of ER overexpression, or even the better prognosis associated with ER-positive breast cancers. With a significant minority of breast cancers showing some form of p53 mutation [at least 30 have been described\(^6\)], developing assays which can characterise this evidently non-superficial interaction may be useful for the stratification and monitoring of patients to therapeutic interventions.

Figure 2.29: Results of the p53-ERα spatial multiplexing experiment. Spot radius corresponds to the number of cells lysed in each chamber, the smallest points being a single cell, the largest 4 cells. Although a strong p53+ERα signal is only measured from a single cell, the experiment suffices to show that the method is a viable route for obtaining MAC chip protein measurements for two proteins simultaneously. [Inset] Detail of antibody chambers in the multiplex MAC chip.

2.19. Discussion

In this chapter, we have described the attempts to develop a MAC chip single cell assay for the biomarker protein estrogen receptor alpha. We have attempted to detect ERα in single cells under a wide range of assay conditions, however we have only been able to measure ERα signal in the MAC chip assay for a very small minority of MCF7 cells, one of the two most highly expressing ERα+ cell lines\(^6\). We have detected ER more reliably in tamoxifen-treated cells, in which ER-expression is upregulated, though still only in a minority of the population. The inability of the assay to measure basal levels renders it essentially useless for most practical
purposes, as at means there is no scope for making a quantitative comparison between over-
expressing cells with baseline populations, precluding the assay’s use in the interrogation of
ERα in a biomedical or pharmacoproteomic setting. The key point of single cell analysis, is
after all, to see the distribution.

It is useful to discuss the reasons which have been ruled out as the cause of this failure in
measurement. Firstly, through Biacore surface plasmon resonance measurements we know
that it is not due to insufficient affinity of the assay antibodies, the best of which bind their
ERα target with a tightness several orders of magnitude better than the antibodies used in
another, working, single cell assay for the protein p53. Secondly, immunofluorescence imaging
showed that it was not because the MCF7 cells were not expressing ERα, although admittedly
these experiments only proved that a significant proportion of the ERα present had its N-
terminus available to bind, as this is the target epitope of the ab205850 antibody used in the
experiments. Through the development and use of the standard candle MAC chip we have
shown that the printed antibody spots are active during the single cell experiments, although
the nature of flow in the control lanes meant that it was impossible to tightly control the
amount of recombinant protein which had access to the antibody spots. Evidence suggests
that there were at least 10^5 active antibodies in the control spots under non-
saturating condi-
tions. Preliminary evidence suggest that it is not due to the lysis via laser cavitation being ine-
effective, as cell measurements performed using the diffusion of RIPA lysis buffer into the anal-
ysis chambers also failed to measure any protein signal.

What then are the possible reasons for this failure? Firstly, although immunofluorescence
indicated that the cells did contain ERα, the experiments said nothing about the abundance,
nor the degree to which the total amount of protein was available to the sandwich antibody
system. Put simply the ERα itself could be bound up in complexes with other proteins – only
one of the two epitopes needs to be blocked for the presence of the molecule to become
unmeasurable – and so the amount of ‘free’ ERα may be considerably lower than our estimate
of ~500,000 molecules would seem to suggest. ERα is documented to have hundreds of direct,
binary interactions with other proteins

[70] [Figure 2.30]. Furthermore, immunofluorescence ex-
periments are performed at a massive excess of secondary antibody, meaning that most, if not
all, antigen molecules in the cell can be efficiently bound by the secondary. In the MAC chip
assay this not necessarily the case, and would only be true for low ERα copy number, or very
high secondary concentrations. An added consideration is whether the recombinant protein
accurately reflects its wild-type equivalent. In addition to possibly being bound up in complexes
with other proteins, ERα may exhibit post translational modifications which may inhibit the
binding of the primary or secondary antibody: for instance, there are 4 phosphorylation sites
within the target epitope of ab205850. As we have outlined above, at concentrations relevant
to our assay a considerable fraction of ERα may be dimerised, which reduces the available
number of protein units that can be pulled down.
Although we have broadly failed to detect ERα at the sensitivity we require for single cell analysis, the D12-HC20 assay calibration curves we have performed are at least able to experimentally put a loose upper bound on the free ERα in MCF7 cells, of ~364,000 proteins per cell; if cells were expressing more than this then the bulk population ought to have registered on the D12-HC20 MAC chip. Results from the other antibody pair experiments do not contradict this finding, and it is interesting to note that the highest affinity antibodies did not produce the MAC chip with the best limit of detection, showing there are many more things at play in the MAC chip chamber than can be accounted for with simple kinetic considerations. From an assay development perspective, this work is useful because we have been able to describe the experimental difficulties associated with assay chamber miniaturisation [spot crushing/wicking, non-specific binding], demonstrated strong evidence for the non-correspondence of flowchip-based experiments to conventional MAC chambers, as well as demonstrated that a spatial multiplexing approach is a viable method of measuring the protein expression level of multiple proteins in a single MAC chamber.

Figure 2.30: Direct protein interactions of ERα with at least 3 published pieces of evidence.
Chapter 3. High dynamic range single molecule image analysis

‘Counting is the religion of this generation. It is its hope and its salvation.’

– Gertrude Stein

3.1. MAC chip data and the need for a new analysis method

Effective single molecule counting is imperative to the goal of measuring protein copy numbers via the MAC chip, and it is impossible to achieve ‘absolute quantification’ unless the methods are well characterised. During development of the ERα assay new image analysis methods were created to improve the accuracy and reliability of the single molecule counting procedures used in MAC chip experiments.

Firstly, it is useful to consider the specific format of raw MAC chip data. Images are collected as 512×512 pixel 16bit multi-frame image stacks. For further analysis, these images can be classified according to the density of fluorescent molecules recorded in each frame. The idiosyncrasies of MAC chip experiments produce images that conform to any one of three possible types:

1. ‘non-congested’ images: An image is non-congested if all the single molecules in it are separated in space by a distance greater than the diffraction limit of light. In practice this means that the single molecules must all be individually resolved in the image.
This does not necessarily mean there must be zero overlap between molecules, as a single molecule is resolvable right up until the point known as the Rayleigh Criterion [see below].

2. ‘congested’ images: an image is congested if the majority of molecules in the image are unresolvable due to the close proximity of other molecules. Congested images cannot be analysed with the same method as non-congested images.

3. ‘partially congested’ images: For several reasons [irregular printing, analyte concentration, non-homogenous spot binding due to the influence of the position of cellular lysis], many images fall somewhere between definitions [1] and [2].

The original image processing algorithm used for single molecule identification in MAC chip data was based on intensity thresholding. This is a simple technique which relies on the fact that a fluorescent single molecule will be very much brighter than the background intensity level of its local environment, and, when viewed on an EMCCD camera will extend over several pixels with a roughly circular shape, due to diffraction. To illustrate, the default parameters of this old algorithm, widely used within the group for p53 data analysis, defined a single molecule as any image region of 4-9 clustered pixels, with circularity greater than 0.5 and with pixel intensities at least 3 times the standard deviation of the background plus its mean value. The background in this case is defined, on an image by image basis, as the mean intensity level of a 75×75-pixel region in either the top left, top right, bottom right, or bottom left corner of the image – the location chosen such that region defining the background level should avoid the location of the printed antibody spot. The sum of all such regions is then computed to give the single molecule count of the image, and derive the mean single molecule intensity. For experiments with large degrees of binding [semi- to fully congested images], individual single molecules cannot be discriminated and the single molecule count is calculated as the total intensity of the image divided by the mean single molecule intensity. Whilst this method can produce adequate results for simple datasets, with bright, spatially separated, single molecules, and a well-defined background, there are numerous issues which prevent accurate single molecule counts being obtained in many situations.

Firstly, the way the background intensity level of an image is computed is too crude for many experimental images. Non-specific binding of secondary fluorescent antibody to off-spot areas of the microarray slide is a significant problem, and merely because the image area [ROI; region of interest] used for the background calculation is not situated over the antibody spot itself, does not mean that it is free of single molecules. When single molecules are present in this area, the background intensity level is inflated, potentially leading to a pronounced undercounting of the true number of single molecules in the image, as well as an overestimation of the mean single molecule intensity, which consequently leads to undercounting of the number of single molecules in congested images. This is self-evidently an important issue to address when the experimental goal is the accurate quantification of protein copy number.
Secondly, while the algorithm’s clustered pixel and circularity criteria recognise that [in a correctly configured experiment] the photons emitted by a single fluorophore will be distributed across more than one pixel and be roughly circular, this is an oversimplification of what we measure on the EMCCD when we take an image of a single molecule. Indeed, the thresholding algorithm ignores a key piece of a priori information we have about single molecules in 2D images. The theory of diffraction tells us that light from a diffraction-limited point source will spread out and form a characteristic Airy pattern on transmission through a circular aperture, and a true single molecule in our image should therefore have an underlying 2D intensity distribution that corresponds to the Airy function. Hence it is the ‘shape’ of the intensity distribution which should inform us whether a given intensity spike is a single molecule, not just whether it surpasses a given threshold.

Figure 3.1: Single molecules and the Rayleigh criterion. An image of a single molecule is a point source convolved with the PSF of the imaging system. Two simultaneously emitting fluorescent molecules are distinguishable from each other up until the point known as the Rayleigh criterion, where the central diffraction maxima of each molecule coincide with the first diffraction minima of the other. The system is said to be diffraction-limited, and at all points closer together than this, the molecules cannot be told apart. Adapted from reference 72.

3.2. Identifying single molecules: a better way

As stated above the key thing to recognise in optical single molecule imaging is that a true single molecule has a 2D spatial intensity distribution defined by the point spread function [PSF] of the imaging system. Ignoring artefacts or defects within the imaging setup the intensity is given by the Fraunhofer diffraction pattern for light transmitted through a circular aperture, Equation 3.1.

\[ I(\theta) = I_0 \left( \frac{2J_1(ka \sin \theta)}{ka \sin \theta} \right)^2 \]
Where \( I_0 \) is the central intensity maximum, \( f_1 \) is a first order Bessel function, \( k = 2\pi/\lambda \) is the wavenumber of the system, and \( \alpha \) is the radius of aperture.

As seen in Figure 3.2, the central lobe of the intensity function can be closely approximated with a Gaussian of the form:

\[
I(q) \approx I_0 \exp \left( -\frac{q^2}{2\sigma^2} \right)
\]

Where \( q \) is the radial distance from the central maximum, and \( \sigma \) is the one-dimensional RMS width of the Gaussian.

Figure 3.2: The true Airy diffraction intensity [solid line] vs its Gaussian approximation [dashed]. Reproduced from Wikipedia Commons under Creative Commons License

Single molecule experiments are now in widespread use in fluorescence microscopy, and as such there are many existing tools available for identifying single molecule intensity peaks in digital images, and fitting them with a 2-dimensional Gaussian. Most of these derive from superresolution microscopies such as PALM, STORM, and STED etc., some of which themselves have their roots in the astrophysical point-source locating algorithms used in sky surveys.

These techniques use a variety of strategies to temporally separate molecules confined within a diffraction-limited volume that would be otherwise unresolvable with traditional wide-field imaging. In the case of PALM this is with endogenously expressed photoactivatable fluorescent proteins, and for STORM with small molecules that can be either optically or chemically switched from their emission-state to a stable dark-state. It is possible, either through buffer conditions or activation light, to tailor experimental conditions such that only a few molecules are ever fluorescing at any one time. Temporally separated molecules are fitted with a Gaussian, the centroid of which is calculated mathematically, and the positions of all these centroids are plotted in a parameter space scaled with respect to the original image, allowing
features to be resolved in super-resolution. However, we face additional confounding problems with respect to the MAC chip: [1] The MAC chip has a large dynamic range and only at low analyte concentrations can single molecules be reliably separated and counted via super-resolution techniques in our images, and [2] Our fluorophores are non-photoactivatable and thus analyte-dense images cannot be temporally separated in order to measure every molecule, and [3] our secondary antibody molecules may be multiply-labelled, displaying quite a broad intensity distribution compared to e.g. a target protein tagged with GFP.

3.3. Image processing software

All image processing procedures were performed in Fiji/ImageJ. Four pieces of software were written in the ImageJ macro language for the analysis of MAC chip images [See Appendix A]. Two of these macros are concerned with calculating the baseline noise offset [see Section 3.5.2] for non-congested and congested images respectively, and the remaining two compute the single molecule content. These macros process a given set of images with internal ImageJ functions, and where necessary, call externally-compiled ImageJ Java plugins to perform specific image processing tasks or data handling. The two non-native ImageJ plugins which are used by the macros are as follows: all Gaussian peak fitting is performed with the University of Sussex’s GDSC Single Molecule Light Microscopy [SMLM] plugins\textsuperscript{75}; and the Stowers Institute for Medical Research’s plugin ‘hist columns jru v1’, written by Dr Jay Unruh\textsuperscript{76} is used within the localisation counting algorithm to display a histogram, within ImageJ, of the intensity of all the single molecules found during the single molecule counting procedure.

There are multiple ImageJ packages for PALM-style localisation image processing currently available, and many more for other platforms. See, for example, ThunderSTORM, QuickPALM, ClearPALM, DAOStorm, and Octane. A useful repository of single molecule localisation algorithms is located at the EPFL’s Directory of Localisation Microscopy Software\textsuperscript{77}. The SMLM plugins were chosen primarily because [1] they require no additional software other than ImageJ to run, and [2] the output data format includes a table with molecular localisations listed by image frame number, and hence it is easy to extract the pertinent number for a MAC chip experiment, which is the number of single molecules per frame. The SMLM PeakFit algorithm has been performance benchmarked against a large group of other comparable algorithms and has been found to be one of the best performing algorithms across a range of criteria\textsuperscript{78}.

The SMLM package uses the following method for single molecule localisation. Candidate spots are identified in each frame then fit using a local region surrounding the candidate according to Wolter et al [2010]\textsuperscript{79}. The raw image is smoothed and peak candidates are identified using non-maximal suppression, then processed in descending-height order by fitting to a 2D Gaussian in a defined ROI set to a multiple of the estimated Gaussian standard deviation.
Fitting is performed using a non-linear least squares Levenberg-Marquardt method until convergence [i.e. the candidate molecule is accepted] or the maximum iterations is exceeded [and the candidate rejected]. Fitted single molecule peaks are filtered using signal-to-noise, width, precision and coordinate shift criteria. Processing is terminated when a set number of consecutive candidate peaks fail to achieve convergence.

### 3.4. Overview of non-congested counting algorithms

The overall image processing procedure for non-congested images, with both the thresholding algorithm and with localisation counting, is shown in the flow chart in Figure 3.3. Figure 3.4 explains the key steps of the threshold algorithm. The Gaussian localisation algorithm is more complex, and is explained in depth in the following sections.

![Non-congested image processing flowchart](image)

Figure 3.3: Comparison flowchart of the image processing steps for the old and new single molecule counting algorithms
Figure 3.4: Key steps in the thresholding algorithm. The raw image [A] is blurred with a large-radius [50 pixel] Gaussian filter resulting in [B]. The original image [A] is then divided by the blurred version of itself [B] to give an approximately normalised image [C]. A region of [C] is then defined as a ‘background’ area and a histogram of the pixel intensities is calculated [inset] along with the mean and standard deviation. A threshold corresponding to the mean + 3 standard deviations of the background region is set on image [C], and the above-threshold regions are turned into the binary mask seen in [D]. The masked regions can be analysed for their size and shape properties. A ‘single molecule’ is defined in this case as a region of 4-9 clustered, above-threshold pixels, with circularity greater than 0.5. These definitions are designed to filter out small-scale, random intensity spikes due to camera noise, and larger scale features such as agglomerated single molecules or areas of scattered light.

3.5. Details of processing workflow for localisation counting algorithm

In the following sections, we will discuss in detail the stages of the 2D Gaussian-based single molecule localisation counting algorithm.
3.5.1. Image flattening and background subtraction

Raw images must have several processing steps performed on them prior to peak identification and single molecule counting. These are image flattening, background subtraction, and baseline noise offset compensation. The role of image flattening is to compensate for the non-uniform illumination of the excitation laser [Figure 3.5A]. Due to its Gaussian intensity profile the photon flux of the excitation laser is lower at the periphery of image, so we must normalise the image so that a fluorophore excited at the image edge and an identical fluorophore excited in the centre have the same apparent brightness. Practically this is done by dividing the raw single molecule image by an image of the laser profile, which can either be measured with blank samples, or generated from a series of single molecule images from the experimental run that is being analysed. In practice, the latter is often easier, and the new algorithm generates the laser profile from a series of single molecule images input by the user. These images should be from the same experimental run as is to be analysed, and be sparsely populated with single molecules, or empty. In practice, these sparse single molecule images can usually be guaranteed in a MAC chip dataset by keeping several MAC chip analysis chambers without a capture antibody spot. Five has been found to be enough images with which to accurately generate the laser profile, without unduly reducing the number of antibody spots in a MAC chip usable for single cell analysis. The algorithm generates a close approximation of the laser profile by creating an image stack from the sparsely populated images opened by the user, then performing a median intensity projection through the stack, followed by a Gaussian blur [default $r = 50$ pixel], to smooth out discontinuities in the resultant image [Figure 3.5B]. A final step subtracts the camera bias from the profile [100 analogue-digital units [ADU] for the Andor iXon 897].

The method works because the only single molecules present in these images should be those that are non-specifically bound to the uniform surface of the functionalised coverslip, and their positions will be random. The likelihood of there being single molecule intensity information in successive frames of the stack is relatively low and therefore when we take the median pixel value of the stack we remove the bright outliers, and generate an image with a 2D intensity profile approximating the underlying excitation profile that is present in all images. Image artefacts will be produced downstream if the images from which the profile is generated are too densely populated with single molecules, or contain repeating motifs that are not present in the data images.

In general, the flattening process does not produce a perfectly uniform intensity profile across the image [Figure 3.5E]. This is because some intensity variations in images are produced neither by single molecules, nor are they the result of the nonuniform illumination of the laser profile. Two possible sources for these intensity variations are scattered laser light from the interaction of the excitation beam with the PDMS walls of the microfluidic, or defocused light from intrinsic autofluorescence in either the sample or antibody spot itself.
Whatever the source of these variations, they can be removed via the application of a rolling ball background subtraction of an appropriate radius [Figure 3.5F]. The rolling ball algorithm is designed to remove image features which have a spatial frequency larger than the radius of the ball itself. Conceptually, the rolling ball works as follows: any 2-dimensional image can be thought of as a 3-dimensional surface, where a third height dimension is defined by the pixel intensities at each point in the image. A virtual ball can be rolled across the underside of this surface, and the whole volume that is reachable by the ball defines a hull which is the background. Areas smaller than the ball itself are inaccessible to it and so these do not form part of the background volume and are not subtracted. Imagine two balls, a golf ball and a beach ball, rolling on the undulating hills of a golf course [the ‘background’], and the holes themselves are the objects we are interested in. The golf ball can fall into all parts of the course, the fairways, the greens, the bunkers and the holes, meaning with this ball our background subtraction would remove the very information we are interested in. The beach ball on the other hand cannot fall into the holes, meaning the information we are concerned with is preserved through the background subtraction process. Make the ball even larger still [e.g a zorbing ball], and the ball may not efficiently roll into the bunkers, creating artefacts or false positives in the final image. The key is to choose a ball that is of the appropriate size to remove background but leave object pixel regions [in our case single molecules] intact. By choosing a suitably large value [50 pixels] for the radius we can ensure that we remove large-scale unwanted features from our single molecule data whilst not affecting the relative intensity of single-molecules [which typically have a radius of only a few pixels]. The mean single molecule intensity calculated by the localisation algorithm is unaffected by the performance of rolling ball background subtraction with these parameters.
3.5.2. Baseline noise offset compensation

The next stage of image processing introduces a new, key parameter: the baseline noise offset, or BNO. The BNO is a term introduced to describe the fact that the 32bit-float images that result after image-flattening and background subtraction, have intensity information present in pixels that essentially have ‘nothing’ in them in terms of the presence of single molecules [Figure 3.6]. If we measure the intensity profile across our flat, background subtracted image we can see the profile is noisy, and varies randomly around an arbitrary level, close to but not exactly zero. We designate this noise floor the ‘baseline noise level’ and introduce a factor, the BNO, equivalent in value to the mean of the baseline noise level, which we subtract from every
image so that the intensity contribution from all dark pixels in the image averages to zero. Formally,

\[ BNO = \frac{1}{i} \sum_{i} P_i \]

where \( P_i \in \mathbb{D} \), is the grey value of the \( i \)th member of the set \( \mathbb{D} \) of non-single molecule-containing pixels.

The BNO is critical for establishing a convergence between single molecule counting in the congested and the non-congested regimes. Without compensating for the BNO the congested counting process dramatically overcounts the number of single molecules present in an image. This is because, even though the intensity contribution of any single dark pixel is very low, when that small value is multiplied across the whole image [512×512 pixels in the case of this data], the net contribution can be large and will unduly bias the calculated number of molecules towards a higher value. For example, consider the case where a set of images has a BNO of 0.049 – a fairly typical value for single molecule experiments. The total intensity in the image purely due to noise is then approximately 12,845 grey values. Considering that an average single molecule usually has an integrated intensity somewhere in the range 1 – 10 grey values, then if we do not subtract the BNO, our algorithm is liable to overcount congested images by some 1,200 – 12,000 molecules.

Although we formally define the BNO per Equation X, this is not how the BNO is calculated in the program’s code. Rather than measure and take the average of all non-single molecule pixels, the code instead takes a sample of pixels, which is both simpler to code, and quicker to run. Every flattened, background-subtracted image frame that is being analysed for single molecules has 4 straight line profiles traced round the periphery – though not the very edge – and the pixel intensities measured. The reason the periphery is chosen for the profiles is due to the placement of the antibody spot, which is normally in the centre of the field of view, and thus where we would expect to encounter the most single molecules [antibody-antibody cross reactivity is the overwhelming cause of background single molecule information in MAC chip assay images taken without the presence of analyte, so this is true in all cases, except where a chip has malfunctioned]. By measuring the intensity profile at the periphery, we minimise the chances of incorporating single molecule information into our BNO measurement, and thus skewing it to a higher value. The BNO is then calculated as the mean intensity of all pixels measured across the 4n profiles in an n-image stack. The code outputs both the mean and standard deviation of this sample of the non-single molecule pixels at this point; a high standard deviation is a good indication that fluorescent single molecules may be present in the line profiles used to calculate the BNO.
Figure 3.6: Illustration of the Baseline noise offset. Profile is taken from bottom left to top right. As can be clearly seen, the noise floor of the image has a finite value, and this is generally true for all images. Subtracting the BNO from every pixel produces a resultant image with a noise floor that averages to zero, and hence one in which the only net contribution to the total integrated intensity is from single molecules.

3.5.3. PSF estimation

After BNO compensation, we are left with a series of images in which the only net contributor to the total image intensity is the fluorescence from single molecules. The next stage is to estimate the point spread function of the microscope, so that the least-squares Gaussian fitting routine which follows later has an appropriate reference for what a true single molecule should look like. This is performed by the PSF Estimator plugin of the GDSC SMLM toolset. This plugin makes its best guess as to the true profile of a single molecule by randomly selecting N peaks [set to 1000 by default] in the image and least-squares fitting the intensity of the surrounding pixels and calculating the average of the Gaussian parameters of each peak. The estimator uses the starting configuration to fit N peaks taken from randomly selected frames.
in the image stack. The average of the fitted parameters is then used as the starting parameters to perform fitting again. This iterates until the Gaussian parameters do not significantly change.

3.5.4. Single molecule counting by Gaussian fitting

The image processing code next calls the GDSC SMLM Peakfit algorithm which tests every local intensity peak in every image frame for its convergence to the ideal Gaussian profile of a single molecule identified by PSF estimation. Two factors, signal and width [see below], control the degree of flexibility in the fit and the user is asked to input these in the dialogue which runs when the macro begins. The number of single molecules identified in each image is printed to the FIJI Log window in the order that the images were opened into FIJI. The average single molecule intensity is also printed to the Log window, and corresponds to the arithmetic mean of the integrated pixel intensities beneath the 2D Gaussian functions used to fit the single molecules [it is derived from the Signal column of the Fit Results window produced by the Peakfit algorithm]. Figure 3.7 shows an original TIRF image of a non-congested spot next to its Gaussian peak-fitted reconstruction.

![Figure 3.7: Original TIRF image [left] and Gaussian peak-fitted reconstruction [right].](image)

3.6. Analysis of congested images

An efficient MAC chip assay may bind tens or even hundreds of thousands of antigen molecules if the protein target is highly expressed. Such numbers push the single molecule fluorescence in the antibody spot into the so-called congested regime [Figure 3.8]. This is a key aspect of MAC chip data that differentiates it from most other forms of optical single molecule experiment, and in consequence the Gaussian peak fitting routines used in localisation microscopy are no longer applicable.

Some obvious questions are raised at this point. At what point does an image become ‘congested’? At what point should we stop using the localisation counting algorithm to count
single molecules? We shall address these important issues in later sections. For the time being we shall concern ourselves only with how best to analyse such images.

A companion algorithm to the localisation method based on Average Single Molecule Intensity analysis [ASMI] was developed to accurately quantify single molecule content of congested images. The image processing workflow for congested images with ASMI method is shown in Figure 3.9.

Figure 3.8: A congested antibody spot. The thousands of individual molecules present in the image prevents the resolution of any individual one.
There are key similarities between the congested image processing workflow and the non-congested one outlined above. Congested images must again be flattened to compensate for the laser profile, background subtraction must also be performed, and a BNO compensation factor must be derived and applied – however, the implementation of the latter two steps is different for congested images compared to non-congested ones.

The very nature of a congested image means we can no longer 'see' an individual single molecule, but it is critical that the mean single molecule intensity of the congested image corresponds exactly with the mean single molecule intensity of the non-congested image. This correspondence, while seemingly trivial, is actually hard to engineer, as the image processing parameters suitable for the non-congested image are not suitable for processing the congested image. This is a unique quirk of the high dynamic range single molecule data of the MAC chip.

The ASMI method replaces its counterpart threshold algorithm. The threshold algorithm for congested images [henceforth referred to as the threshold-congested algorithm] works as follows: [1] first the images are flattened [every image to be analysed is divided by the blurred [50px] average-intensity projection of a set of non-congested images], [2] a threshold is set from an off-spot ROI defining the intensity which a pixel must reach to be considered single molecule information, [3] this threshold is used to create a binary mask image corresponding to all the pixels which contain signal above background, [4] the original spot image and the binary mask are multiplied together, removing the intensity data of pixels which do not reach the threshold, [5] the total integrated intensity of the resultant image is calculated and divided...
through by the mean single molecule intensity as calculated by the thresholding algorithm, giving the total number of single molecules in the image.

3.6.1. Congested image flattening

This is performed as for non-congested images. Congested images are flat-fielded by dividing them by an artificial image of the laser intensity profile, itself generated from a series of non-congested images ideally taken from the same experimental run as the congested images being analysed. If this latter condition is not fulfilled [i.e. there are no non-congested images from the same experiment, and a set of such images is used from another experiment] there is a greater chance that artefacts may be produced in the flattened image. It is important to note however, that excitation laser intensity can fluctuate from experiment to experiment, meaning that if images from a separate experiment are used to flatten a set of congested images then it is possible that the mean single molecule intensity for the congested and non-congested data may not be equal, potentially leading to over- or under-counting of single molecules in the congested image. Additionally, using images from different experiments can generate BNOs that are larger or smaller than would otherwise be expected, though background subtraction dampens this effect.

3.6.2. Congested image background subtraction

Rolling ball background subtraction is performed on flattened congested [and semi-congested] images to remove image intensity information not due to the presence of single molecules, the same as non-congested images. For congested images the rolling ball radius is much larger [512px] than for non-congested images. This is because the 50px radius rolling ball used for non-congested analysis is smaller than the typical extent of an antibody spot in an image, which are typically several hundred pixels across. If this spot ‘lights up’ with fluorescence from single molecule binding, then when we apply [50px] rolling ball background subtraction, we will remove a considerable proportion of the intensity information from the antibody spot region itself, and thus artificially lower the single molecule count. We circumvent this by increasing the radius of the rolling ball to much larger than the diameter of an antibody spot.

3.6.3. Congested image BNO compensation

Since a different radius rolling ball is used for congested images the baseline noise offset necessary to make the noise level average to zero is in all cases subtly different to that for a given non-congested image. Thus, a BNO derived for single molecule localisation analysis cannot be used for congested images in the same dataset. Nevertheless, the BNO to be applied to a set of congested images should always be calculated from non-congested images, since using congested spot images is liable to introduce high numbers of single molecules into the peripheral
intensity profiles which are used to calculate the BNO. If possible these should be from blank chambers or off-spot regions from the same acquisition run as the congested data to be analysed. For highly congested images the relative impact of the BNO compensation is small, however it is important for improving the correspondence of the congested counting method to the Gaussian localisation method in the low to middle copy number regimes. Its inclusion lowers the point at which it becomes more accurate to measure a semi congested image via intensity analysis compared to via Gaussian fitting [See Algorithm performance].

3.7. Algorithm performance: simulated images & validation

Simulated single molecule images were generated covering the non-congested through to highly congested regimes [approximately 200–1×10⁶ bound molecules] to validate the performance of the new single molecule counting algorithm compared to the thresholding algorithm. Simulated images were created with the GDSC SMLM ‘Create Data’ plugin in ImageJ. Simulated Gaussian peaks were matched to the mean measured PSF parameters measured via the PeakFit algorithm during analysis of MAC chip data. Camera settings [e.g. gain and quantum efficiency for 525 nm light] were also matched where the data was available. Illumination light was simulated with a radial fall-off to simulate the darker edges present in a MAC chip TIRF image, the edges being set to 50% of the central maximum. Illumination and background light were subject to Poisson noise and a Gaussian noise model was used to simulate camera read noise. Fluorophores were modelled as point particles with a uniform photon emission distribution of 2000 photons/s and subject to a point spread function with a 245.7nm standard deviation. Fluorophore generation was confined to a mask area within a 512x512 pixel image, representing a 138x138μm field-of-view as in MAC chip experiments, and corresponding to 3 different cases: [1] an ‘ideal’ 100-micron diameter circular spot, [2] the outline of a real ERα antibody spot with a halo of small scattered antibody blobs, and [3] another real antibody spot, this time displaying the coffee-ring effect, where the outside edge of the antibody spot features vastly more fluorescence than the central region [Figure 3.10]. These masks were chosen to be representative of the spread of quality that is often present in printed capture spots.

Each simulated image consisted of two components, the first being the analyte binding, which was restricted to the area of the mask, and the other being the non-specifically bound component, which consisted of N molecules, unconfined to the mask, and randomly distributed in the 512x512 pixel image. For the case of the ideal 100-micron spot, 6 levels of non-specific binding were tested: 50, 150, 250, 300, 500 and 1000 molecules. For the two more realistic masks, only low [150] and high [1000] levels of NSB were evaluated.
3.7.1. An ideal 100\(\mu\)m antibody spot

Figure 3.11 shows the performance of the localisation and ASMI counting methods in comparison to the threshold and threshold-congested methods for the ideal 100\(\mu\)m circular spot. Performance is measured in terms of the ‘counting efficiency’ or the ratio of the number of single molecules detected to the number of simulated molecules in the image, perfect performance equalling 1, and no molecules being detected equalling 0. Performance of the localisation algorithm was evaluated with a low signal factor [i.e. the lowest acceptable signal-to-noise ratio per molecule] of 1.5.

There are several important trends to describe. Directly comparing localisation [black curve] and thresholding [blue curve] we see that although localisation produces more false positives at low molecule numbers [see following section], as single molecule numbers increase the localisation method outperforms thresholding by a considerable margin, maintaining superior efficiency well into the region where it becomes desirable to use ASMI or threshold-congested methods. As non-specific binding increases the performance of the localisation algorithm is roughly stable, however the thresholding method sees a marked drop in performance at higher levels of NSB. This is due to the increased likelihood of single molecules being present in the off-spot ROI used to calculate the threshold intensity, and thus a higher threshold being set, meaning only the very brightest molecules will be counted by the algorithm. In the semi-congested regime [1000-10,000 molecules] a surprising result is how unstable the
threshold-congested counting method is, showing jumps in counting efficiency of >20% between images which differ by only 100 simulated molecules. The stability of the ASMI counting method in this transition regime is vastly superior: if we define the stability of the algorithms as the standard deviation of the counting efficiency, then the average stability of the ASMI algorithm for all measurements over 1000 molecules, for all levels of NSB, equals 2.7% compared to 20% for the threshold congested method [Figure 3.12]. Stability also considerably worsens with NSB level for the threshold-congested method. The likely reason for the instability shown by the threshold congested method in this regime is the lack of a background removal step after image flattening in the algorithm, and the instability of the threshold itself: NSB positions are randomised in the simulation, so the threshold region may contain more or less fluorophore in each frame and thus produce a relatively higher or lower threshold. This is a situation representative of real life data. Directly comparing ASMI [red curves] and threshold-congested [pink curves] methods in the highly-congested regime [>10,000 molecules] we see that in all cases the ASMI method produces a superior counting efficiency. The threshold-congested method is prone to overcounting by up to 30% from the true value, although the algorithm does reach a high-end plateau earlier than the ASMI method. The ASMI plateau is preceded by a slight [~5%] dip in counting efficiency caused by the rolling-ball background subtraction that is necessary to produce convergence with the localisation method at low molecule numbers and increase stability in the semi congested regime.

As stated above, these benchmark tests were run with a minimum signal to noise ratio of the localisation algorithm of 1.5. This is essentially an arbitrary value, and as can be seen from the figures causes a degree of overcounting at very low molecule numbers. Increasing the minimum signal to noise ratio can rectify this, however also results in a concomitant increase in the mean single molecule intensity [Figure 3.13]. Increasing the minimum SNR also hastens the drop-off in counting efficiency – this makes sense because the higher the ratio, the smaller the proportion will be of molecules that are sufficiently bright. An obvious problem to note here is that rectifying overcounting in the non-congested regime will lead to more pronounced undercounting in the congested regime. We trade accuracy in one regime for accuracy in another, and there is hence a sense of arbitrariness about quantification with the MAC chip that means ‘absolute quantification’ is far from absolute.
Figure 3.11: Comparison of single molecule counting algorithm performance for an ideal 100-micron circular antibody spot. The localisation + ASMI method consistently outperforms the threshold/congested threshold counting across a range of non-specific binding.
Figure 3.12: Comparison of the variability of the old and new counting methods. The variability increases when non-specific binding increases, however the trend is much more pronounced for the threshold congested method. A box and whisker plot of the counting efficiency standard deviation [right panel] highlights the improvement the ASMI counting makes over the threshold congested method: the mean variation of the threshold congested algorithm is almost 20%, whereas mean variation is only 2.7% for ASMI.

Figure 3.13: The effect of signal factor on the mean single molecule intensity and the counting efficiency [NSB=150].

3.7.2. Realistic spot morphologies

Figure 3.14 and Figure 3.15 show counting algorithm efficiencies for the two more realistic spot morphologies, for low [150 molecules] and high [1000 molecules] levels of non-specific binding. In all these cases, we see similar characteristics to above. The localisation-ASMI method performs better than the threshold-based algorithms in every case, producing greater
stability in every counting regime, as well as superior counting efficiency. For a larger spot, the congested-plateau occurs at a higher number of bound molecules.

Figure 3.14: Counting algorithm efficiencies for a large, asymmetric spot generated from Mask 2 in Figure 3.10B. Localisation and ASMI are performed with a signal factor of 1.5, although for the NSB=150 case, the black and red dotted curves show the case for a signal factor of 30.

In the case of the coffee ring spot [Mask 3, Figure 3.10C] we observe a catastrophic failure of the threshold congested algorithm under standard operating conditions [pink curves Figure 3.15]. This occurred because the region [75×75 pixels in the top left of the image, Figure 3.16] used to set the threshold in the algorithm contained a large amount of single molecule data, and thus the threshold was set too high to accurately measure the single molecule content. Rerunning the algorithm with a different threshold region [bottom right 75×75 pixels] circumvented this problem and produced similar results to the other benchmarking tests [Figure 3.15, green curves], with the exception that at an extremely high number of bound molecules the algorithm failed once more – and again due to the accumulation of a sufficiently high number of molecules within the threshold region.
Figure 3.15: Counting algorithm efficiencies for a large spot with coffee ring effect, generated from Mask 3 in Figure 3.10C.

This highlights the critical flaw of the thresholding method, and how essential the move away from the method is for MAC chip data analysis. Many things can occur in a MAC chip experiment which place sufficient fluorescence intensity within the threshold region to disrupt the algorithm: for instance, [1] the antibody spot may be exceptionally or unexpectedly large, [2] excess antibody may adsorb to the surface surrounding the antibody spot when the device is filled, and [3] scattered light may be present due to e.g. the interaction of the excitation laser with the PDMS walls of the analysis chamber. Moreover, these artefacts may affect images from a single experiment to different degrees, and in different spatial locations, meaning that every dataset requires manual assessment of the images prior to thresholding. Accounting and compensating for all these scenarios is impractical for large datasets. The localisation-ASMI method requires no threshold to be set and thus is much more robust to deal with all kinds of single molecule data as shown here in the counting efficiency curves.
Figure 3.16: Effect of threshold region on single molecule counting. Choosing an unsuitable area to set the threshold dramatically affects the single molecule count. The solid pink region was used as the threshold which lead to catastrophic failure of the counting algorithm in Figure 3.15; exchanging this for the dashed-green region produced better results, but still failed at high numbers of bound molecules.

3.8. Future improvements

What underlies the failure to generate perfect single molecule counts, is that we have failed to isolate the pure single molecule information in our non-congested and congested images, and failed in our aim of producing a 1:1 correspondence between the intensity of a single molecule in the non-congested regime to its intensity in the congested regime, once all other sources of intensity are compensated for. We could avoid this situation if localisation counting methods could be used in all regimes, for example using dSTORM methods to temporally separate the spatially-unresolved bound molecules in the semi and fully-congested regimes. Technically this ought to be possible with the use of secondary antibodies conjugated to photoswitchable fluorescent dyes, or as Vogelsang et al have demonstrated, with standard fluorescent dyes under well-tuned redox buffer conditions. By choosing the correct imaging conditions [such as readout laser intensity, activation with near-UV light, and the presence of a redox control system e.g. a thiol + oxygen scavenger system] it is possible to control the duty cycle of the fluorescent probes [the ratio of time spent in the fluorescent state versus time spent in the dark state], and thus control the fraction of fluorophores emitting at a given time point. Under this scheme, a MAC chip experiment would be left to reach equilibrium prior to imaging the antibody spot, and MAC chip data would then be taken as a high frame rate image stack until all bound fluorophores were bleached. Ideally secondary antibodies would be single conjugated with an easily photoswitched dye e.g. Atto-488, Alexa-488, or Alexa647, although in most cases commercially available antibodies may be conjugated with several dye molecules, which complicates the images process, as it is more difficult to achieve acceptable rates of photoswitching, and the close proximity of molecules gives rise to a significant degree of fluorophore self-quenching. Nevertheless, if it were possible to maintain a constant level of fluorophore activation during readout of a congested spot, e.g. 500 molecules per frame, it would be possibly to tune the localisation software such that as close to 100% of molecules are identified, and single molecule counting efficiency is constant across the acquisition. It should be possible to design a MAC chip that allows the introduction of an optimised photoswitching buffer after the assay has reached equilibrium, allowing efficient readout.

3.9. Conclusions

In this chapter, we have described the new approaches made to the single molecule counting methods used to analyse MAC chip datasets. A new set of algorithms based on single molecule localisation were written in the ImageJ macro language, and the performance of these was
evaluated against simulated MAC chip image sets designed to approximate a variety of experimental data types. Direct comparisons of these with the previously established [thresholding-based] algorithms showed that in all cases the localisation-based algorithms outperformed the old method in terms of counting efficiency, particularly in the so-called semi-congested regime. The new algorithms converge in the non-congested regime, and the stability [as measured by the standard deviation of the counting efficiency] of the ASMI algorithm is roughly an order of magnitude better than the previous methods, consistently across datasets with non-specific binding up to 1000 molecules per field of view. The new methods are not susceptible to failure due to non-uniform or local single molecule information away from the antibody spot in the way that threshold techniques are, and do not require the spot morphology to be inspected prior to analysis. We have outlined a method for using localisation-based methods to study precisely protein-protein interactions, and thus enhance the information provided by the MAC chip beyond simple copy number.

The benchmarking tests we have performed emphasise the desirability of limiting non-specific binding in MAC chip systems, as higher levels of NSB generally lead to a reduced accuracy in the semi- and fully-congested regimes, as it is more challenging to accurately compute the mean single molecule intensity from a high-NSB image. On the other hand, it is important NSB is not reduced to zero, as sufficient molecules are required to compute the mean single molecule intensity [this is especially true if post-lysis analyte binding is expected to enter the semi- or fully-congested regime]. The dependence of counting efficiency on NSB levels also underlines the need for consistency of NSB across MAC chip experiments: experimental protocols should therefore be optimised with this in mind.

In Section 3.6 we raised the question ‘at what point does an image become congested?’, and thus at what point should the experimenter switch from a non-congested counting method to the congested version. The benchmarking tests of this chapter indicate that the answer is surprisingly low: approximately 1000-1500 bound molecules. Fortunately, the approximate convergence of localisation and ASMI at low molecule numbers means that this transition is not as critical as for the threshold and threshold-congested methods. As the counting efficiency of the localisation method only worsens with increased numbers of bound molecules, it is probably prudent to switch to ASMI counting at the lower end of this range. The exact switch-over number will differ slightly for different spot morphologies, so a better way of expressing this is in terms of density of the observed molecules: 1000 bound molecules in these simulations corresponds to a density of ~0.05 molecules/μm². This provides a useful rule of thumb for assessing the optimum algorithm to use for a given dataset, and may be applied to local regions of apparent congestion in images where binding is non-homogeneous.

As stated in the introduction of this chapter, one of the principle goals of MAC chip technology is absolute quantification: precisely how many proteins of type X are present in the cell being analysed. The localisation-ASMI method undoubtedly improves the overall accuracy
and reliability of single molecule counting, and hence our capability for both relative and absolute protein quantification, though the approach is still not perfect. The principle reason for this is the semi-arbitrary nature of the signal factor used to implement the localisation algorithm: there is a range of values this factor can take while still producing reasonably accurate single molecule counts. Furthermore, counting efficiency descends linearly with molecular density, but is also intricately associated with the signal factor used to fit the localisations. The resulting situation is that we can tailor our signal factor to produce a perfect counting efficiency [or at least one that is off by a known factor] at a particular density of bound molecules, but our accuracy will be worse for images with higher or lower densities. The signal factor we choose alters our mean single molecule intensity, and this in turn will change the result of the ASMI algorithm for congested images. A higher signal factor may address overcounting in the noncongested regime, but it also increases the mean single molecule intensity, which will reduce the ASMI count for congested images. We trade accuracy in one regime for accuracy in another, and as we remark above, there is an inherent sense of relativity regarding quantification with these algorithms. The question is whether this inhibits our ability to perform absolute quantification, but the answer is in fact not. Provided the assay in question can be calibrated with known amounts of recombinant analyte then absolute quantification is relatively straightforward, as these systematic errors drop out during calibration. If an assay cannot be calibrated, then with the new algorithms, and with the parameters chosen in these simulations it ought to be possible to get within ±5-10% of the true value for most levels of analyte binding under a range of experimental conditions, as the benchmarking tests here indicate.
Chapter 4. Strategies for isolation, concentration and quantitative proteomic analysis of circulating tumour cells

*Blood is a juice of rarest quality.*

– Johann Wolfgang von Goethe

Faust, l. 4. 214.4.1.

4.1. Introduction

Circulating tumour cell [CTC] isolation and analysis is one of the most difficult challenges in contemporary oncology. This is mainly due to the sheer scarcity of CTCs themselves: with only a handful of these cells existing in blood samples that can contain many billions of erythrocytes and hundreds of thousands of leukocytes, the challenge of isolation is self-evidently a great one. Add to this the exquisite sensitivity required to analyse a cell on an individual basis and the challenge is greater still. The MAC chip is designed to be able to analyse rare cells on an individual basis and so could potentially be used to analyse CTCs for protein copy number, however for this to be feasible a technological bridge must be built between CTC isolation technology [which is a deep and complex field in its own right] and the MAC chip platform. This chapter will cover two distinct but connected pieces of research, in two sections: [1] the
in-house recreation of a published CTC isolation method, the ‘spiral biochip’, and [2] the attempted development of a bridging technology to allow the output of the spiral biochip to be introduced to the MAC chip.

To analyse CTCs with the MAC chip we must consider the output of the various possible methods of CTC isolation, and how best we might couple them into the chip itself. The nature of the MAC chip technology places certain restraints on the nature of the sample which we introduce into it. Firstly, all cells introduced to the MAC chip must not be fixed. This is because the analysis stage requires that the cellular protein under interrogation must be free to diffuse and to bind to the antibody capture spot in the analysis chamber. Fixation with aldehydes causes a cell’s proteins to crosslink with each other, while alcohols will cause denaturation and precipitation, preventing the MAC chip from measuring accurate copy numbers. Because cell selection in the MAC chip is performed manually with the optical trap we require that the purified CTC sample is ideally in as small a volume of buffer as possible. If this criterion is ignored then it may take an impractically large amount of time to process the entire sample, and thus become impossible to manually identify individual CTCs within the reservoir channel of the MAC chip. Slow processing increases the likelihood of the CTCs entering apoptosis prior to analysis as well as increasing the chances that the CTCs’ protein expression profiles no longer represent their in vivo state. Thirdly, the isolation efficiency of the device must be good. Typically, CTC isolation methodologies are validated with cells from cancer cell lines spiked into healthy blood samples. For example, a methodology that manages to recover 95 cells from a sample that has had 100 cells spiked into it would have an isolation efficiency of 95%; the inherent scarcity of CTCs in peripheral blood makes maximising this parameter essential. Finally, since CTCs are isolated from whole blood it is important for the CTC recovery process to diminish the components of the blood which do not derive from the patient’s tumour[s], including erythrocytes, leukocytes, platelets and other minority components.

We can summarise the constraints as follows:

1. Viable, non-fixed cells in input and output
2. Fast isolation
3. High isolation efficiency
4. High purity
5. Low volume of buffer in final solution

Unfortunately, there is no single technology which can provide for all five of these criteria. For reasons outlined in the following sections we decided to use a hydrodynamic, size-based
CTC isolation method known as the spiral biochip, and attempted to develop another hydro-
dynamic, deterministic cell trapping method to enable the output cells of the spiral biochip to
be analysed with MAC chip methods [Figure 4.1].

Figure 4.1: Overview and aim of the work in Chapter 4.

PART 1: CTC ISOLATION AND THE SPIRAL BIO-
CHIP

4.2. CTC isolation methods

CTC isolation methods fall into several categories, based on the characteristics of the isolation
method. The most established are size-based detection and immunological enrichment via
marker proteins, though some other methods have been described [e.g. deformability\textsuperscript{85}, or
dielectrophoretic field-flow fractionation based on cell membrane capacitance\textsuperscript{86}]. Isolation
methods tend to be tuned to identify cells that meet a small set of specific criteria, and hence
have blind spots, and miss other cells that may be pertinent for the analysis to be undertaken.
Current research indicates that CTCs likely defy a single categorisation, and no single biological
or physical standard can be used to define all types of CTCs.\textsuperscript{87}

The first widely used, and so far, only FDA-approved system for CTC isolation and enu-
meration from cancer patients was CellSearch. CellSearch is a commercial semi-automated
platform with disposable cartridges that perform an immunomagnetic separation of cancer
cells from regular blood components using magnetic particles coated with anti-EpCAM antibody. A whole blood sample is preprocessed to remove plasma, then the cocktail of blood components which remains is incubated with ferrofluid nanoparticles conjugated to EpCAM antibodies which target epithelial cells, and a high magnetic field is applied to magnetically separate the CTCs from the other remaining blood cells. Fluorescently-labelled anti-cytokeratin antibodies are introduced which bind only and highlight the epithelial cells, while fluorescent anti-CD45 antibodies are added to highlight leukocytes which might be contaminating the sample. The DNA stain DAPI is added to identify nucleated cells. The sample is then scanned and candidate CTCs [i.e. DNA- and cytokeratin-positive cells] are automatically displayed to a trained operator for final review.

CellSearch has several drawbacks: it has high initial setup and ongoing costs, it is laborious, time consuming, and can vary considerably in its CTC recovery efficiency. Fixation means cells are dead on analysis, and it is also restricted to EpCAM-positive cells meaning a whole subset of potentially dangerous EpCAM-negative cells is missed in the analysis. EpCAM expression can vary wildly between disseminated tumour cells from the same source, as well as between tumour types, and targeting only EpCAM may potentially miss cells which have lost EpCAM expression due to the epithelial-to-mesenchymal transition, a change in phenotype that has been hypothesised to be responsible for the production of a dangerous subset of cells that are more prone to intra- and extravasation from the blood stream. The drawbacks of CellSearch along with the advent of microfluidics has spurred much innovation and progress in the field of CTC isolation, with many unique technologies being published, and several being turned into commercially available products.

Two of the most reported microfluidic isolation methods are the CTC chip and its successor the Herringbone chip. The CTC chip relies on the interaction of marker proteins expressed on the surface of CTCs with antibodies immobilised to an array of 78,000 microposts under laminar flow conditions that are tuned to maximise cell interaction with the capture substrate. The CTC chip was used successfully in a pilot longitudinal study of CTC levels in prostate cancer patients. The Herringbone chip improves on the first generation by introducing a highly asymmetrical array of chevrons into the upper surface of the microfluidic flow channel, which disrupt the laminar flow lines and cause microvortices which enhance the interaction of the cells with the capture surface. The Herringbone chip has recorded high capture efficiencies of 91.8 ± 5.2%. Both the CTC and Herringbone chips may be used with pure blood, or alternatively with a pre-processing step to remove red blood cells. However, a drawback of the devices is that they can lead to a fair degree of non-specific capture of white blood cells, with purity of the recovered sample varying from 9.2% [for the CTC-chip] to 14.0% [for the Herringbone chip], although this has been improved by Wang et al to 39.4% by turning the discontinuous Herringbone vortex mixer into a smoothly-varying wavy design. Processing with these chips is slow, with the optimum flow rate being around 1ml/hour, meaning
a whole 7.5ml blood sample could take hours to process, or multiple microfluidics would be needed to be used in parallel, increasing reagent costs. Nevertheless, the Herringbone chip was able to be used to measure RNA transcript levels via in-situ hybridisation techniques and provide strong evidence that CTCs in breast cancer patients show dynamic changes in epithelial and mesenchymal composition.

Affinity capture methods can offer high efficiency, purity, and an inherently microfluidic-scale recovery volume, however, they are comparatively slow to process large volumes of blood as they require low flow rates to facilitate binding between immobilised capture antibodies and cell surface marker antibodies. Many of these techniques also require a cell fixation step, which, while discarding the need for a speedy isolation, also precludes their use with the MAC chip. Electro- and magnetophoresis techniques were assessed for our purpose, but ruled out as they were considered to be beyond what was technically possibly in the available time, as there existed no prior experience in the group of incorporating electrodes and other components directly onto a microfluidic device.

A full review of the many and varied types of CTC isolation microfluidics is beyond the scope of this thesis, but there are many such reviews in the literature. See for example.

4.3. The spiral biochip

The reason many surface marker-based devices fail to meet clinical requirements is because they fail to process the blood sample in a reasonable amount of time. Size-based isolation methods allow for faster separation. A notable example of this is the spiral biochip of Hou et al, which uses a technique called Dean Flow Fractionation to continuously separate CTCs from smaller blood components [red and white blood cells, platelets]. The spiral biochip provides antibody-free isolation, ultra-high-throughput [7.5ml blood processed in <10 minutes for a 3× multiplexed design], 99.99% reduction in white blood cell content, and a high recovery rate of >85% tested across multiple cell lines spiked into blood. The spiral biochip has been clinically validated with advanced stage metastatic breast and lung cancers, and has been used in conjunction with immunophenotyping, fluorescence in situ hybridisation and targeted somatic mutation analysis, and ultrasensitive mass spectrometry based methods for further characterisation of CTCs post-isolation.

A pure fluidic method was thought to be the design type with the highest likelihood of success, considering the group’s experience with single-layer, un-valved microfluidic chip design. The spiral biochip consists of only a single spiral microfluidic channel, and for this reason, coupled with the fact that it scores highly in most areas of the design criteria outlined above, it was decided to develop the spiral biochip in-house as our primary method of CTC isolation. The key drawback of the spiral biochip is the large volume of sheath fluid that is used for hydrodynamic focusing at the inlet, which leads to CTCs being recovered in a large volume of buffer. This makes manual cell selection within the MAC chip almost impossible and meant
that an additional processing step was required to concentrate the cells into a manageable volume for MAC chip analysis [See Part 2].

4.3.1. Dean flow

Dean flow is a characteristic effect of particle flow in curvilinear channels and is the effect whereby centrifugal forces give rise to a secondary cross-sectional field flow perpendicular to the primary flow direction\(^9\). This secondary vortical flow allows particles in a curved channel to migrate across streamlines. The magnitude of the dean vortices is given by\(^10\):

\[
De = \frac{\rho U_F D_H}{\mu} \sqrt{\frac{D_H}{2R_C}} = Re \sqrt{\frac{D_H}{2R_C}}
\]

where \(\rho\) is the fluid density, \(U_F\) is the average flow velocity, \(\mu\) is the viscosity of the fluid, \(R_C\) is the radius of curvature of the path of the channel, \(D_H\) is the channel hydraulic diameter and \(Re\) is the flow Reynolds number (the ratio of inertial to viscous forces). Dean flows create a drag force on particles which pushes them along the direction of flow inside the vortex. The magnitude of the dean force is:

\[
F_D = 3\pi\mu U_{Dean} a_c
\]

Where \(U_{Dean} = 1.8 \times 10^{-4}D_e^{1.63}\) is the Dean velocity, and \(a_c\) is the cell diameter. Particles in a curvilinear channel also experience an inertial lift force \(F_L\) due to shear and proximity to the flow boundary wall:

\[
F_L = C_L \rho G^2 a_c^4 = \frac{2\rho U_F^2 a_c^4}{D_H^2}
\]

Where \(C_L\) is the lift coefficient, \(G\) is the shear rate of the fluid, and \(U_F\) is half the maximum fluid velocity.

Both these forces are dependent on particle diameter, and it follows the balance between the inertial lift forces and Dean forces can be tuned to separate particles of different sizes. The spiral biochip capitalises on this to cause the separation of CTCs from smaller blood cells at its outlet channels [See Figure 4.2].
Figure 4.2: The spiral biochip. Whole or processed blood is introduced via the inlet at the inner part of the spiral channel. A sheath fluid concentrates this at the outer wall of the channel, and the centrifugal forces that particles of difference diameters experience in the curved flow cause them to separate out in the lateral direction. Tuning the geometry of the spiral allows a cut-off to be set such that CTCs all emerge down one outlet, while smaller white and red blood cells exit through the other.

4.4. Recreating the spiral biochip

A technical challenge was immediately encountered as there is considerable ambiguity in the published literature on the spiral biochip regarding the precise height and tolerance required on the main spiral channel to perform effective isolation of CTCs. For example, in the original paper describing the spiral biochip the z-height of the channels is stated to be 160µm, but the COMSOL flow modelling performed to characterise the Dean flow which is responsible for the separation of particles of different sizes is performed on a channel with a z-height of 155µm. Correspondence with the authors confirmed that 160µm was indeed the target z-height which should be aimed for, with a z-tolerance of 1-2µm. However, recently the manufacture of the spiral biochip has been described in Nature Protocols, where a z-depth of ‘~170µm’, and ‘~165±5µm’ is stated to be the target depth of the deep reactive ion etch which produces the channel for the spiral chip. In the same paper, ‘Incorrect channel height (i.e., it must be 165–170 µm), which can be the result of improper DRIE [Deep Reactive Ion Etching],’ is given as the reason for incorrect focussing of cells of different sizes at the outlet of the device, and measuring a 1-5µm tolerance on the PDMS channel height is stated to be a critical step. This ambiguity is troubling from a fabrication perspective, although it may not explicitly be a contradiction if it is the variation in z-height of the channels which is the truly critical parameter, rather than the mean depth. Put another way, there may be several mean channel heights which produce an effective separation of CTCs, but only if the variation across the entire ~10cm length of the channel does not exceed 1-2µm, or possibly up to 5µm.

Only the information from the 2013 Scientific Reports paper [i.e. z-depth 160µm, no indication of tolerance] was available at the time it was decided to attempt to recreate the spiral biochip. However, it was considered worth an attempt as the promise of such extremely fast, label-free isolation was great, and was considered to provide greater compatibility with downstream analysis in the MAC chip, as well as the fact that the additional funding this project
received from CRUK allowed a higher risk, higher reward strategy to be attempted. Previous [unpublished] results within the group [experiments performed by Dr Mattia Terenghi, Marie Curie Fellow] produced weak recovery rates in spiked blood, and real patient samples, with antibody-based isolations, and were also slow processes which could take >4 hours to complete. It was thus considered advantageous to attempt a strategy which avoided these pitfalls and produced better recovery rates, faster.

Several iterations of photomask were produced, as the exact dimensions of the inlet and outlet channels of the spiral biochip were not published in the original paper, nor were they provided by the authors when asked in correspondence. A slightly modified design of the biochip has since been released into the public domain as an AutoCAD file. The spiral biochip was initially recreated using standard Su-8 2075-based photolithography. Su-8 2075 can produce layers of photoresist between 150-170 microns thick when spun at approximately 1400-1700rpm. In the first instances, Su-8 2075 was spun as a single layer of resist between 1000-1650 rpm, to check the range of thicknesses the Su-8 might be able to produce [Figure 4.3]. Su-8 was processed according to the Microchem guidelines for layers of specific thickness, with respect to bake times and temperatures, exposure times, and hard bake processing. All other settings for the processing of the resist were kept constant for the comparisons [i.e. ramp up/down accelerations]. Channel heights were measured on a Dektak 6M stylus profilometer. The film thickness plateaued at around 140µm, short of the target of 160µm. Initially this was suspected to be due to a possible measurement error i.e. the measurement height might have exceeded the calibration range of the profiler, however this was ruled out as the Dektak machine was checked and shown capable of measurements up to 2620 Å [262µm].

Figure 4.3: Experimental channel thicknesses for the spiral biochip overlaid on spin speed-thickness curve for Su-8 2000 series. Measurements are the mean ± SD of 5 channel depths as measured on a Dektak contact profiler.
Subsequently Su-8 2075 was deposited in a double layer, whereby the first layer was spin coated, then soft baked, then a second layer was deposited on top of the first, then soft baked in turn, prior to exposure. Producing a thick layer of Su-8 from two thinner layers in this way has the advantage that the layer thickness is less sensitive to variations in spin speed at higher rotation speeds, leading to greater reproducibility. Again, the channel heights of the Su-8 mould were measured with a contact profilometer, and the results of these measurements are shown in Figure 4.4.

While the double-coating technique indicates that it is possible to fabricate a mould with a mean z-height of 160 microns with Su-8-2075, it is also clear from these measurements that the thicknesses of Su-8 films produced from spin coating are non-uniform over the length scales of the features of the spiral biochip. Significantly, the variation displayed in all measurements is larger than the tolerance [1-2µm] said to be required for effective CTC isolation. Su-8 spin coating lithography was subsequently abandoned as a strategy for manufacturing the biochip.

![Figure 4.4: Su-8 2075 channel height variation for three spiral biochips manufactured via a double-coating process on a single wafer. Mean channel heights are indicated by the grey vertical line, whiskers indicate ± 1 standard deviation.](image)

4.5. Experiments with unoptimized chip

The z-height of the channels of the double-coated spiral biochip 1 mould [Figure 4.4] are at least in the right ballpark however, and it was thus decided to attempt to perform a series of isolation experiments with a PDMS chip created from this mould, to see if the device was capable of any useful level of CTC enrichment, despite the unoptimized geometry.
A series of bead experiments were performed where a sample of mixed size beads was passed through the spiral biochip at various inlet as well as sheath flow rates. The composition of the inlet bead solution was measured via haemocytometer slides, and again at both outlets. The fractional composition at each of these stages was determined via a custom image segmentation algorithm. However, none of these experiments produced an effective separation – typical data is shown in Figure 4.5. Secondary experiments with a high frame rate [HFR] imaging system were performed with the device in operation using a mixture of 15µm and 6µm polystyrene beads [Sigma] to represent CTCs and smaller blood components respectively, as in the original biochip paper°. Using this system, it was possible to directly observe the trajectories of the different size particles, confirming that CTCs and WBCs were not separating along the necessary flow lines at the outlets, resulting in heterogeneous solutions leaving the CTC and WBC outlets.

A trial experiment was also performed with MCF-7 and MDA-MB-231 cells respectively spiked [50,000 cells/ml blood] into healthy blood samples, to test the chip under more realistic operating conditions, which produced poor separations similar to the bead experiments [Figure 4.6]. Although no positive staining steps were undertaken to identify the spiked MCF7 cells via e.g. EpCAM expression, it is clear from the images that a mixture of many small and large cells exited the spiral biochip from both outlets.
Figure 4.5: Measured composition of inlet and outlet solution of spiral biochip with a mixed bead solution [6μm and 15μm-diameter]. In a correctly optimised system the waste outlet should contain 100% of the small beads, and the CTC outlet 100% of the larger beads. The size of the beads is slightly inflated from the true size because of brightfield glare through the transparent beads, and an image processing artefact due to the thresholding used to binarise the raw image data and identify the circular beads automatically. The CTC outlet produces a modest enrichment of the CTC fraction, however is still highly contaminated, containing a majority of the smaller beads.
4.6. Attempt to outsource production

Once it became apparent that production of the spiral biochip would not be possible with the equipment in the in-house cleanroom, an external company specialising in microfluidic production, lithography, or MEMS engineering was sought to facilitate production of the mould to the required tolerance. An order was placed with NIL Technology [Denmark] to produce a nickel shim of the spiral biochip in September 2015, using a Bosch process isotropic plasma deep-etching technique. A lead time of ~1 month was quoted, however, after a sequence of delays the order was cancelled by the company in March 2016 due to an unfixable technical fault with the deep-etching equipment critical for mould production [Figure 4.7]. The nature of the fault led to the reactive species of the process plasma attacking the sidewalls of the
channel past a certain depth on certain process cycles. This produced an extremely rough surface the deeper the etch went into the Ni wafer. As can be seen from the SEM images the roughness of the surface verges on porosity in places, which is worse from a technical perspective, as it would affect the Ni plating and severely hinder the replication done with the Ni shim when producing microfluidics.

At this point, the decision was made to abandon the spiral biochip entirely, and look to integrate the cell trapping/concentrating devices of the next section with other methods of CTC isolation.

Figure 4.7: SEM images of the morphological defects in the sidewalls of the Ni shim of the spiral biochip produced by NIL Technology.
PART 2: BRIDGING TECHNOLOGY

4.7. Microfluidic designs for rare cell concentration

Concurrently with the work of the previous sections, and with the proviso that an operational spiral biochip mould was fully expected to be produced by the contracted company once the contract was signed, we attempted to produce an intermediate microfluidic device which would make the output of the spiral biochip device compatible with the MAC chip.

The key problem to address is how to concentrate an extremely dilute solution of pure CTCs, containing as little as 1 – 10 cells in several millilitres of blood, into ideally less than 100 microlitres, without losing any of the CTCs in the process. Centrifugation is used routinely in biological protocols for concentrating cell samples and resuspending cells in alternative buffers for a particular downstream purpose. However, in the case of CTCs there may be as few as a couple of cells in the entire sample. Aspiration of the fluid that contained the cells in suspension prior to centrifugation relies on the experimenter’s ability to visibly locate [usually unaided by magnification] the position of the cell pellet, which if there is only a handful of cells would be almost impossible. The act of aspiration may itself disturb the CTCs and possibly lead to their accidental removal, and a centrifugation step thus has the potential to be quite lossy.

There are several advantages to a microfluidic approach: [1] rational design rules based on the physics of laminar flow may be followed to maximise cell recovery, [2] a microfluidic design may be directly incorporated on-chip with both the MAC chip microfluidic [or features thereof] and the spiral biochip, and [3] microfluidic cell trapping is a mature field in its own right\textsuperscript{102}, and many designs already exist in the literature, any of which could potentially be incorporated into the setup.

4.7.1. Microfluidic cell trapping methods

Important early work in microfluidic hydrodynamic cell trapping was performed by Di Carlo et al, who developed one of the first hydrodynamic single cell trapping arrays. Using this device they were able to create arrays of single adherent cells and dynamically control perfusion of fluid through the device, allowing nuanced control over culture conditions and the elucidation of novel single cell enzyme kinetics\textsuperscript{103,104}.

An important concept in hydrodynamic trapping is minimising the resistance path through the trapping obstacle compared to its bypassing route [Figure 4.8]. Cell trapping specifically exploiting this concept has been implemented by Tran et al\textsuperscript{105}, Jo et al\textsuperscript{106}, Kazayama et al\textsuperscript{107}, Lin et al\textsuperscript{108}, Benavente-Babace et al\textsuperscript{109}, and many others. Important formalisms were introduced by Xu\textsuperscript{110} and Guan et al\textsuperscript{111} for maximising the trapping efficiency for a particle of a defined diameter. The principles of Guan trapping are outlined in Figure 4.9 and Figure 4.10.
Guan trapping theoretically allows for 100% trapping efficiency. In their paper, Guan et al experimentally demonstrate the ability of a 5-trap design to trap the first 5 cells to pass through the device’s channels. They were then able to elute these cells such that a new solution containing only 5 cells was produced. Finally, they demonstrated that by re-flowing this dilute solution of 5 cells back through the same microfluidic, they could recapture all 5 cells, with no loss, with excellent reproducibility. The ability to capture rare cells without loss from a dilute solution is extremely attractive for the problem of CTC capture from the diluted output of the spiral biochip.
Figure 4.9: Overview of the design of Guan traps. [Upper] Four traps in series. Each loop/dumbbell structure comprises a single cell trap. [Lower] Cells above a certain size have a 100% probability of entering the first trap in a sequence, and the cell behind is diverted round a bypass loop, whereupon it flows into the following trap.

Figure 4.10: Illustration of the theory of Guan trapping. A spherical particle in flow follows its centre of mass streamline. A cutoff radius is defined as the distance from the outside wall of the inlet channel to the outermost streamline that follows the path through the central trap, as opposed to the bypassing loop; any particle with
radius greater than $R_{\text{cutoff}}$ will follow the trapping path, whereupon the flow through the central trap is blocked, and all subsequent flow [including the next particle] is diverted round the bypassing loop. By engineering the geometry of the inlet channel and bypassing loop correctly it is possible to tune the cutoff radius [and the streamline distribution] to a value suitable to the size of cells that are desired to be trapped.

4.8. Design concepts

The parameters which guided the design of the 2nd stage cell concentrator are summarised in Figure 4.11. Of overarching importance is the ability of the microfluidic to deal with a high inlet flow rate, as the device was to be placed directly in series with the spiral biochip, whose outlet flow rate is approximately 1ml/minute. Of equal importance, the device should output a volume suspended cells no more than $\sim 100\mu l$, as higher volumes than this would make use with a standard MAC chip challenging. Ideally the suspension volume should be less than this, as lower volumes make the manually-performed optical cell trapping required in the MAC chip easier, if there is only a limited number of available cells.

Alternatively, MAC chip-style analysis chambers may be able to be incorporated into the trapping microfluidic itself. In this case, cell trapping, dilution, and analysis all take place within the same microfluidic. A further challenge that the cell concentrator must provide a solution to is flow-velocity reduction. A high volumetric flow rate in a narrow microfluidic channel necessarily involves high flow velocities, and if the flow velocity is too high, the pressure that the cell experiences when it encounters a trapping obstacle will cause it to deform and squeeze through. Additionally, extended exposure to such high velocities may expose the cells to shear-stresses which may damage or lyse them\textsuperscript{113}. The final requirement of the concentrator is that it must be robust to clogging, for example if cell clusters, or detrimental blood components ['gunk'] make it through the CTC isolation stage.

A device where the cells are trapped in position by the flow of the fluid itself provides the additional advantage that the buffer fluid can be replaced, while keeping the cells trapped in known locations. By replacing the fluid with buffers containing e.g. a fluorescent antibody marker, or a viability stain, live CTCs can be identified into subpopulations prior to analysis [e.g. mesenchymal or epithelial].
4.9. Guan trap-based deterministic trapping designs

The Guan methodology described above is a specific attempt to address the lack of quantitative design criteria in single cell hydrodynamic trapping designs, and produce definitive rules for designing traps with maximal efficiency for a given trapping problem. The Guan method potentially allows for 100% trapping efficiency\textsuperscript{[11]}. An added advantage of using Guan traps is that a single design could be used for both cell trapping and elution into a standalone MAC chip, as well as cell trapping and analysis in the same hybrid device, as the addition of MAC chambers upstream of the trapping channels was not expected to affect the efficacy of the traps themselves. Though the Guan trap chips could not intrinsically cope with a high flow rate, this was surmountable through the use of a manifold splitter between the spiral biochip and multiple Guan trapping chips. Though it was not known at the time, the available lithography equipment would severely hinder the manufacturing of all devices described in the following sections; this will be discussed in detail in section 4.11. A variety of non-Guan trap based microfluidic concentrators are described in Appendix B.

4.10. Validation of Guan trap methodology

A series of simple microfluidic designs were initially created to test the Guan-trap methodology for the cases of polystyrene beads and MCF7 cells, and to confirm the parallelisability of the method, for high-throughput sample processing. Modification of the original Guan-design was required because MCF7 cells, and expected CTCs, are larger in diameter than the white blood cells which were used to demonstrate the trapping principle in the original paper. The published chip is 18µm in height, however as we can see from Figure 4.12, this is not deep enough to accommodate a considerable fraction of the MCF7 cells, the largest of which can be 30
microns or more in diameter. Using the 18µm chip with MCF7 cells, and possibly CTCs, would likely lead to failure of the device by clogging, or lysis of the larger cells through sheer forces, which we clearly wanted to avoid. Increasing the height of the channels leads to other issues however, for instance increasing the probability of multiple cell occupancy in a single trap [Figure 4.13].

Figure 4.12: Measured MCF7 cell diameters. Mean cell diameter is 16.5µm, necessitating an increase in channel height from the 18µm used in the original Guan trap design to use it to trap these cells.

Figure 4.13: Geometric considerations for increased height Guan traps. Single occupancy of trapping points is linked to the proportion of streamlines that are blocked when a cell is positioned in the trap. For a 10µm bead or WBC in the original Guan trap 54% of streamlines are blocked during a trapping event. If we increase the height of the microfluidic to 33µm to accommodate the largest MCF-7 cells, then the percentage of streamlines blocked for the smallest cells is only 37%. For average-sized and very large cells the percentage blocked is 49% and 83% respectively. The lower this value is, the more likelihood there is of a secondary cell being caught on a streamline which takes it into the trap which already contains a cell.

Another factor to assess was whether the Guan traps could be utilised with pump-driven flow, and if so at what flow rates, as in the original demonstration bead and cell solutions are introduced into the microfluidic via passive, gravity-driven flow. This is intrinsically linked to the layout of the chip, which must be designed such that for a given inlet flow rate, particle flow velocities in trapping regions are low enough to prevent high sheer stresses, deformation
of the cells through the traps themselves, or even lysis. A strategy of inlet bifurcations was adopted to mitigate this issue.

A key microfluidic in these validation experiments was the ‘pizza shovel’ [Figure 4.14]. Featuring a bifurcating inlet leading to multiple lanes of parallel Guan traps, the device was able to be used to trap beads from solution pumped into the inlet, provided the PDMS superstructure was plasma bonded to the glass substrate. A version of this design with a 15µm z-height was capable of trapping 10µm diameter beads, while a deeper chip with a 27.5µm z-height was proven capable of effectively trapping 15µm beads in series. 27.5µm height was chosen as a compromise between capturing most sizes of MCF-7 cell, inhibiting multiple occupancy of trap sites, and ease of manufacture [deeper chips with narrow features are harder to manufacture].

The cell concentration microfluidics were found to require plasma bonding of the PDMS channels to the glass substrate. Without plasma bonding the integrity of the device was insufficient to withstand the internal pressures caused by the flow, resulting in leaks, primarily at the inlet. This was an important result for any final device incorporating MAC chambers, and printed antibody spots, because typically MAC chips are not plasma bonded together. The fine tolerance in the manual alignment required to position antibody spots into analysis chambers, coupled with the short-lasting effects of plasma activation [~30-60s], introduced an added challenge at this stage to the development of the combined CTC concentration and analysis microfluidic [See Section 4.12, Mature designs and testing].
Figure 4.14: The ‘pizza shovel’ microfluidic incorporates a bifurcating inlet and an array of parallelised Guan-traps emptying into a low pressure sink before the outlet. The asymmetric design is due to concerns that the narrow trapping channels [25µm] would lead to very high hydraulic resistance, which in the end proved unfounded. [Bottom] 15-µm beads [position indicated with red arrow] sequentially trapped down one lane of the pizza shovel [z-height 27.5µm], with single occupancy in all traps.

4.11. Fabrication challenges

At first none of the Guan-trap based designs described above displayed effective trapping either of beads, or cells, under any conditions. This was thought to be due to defects in the PDMS in the cell trapping regions of the microfluidics, however this was difficult to confirm
with standard optical microscopy due to the transparent nature of PDMS and the small scale of the features that needed to be examined. Scanning electron microscopy [SEM] was subsequently performed at the Harvey Flower Microstructural Characterisation Suite [Imperial College London] on the PDMS channels of the microfluidics to examine for defects, and it was discovered that in all cases, the trapping structures were incompletely formed, or entirely absent, and this was also true of the Su-8 moulds [Figure 4.15]. What seemed to be happening is that the Su-8 structures in the mould were broadening out during the lithographic process, and this had a devastating impact on the formation of the small-scale features present in trapping regions, where linear dimensions could be <10µm.

It was realised that this phenomenon was attributable to diffraction of the exposure light used to polymerise the Su-8 on transmission through the transparent slits of the photomask. The simple Fraunhofer model of diffraction shows that the broadening of light at a single slit is proportional to the wavelength and the distance from the aperture, and inversely proportional to the width of the slit:

$$y \approx \frac{m\lambda D}{a}$$
Where $y$ is the linear displacement from the central maximum at which intensity is at a minimum, $m$ is the order of diffraction, $D$ is the distance from the aperture, and $a$ is the aperture width, and $\lambda$ is the wavelength of light passing through the slit.

It follows then that as we try to reduce the feature sizes in our photomask [i.e. $a$ decreases] we will witness the impact of diffraction on the achievable resolution. The wavelength dependence is interesting because it indicates that the diffractive broadening can be minimised by using shorter wavelengths for the lithographic exposure. The standard light source for soft lithography is a mercury arc lamp. This is a broadband light source [inset Figure 4.16] with strong emission lines in the ultraviolet and additional intensity peaks in green/yellow part of the visible spectrum. Consequently, experiments were performed where the exposure wavelengths were filtered around the 365nm mercury I-line to reduce the effect of diffraction of longer wavelengths. Filtering of short wavelengths under 350nm is also advantageous due to rapidly increasing actinic absorbance below this wavelength, which leads to overexposure. Doing so enabled the finer structures in the designs to be more accurately manufactured, although imperfections in the highest resolution patterns were still apparent in some cases [Figure 4.17].

The reason the i-line filter did not fully solve the resolution problem is because wavelength is not the only parameter the diffraction effect depends on. Another important contribution is the boundary effect due to the changes in refractive index between the glass photomask, the su-8 substrate, and the interstitial air gap\textsuperscript{15}. We attempted to diminish the effect of the air gap by, for example, manually removing the raised edge-bead of photoresist created at the periphery of the wafer during spin coating, and experimenting with a layer of refractive index matched fluid between the resist [Su-8 2035 @365nm, $n\sim 1.63$] and the photomask [soda lime, $n=1.520$] during exposure e.g. glycerol [$n=1.4722$] or Nikon immersion oil Type A [$n=1.515$], however the results of these experiments were inconclusive as to whether there was measurable improvement in quality of the features in the final developed wafers, compared to using only the i-line filter. Ultimately, the most reliable way to remove the air gap during exposure is to use a vacuum-contact mask aligner, which uses a vacuum seal and mechanical pressure to create a perfect contact between the photomask and the wafer, however these are highly expensive pieces of equipment and one was not available to use in these experiments.
Figure 4.16: Diffraction and high resolution photolithography. [A] Diffraction of the exposure light on transmission through the transparent portions of the photomask results in some of the photoresist that lies under the opaque portions of the photomask to be crosslinked as well as the target areas. Feature size broadening is wavelength depended: longer wavelengths diffract at wider angles for a given slit diameter, leading to broader features in the developed mould relative to shorter wavelengths. [B] Development can reveal structures which are significantly broader than those intended by the mask. For a polychromatic light source, the longest wavelengths will dictate the resolution. Filtering the light source will reduce wavelength-dependent effects and can improve resolution. [C] In the case where slits are very close together [very fine features], the broadening can cause features to merge together [see Figure 4.15]. [Inset] Mercury arc lamp spectrum with major emission lines indicated [adapted from Chroma technical resource116].
4.12. Mature designs and testing

Having resolved the problems with the lithographic process and demonstrated the ability of Guan traps to trap beads and cells in designs that incorporate a small number of MAC chambers, more complex microfluidics were developed. These designs increased the number of MAC chambers and cell traps, and were designed to cope with higher inlet flow rates, while still maintaining favourable conditions for cell trapping at the trapping locus.

4.12.1. The spinning top chip

The spinning top chip develops from the pizza shovel design by incorporating a MAC chamber in an upstream perpendicular channel to each Guan trap in the parallel array [Figure 4.18]. The chip is designed with the idea of using an optical trap to move the hydrodynamically trapped cells from the Guan traps to the MAC chambers. The spinning top chip is thus a hybrid concentration and analysis device, though once again could still be used only for trapping and subsequent elution.
Optical trapping was first demonstrated in this device with $15\mu m$ polystyrene beads [Figure 4.19], using a chip pre-blocked with a solution of 4% PBSA. Without blocking, static/attachment forces prevented the bead from being removed from the trapping locus with the laser, so the pre-blocking of all such devices is critical for their correct operation. The achievable inlet flow rate was unknown at the time of design, but the bifurcating inlet is designed to maximise it, as in the pizza shovel chip above.
4.12.2. Assembly

It was known from experiments with the pizza shovel and other Guan-trap containing designs that plasma bonding of the PDMS superstructure to the glass coverslip would be required to maintain the integrity of the chip under flow, however the spinning top chip is the first design for which real MAC chip-style printed antibody capture spots were attempted to be incorporated into the chip during flow rate testing. This presented an extra challenge as a precise alignment is required to align the PDMS chambers up with the printed spots [See Chapter 2], yet the activated surface that enables the bonding to take place lasts only approximately 30s after the surface is exposed to the plasma, leaving only a small window in which to assemble
the chip\textsuperscript{117}. A method to perform this fast plasma bond was developed whereby the PDMS and the antibody slide were pre-aligned on the jig, brought as close as possible to contact whilst maintaining alignment of the spots and the chambers, then the entire PDMS holder was removed from the jig and placed in the plasma oven. After exposure, the PDMS holder was removed from the plasma oven and quickly replaced on the jig. In most cases the alignment required only slight adjustment thanks to the pre-alignment step, and the PDMS and antibody slide could then be brought into contact.

The downside of this procedure is that only the PDMS can be exposed to the plasma. The antibody slide cannot, because [1] removing both slide and PDMS holder from the rig will destroy the pre-alignment necessary to bond the surfaces in sufficient time, and [2] because the plasma itself will attack and possibly destroy antibody spots. SSC-betaine containing spots, which exist in a gel phase, were destroyed during a test 50 second plasma exposure. ArrayIt containing spots, which exist in a crystalline phase, were not destroyed, but may have been etched or damaged; assessing the impact of this on their ability to bind analyte was not performed, as it was decided to not be a viable strategy for obtaining the quick, precise alignment required by the MAC chambers.

It has been reported that plasma bond integrity can be improved through heating the bonded surfaces immediately after contact, and may also be improved via the addition of a weight or device to keep the surfaces held together while the bond matures\textsuperscript{118}. Accordingly, both these steps were incorporated into the plasma bonding protocol. Heating the chip to improve the bond raised a further issue: capture spot stability with temperature.

Temperatures of 60\textdegree C have been used in the literature to improve bond strength, however, biological macromolecules such as DNA and proteins, including IgGs, are susceptible to denaturation with elevated temperature, so it is reasonable to question what effect this heating is likely to have on our capture agent. Vermeer and Nord investigated the thermal stability of immunoglobulin and found that it has two thermal transition temperatures for denaturation, corresponding to the F\textsubscript{ab} and F\textsubscript{c} regions respectively\textsuperscript{119}. The F\textsubscript{ab} antigen binding domain is the most temperature sensitive and has the lower transition temperature, which was measured to be around 60-70\textdegree C under various conditions, indicating significant unfolding might be expected for the F\textsubscript{ab} regions of our capture antibody with a bonding temperature of 60\textdegree C. The transition peak is steep however, and in fact circular dichroism measurements indicated that the IgG’s secondary structure remains quite stable between 20 and 55\textdegree C, and the consequent proportion of denatured IgG is low. On this basis, a temperature of 55\textdegree C was decided on for MAC chip bonding purposes. The finalised assembly protocol was as follows: [1] PDMS was sonicated in detergent [1\% Alconox] for at least 1 hour, [2] PDMS was then rinsed copiously in isopropanol and dried with nitrogen gas, [3] printed antibody slide and PDMS piece were pre-aligned on alignment jig, [4] a brief plasma cleaning step was performed where the PDMS piece [attached to the alignment rig holder] was exposed to the plasma for 50s, [5] a relaxation
step of ~30s to allow cleaned, activated PDMS surface to relax, [6] 50s plasma exposure, [7] fast final alignment and bond, [8] the bonded chip was placed in a dish, and sandwiched between dust-free cloth, with ~1kg weight on top and placed in oven at 55°C for 45 minutes. Even with this protocol however, device failure rate was high, at around 60-70%.

4.12.3. Flow rate testing

Flow rate investigations with solutions of 4% BSA indicated that the spinning top chip could reproducibly withstand flow rates up to 100μl/min without leaking. Additionally, trapping of 15μm beads in the Guan traps was demonstrated at flow rates up to 30μl/min. For cells, 30μl/minute was shown to be too fast to effect trapping. At this flow rate, cells deformed and squeezed through the trapping locus, however, at the lower rate of 10μl/min, cells were stably held in the traps without issue. Trap clogging [Figure 4.20] was observed in some instances, however it was unclear whether this was due to multiple single cells arriving at the trap separately and building up the blockage sequentially, or whether clumps of cells entered the trapping lane together, then became lodged in the trap. Cell clumping is frequently observed with MCF7 cells in the MAC chip so there is a strong possibility this was due to the latter. Our observation of multiple occupancy in the Guan traps under some conditions suggests that the analytical criterion that underpins the trapping concept is only suitable for cells which have a narrow size distribution.

Figure 4.20: Trap clogging observed with MCF-7 cells.
4.12.4. Device testing with CTC-microtubes

Although we were unable to test any of the devices with the output of the spiral biochip, experiments were undertaken where the elute from another CTC isolation platform, the antibody functionalised microtubes of Hughes et al\textsuperscript{120}, was passed through the spinning top, to see if the device was capable of capturing MCF7 cells that had been spiked into a blood sample. Although prior validation experiments with these tubes had shown them to have recovery rates of \(~80\%\) for \(~500\) cells spiked into \(7.5\text{ml}\) blood, it was found that the purity of the recovered cell fraction was inadequate when the eluted volume was connected directly to the spinning top. An inhomogeneous mixture of residual blood components caused clogging, as well as possibly fragments from the interior matrix which supports the antibody capture surface on the interior of the MicroRenathane tubing [Figure 4.21].

Figure 4.21: [A-I] Clogging observed in spinning top chip with CTC-enriched output of the CTC capture tubing.
4.12.5. Spinning top with debris filter

To address the problem of trap clogging with cell clusters, or inhomogeneous gunk from blood samples, variations of the spinning top chip were designed that incorporated microfluidic size-exclusion filters upstream of the trapping regions. Three such filters were designed and tested [Figure 4.22]. These filters, designed to capture large debris, also had to be robust to clogging, hence the filter stage in each design is large by microfluidic standards. All filters were crude micropost designs, similar to Xu et al or Di Carlo, although specifically not hydrodynamically optimised, as optimisation requires knowledge of object size, and can only be done for single cells, whereas the gunk which we desired to filter out was of unknown size [albeit larger than the 27.5×25μm cross section of the Guan trapping lanes], and of possibly highly amorphous composition. Filters with critical dimensions of 40μm and 30μm were used. Although 40μm is sufficiently large to let some outsize particles through to the spinning top, it was considered suitable to filter very large debris or agglomerations of cells, whilst also leading to a lesser hydraulic resistance than a smaller filter would. This latter point is important as the addition of the filter increases the overall resistance in the chip, requiring a higher pressure to pass fluid through it, and the higher this pressure is the stronger the requirements on the integrity of the chip and the plasma bond holding it together.
The filter stage in this device consists of two parallel regions of square 40μm microposts, each column offset from the next by 40μm in the x and y directions. To prevent clogging, the filter was thought to need a large cross sectional area so that the flowlines from the inlet could disperse over as many microposts as possible, and so that debris did not over-accumulate in any single area, preventing the passage of smaller cells into the downstream traps. Two parallel filters were chosen over one large one in this case as there was concern about how effective fluid flow would be in such a wide device, as well as the need to incorporate more support posts into the design, as wide aspect ratio channels can lead to warping of PDMS, which in our case could cause shear stresses on cells as they pass through, potentially damaging them, or leading to more clogging with larger debris. [Middle panels] In this design a single filter with three stages of square posts that have progressively smaller apertures [50μm, 40μm and 30μm] is used. It was thought that a progressively staged filter such as this would further prevent clogging by separating large debris of different sizes along the axis of the flow, rather than all sizes being allowed to accumulate in the same section. [Bottom panels] It was acknowledged that a drawback of the first and second filter design was that, due to the parabolic velocity profile of a fluid under laminar flow, much more debris would pass through the centre of the filter than the edges, again possibly leading to a clog point at the very centre of the design. To tackle this an angular filter was designed whereby a 40μm circular post filter region was rotated by 69° in the x-y plane and positioned such that the entering streamlines would hopefully disperse more evenly along the width of the trap.
To further reduce the amount of debris, a white blood cell depletion step was added to the microtube protocol, whereby an additional microtube was used, functionalised with anti-CD45 and anti-E-selectin antibodies. Testing with the three-filter spinning top chips showed that the addition of the filters could reduce the amount of gunk that was entering the cell-trapping and analysis region. Elution was performed at 10μl/min after drawing Accutase detachment solution into the microtubes. Trapping of MCF7 cells that had come through the blood-spiking and microtube enrichment process was observed in a handful of traps [Figure 4.23]. The combination of WBC-depletion and the upstream filter reduced the presence of the inhomogeneous gunk in the channels and trapping locations, though some large debris still persisted. The main problem with the filter designs was that after several minutes of continuous flow the device would delaminate and spring a leak, meaning it could no longer be used. This was ascribable to the presence of the filters, as the devices failed under the same test conditions as had been successfully demonstrated for the device without filter. The filter enlarged the surface areas of the devices, increasing the difficulty of creating a strong, even bond holding the devices together.
Figure 4.23: Trapping results with the 40μm square array spinning top. [a-b] MCF7 cells singly trapped in Guan trap. [c-e] debris stuck in traps. [f] optical trapping caused an ignition event at the PDMS-fluid boundary, destroying much of the PDMS structure close to the ignition site, yet fortuitously [g] blasting the MCF7 cell that was being attempted to be trapped into the analysis chamber. [h-i] Delamination and failure of the device.

4.13. Discussion

In this chapter, we have discussed the various strategies that were developed to attempt the analysis of CTCs in a MAC chip style environment. A potentially useful development of the MAC chip has been described which incorporates on-chip hydrodynamic trapping to fish out cells from a dilute solution, with a high theoretical trapping probability for all cells above a certain diameter.

The Guan trapping technique has been experimentally demonstrated for real mammalian cancer cells [MCF7] with diameters and a size distribution larger than the white blood cells used in the original work, and thus the work here represents an adaptation of the Guan method to an experimental system which more closely represents circulating tumour cells. We have successfully demonstrated the use of Guan-trapping in combination with optical trapping to deliver microbeads into a MAC chamber, although we were unable to demonstrate this for MCF7 cells, due to the enhanced binding of the cell surface to the PDMS trapping structures. It should be noted that we have observed that MCF7 cells are particularly prone to adhesion during optical trapping in standard MAC chip experiments, compared to cell lines such as MDA-MB-231 or dissociated xenograft tumour material [as in Chapter 5], and this should therefore not entirely rule this out as a viable strategy. The difficulty of optically trapping the MCF7 cells in a narrow channel led directly to the ignition event which destroyed the chip in Figure 4.23. Again though, this edge effect has been observed in standard MAC chip channels \(w=35\mu m, 50\mu m\), and seems to be a general risk of optical trapping in a restricted geometry, so this also may not preclude the use of the Guan traps in combination with the MAC chip. We have performed trapping of MCF7 cells in the Guan traps and then released them by raising the flow rate to 30μl/min, and it therefore is possible that the Guan traps could be used in isolation to concentrate a solution of cells and then release them into a reduced volume.

As we have described above, these microfluidic devices were designed to operate in series with the spiral biochip, however our inability to reproduce a working spiral biochip either internally or through outsourcing, made a lot of these devices here sort-of orphan technologies once the spiral biochip was finally abandoned. The chips were designed to be used with a pure sample, and as experiments with the cells that had been through microtube process showed, with a dirty sample they were far from optimal. Although the volumes of fluid processed, and the speed at which they were processed, were not sufficient to allow the spiral biochip to directly couple to the spinning top or its variants [even using a manifold to split the flow], we
have nevertheless observed trapping of a small number of MCF7 cells isolated from spiked blood, indicating that the strategy was not entirely misplaced.

In 2016 a Nature Protocols paper appeared indicating that the spiral biochip could in fact be successfully used with a centrifugation step to concentrate cells for downstream analysis\textsuperscript{101}, though the practicalities of such a step were not evinced. This calls into doubt the decision to avoid centrifugation by designing a deterministic trapping device to concentrate the dilute CTCs at the output of the spiral biochip. Nevertheless, this information was not available at the time, and the microfluidic concentrator work was embarked upon as it was thought to be necessary step for introducing CTCs to the MAC chip. Our strategy has at least allowed an exploration of the experimental space at the confluence of hydrodynamic cell trapping and single cell proteomics.

The limits of the soft lithography process were also encountered in the Chemistry Dept. cleanroom, meaning that for this work to be extended, or for any microfluidics work requiring comparable or finer structural resolution, searching for an alternative manufacturing facility would be a prudent early step.

The central challenge to overcome in any future work to incorporate the MAC chip with CTC isolation or hydrodynamic cell capture technology into a single-stage device, is the requirement of a strong bond to hold the antibody capture slide to the PDMS channels of the device. In this work, we had difficulty performing this reproducibly, for which there are several possible reasons. Firstly, the process gas used to create the activation plasma was not controlled. The composition of the gas was room air, which is predominantly nitrogen and oxygen, but the exact proportion of components can fluctuate with environmental conditions [e.g. ambient humidity levels, dust] and this may affect the quality of the surface activation. Adapting the plasma oven to work with a process gas e.g. O\textsubscript{2} or Argon, may allow superior activation quality and reproducibility. Secondly the plasma bond was performed on an open bench under standard lab conditions, which means there was the possibility of fouling due to dust or other microscopic contaminants [for instance the microarray dehumidifier fan has been observed to occasionally blow contaminants onto glass coverslips during operation]. Performing this step, as well as microarray slide printing, in a cleanroom environment would decrease the likelihood of surface contaminants leading to bond-failure. The manual device alignment leads to precious delays in the time critical stage after plasma activation and a concordant decrease in the final bond strength. Increasing the stability of the alignment jig would allow this to be performed faster. Alternatively, it may be possible to immobilise the capture agent in the MAC chamber through a flow chemistry protocol, in a manner akin to the antibody barcoding procedure of the Heath group\textsuperscript{121}. This would allow the PDMS channels and the coverslip to be bonded prior to deposition of the capture surface and would thus do away with the requirement of the alignment of the microarrayed spots to the PDMS chambers. PEG-neutravidin surfaces are already routinely used to prevent non-specific binding of secondary antibodies, so
it is possible a flow-protocol using a biotinylated primary antibody could be used to create the capture surface in a pre-assembled CTC isolation and analysis chip.

In summary, whilst the MAC chip is a suitable platform for the analysis of rare clinically-derived cells such as CTCs if they can be delivered into the chip itself, the work presented here was ultimately unable to deliver on this ambition. Nevertheless, these investigations have been a worthwhile exploration of the experimental space concerning CTC isolation and single molecule protein analysis, and the work will serve to inform any future attempts at CTC analysis with the MAC chip.
Chapter 5. Translational single cell proteomics: multiplexed single cell protein expression analysis in solid tumours using the MAC chip

QUINCE:
Bless thee, Bottom, bless thee! Thou art translated.

– William Shakespeare
A Midsummer Night’s Dream, Act III. Scene I.

The work in this chapter first appeared in the paper *Multiplexed single cell protein expression analysis in solid tumours using a miniaturised microfluidic assay* [Magness et al, Convergent Science Physical Oncology, 2017]122. Figures and text are reproduced with the express permission of IOP Publishing Limited.

5.1. Chapter introduction

The aim of this thesis has been to develop the MAC chip such that it is able to be used to study precious clinical material, particularly circulating tumour cells. Though we were unable to achieve this with circulating tumour cells, single cell proteomics is still a field in relative infancy and as such there is a potential wealth of untapped information that can be gleaned from biological material whose extraction is less of a technical challenge. Accordingly,
a collaboration was set up with Dr Marco Gerlinger and the translational oncogenomics group at the Institute of Cancer Research, London, to study single cell protein copy number heterogeneity in quasi-clinical material. This material consisted of dissociated tumour cells extracted from mouse xenografts derived from colorectal cancer patients.

The group has previously developed a MAC chip protein assay for the tumour suppressor p53 and has tested and validated it on a range of cell lines\textsuperscript{43,58,123}. Fellow Single Cell Proteomics group member Jim Squires developed an extension of this assay allowing the simultaneous measurement of p53 and phosphorylated p53 [at serine-15] through the use of multiple secondary antibodies labelled with spectrally distinct fluorophores. See Chapter 2 for an in-depth discussion of the MAC chip platform. The p53 assay is quantitative and robust\textsuperscript{45}, which allows it to provide a window into protein expression and modification heterogeneity both within and between cell types\textsuperscript{1}.

In this chapter, we provide feasibility data and describe the workflow for the single cell proteomic and phosphoproteomic analysis of tumour samples with a size similar to that of typical cancer core biopsies. This is a proof-of-principle which shows that translational single cell proteomics may be performed effectively on clinically-derived material with the MAC chip, opening the way for routine monitoring of patient protein phenotypes at the single cell level. The processing we will outline involves tumour disaggregation into a single cell solution, cell sorting with optical tweezers to select only live cells of a chosen type then transferring the cells to MAC chip analysis chambers for lysis and the subsequent detection of single molecule binding events.

5.2. Methods

Single cell experiments were performed as described in Chapter 2. All experiments in this chapter were performed in 4.5 nl standard candle MAC chips, as this is the usual chip geometry used with the p53 assay. The internal volume of the main reservoir channel on this device is approximately 3.3 µl and hence this is the approximate lowest volume that may be processed by the chip and still efficiently filled using the optical trap. Figure 5.1 shows example multiplex single molecule data from a p53 + p53-S15 xenograft experiment, where a single anti-p53 antibody spot has been imaged with two secondary antibodies, one 647-labelled for total p53 detection, the other 488-labelled for p53-S15 detection.
Figure 5.1: Example TIRF readout of single molecule data for a multiplex p53 MAC chip xenograft experiment. A single p53 antibody spot viewed under 488 illumination [panels A & B] and 647 nm illumination [panels C & D]. There is a time delay of a few minutes between 488 and 647 channel acquisitions, though with the assay at chemical equilibrium the relative extent of binding between the phospho-p53 and p53 channels is unaffected. The white dotted line approximates the perimeter of the printed antibody spot.

5.2.1. Xenografts and processing

Patient-derived tumour xenografts [PDXs], where human tumour tissue is extracted from a patient and implanted under the skin, or in the organ type in which the tumour originated, of immunodeficient mice, are becoming a widely adopted model system in cancer research [Figure 5.2]. PDXs offer a series of advantages over other model cancer systems such as cancer cell lines and genetically engineered mice [GEMs]. For instance, PDX models, consist of actual human tissue, and accordingly display the same population complexity displayed in the human tumour. As Lai et al summarise, it is known that PDX models of a broad range of cancers, including chronic lymphocytic leukaemia, large B cell lymphoma, pancreatic cancer, colorectal cancer, gastric cancer, high-grade serous carcinoma, and intrahepatic cholangiocarcinoma are
biologically stable and accurately reflect the patients’ tumours with regard to histopathology, gene expression, genetic mutations, inflammation, and therapeutic response.\textsuperscript{124} The application of PDXs ranges from the study of the basic biology of cancer, tumorigenesis and metastasis, to important pre-clinical cancer research in terms of drug screening, biomarker identification and ultimately the guidance of therapy. A human tumour expanded into multiple mouse hosts can be treated simultaneously with multiple combinatorial drug interventions, the response to which can be monitored, thus allowing the physician to accurately predict patient response to therapeutic intervention. PDXs are superior to cancer cell lines for preclinical drug development because cancer cells lines do not accurately recapitulate the conditions inside a malignant tumour\textsuperscript{125}, and are also subject to evolutionary pressures associated with the artificial nature of the culturing environment, which can alter their genetic makeup and may take their biology further away from that of the system they are supposed to represent\textsuperscript{126}. Compared to GEMs, PDX models are also much quicker to set up, taking only several weeks to establish to the point that drug efficacy trials may take place, compared to up to a year for the equivalent stage in GEMs\textsuperscript{127}.

![Figure 5.2: Overview of xenograft/xenotransplantation procedure.](image)

Xenografts were established in the Tumour Profiling Unit at the Institute of Cancer Research from biopsies acquired through the ProspectC and ProspectR colorectal cancer trials at

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the Royal Marsden Hospital [London, UK]. Each trial had been approved by an ethics com-
mittee and all patients provided written informed consent before trial participation and biopsy.
All tumours were genotyped for TP53 mutations. Mutations are defined according to the main
Ensembl transcript for TP53, ENST00000269305.4. A small piece from each biopsy was
grafted subcutaneously or under the kidney capsule of CD1 nude female mice. Xenografts
included in these experiments were further passaged in the flanks of mice between 1 and 4
times before use. The patient-derived colorectal cancer xenografts [PDXs] were then harvested
and dissociated into single cell suspensions in a gentleMACS Octo dissociator using the human
Tumour Dissociation Kit [both Miltenyi Biotec] according to the manufacturer’s instructions.
Mouse cells were depleted from the cell suspension with the Miltenyi Mouse Cell Depletion
kit which contains a mixture of antibodies recognising mouse cells, to remove mouse material
from a homogeneous sample via immunomagnetic labelling. Cell viability was checked after
the mouse cell depletion with trypan blue staining on a Countess automated cell counter [Invi-
trogen]. The viability of the cancer cells in the single solution varied over a wide range
from 1% to 78%. Dissociated cells were routinely resuspended at a concentration of $2 \times 10^6$
total cells per ml in DMEM/F12 media +10% FBS for transport to the analysis laboratory,
and kept on ice until being introduced into the MAC chip.

In order to distinguish viable from non-viable cells during MAC chip loading, the sample
was incubated with 0.04% trypan blue [Life Technologies] in media, or spun down and resus-
pended in PBS with LIVE/DEAD® Fixable Violet Dead Cell Stain Kit [ThermoFisher Scien-
tific], on ice for 30 minutes prior to loading of the sample into the MAC chip. Incubation
in PBS must take place for the LIVE/DEAD stain as the presence of extraneous proteins
inhibits the efficiency of the dye coupling reaction. After incubation in PBS, cells were resus-
pended in 4% BSA in PBS for introduction to the MAC chip. Initial experiments were per-
formed with trypan blue, however it has previously been shown that this may increase cellular
p53 levels in epithelial cells and we subsequently changed to the LIVE/DEAD stain. The
final two PDX derived cell suspensions were also incubated with Alexa-555 labelled anti-Ep-
CAM antibody [clone VU1D9, New England Biolabs UK] to test the feasibility of cell surface
marker specific cell selection within the MAC-chip. The specific wavelength of the labelling
dyes selected will depend on the exact set up of the operator’s system; here we have chosen
dyes compatible with the excitation and emission bands of the LF405/488/532/635-A-000
filter [Semrock] and which do not lead to crosstalk with the fluorescent 488 and 647 detection
fluorophores.

On average around 40 µg of solid tumour was processed per microfluidic chip, an esti-
mated value arrived at by multiplying the processed volume of cell suspension [approx. 10µl],
the cell seeding density and an approximate cell mass of 2.29 ng.
5.2.2. On-Chip Cell Selection and Sorting

A disaggregated tumour sample can be highly heterogeneous in terms of the output material, and may contain erythrocytes, dead cells and other debris that are unwanted in the final single cell analysis. To select only the cells of interest we used the custom-built, manually operated optical trap to distinguish the target cells through the visual fluorescent cues established during earlier labelling steps. Experience shows LIVE/DEAD incubation makes discrimination of viable cells easier than with trypan blue.

Here we labelled for live cells, and also those that expressed the cell surface marker protein EpCAM [Figure 5.3 & Figure 5.4], a widely used maker for cells of epithelial origin, but the selection marker depends on the ultimate goal of the experiment, and can be chosen accordingly [e.g. anti-vimentin antibodies for mesenchymal or EMT-like subpopulations]. Incorporating more markers into this discrimination step would potentially allow complex subpopulations to be identified.

The cell loading process can be completed within ~30-45 minutes, depending on the sample. The total time from cell loading to completion of cell lysis was approximately 1-1.5 hrs. Factoring in the time for the tumour dissociation and mouse cell depletion steps [1.5 hrs] and the subsequent fluorescent labelling step, the total time from the taking of the biopsy to single cell protein analysis could be as low as three hours.

Figure 5.3: [left] Tile image showing the composition of a disaggregated sample as viewed in the MAC chip without staining; [right] using a fluorescent viability stain to select only live cells. [Arrows] Dead and dying cells accumulate fluorescent dye through their compromised membranes allowing live cells to be confidently identified. [Dashed rectangle] A repeated artefact due to the presence of dirt somewhere in the brightfield microscope optics.
5.2.3. Target analytes

p53 and phospho-p53 [serine-15] levels are relevant in a variety of human cancers, including colorectal cancers\textsuperscript{130}. Mutations of TP53 are commonly observed in colorectal cancer, with five “hot-spot” mutations at codons 175, 245, 248, 273 and 282 accounting for over 40\% of cases\textsuperscript{131}.

p53 function is modulated significantly through post-translational modification and serine 15 phosphorylation is considered to be a key modification in the activation of p53, which acts through a variety of modes to activate and stabilise p53, as well as masking the nuclear export signal on p53, preventing its export out of the nucleus\textsuperscript{132}.

5.3. Results

Dissociated cancer cell from nine colorectal PDXs were subjected to single cell protein analysis: 5 from freshly culled mice while the remainder were taken from frozen stocks of disaggregated cell suspension obtained during previous rounds of harvesting and thawed immediately prior to the MAC chip experiment. All tumours had been genotyped for TP53 mutations before samples were used for any experiments.

Multiple samples from the same PDX lines were examined in three cases. PDX-98-P4 and PDX130-P2 were repeated using thawed material of the originally tested xenograft. These samples are indicated with the addition of an asterisk as PDX-98-P4* and PDX130-P2*. A single PDX, PDX97, was repeated using fresh material from a different mouse to the one originally tested, though the source of the xenograft was the same patient and the xenograft had undergone the same number of passages through mouse hosts. We denote these PDX97-
P2 and PDX97-P2** respectively. These repeat experiments demonstrate the robustness of the methodology in analysing different sources of material. A summary of the PDX information is provided in Table 5.1. Mean human cell viability in the dissociated samples was 38% [Figure 5.5].

Table 5.1: Summary of PDXs used in experiments. A single asterix* indicates a PDX experiment using frozen material; a double asterix** indicates a fresh repeat of a xenograft with the same PDX identifier.

<table>
<thead>
<tr>
<th>Sample</th>
<th>P53 mutation status</th>
<th>Xenograft source</th>
<th>Assay</th>
<th>Selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDX97-P2</td>
<td>—</td>
<td>Fresh</td>
<td>p53</td>
<td>None</td>
</tr>
<tr>
<td>PDX98-P4</td>
<td>—</td>
<td>Frozen</td>
<td>p53</td>
<td>Trypan blue</td>
</tr>
<tr>
<td>PDX136-P2**</td>
<td>—</td>
<td>Fresh</td>
<td>p53</td>
<td>Trypan blue</td>
</tr>
<tr>
<td>PDX156-P2**</td>
<td>—</td>
<td>Fresh</td>
<td>p53</td>
<td>Trypan blue</td>
</tr>
<tr>
<td>PDX73-P2**</td>
<td>—</td>
<td>Frozen</td>
<td>p53</td>
<td>Trypan blue</td>
</tr>
<tr>
<td>PDX106-P2*</td>
<td>—</td>
<td>Frozen</td>
<td>p53 + phospho-p53</td>
<td>Trypan blue</td>
</tr>
<tr>
<td>PDX97-P2**</td>
<td>—</td>
<td>Frozen</td>
<td>p53</td>
<td>Trypan blue</td>
</tr>
<tr>
<td>PDX136-P2**</td>
<td>—</td>
<td>Frozen</td>
<td>p53 + phospho-p53</td>
<td>Trypan blue</td>
</tr>
</tbody>
</table>

Table 5.1: Summary of PDXs used in experiments. A single asterix* indicates a PDX experiment using frozen material; a double asterix** indicates a fresh repeat of a xenograft with the same PDX identifier.
Single cells from all 9 samples were analysed for p53 protein expression and of these a subset of 4 were also subjected to the additional phosphorylated p53 [S15] measurement. Two 55-chamber Standard Candle MAC chips were used per sample, into which cells were loaded and lysed sequentially. The only exception was PDX130-P2, which was loaded into 3 Standard Candle MAC chips.

![Graph showing recovered human cell viability from dissociated tumours.](image)

**Figure 5.5:** Recovered human cell viability from dissociated tumours.

### 5.3.1. p53 measurements

Single cell p53 expression varied markedly between samples [Figure 5.6]. The four dissociated xenografts measured in the MAC chip that did express p53 derived from two patients, and xenograft material from each of these patients was tested twice: once as a freshly harvested tumour and once as thawed cryopreserved cells [Figure 5.7]. All four samples showed a similar distribution of expression, with the majority of cells showing low or undetectable ‘basal level’ of expression, and a few outlier cells expressing comparatively larger quantities of p53. The repeated samples PDX-98-P4 and PDX130-P2 showed similar distributions between samples, though there was heterogeneity in the highly expressing cells. In total outlier cells represented between 1.7% and 5.8% of the total single cells analysed [Table 5.2]. Relative levels of p53 expression in outlier cells can be several orders of magnitude higher than the mean level of expression in the basal distribution [Figure 5.8]. In the most extreme case, an outlier cell produced a p53 response in the assay some $493 \times$ higher than the mean of the basal population. Even within these 4 expressing PDXs there were high proportions of cells [8, 54, 62 & 94%] in which p53 could not be measured. It is likely that these cells express a low level of p53 which is below the detection limit of the MAC chip assay.
Figure 5.6: Heterogeneous single cell p53 signal in human colorectal cancer mouse xenograft cells. \( n \) = total number of cells analysed in experiment. p53 expression is instead given as a background-corrected value corresponding to the increase in single molecule binding at the antibody spot after lysis of the cells.

Figure 5.7: p53 expression distribution in four PDX samples that contained cells with high p53 expression levels. Note the different scales of the x and y-axes. [Inset graphs] Zoom of low expressing region, below 500 counted proteins.
Figure 5.8: Outlier cells [red vertical bars] express many multiples of the mean basal expression level [black vertical bars]

Outlier cells from both freshly harvested xenograft samples expressed more p53 than their frozen counterparts, with the mean expression of the frozen outliers reducing to 20% and 34% of PDX98-P4 and PDX130-P2 respectively. Trypan blue was used as the viability indicator for the earlier fresh experiments and as has already been noted, this can generate a short term increase in p53 expression levels. Alternatively, the freeze-thaw cycle may affect p53 levels and this will require further study. Additional complexity is added to interpretation of the results when one considers the basal cells: in PDX98-P4 the mean basal expression dropped for the frozen material, whereas for PDX130-P2 it increased.
Table 5.2: Numerical summary of p53 as quantified by the MAC chip assay in PDX samples. Clearly the presence of a large number of samples below the sensitivity of the assay skews the basal mean, and is meant purely to illustrate the extreme nature of the outlier cells.

<table>
<thead>
<tr>
<th>Basal cells</th>
<th>Outlier cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells</td>
<td>Mean</td>
</tr>
<tr>
<td>PDX98-P4</td>
<td>71</td>
</tr>
<tr>
<td>PDX98-P4*</td>
<td>49</td>
</tr>
<tr>
<td>PDX130-P2</td>
<td>132</td>
</tr>
<tr>
<td>PDX130-P2*</td>
<td>59</td>
</tr>
</tbody>
</table>

5 PDX samples derived from 4 separate patients showed no detectable p53. To confirm the assay was functional in these cases a positive p53 control was added to the chip via the secondary inlet [Figure 5.9]. For PDX156-P1 this was likely due to the truncating TP53 mutation at R342 which prevents formation of the full length protein. This truncation leads to the loss of one of the antibody binding epitopes required for the sandwich assay. The p53 assay is an antibody sandwich system which requires the anti-p53 antibodies’ respective N- and C-terminal target epitopes to be available to bind in order for a signal to be generated at the surface of the coverslip. As the necessary C-terminal epitope is not present in p53 with a R342 truncation, the assay will fail, and no protein can be measured even if the variant is highly expressed. We have observed similar null signals for the p53 MAC chip assay with p53-knockout cells of the HCT116 colorectal carcinoma cell line [data not shown]. However, lack of expression in the remaining three cases could not be explained by p53 mutations alone. For the only PDX which was analysed on two different occasions [PDX97-P2; each time as freshly harvested tumour material] no p53 signal was measured either time the assay was performed.

It is interesting to note that PDX73-P2 harboured the same R175H p53 mutation as PDX130-P2 and yet had unmeasurable levels of p53; a confirmation that protein expression assays can reveal information which cannot be assessed by sequencing the gene. R175H and other missense mutations typically occur in the DNA binding domain of p53 and therefore prevent p53 from binding to its target genes. However, it has been shown that specific missense mutations, including R175H, have additional gain of function effects that can induce resistance to chemotherapy agents and result in highly metastatic cancer.\(^{133}\) R175H mutants usually overexpress p53, though the mutant tetramers are inhibited in their binding to the target genes.\(^{134}\) This documented overexpression in R175H mutants is a tempting explanation for the comparatively high basal level of PDX130 samples, however the unmeasurable levels of p53 in PDX73 leave this an open question at this stage. Mutant p53 tetramers can still interact with other proteins via the transactivation domains, and it has been shown that these
interactions may inhibit other tumour suppressor proteins, leading to increased resistance to chemotherapy.\textsuperscript{135,136}

Figure 5.9: Introduction of positive p53 control into control lanes of the Standard Candle chip confirms the MAC chip assay is operational when no p53 is measured in the sample. p53 positive MDA-MD-231 lysate at 1mg/ml total protein [\textasciitilde 8 cells per nl] is introduced to the chip via the secondary inlet after the single cell experiment is performed. This is not performed concurrently with the main experiment in order to avoid the possibility of diffusion of the target analyte from the main reservoir into the single cell analysis chambers and artificially increasing the signal measured from the sample cells. Single molecule counts are registered prior to lysis due to non-specific binding of the fluorescent secondary antibody to the primary antibody and surrounding functionalised coverslip.

5.3.2. phospho-p53 [S15] measurements

Multiplexed measurements were conducted on four PDX samples derived from four distinct trial patients: three from archived frozen stocks [PDX73-P2, PDX98-P4* & PDX130-P2*], and the other a freshly harvested tumour [PDX97-P2**]. Similar to total p53 quantification, samples varied in the quantity of phosphorylated p53 molecules that were detected [Figure 5.10]. Firm conclusions cannot be made due to the small number of samples tested, but we note with caution that it may already be possible to identify three distinct patterns within the analyte distributions – that of p53/phospho-p53 [+/+]; [+/−], and [−/−]. Interestingly phospho-p53 levels in PDX130-P2* display a long-tailed distribution as observed for p53 [Figure 5.11A]. Figure 5.11B shows the trend of phospho-p53[S15] versus p53 in single cells from PDX130-P2*. The lack of overlap in phosphorylation levels between the two MAC chips may be a consequence of the significant time delay between lysis in the two assays.
Note that there is no inconsistency in measuring phospho-p53 in the assay when no p53 is registered as the signal is not calibrated for absolute quantification; what it reflects is that the anti-pS15-p53 secondary antibody is of higher affinity than anti-p53 secondary, and also has a lower rate of non-specific binding to the printed primary p53 antibody.

Figure 5.10: p53 and phospho-p53 responses of the PDX samples which underwent multiplexed MAC chip analysis. The emergence of categorical response types may be visible in multiplexed data, p53/phospho-p53 [−/−] as in [A] and [B]; [+/−] as in [C]; and [+/+] as in [D].

Figure 5.11: [A] Phosphorylated p53 distribution of PDX130-P2* and [B] relationship between phospho-p53 and p53 in single cells. Each data point represents a single cell from PDX130-P2* lysed in the MAC chip. Red data points = MAC chip 1, Blue data = MAC chip 2, cells lysed approximately 4-5 hrs after MAC chip 1.
5.4. Discussion

In this chapter, using patient-derived colorectal cancer xenografts, we have demonstrated a practicable workflow for single cell proteomics in clinically relevant samples and thus a potential translational route for single cell proteomics into medical diagnostics. Using the MAC chip we measured the expression of the tumour suppressor protein p53 and of its post-translationally modified form phosphorylated at serine-15. Aberrant expression of these has commonly been found in colorectal cancers and has been investigated before for prognostic significance. Our results show that the MAC technology is viable for quantitatively assessing protein expression and phosphorylation at the single cell level in microscopic amounts of clinically relevant tumour material. Thus, the MAC chip could become a useful tool in therapeutic-associated single cell protein analysis. We also found dramatic variability of p53 and phosphorylated p53 quantities between individual cancer cells from the same sample, demonstrating the power of this single cell technology to study functional intratumour heterogeneity.

The heterogeneity we have observed within and across samples is notable, though the data itself is early stage, and it is not yet possible to develop a firm biological narrative. A more comprehensive investigation of this is required in order to fully describe the phenomenon and assess its implications. Demonstrating these protocols and workflow on xenograft tissue suggests that our method can also be directly applied for single cell analysis of primary tumour material, without a need to expand the tissue in a host organism.

Basal expression in several PDX samples was below the limit of what was quantifiable by the assay. This was surprising as for the p53 and phospho-p53 assay a 4.5 nl chamber is sufficient to allow the reliable detection and quantification of p53 in both BE [highly expressing] and MCF-7 [low-expressing] cell lines\(^{58}\). At ~1–2×10^2 molecules the limits of detection in these experiments were in line with previously published values for the p53 assay, and were constrained by the variation in non-specific binding of the secondary antibody\(^{43,45}\). For low-abundance proteins the dimensions of the chamber may be reduced so as to concentrate the analyte and increase the favourability of binding at the antibody capture spot\(^{58}\). Migrating to a MAC chip with smaller chambers may therefore confer a gain in assay sensitivity that allows discrimination of the p53 expression in the low level basal populations.

The PDX experiments above were all performed using multiple MAC chips for each sample. This makes the protocol slower, as switching chips takes time, and more prone to error during implementation, as the process of removing the pump from the MAC chip, and the MAC chip from the TIRF microscope can be difficult. This can easily be rectified by increasing the number of analysis chambers in the microfluidic from the current 55, to 110, or even more. The number of chambers on chip is essentially arbitrary and only limited by the availability of space on the coverslip and the amount of time the experimenter can spend manually optically trapping the cells in the chambers. For the purposes of samples such as those tested here,
considering the low incidence of the outlier cells, the more chambers on a single chip that can be utilised, the better.

The limiting step in the current protocol for microscopic sampling of a solid tumour is the dissociation procedure, which according to the manufacturer is recommended for tumours down to 0.01g [~10 mm³]. The actual tumour volume processed on a single MAC chip is around 200x smaller than this, and so in the future therefore it may be desirable to develop a microfluidic tumour cell dissociator capable of working in series with the MAC chip and efficiently handling and dissociating tumour volumes less than those capable by the current state of the art in commercial kits. We note that recently others have already begun to prototype such devices\textsuperscript{137}.

A decrease in the expression level of p53 in the outlier cells in the cryopreserved samples was noted above. This may be due to inherent differences between fresh and cryopreserved material, but it should be noted that other physical factors could account for the discrepancy in the measurements, for instance variations in the mean antibody spot area between chips, which may vary depending on microarray printing conditions such as relative humidity, pin head fouling, surface hydrophobicity and contact angle, as well as MAC-chip specific issues with the printed spot such as damage or partial destruction during alignment of the microarray slide with the PDMS channels of the microfluidic. These factors would not account for the variation in the basal cells, which decreased for one sample and increased for the other. If cryopreservation does lead to analyte copy number variations, then a standard protocol would address this in clinical usage, e.g. using only fresh biopsy material for analysis. Nevertheless, we have demonstrated that single cell protein analysis can be successfully performed on samples that have been cryopreserved.

It has been proposed previously that p53 expression can be used as a biomarker for prognostic outcomes in cancer patients, though studies are conflicting on its usefulness\textsuperscript{138}. Although overexpression is commonly observed in 60-80\% of colorectal carcinoma, few studies have shown any statistical significance between p53 overexpression and survival outcomes\textsuperscript{139}. Importantly, bulk quantification assays applied to cell lines or cancer samples lack the ability to discriminate whether protein expression is altered across all cells or whether this is driven by heterogeneous changes affecting only a fraction of cells in a sample. Single cell analysis in a MAC chip confers several advantages that may get around these shortcomings. Firstly, the sub-populations within the cell population can be selected using optical trapping based upon cell surface biomarkers, such as EpCAM, as demonstrated in this work. Secondly, an activated form of p53, pS15-p53 can be detected and its ratio to total p53 levels quantified. Thirdly, single molecule-sensitive readout may provide the sensitivity required to differentiate between clinically relevant p53 responses. Finally, the MAC chip can work with only a few hundreds of cells, and so can be applied to rare samples in a way that more materially demanding methods cannot.
By detecting single cell pS15-p53 we have identified an activated subset of p53 that is key to its cellular function, and as such the MAC chip can be considered an activity assay. The p53 activation pathway is exceptionally complex, and fully accounting for the expression levels observed in the samples analysed here is impossible given the limited size of the cohort. However, we note that an individual cell that expresses high levels of p53 without any S15 phosphorylation, may have a completely different phenotypic effect to a cell that expresses low levels of p53 but which is highly phosphorylated, and therefore highly active\textsuperscript{131,135,140–147}. Additionally, phosphorylation in mutated isoforms can have different functional implications to that of activated wild-type protein, and there is already evidence to support the idea of differential gain of function with respect to mutant p53 phosphorylation\textsuperscript{148}. This may be something the multiplexed MAC chip can directly measure in terms of relative or absolute copy number ratios, and as such the technology may allow elucidation of the causes of phenotypic effects seen in different cancer samples. Implementing this methodology into a clinical trial of greater scope has the potential to identify clinically meaningful patient subtypes with respect to p53 where other methods have not.

We have demonstrated the work here on PDX samples, but the protocol can be applied to disaggregated clinical tumour material of almost any source, and is particularly suited to scarce samples limited by the quantity of cells available. The small demand for primary material and the ability to work with extremely low viability samples means that the MAC chip is an obvious candidate assay for the proteomic profiling of cytology samples acquired through fine needle aspirates [FNAs]. The low number of cells required also means that for larger sources of clinical material, the MAC profiling could easily be done in parallel to other types of assays without placing a significant restriction on the quantity of material available. A MAC chip assay can in principle be developed for any protein of interest provided there are antibodies [or other capture agents such as DNA or small molecule covalent binders] of sufficient affinity available for that target. Because p53 is not a routine biomarker in therapeutic oncology, the results here should be viewed as a proof-of-principle which shows the potential of the technique for translational single cell proteomics. Development of other assays would allow quantification of proteins with immediate clinical relevance, such as steroid hormone or growth factor receptors. For example, a HER2 assay could examine expression level heterogeneity in HER2 positive breast or gastric cancer and could be assessed for correlation with treatment outcomes for trastuzumab, an anti-HER2 antibody drug, which is routinely applied to these patients. Furthermore, receptor expression heterogeneity in circulating tumour cells [CTCs] has been identified as a possible mechanism of acquired resistance to treatments targeting estrogen receptor and epidermal growth factor receptor [EGFR] in breast and colorectal cancer respectively\textsuperscript{149,150}. Characterising this heterogeneity with high resolution is important for the identification of clear subpopulations, and this is something MAC chips can address in CTCs as well as the tumour itself. Others have already envisaged individualised therapy programs...
informed by single cell proteomics and test panels of anti-cancer agents. Ex vivo screens of routinely used and experimental therapeutic compounds are possible in the MAC chip through an extension of the described protocol that incorporates cell drugging within the microfluidic chip prior to analysis. Potentially this could be a vital approach to guide drug selection for personalized cancer therapy as it would allow patient material to be assessed for heterogeneous single cell responses with respect to the drug and enable targeted therapeutic interventions which take into account the inherent heterogeneity of the tumour.

While producing assays of sufficient sensitivity for multiple analytes is challenging, multiplexed analyses as demonstrated here may allow the patient stratification space to be defined with even greater clarity. It may well emerge that it is the relative degree of these responses that is critical for the development of strategies for therapeutic intervention, and it is this that the high-resolution proteomic data that MAC chip analysis provides is uniquely situated to address.

To conclude, we have demonstrated that the MAC chip workflow is a practical method for the acquisition of single molecule-resolution single cell data in very small quantities of clinical tumour samples. Expanding the repertoire of analytes for which there are MAC chip assays beyond p53 and its phosphorylated form must now be a priority if the technique is to translate to the oncology clinic. Longitudinal studies are now required where tissue heterogeneity is monitored with the MAC chip through the course of treatment such that the clinical utility of the platform can be assessed. The uniquely quantitative single cell information provided in such studies may reveal insights unobtainable by other methods.
Chapter 6. Conclusions & future work

“If a victory is told in detail, one can no longer distinguish it from a defeat.”

– Jean-Paul Sartre

6.1. General conclusions

In this thesis we have developed methods for the application of single cell proteomics to clinically relevant samples in oncology. We have done this through two routes. The first focussed on technological development of the MAC chip platform, both with the attempted development of a single cell assay for the breast cancer biomarker protein estrogen receptor alpha, and with the integration of the MAC chip format into a multiple-stage microfluidic device for the isolation, concentration, and analysis of circulating tumour cells. The second route focussed on the development of an efficient protocol for the single cell analysis of disaggregated tumour samples, and demonstrated that this method could be used to discover considerable heterogeneity in protein expression among the tumour cells of the xenografts which were analysed. Along the way we have experimentally demonstrated multiple modalities of multiplexed measurement with the MAC chip, and redeveloped the single molecule counting algorithms used to analyse MAC chip TIRF image data, leading to more accurate protein counting in single cells, which is the goal of the MAC chip platform.

As we have seen in Chapter 5, a simple improvement to the MAC chip could readily be performed which would make the method easier to implement with samples such as clinical tumour material, where the maximum yield of analysed cells is important, as the possibility of repeats may be limited by the availability of biopsies. Simply doubling the number of analysis chambers in each device by having analysis lanes branch from both sides of the main cell
reservoir would prevent the need to manufacture multiple chips for each experiment [reducing reagent costs] and reduce the labour involved in changing over chips during experiments. This should be done prior to any further work which uses the MAC chip to study expression levels in clinical or quasi-clinical samples.

6.2. Dual colour & multiplexing

The dual colour p53/phospho-p53 multiplexing experiments performed on the xenografts of Chapter 5 were able to show dramatic degrees of heterogeneity in protein expression in the disaggregated tumours. This interesting result should be pursued by integrating the p53-phospho-p53 assay into a broader study, and using the heterogeneity to identify expression profiles of clinically relevant patient subtypes. As this work was only performed on patient derived xenografts, an important step would be to perform the same workflow directly on patient biopsies. The extremely small sample requirement of the workflow means that the MAC chip analysis can be done in parallel to, and without having a detrimental effect on, other analysis techniques, so incorporation into an already established study should not be that difficult.

Having shown that the dual colour spatial multiplexing experiments performed in Chapter 2 were a viable method of multiple analyte quantification, these experiments should be followed up for a more robust pair of protein assay systems, where signal can be more easily distinguished from noise. Altering the geometry of the MAC chamber would allow more than 2 antibody spots to be printed down inside the chamber, and thus further increase the multiplexing capability of the assay. If it can be shown that the degree of cross-reaction between the primary antibodies is negligible, then multiplex experiments may be performed with multiple fluorescent secondaries of the same wavelength [e.g. 488]. The potential level of multiplexing would then be equivalent to \( N_{\text{spots}} \times N_{\lambda} \) where \( N_{\text{spots}} \) is the number of printed primaries, and \( N_{\lambda} \) the number of excitation wavelengths available on the TIRF system. For the group setup, with a dual spot, the potential level of multiplexing is thus 8 different proteins, binding partners or post translational modifications. The greater degree of multiplexing available, the subtler the discriminations that may be made in the phenotypic space, and thus the greater the usefulness of the assay for the stratification of patient subtypes in translational research and personalised medicine. Using modified methods, the MAC chip would be capable of directly measuring protein-protein interactions [see Section 6.4].

6.3. CTCs and the MAC chip: a route forward

The three-stage CTC system using the spiral biochip in conjunction with a cell concentrating microfluidic to deliver CTCs to the MAC chip proved to be a dead end. Even so, there has been continued improvement in CTC isolation and analysis methods in the published literature throughout the duration of this project, much of it with direct relevance to the work described
here. For instance, the CTC-iChip now incorporates hydrodynamic cell sorting, inertial focusing and magnetophoretic separation into a compound device\textsuperscript{152}. It has been used to profile HER2 expression in estrogen receptor positive/HER2-negative breast cancer CTCs, showing that subpopulations of CTCs exist with different receptor profiles from the primary tumour classification, and display heterogeneity with respect to proliferative potential and resistance to cytotoxic chemotherapy\textsuperscript{153}, thus demonstrating that developing assays capable of measuring breast cancer biomarkers in CTCs is a worthwhile strategy.

Other novel isolation methods have been described which may lend themselves more easily to incorporation with the MAC chip. A recent hydrodynamic isolation method known as the Vortex chip lends itself directly to incorporation with the MAC chip. The vortex chip uses a pinched flow methodology to trap larger cells in local vortices whilst allowing red and white blood cells to pass through, and is exceptionally high throughput [8ml whole blood per minute]. Importantly the trapping mechanism does not require high resolution lithography or extremely tight control of the z-depth, and so would be well within the technical means of the production facilities in the Chemistry department. As shown in Figure 6.1, the Vortex chip could be implemented with standard MAC chambers, provided the plasma bonding issues described in Chapter 4 are resolved, or alternatively implemented with a valved system that allows the antibody capture surface to be homogeneously deposited down a valve-isolated, parallel analysis lane. Alternatively, a standalone vortex chip could release isolated CTCs in a small volume directly into the MAC chip.

The vortex-chip method has been used in conjunction with the new technique of single cell western blotting to measure protein expression in CTCs. Capable of analysing a panel of over 10 separate protein analytes from $\sim10^3$ cells simultaneously in 4 hours, the scWestern is both high throughput and highly multiplex. For isolated CTCs eluted from the vortex chip, the scWestern technique was able to identify variations in protein expression for a panel of 8 individual proteins, including estrogen receptor alpha\textsuperscript{154}. For rare-cells, the scWestern requires a manual micropipetting step to transfer individual CTCs to the microwells prior to electrophoresis. This manual loading step is similar to the optical trapping step in the MAC chip. However, if trapping could be performed straight from the vortex chambers into MAC chambers on a single microfluidic then throughput could potentially be higher, and the potential for cell loss is decreased, as cells remain inside the microfluidic at all times with no elution step.

As the vortex chip is well within the technical capabilities of the group’s fabrication facilities, and the design files have already been produced, an obvious future direction for this work would be to manufacture a vortex chip/hybrid vortex-MAC chip, and test the isolation and analysis workflow with cell lines spiked into healthy blood.
Figure 6.1: Proposed hybrid Vortex/MAC chip for CTC analysis. [upper] Schematic of the Vortex Chip. [Centre] Vortex chip with standard MAC chambers [adapted from155]. The static lanes are positioned so as to minimally perturb the vortex creation in the main flow chamber. [Bottom] Vortex chip with flow chemistry-based capture surfaces. Using valve layers the capture chambers can be isolated from the vortex lane and allow the surface to be functionalised only in the capture lanes. Valve layer 1 would be activated during the vortex flow, then relaxed to allow optical trapping of CTCs into the analysis chambers; valve layers 1 & 2 would...
be simultaneously activated to isolate the analysis chambers and prevent diffusion of the analyte out of the chamber.

6.4. Extending the MAC chip method: direct measurement of protein-protein interactions via superresolution colocalisation

The improvements to single molecule counting we described in Chapter 3 provide new methodological capabilities to the MAC chip platform. Using superresolution single molecule localisation methods it is now possible to directly measure protein-protein interactions in the MAC chip. Consider the situation where an antibody spot is printed down that targets a cellular protein [protein A], which binds directly to another cellular protein [protein B]. The standard MAC chip assay for protein A will pull down a proportion of the complexed protein, however, if the assay uses only a fluorescent secondary targeting protein A, then we will be unaware of it. Introducing a secondary fluorescent antibody for protein B, emitting at a different wavelength to the detection antibody for protein A, would allow us to directly measure the fraction of AB complexes there are in the cell relative to the total amount of protein A. If we used a mixed primary antibody spot, containing antibodies against both protein A and protein B, we could measure the total protein A and B, as well as the fraction that is complexed. Gaussian localisation methods allow routinely for localisation precisions in the tens of nm-regime, and can be improved with specialist methods to 1-2 nm, and even below\(^156\).

Saturation experiments with recombinant protein reveal that as few as 1% of the printed primary antibodies in a MAC chip primary antibody spot may be active, assuming a theoretical maximum packing density of 16nmol/m\(^2\)\(^157\). Given approximately \(5 \times 10^6\) active antibodies in a 100µm circular spot, on average an active primary antibody should occupy 1570 nm\(^2\), providing a linear distant between antibodies of approximately 40 nm, if we accept the admittedly unrealistic assumption that the antibodies are evenly distributed on a square grid. Thus, this usually unfavourable characteristic of the MAC chip benefits us here because it means we can accept a more lenient condition on our localisation precision to decide whether two proteins are complexed or not. The horizontal displacement between two non-complexed secondary antibodies of the two different wavelengths will be much greater than the horizontal displacement between two such molecules which are bound to the same complex [i.e. \(l_1 > > l_2\)] as in Figure 6.2.

Consequently, by performing colocalisation on superresolution data from two channels, and setting a lateral displacement cut-off we can obtain accurate information on whether two given proteins are likely to be bound to one another.
Figure 6.2: Illustration of the proposed method for quantifying protein-protein interactions in the MAC chip with superresolution detection.

There are several caveats worth outlining with this approach. Successful observation of complex AB is impossible if the binding of the two proteins obscures either of the epitopes recognised by the two secondary antibodies. Likewise, if immobilisation at the surface sterically hinders the formation of the large pentameric complex. Provided this is not the case, the ability of the assay to measure the protein-protein interaction depends on the stability of the AB complex itself. If the complex is likely to dissociate on a timescale less than that for the protein A-capture antibody system to reach equilibrium, then at the very least the measured number of complexes will not correspond to the true number in the cell at the time of lysis, and may lead to none being measurable at all. Microscope stability is especially important, as taking measurements in two different channels produces a delay between observations of protein A
and protein B, allowing for the possibility of some environmental effect producing a spatial shift between the channels which might frustrate the colocalisation analysis.

These issues are surmountable, and this could be a promising augmentation of the MAC chip experimental process, and one of the few techniques that could directly measure and quantify protein-protein interactions at the single molecule level in single cells. Ratiometric analysis of protein to bound complex may provide insight into cellular regulatory pathways, for example the relationship between p53 and binding partner MDM2.

6.5. Summary

In summary, we have introduced numerous improvements to the MAC chip technology, and investigated ways for the MAC chip to be applied to circulating tumour cells as well as solid tumours. Due to its inherent methodological and sensitivity challenges, quantitative proteomic profiling of single cells is not a routine or simple measurement to make. Single cell proteomics and circulating tumour cell analysis is still at the leading edge of biological research, and consequently there is still plenty of room for novel techniques to make an impact in the field. The MAC chip, and particularly the multiplexed MAC chip is a useful single cell proteomic technology that can be applied to small amounts of clinical and clinically-relevant material in cancer research, as has been demonstrated in this thesis. Although CTC analysis in the MAC chip was not possible in conjunction with the isolation methods tried here, continued improvements in the field of CTC isolation mean that it should not be ruled out as a possibility.

The compound developments of the MAC chip, data analysis methods, and experimental protocols for disaggregated tumour cells we have described are all crucial for the future application of the MAC chip in translational cancer research, and useful additions to the field of single cell analysis, and the study of protein expression heterogeneity in general. Though we have fallen short of it, the work here nevertheless provides a solid stepping stone towards a MAC chip-based liquid biopsy.
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Appendix A: Image analysis codes

A.1. Baseline noise offset calculator for non-congested images

//Macro to calculate the pixel gray-value offset required to accurately measure
//single molecule numbers in the non-congested regime
//Alastair James Magness 25/11/2015
//create dialogue for input of algorithm parameters

Dialog.create("Congested Fitting Parameters");
Dialog.addMessage("Please enter the following fitting parameters
\n\NOTE:
Please run localisation counting program on non-congested,\ndata before running
this script. Enter\naverage SM intensity in dialogue below\n\n");
Dialog.addNumber("Number of images to be counted for single molecules:", 5);
Dialog.addNumber("Number of field-flattening images:", 5);
Dialog.addCheckbox("Run in batch mode?", false)
Dialog.show();

n_SMImages = Dialog.getNumber();
n_bgImages = Dialog.getNumber();
batch=Dialog.getCheckbox();
if (batch==true) setBatchMode(true);

//create new array for image indexing
imgArray = newArray(nImages);

//get ImageID open stacks
for (i=0; i<nImages; i++) {
    selectImage(i+1);
    imgArray[i] = getImageID();
}

//set up loop extents
spot_nImages = imgArray.length;
slice_final = nSlices();
user_nImages=n_SMImages+n_bgImages;

//duplicate final frame from spot stacks into separate image
for (i=0; i<spot_nImages; i++) {
    selectImage(imgArray[i]);
    setSlice(nSlices());
    //setSlice(6);
    run("Duplicate...", "title=spot");
    rename("spot " + i+1);
}

for (i=0; i<spot_nImages; i++) {
    selectImage(imgArray[i]);
    close();
}

//get ImageIDs for open spot frames
for (i=0; i<spot_nImages; i++) {
    selectImage(imgArray[i]);
    imgArray[i] = getImageID();
}

//Divides all frames by a median-projected background average image created
//from the input images
//Turns individual projection profile-flattened images into stack for PSF optimisation
a=spot_nImages-n_bgImages;
b=spot_nImages;
run("Images to Stack", "name=Stack title=[] use");
selectWindow("Stack");
run("Duplicate...", "title=Stack-1 duplicate range=nSlices");
run("Make Substack...", "delete slices=a-b");
selectWindow("Stack");

for (i=0; i<n_SMImages; i++) {
    setSlice(1);
    //setSlice(6);
```java
run("Delete Slice");
}
selectWindow("Stack-1");
for (i=0; i<n_bgImages; i++) {
    setSlice(nSlices);
    //setSlice(6);
    run("Delete Slice");
}
selectWindow("Stack");
run("Image Background", "bias=100 blur=50");
selectWindow("Mask");
close();
imageCalculator("Divide create 32-bit stack ", "Stack-1","Background");
selectWindow("Background");
close();
selectWindow("Stack");
close();
selectWindow("Stack-1");
close();
selectWindow("Result of Stack-1");
run("Subtract Background...", "rolling=50 stack");
//Here we measure the intensity profile across the perimeter of the flattened image and output the mean //pixel level to the Log Window
run("Clear Results");
slicenumber=nSlices;
for (i=1; i<=slicenumber; i++) {
    makeLine(0, 512, 0, 0);
    run("Measure");
    makeLine(0, 511, 511, 511);
    run("Measure");
    makeLine(511, 0, 511, 511);
    run("Measure");
    makeLine(512, 0, 0, 0);
    run("Measure");
}
run("Summarize");
selectWindow("Results");
bline_offset = getResult("Mean", 4*slicenumber);
offset_SD = getResult("Mean", 4*slicenumber+1);
print("Baseline Noise Offset = ", bline_offset," +/- ", offset_SD);
print("\nWhen prompted enter the mean value into the ASMI program in addition \nto the Mean SM intensity derived from localisation counting\n
A large SD may mean a portion of your SM images have too many single molecules corrupting the noise level data,\n\nremove these images and try again\n");
selectWindow("Log");
```

A.2. Baseline noise offset calculator for congested images

//Macro to calculate the pixel gray-value offset required to accurately measure //single molecule numbers in the congested regime
//Alastair James Magness 25/11/2015
//create dialogue for input of algorithm parameters
Dialog.create("Congested Fitting Parameters");
Dialog.addMessage("Please enter the following fitting parameters\n
\nNOTE: Please run localisation counting program on non-congested,ndata before running this script. Enter\naverage SM intensity in dialogue below\n\n");
Dialog.addNumber("Number of images to be counted for single molecules: ", 5);
Dialog.addNumber("Number of field-flattening images: ", 5);
Dialog.addCheckbox("Run in batch mode?", false)
Dialog.show();
n_SMImages = Dialog.getNumber();
n_bgImages = Dialog.getNumber();
batch=Dialog.getCheckbox();
if (batch==true) setBatchMode(true);
//create new array for image indexing
imgArray = newArray(nImages);

//getImageID open stacks
for (i=0; i<nImages; i++) {
    selectImage(i+1);
    imgArray[i] = getImageID();
}

//set up loop extents
spot_nImages = imgArray.length;
slice_final = nSlices();
user_nImages=n_SMIImages+n_bgImages;

//duplicate final frame from spot stacks into separate image
for (i=0; i<spot_nImages; i++) {
    selectImage(imgArray[i]);
    setSlice(nSlices());
    setSlice(6);
    run("Duplicate...", "title=spot");
    rename("spot " + i+1);
}

for (i=0; i<spot_nImages; i++) {
    selectImage(imgArray[i]);
    close();
}

//get ImageIDs for open spot frames
for (i=0; i<spot_nImages; i++) {
    selectImage(i+1);
    imgArray[i] = getImageID();
}

//Divides all frames by a median-projected background average image created from the input images
//Turns individual projection profile-flattened images into stack for PSF optimisation
a=spot_nImages-n_bgImages;
b=spot_nImages;
run("Images to Stack", "name=Stack title=[] use");
selectWindow("Stack");
run("Duplicate...", "title=Stack-1 duplicate range=nSlices");
selectWindow("Stack");
for (i=0; i<n_SMImages; i++) {
    setSlice(1);
    //setSlice(6);
    run("Delete Slice");
}
selectWindow("Stack-1");
for (i=0; i<n_bgImages; i++) {
    setSlice(nSlices);
    //setSlice(6);
    run("Delete Slice");
}
selectWindow("Stack");
run("Image Background", "bias=100 blur=50");
selectWindow("Mask");
close();
imageCalculator("Divide create 32-bit stack", "Stack-1", "Background");
selectWindow("Background");
close();
selectWindow("Stack");
close();
selectWindow("Stack-1");
close();
selectWindow("Result of Stack-1");
run("Subtract Background...", "rolling=512 stack");

//Here we measure the intensity profile across the perimeter of the flattened image and output the mean
//pixel level to the Log Window
run("Clear Results");
slicenumber=nSlices;
    for (i=1; i<=slicenumber; i++) {
        makeLine(0, 512, 0, 0);
        run("Measure");
        makeLine(0, 511, 511, 511);
        run("Measure");
        makeLine(511, 0, 511, 511);
        run("Measure");
        makeLine(512, 0, 0, 0);
        run("Measure");
    }
run("Summarize");
selectWindow("Results");
bline_offset = getResult("Mean", 4*slicenumber);
offset_SD = getResult("Mean", 4*slicenumber+1);
print("Baseline Noise Offset = ", bline_offset,"+/-", offset_SD);
print("When prompted enter the mean value into the ASMI program in addition \
to the Mean SM intensity derived from localisation counting");
print("A large SD may mean a portion of your SM images have too many single 
molecules corrupting the noise level data,\nremove these images and try 
again");
selectWindow("Log");

A.3. Single molecule localisation counting

/*PROGRAM TO COUNT MOLECULES BY LOCALISATION 
//GAUSSIAN FITS INTENSITY PROFILES OF ALL PEAKS IN IMAGE
//USING UNIVERSITY OF SUSSEX GDSC PLUGINS [INSTALL FIRST]
//TO INSTALL GO TO HELP>>UPDATE FIJI>>MANAGE UPDATE SITES, THEN SELECT THE TICK
//BOX FOR BOTH GDSC AND GDSC-SMLM,
//then ADD, and APPLY CHANGES. FIJI WILL KEEP THE NECESSARY PLUGINS UP TO DATE
The code will not run unless the location of the temporary config file refer-
ence in line 150
is changed to a usable location on your own computer. This can be done simply
by changing "AM8812"
to your own user/computer name. */

print("\Clear")
run("Clear Results");

//check Fit Results window is not open
check=isOpen("Fit Results");
    if (check != 0) {
        selectWindow("Fit Results");
        run("Close");
    }

//check SM intensity distribution image window is not open
check=isOpen("Single Molecule Intensity Distribution");
    if (check != 0) {
        selectWindow("Single Molecule Intensity Distribution");
        close();
    }

//create dialogue for input of algorithm parameters
Dialog.create("Fitting Parameters");
Dialog.addMessage("Please enter the following fitting parameters:\n\nNOTE:
Changing exposure time will NOT affect the SM count,\nit will only affect the 
time scales of some of the more advanced\nfunctions of the PeakFit algorithm\n\n");
Dialog.addNumber("Number of images to be counted for single molecules:", 55);
Dialog.addNumber("Number of field-flattening images:", 5);
Dialog.addNumber("Exposure time of images:", 1);
Dialog.addNumber("Signal Factor:", 1.5);
Dialog.addNumber("Width Factor:", 1.1);
Dialog.addNumber("Baseline Noise Offset:", 0.1);
Dialog.addChoice("Reconstruction type", newArray("PSF", "Localisations", "Frame number", "Localisations (width=precision)", "Signal intenisty", "Signal intenisty");

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(width=precision), "Localisations (width=av.precision)", "Signal (width=av.precision)", "Fit Error"));
Dialog.addChoice("Results table type", newArray("Uncalibrated", "Calibrated"));
Dialog.addCheckbox("Display SM intensity distribution?", true);
Dialog.addCheckbox("Run in batch mode?", false);
Dialog.show();

n_SMImages = Dialog.getNumber();
n_bgImages = Dialog.getNumber();
exptime = Dialog.getNumber();
SNR = Dialog.getNumber();
width_fac = Dialog.getNumber();
BNO = Dialog.getNumber();
reconstruction = Dialog.getChoice();
RTable = Dialog.getChoice();
distribution=Dialog.getCheckbox();
batch=Dialog.getCheckbox();
if (batch==true) setBatchMode(true);

//create new array for image indexing
imgArray = newArray(nImages);

//get ImageID open stacks
for (i=0; i<nImages; i++) {
    selectImage(i+1);
    imgArray[i] = getImageID();
}

//set up loop extents
Spot_nImages = imgArray.length;
Slice_final = nSlices();
user_nImages=n_SMImages+n_bgImages;

//duplicate final frame from spot stacks into separate image
for (i=0; i<Spot_nImages; i++) {
    selectImage(imgArray[i]);
    setSlice(nSlices());
    //setSlice(6);
    run("Duplicate...", "title=spot");
    rename("spot " + i+1);
}

for (i=0; i<Spot_nImages; i++) {
    selectImage(imgArray[i]);
close();
}

//get ImageIDs for open spot frames
for (i=0; i<Spot_nImages; i++) {
    selectImage(i+1);
    imgArray[i] = getImageID();
}

//Divides all frames by a median-projected background average image created from the input images
//Turns individual projection profile-flattened images into stack for PSF optimisation
a=Spot_nImages-n_bgImages;
b=Spot_nImages;
run("Images to Stack", "name=Stack title=[] use");
selectWindow("Stack");
run("Duplicate...", "title=Stack-1 duplicate range=nSlices");
selectWindow("Stack");

for (i=0; i<n_SMImages; i++) {
    setSlice(1);
    run("Delete Slice");
}

selectWindow("Stack-1");

for (i=0; i<n_bgImages; i++) {
    setSlice(nSlices);
    run("Delete Slice");
}
selectWindow("Stack");
run("Image Background", "bias=100 blue=50");
selectWindow("Mask");
close();
imageCalculator("Divide create 32-bit stack", "Stack-1","Background");
close();
selectWindow("Stack");
close();
selectWindow("Stack-1");
close();
selectWindow("Result of Stack-1");
run("Subtract Background...", "rolling=50 stack");
run("Subtract...", "value=BNO stack");

//PEAK ESTIMATOR
run("PSF Estimator", "initial_stddev0=1.000 initial_stddev1=1.000 initial_angle=0.000 number_of_peaks=1000 p_value=0.0100 update_preferences log_progress iterate_histogram_bins=100 spot_filter_type=Single spot_filter=Mean smoothing=0 search_width=2 border=1 fitting_width=3 fit_solver=[Least Squares Estimator (LSE)] fit_function=Circular fail_limit=5 include_neighbours_neighbour_height=0.90 residuals_threshold=0.10 shift_factor=0.27 signal_strength=0 min_photons=0 width_factor=2 fit_criteria=[least-squared error] significant_digits=5 coord_delta=0.0001 lambda=10.0000 max_iterations=20");
run("Stack to Images");

//get ImageIDs for open spot frames
for (i=0; i<nImages; i++) {
    selectImage(i+1);
    imgArray[i] = getImageID();
}

/**********
//PEAK FIT
**********/

//runs peak fitting algorithm from GDSC SMLM package [psf estimator must be run first]
//increasing width factor increases SM count; this plateaus according to the size of the estimated PSF
//plateau starts around 3 for ER images with dylight 488
//Signal strength is other important parameter, count decreases exponentially as a function of input signal strength
//user must input appropriate value which is obtained from calculating the average signal to noise ratio
//from the Fit Results table manually
print("\nSM Count by Localisation [Gaussian Fitting]\"");
if(reconstruction=="PSF")
    for (i=0; i<n_SMImages; i++) {
        selectImage(imgArray[i]);
        run("Peak Fit", "config_file=C: Users Alastair gdsc.smlm.settings.xml calibration=107 gain=37.70 exposure_time=exptime initial_stddev0=1.000 initial_stddev1=1.000 initial_angle=0.000 spot_filter_type=Single spot_filter=Mean smoothing=0 search_width=2 border=1 fitting_width=3 fit_solver=[Least Squares Estimator (LSE)] fit_function=Circular fail_limit=5 include_neighbours neighbouring=0.90 residuals_threshold=0.10 duplicate_distance=0.20 shift_factor=2.33 signal_strength=SNR min_photons=0 width_factor=width_fac precision=0 results_table=Uncalibrated image=PSF weighted equalised image_precision=1 image_scale=1 results_dir=[] slice");
    }
else if (reconstruction=="Signal intensity")
    for (i=0; i<n_SMImages; i++) {
        selectImage(imgArray[i]);
        run("Peak Fit", "config_file=C: Users am8812 GDSC_settings gdsc.smlm.settings.xml calibration=107 gain=37.70 exposure_time=exptime initial_stddev0=1.000 initial_stddev1=1.000 initial_angle=0.000 spot_filter_type=Single spot_filter=Mean smoothing=0 search_width=2 border=1 fitting_width=3 fit_solver=[Least Squares Estimator (LSE)] fit_function=Circular fail_limit=5 include_neighbours neighbouring=0.90 residuals_threshold=0.10 duplicate_distance=0.20 shift_factor=2.33 signal_strength=SNR min_photons=0 width_factor=width_fac precision=0 results_table=Uncalibrated image=PSF weighted equalised image_precision=1 image_scale=1 results_dir=[] slice");
}
if (reconstruction=="Localisations")
  for (i=0; i<n_SMImages; i++) {
    selectImage(imgArray[i]);
    run("Peak Fit", "config_file=C:\Users\am8812\GDSC_set-
inggs\gdsc.smlm.settings.xml calibration=107 gain=37.70 exposure_time=exptime initial_stddev=1.000 initial_angle=0.000 spot_filter_type=Single spot_filter=Mean smoothing=0 search_width=2 border=1 fit-
ting_width=3 fit_solver=[Least Squares Estimator (LSE)] fit_function=Circular fail_limit=5 include_neighbours neighbour_height=0.90 residuals_threshold=0.10 duplicate_distance=0.20 shift_factor=2.33 signal_strength=SNR min_photons=0 width_factor=width_fac precision=0 results_table=RTable image=Localisations weighted equalised image_precision=1 image_scale=1 results_dir=[] slice");
  }
else if (reconstruction=="Frame number")
  for (i=0; i<n_SMImages; i++) {
    selectImage(imgArray[i]);
    run("Peak Fit", "config_file=C:\Users\am8812\GDSC_set-
inggs\gdsc.smlm.settings.xml calibration=107 gain=37.70 exposure_time=exptime initial_stddev=1.000 initial_angle=0.000 spot_filter_type=Single spot_filter=Mean smoothing=0 search_width=2 border=1 fit-
ting_width=3 fit_solver=[Least Squares Estimator (LSE)] fit_function=Circular fail_limit=5 include_neighbours neighbour_height=0.90 residuals_threshold=0.10 duplicate_distance=0.20 shift_factor=2.33 signal_strength=SNR min_photons=0 width_factor=width_fac precision=0 results_table=RTable image=Frame number weighted equalised image_precision=1 image_scale=1 results_dir=[] slice");
  }
else if (reconstruction=="Signal (width=precision)"
    for (i=0; i<n_SMImages; i++) {
      selectImage(imgArray[i]);
      run("Peak Fit", "config_file=C:\Users\am8812\GDSC_set-
inggs\gdsc.smlm.settings.xml calibration=107 gain=37.70 exposure_time=exptime initial_stddev=1.000 initial_angle=0.000 spot_filter_type=Single spot_filter=Mean smoothing=0 search_width=2 border=1 fit-
ting_width=3 fit_solver=[Least Squares Estimator (LSE)] fit_function=Circular fail_limit=5 include_neighbours neighbour_height=0.90 residuals_threshold=0.10 duplicate_distance=0.20 shift_factor=2.33 signal_strength=SNR min_photons=0 width_factor=width_fac precision=0 results_table=RTable image=Signal (width=precision) weighted equalised image_precision=1 image_scale=1 results_dir=[] slice");
  }
else if (reconstruction=="Localisations (width=precision)"
    for (i=0; i<n_SMImages; i++) {
      selectImage(imgArray[i]);
      run("Peak Fit", "config_file=C:\Users\am8812\GDSC_set-
inggs\gdsc.smlm.settings.xml calibration=107 gain=37.70 exposure_time=exptime initial_stddev=1.000 initial_angle=0.000 spot_filter_type=Single spot_filter=Mean smoothing=0 search_width=2 border=1 fit-
ting_width=3 fit_solver=[Least Squares Estimator (LSE)] fit_function=Circular fail_limit=5 include_neighbours neighbour_height=0.90 residuals_threshold=0.10 duplicate_distance=0.20 shift_factor=2.33 signal_strength=SNR min_photons=0 width_factor=width_fac precision=0 results_table=RTable image=Localisations (width=precision) weighted equalised image_precision=1 image_scale=1 results_dir=[] slice");
  }
else if (reconstruction=="Localisations (width=av.precision)"
    for (i=0; i<n_SMImages; i++) {
      selectImage(imgArray[i]);
      run("Peak Fit", "config_file=C:\Users\am8812\GDSC_set-
inggs\gdsc.smlm.settings.xml calibration=107 gain=37.70 exposure_time=exptime initial_stddev=1.000 initial_angle=0.000 spot_filter_type=Single spot_filter=Mean smoothing=0 search_width=2 border=1 fit-
ting_width=3 fit_solver=[Least Squares Estimator (LSE)] fit_function=Circular fail_limit=5 include_neighbours neighbour_height=0.90 residuals_threshold=0.10 duplicate_distance=0.20 shift_factor=2.33 signal_strength=SNR min_photons=0 width_factor=width_fac precision=0 results_table=RTable image=Localisations (width=av.precision) weighted equalised image_precision=1 image_scale=1 results_dir=[] slice");
  }
else if (reconstruction=="Signal (width=av.precision)"
    for (i=0; i<n_SMImages; i++) {
      selectImage(imgArray[i]);

run("Peak Fit", "config_file=C:\\Users\\am8812\\\GDSC_settings\\gdsc.smlm.settings.xml calibration=107 gain=37.70 exposure_time=exptime initial_stddev=1.000 initial_stddev=1.000 initial_angle=0.000 spot_filter_type=Single spot_filter=Mean smoothing=0 search_width=2 border=1 fit_width=3 fit_solver=[Least Squares Estimator (LSE)] fit_function=Circular fail_limit=5 include_neighbours neighbour_height=0.90 residuals_threshold=0.10 duplicate_distance=0.20 shift_factor=2.33 signal_strength=SNR min_photons=0 width_factor=width_fac precision=0 results_table=RTable image=[Signal weighted equalised image precision=1 image_scale=1 results_dir=[] slice");

else if (reconstruction=="Fit error")
for (i=0; i<n_SMImages; i++) {
    selectImage(imgArray[i]);
    run("Peak Fit", "config_file=C:\\Users\\am8812\\\GDSC_settings\\gdsc.smlm.settings.xml calibration=107 gain=37.70 exposure_time=exptime initial_stddev=1.000 initial_stddev=1.000 initial_angle=0.000 spot_filter_type=Single spot_filter=Mean smoothing=0 search_width=2 border=1 fit_width=3 fit_solver=[Least Squares Estimator (LSE)] fit_function=Circular fail_limit=5 include_neighbours neighbour_height=0.90 residuals_threshold=0.10 duplicate_distance=0.20 shift_factor=2.33 signal_strength=SNR min_photons=0 width_factor=width_fac precision=0 results_table=RTable image=[Fit error weighted equalised image precision=1 image_scale=1 results_dir=[] slice");
}

//Tidy up. Create two stacks, Closes joint stack of original+reconstruction image, renames stacks
run("Images to Stack", "name=Stack title=[] use");
run("Stack Splitter", "number=2");
close("Stack");
selectWindow("stk_0001_Stack");
rename("Original");
selectWindow("stk_0002_Stack");
rename("Reconstruction");

//We now select the window containing the list of localised molecules found by the Peak Fit algorithm and return
//the mean signal intensity of the molecules
selectWindow("Fit Results");
summary = split(getInfo(), ";n");
SM_total=summary.length-1;
Signal_array=newArray(SM_total); //create new array to house the signal intensities values for every molecule
for (i=0; i<SM_total; i++) {
    values = split(summary[i+1], ";t");
    Signal_array[i] = values[9]; //signal is the 8th column in the results table, loop iterates from 0
} //print(Signal_array[i]);

Array.getStatistics(Signal_array,min,max,mean,stdDev);
mean_SMint=mean;
print("Mean SM intensity = ", mean_SMint);
print("Total SM identified =", SM_total); //should be the same as the number of molecules in the Fit Results windows

/**********
* Here we make a histogram of the single molecule intensities; this allows us
to decide which measure of the average single molecule intensity
* to use when analogue counting. Heavily skewed distributions imply the use of
the median rather than mean SM intensity.
**********
if (distribution==true) {
    run("hist columns jru v1", "windows=[Fit Results] y=Signal");
}

selectWindow("Fit Results Histogram");
rename("Single Molecule Intensity Distribution");
print("\nPeak Fit Algorithm Parameters:");
print("\nSignal Factor " + SNR);
print("\nWidth Factor " + width_fac);
print("Reconstruction type " + reconstruction);
print("Baseline Noise Offset " + BNO);
A.4. Average single molecule intensity counting

//PROGRAM TO COUNT MOLECULES BY AVERAGE SINGLE MOLECULE INTENSITY ANALYSIS
//Please run AJM Single Molecule Localisation Count V2.5 and AJM Baseline Noise Offset Calculator BEFORE
//running this program, as you will be asked to input information provided by these other programs
//in this program.

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print("\nClear")
run("Clear Results");

//check Fit Results window is not open
check=isOpen("Fit Results");
if (check != 0) {
   showMessage("Warning", "Please close the Fit Results window before running the macro. Failure to do so will result in inaccurate results for the mean SM intensity.");
   selectWindow("Fit Results");
   run("Close");
}

//check SM intensity distribution image window is not open
check=isOpen("Single Molecule Intensity Distribution");
if (check != 0) {
   selectWindow("Single Molecule Intensity Distribution");
   close();
}

//create dialogue for input of algorithm parameters
Dialog.create("Congested Fitting Parameters");
Dialog.addMessage("Please enter the following fitting parameters:

NOTE: Please run localisation counting program on non-congested, non-data before running this script. Enter the average SM intensity in dialogue below.

Number of images to be counted for single molecules: ", 55);
Dialog.addNumber("Number of field-flattening images: ", 5);
Dialog.addNumber("Average single molecule intensity: ", 100);
Dialog.addNumber("Baseline Noise Offset: ", 1);
Dialog.addCheckbox("Run in batch mode?", false)
Dialog.show();

n_SMImages = Dialog.getNumber();
n_bgImages = Dialog.getNumber();
mean_SMint = Dialog.getNumber();
bline_offset = Dialog.getNumber();
batch=Dialog.getCheckbox();
if (batch==true) setBatchMode(true);

//create new array for image indexing
imgArray = newArray(nImages);

//getImageID open stacks
for (i=0; i<nImages; i++) {
   selectImage(i+1);
   imgArray[i] = getImageID();
}

//set up loop extents
spot_nImages = imgArray.length;
slice_final = nSlices();
user_nImages=n_SMImages+n_bgImages;

//duplicate final frame from spot stacks into separate image
for (i=0; i<spot_nImages; i++) {
   selectImage(imgArray[i]);
   setSlice(nSlices());
   //setSlice(6);
run("Duplicate...", "title=spot");
rename("spot " + i+1);
}

for (i=0; i<spot_nImages; i++) {
    selectImage(imgArray[i]);
    close();
}

//get ImageIDs for open spot frames
for (i=0; i<spot_nImages; i++) {
    selectImage(i+1);
    imgArray[i] = getImageID();
}

//Divides all frames by a median-projected background average image created from the input images
//Turns individual projection profile-flattened images into stack for PSF optimisation
a=spot_nImages-n_bgImages;
b=spot_nImages;
run("Images to Stack", "name=Stack title=[] use");
selectWindow("Stack");
run("Duplicate...", "title=Stack-1 duplicate range=nSlices");
//run("Make Substack...", "delete slices=a-b");
selectWindow("Stack");
for (i=0; i<n_SMImages; i++) {
    setSlice(1);
    run("Delete Slice");
}

selectWindow("Stack-1");
for (i=0; i<n_bgImages; i++) {
    setSlice(nSlices);
    //setSlice(6);
    run("Delete Slice");
}

selectWindow("Stack");
run("Image Background", "bias=100 blur=50");
selectWindow("Mask");
close();
imageCalculator("Divide create 32-bit stack", "Stack-1","Background");
selectWindow("Background");
close();
selectWindow("Stack");
close();
selectWindow("Stack-1");
close();
selectWindow("Result of Stack-1");
run("Subtract Background...", "rolling=512 stack");
run("Subtract...", "value=bline_offset stack");

//measure total intensity of image
selectWindow("Result of Stack-1");
slicenumber=nSlices;
for (i=1; i<=slicenumber; i++) {
    setSlice(i);
    run("Select All");
    run("Measure");
}

//get integrated intensity from results windows for each frame and divide by average single molecule intensity to check consistency in non-congested and congested counting
int_densityarray = newArray(slicenumber); // create new arrays to house integrated intensity values
congested_countarray = newArray(slicenumber);
print("Single Molecule Count by Average Single Molecule Intensity Analysis:
\n\n"); print("Spot", "SMcount");

//for loop grabs integrated intensity data from results windows, divides by mean single molecule intensity and prints to log window
for (i=0; i<slicenumber; i++) {

selectWindow("Results");
int_densityarray[i] = getResult("RawIntDen", i);
congested_countarray[i] = int_densityarray[i]/mean_SMint;
print(i+1, congested_countarray[i]);
}
print("\nMean SM intensity used: ", mean_SMint);
print("\nBNO used: ", bline_offset);
selectWindow("Log");
Appendix B: Miscellaneous microfluidics

B.1. Randomised c-sieve

The randomised c-sieve is a large microfluidic that uses wide inlet channels [3.2mm wide] to cope with the high flow rate output of the spiral biochip. It is an example of a brute force methodology for trapping and concentrating cells. The initial channel bifurcates progressively, which reduces the volumetric flow rate by a factor of 5.5 in each of the cell trapping regions, and consequently reduces the mean flow velocity by the same factor. The 16 trapping regions consist of 1,854 cell traps with randomised positions in the transverse plane, for a total of 29,664 individual cell trapping points in the whole microfluidic. The design rationale uses a probabilistic argument for the placement of the traps, which goes as follows. A particle in laminar flow will always follow its centre of mass streamline; at the inlet, there are in principle an almost infinite number of potential streamlines a particle could follow through the device, however the presence of traps and the finite size of a CTC restrict the number of available path endpoints.

At each row of traps a fraction of streamlines, and thus the probability of a cell’s centre of mass streamline passing through a trapping location is equal to $\frac{Nd}{w}$, where $N$ is the number of traps, $d$ is the trap diameter, and $w$ is the diameter of the channel. For each of the 16 channels in the randomised concentrator $N=9$, $d=31\mu m$, and $w=1106\mu m$, giving a total probability of trapping at the first set of traps of $~0.25$. By having so many layers of traps, randomly arranged, the centre of mass streamline of any CTC which enters the device ought to pass through a cell trap at at least one stage of its progress through the microfluidic. Redundancy is built into the design: the act of trapping a CTC in any given trap prevents that trap from trapping the next CTC that comes in on the same or a closely proximate flow line, and as such the device contains multiple stages of random traps which all address the same inlet flow lines. The high total

Figure B.1: The randomised c-sieve concentrator. [left panel] Overview of the whole microfluidic. A single inlet bifurcates to the trapping region. A series of 4 outlets reduce space and remove unnecessary hydraulic resistance. [right panel] Detail of the randomised trapping posts within each of the 16 trapping regions.
number of traps allows for samples with very high numbers of CTCs, while still ideally producing single occupancy in any trap. At the time the device was designed it was not possible to perform fluid flow simulations of the chip to confirm whether the design was optimised to have the highest trapping probability for a given number of cells and the minimum number of traps. However, it was felt the design strategy was reasonable, and the device proceeded to the manufacturing stage. It was acknowledged that the design might be ‘overkill’, however.

In the laminar flow regime, the Navier-Stokes equations are time-reversible, and thus provided cells do not stick to the trapping structures, it ought to be possible to recover the cells from the device by reversing the flow direction; the volume the cells are suspended in could theoretically be minimised to the internal volume of the microfluidic.

The total internal volume of the randomised c-sieve microfluidic, and thus the potential minimum cell recovery volume, for a version with z-height of 45 microns [chosen arbitrarily but with the aim of accommodating anything up to the largest cells, and limiting the hydraulic resistance in the large device], was 27µl.

B.2. Grille concentrator

Like the randomised c-sieve, the grille concentrator, shown in Figure B.2, utilises a similar bifurcating design with large diameter inlet channels to slow cells down by the time they reach the trapping region. The difference with this design is that instead of using individual traps to capture cells on different flowlines, all flowlines are directed through a central sieve, or grille-like structure, the critical dimension of which is narrower than the diameter of a cell [6µm for the channel structure, vs 10+µm for a cell]. Several things must be noted about design choices for the device. Firstly, the parallelised trapping region was realised to be a risk, as the narrow trap channels would create a high resistance to flow necessitating a high inlet pressure, and thus increasing the risk of the chip bursting during operation if plasma bonding to the substrate was not completely effective. Further to this the region increases the risk of clogging, which also increases the risk of chip leaks. However, the highly-parallelised nature of the central trapping regions was designed with a view to mitigating both issues.

The central grille contains 576 individual trapping locations, distributed across 32 550µm channels, which provided the sample has low numbers of CTCs [as expected], ought to be sufficient to singly trap the CTCs isolated with the spiral biochip, provided they are evenly distributed over the inlet flow lines.
B.3. CTC capture spiral

The CTC capture spiral [Figure B.3] was the first combination device designed to both trap CTCs and analyse them in a single device. The complex device features two interconnected, radially offset spiral channels, and utilises centrifugal forces and the pressure gradient formed between the high pressure inner channel and low pressure outer reservoir to move cells to the outside of the inner channel and into cell trapping pockets, from where the cells may be moved via optical trap into a perpendicular channel containing a MAC chip-style analysis chamber.
Using dual spiral channels of this kind to trap biological cells was first described by Ramesh in 2010.

Figure B.3: Schematic of the CTC capture spiral microfluidic. Cell trapping occurs due to a centrifugal migration of cells towards the outside wall of the inner channel. The narrow channels which connect this inner spiral channel to a lower-pressure outer spiral cause a suction effect which draws the cells that have migrated to the outside of the inner channel into the apertures of the trapping channels. As these apertures are narrower than the cells themselves, the cells cannot pass through and become immobilised at the constriction point. An optical trap can then be used to move the trapped cells into the MAC chip-style analysis chamber.
Ultimately the CTC spiral had several fundamental flaws which led to its abandonment as a viable strategy for CTC concentration and analysis. The first of these was the propensity of the inner part of the PDMS spiral to delaminate from the glass substrate, causing a catastrophic failure in the integrity of the chip. This would occur at flow rates of 1-10µl/min, much lower than that required to process the output of the spiral biochip, or process a raw blood sample in a reasonably fast time period. It may be possible to make the CTC spiral more robust to leaks by changing the geometry of the spiral, e.g. by thickening the separating wall between the inner and outer spiral channel, however this was not pursued due to the second issue with the design, shown in FIG X, whereby multiple beads could become lodged in the same trapping position. This issue is caused by flow rates which are too high, and processing a sample with a concentration of beads/cells that is too high. Whilst the latter is not likely to be an issue when processing a blood sample that has been pre-enriched with the spiral biochip, the former is unresolvable as fast processing is critical, and as such this is a terminal design flaw for our purpose.

B.4. Radial concentrators

The radial devices [Figure B.4] provide an elegant solution to the problem of coupling a high flow rate into devices with narrow inlet channels. Essentially these chips remove the requirement of having a manifold splitter to split the output of the spiral biochip into multiple microfluidics, and instead have multiple versions of the same device arranged radially on a single glass substrate. The difficulty with these designs is that they require large coverslips, which complicates the PEG/Neutravidin functionalisation, or requires custom functionalised coverslips to be purchased commercially, increasing cost. Using a non-standard coverslip size also causes compatibility issues with the microarrayer. However, none of these issues is inherently insurmountable.
Figure B.4: High-throughput radial microfluidics, which solve the high flow rate problem by utilising an inlet manifold with a symmetrical design leading to progressively bifurcating flow channels to slow the velocity of cells down before the critical trapping locations.