Tailored bio-molecular screening methodologies for selection of improved antibody lead clones for diagnostic and therapeutic applications.

A thesis submitted for the degree of Ph.D.

By

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August 2015

Based on research carried out at:

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Declaration

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Acknowledgements

Firstly, I would like to thank Dr. Paul Leonard for his unending support and encouragement during the course of my study. I have learned, and continue to learn, an immeasurable amount from him.

Secondly, I would like to thank Professor Richard O'Kennedy for his advice throughout the entire process.

Thirdly I want to mention my colleagues and friends throughout the School of Biotechnology, the National Centre for Sensor Research and the Biomedical Diagnostics Institute. They have made my time in DCU not only bearable but enjoyable. A special mention to Leanne, Niamh, Ruth, Jenny and Bincy, as well as my DiCAST team members Brian Manning, Barry O'Donnell and Brian O'Reilly.

I would also like to acknowledge the Biomedical Diagnostics Institute for their support, both financial and otherwise, throughout my time there.

Finally, I want to thank my friends and family who manage to be totally and utterly supportive of all my endeavours without having any idea what it is I actually do!

Research Outputs

Patents:

Paul Leonard, Ivan Dimov, Richard O' Kennedy and **Valerie Fitzgerald**. DiCAST: Direct Clone Analysis and Selection Technology. PCT Patent Application No. PCT/EP2011/062015.

Invention Disclosure Forms:

Valerie Fitzgerald and Paul Leonard. (2011). Demonstration of in-solution binding methods in a micro-pore array.

Publications:

Valerie Fitzgerald, Brian Manning, Barry O'Donnell, Brian O'Reilly, Dermot O'Sullivan, Richard O' Kennedy and Paul Leonard. (2015). Exploiting highly ordered sub-nanolitre volume microcapillaries as microtools for the analysis of antibody producing cells. Analytical Chemistry 87(2); 997-1003. (Cover Article, please see page vi)

Valerie Fitzgerald and Paul Leonard. (2014). Chapter 13: Next Generation Immunoassays. In Immunoassays – Development, Applications and Future Trends, edited by C. Murphy and R. O'Kennedy, Pan Stanford Publishing, *In press*.

Valerie Fitzgerald and Paul Leonard. (2015). Advanced tools to harness the human antibody response for vaccine development and immunotherapeutics. Review Article. *In preparation.*

Awards:

Outstanding Graduate Researcher Award 2015. Awarded by the Faculty of Science and Health, Dublin City University, Dublin, Ireland.

Shortlisted for the President's Award for Innovation 2015. (https://www.dcu.ie/invent/new/2015/april/dcuinnovationawards.shtml).

Innovation of the Year 2014. DiCAST- Innovation in biological drug discovery. Awarded at The Irish Lab Awards 2014, Dublin, Ireland. (http://labawards.ie/shortlist.php).

Industry Funded Projects:

<u>GE Healthcare</u>

Field trial agreement for evaluation of pre-release Biacore 4000 software and collaboration project for the application notes to coincide with the launch of the new instrument.

<u>BBI Solutions Ltd.</u>

Full commercial evaluation of the DiCAST technology with a number of external customers (2014).

Oral Presentations:

Direct Clone and Analysis Selection Technology (DiCAST). **Valerie Fitzgerald**. Oral presentation given at School of Biotechnology Research Day 2013, Dublin City University, Dublin.

Poster Presentations:

Valerie Fitzgerald, Brian Manning, Barry O'Donnell, Dermot O'Sullivan, Brian O'Reilly, Richard O'Kennedy and Paul Leonard. (2014). Direct discovery of monoclonal antibody secreting cells using densely packed microcapillary arrays. Poster presented at Human antibodies and hybridomas (HAH) conference-2014, Vienna, Austria.

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pubs.acs.org/ac
analytical chemistry
January 20, 2015 Volume 87 Number 2
Exploiting Highly Ordered Subnanoliter Volume Microcapillaries as Microtools for the Analysis of Antibody Producing Cells
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List of abbreviations

3OH	3-OH-C12-HSL-BSA
3OXO	3-oxo-C12-HSL-BSA
AA	Acrylic acid
Abs	Absorbance
ACE-1	Angiotensin converting enzyme 1
ADCC	antibody-dependant cell-mediated cytotoxicity
APS	Ammonium persulphate
APTES	(3-Aminopropyl) triethoxysilane
ASC	Antibody-secreting cell
ATCC	American type culture collection
BDI	Biomedical Diagnostics Institute
bp	base pair
BSA	Bovine Serum Albumin
CDC	complement-dependant cytotoxicity
cDNA	complementary DNA
CDR	Complementarity determining region
cfu	Colony forming units
CK18	Cytokeratin
CRP	C-Reactive protein
cTnI	Troponin I
cTnT	Troponin T
CY3	Cyanine 3
CY5	Cyanine 5
D	Diversity
dAb	Domain antibody
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	Diethylpyrocarbonate
DiCAST	Direct Clone and Analysis Selection Technology
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidyl triphosphates
DTT	dithiothreitol
E.coli	*Escherischia coli*
EBV	Epstein Barr Virus
EDC	(1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride)
ELISA	Enzyme-linked immunosorbant assay
EpCAM	Epithelial cell adhesion molecule
FU	Fluorescence units
FACS	Fluorescence-activated cell sorting
FADS	Fluorecence activated droplet sorting
FBS	Foetal bovine serum
Fc	Constant region of an antibody molecule
FDA	Food and Drug Adminstration
FITC	Fluoroscein isothiocyanate

FLISA	Fluorescence-linked immunosorbant assay
FRET	Förster/Fluorescence resonance energy transfer
GA	glutaraldehyde
GC	Germinal centre
GEHC	General Electric Healthcare
GFP	Green fluorescent protein
HA	Haemagglutinin
HACA	Human anti-chimeric antibody
HAH	Human antibodies and hybridomas
HAHA	Human anti-human antibody
HAMA	Human anti-mouse antibody
HARA	Human anti-rat antibody
HBS-EP	HEPES buffered saline with EDTA and P20 surfactant
HCl	Hydrochloric Acid
HIS	Histidine
HRP	Horseradish peroxidase
HSC	Human string content
HSL	Homoserine lactone
Ig	Immunoglobulin
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISAAC	immunoassay array on a chip
J	Joining
ka	Association constant (on-rate)
KB	Kilobase
KD	Affinity
kd	Dissociation constant (off-rate)
LB	Luria Bertani
LOD	Limit of detection
LOQ	Limit of Quantitation
mAb	Monoclonal antibody
MPO	Myeloperoxidase
mRNA	messenger RNA
MTA	Material transfer agreement
MW	Molecular weight
MWCO	Molecular weight cut-off
NAC	N-acyl-C12-HSL-BSA
NGS	Next generation sequencing
NTA	Nitrilotriacetic acid
OD	Optical density
ONX	Overnight Express
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PCR	Polymerase Chain Reaction
PDMS	polydimethylsiloxane
PEG	polyethylene glycol

PMBC	Peripheral blood mononuclear cell
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
RU	Response units
SB	Superbroth
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOC	Super optimal catabolites
SOE	Splicing by overlap extension
SPR	Surface plasmon resonance
TAE	Tris-acetic acid-EDTA
TEMED	Tetramethylethylenediamine
TEOS	tetraethyl orthosilicate
TMB	3,3', 5, 5'-Tetramethylbenzidine
TRA	Transferrin
V	Variable
VEGFA	Vascular endothelial growth factor A
V_H	Variable region of the heavy chain
V_L	Variable region of the light chain

List of units

°C	degrees Celsius
µg	microgram
µL	microlitre
µM	micromole
µm	micron
g	gram
h	hours
kDa	(kilo) Dalton
kg	kilogram
L	litre
M	Molar
mg	milligram
min	minute
mL	millilitre
mM	millimolar
nm	nanometer
nM	nanomolar
pL	picolitre
v/v	volume per unit volume
w/v	weight per unit volume

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Abstract

Thesis Title: Tailored bio-molecular screening methodologies for selection of improved antibody lead clones for diagnostic and therapeutic applications.

Candidate: Valerie Fitzgerald

The interrogation of highly diverse repertoires of heterogeneous cell populations on a single cell basis increases the likelihood that a cell with unique characteristics will be identified. This thesis describes the development of a new single cell analysis system comprising millions of bundled sub-nanolitre volume bio-incubation chambers for the identification and recovery of target specific antibody secreting cells (ASCs). This **Di**rect **C**lone **A**nalysis and **S**election **T**echnology (DiCAST) is a patented antibody discovery technology that can not only reduce the time and cost for their discovery but also has the potential to find candidates that nobody else can. This newly developed technology is applicable to screening both bacterial and mammalian antibody secreting cells. The implementation and feasibility of this platform in identifying target specific antibodies from bacterial and B cell libraries is investigated in this work. It offers a much enhanced alternative to traditional screening approaches in both fields as well as offering drastically improved throughput and multi-plexing capabilities when compared with competing single cell analysis technologies. In addition to development of this enhanced screening approach, complementary methods for automated (ELISA-based) and high throughput (SPR-based) screening of the selected antibodies were developed and deployed for routine use. These methods can be used to further characterise antibodies selected using DiCAST and aim to improve the overall screening workflow.

Chapter 1

Introduction

1.1 Section Overview

This section serves as a brief overview of the antibody discovery field and the ways in which both traditional and novel, emerging techniques are applied to generate best-in-class monoclonal antibodies. Each of the experimental chapters also includes a more detailed description of the specific areas addressed within the thesis work.

The key characteristics of antibodies, predominantly their high affinity and specificity for a binding partner has led to their utilisation in many varied applications in diagnostic, biotechnology and biomedical fields. The introduction of hybridoma technology in 1975 and the consequent ability to generate monoclonal antibodies (mAbs) led to a surge in antibody research and development. Since then there have been several additional developments in the field such as immortalisation of antigen-specific human B cells, recombinant display technologies, the production of transgenic animals and the ability to directly clone the immunoglobulin (Ig) genes from single B cells (Wang, 2011).

As a result, the field of monoclonal antibody discovery has developed into a thriving industry and had a profound impact by providing a diverse source of therapeutic and diagnostic and research reagents. The global demand for immunoassay testing was nearly $15.6 billion in 2011 and $16.3 billion in 2012. This market is expected to grow to $20.5 billion by 2017 (BCC Research, 2013) with research antibodies reaching $2 billion in 2013 and nearly $2.2 billion in 2014 and the market expected to reach nearly $3 billion in 2019 (BCC Research, 2015b).

Therapeutic use of mAbs has become a major part of treatments in various diseases including transplantation, oncology, autoimmune, cardiovascular, and infectious diseases. With 36 approved mAb products and an unprecedented number of mAb-based therapeutics in clinical trials mAbs have become the fastest growing class of biological drug (Love *et al*., 2013). Initial issues resulting from immunogenicity of murine mAbs have been reduced, or removed, by the development of methods to produce chimeric, humanised and fully human antibodies (Nissim & Chernajovsky, 2008). The global market for antibody drugs was worth nearly $63.4 billion in 2013. This market is expected to grow resulting in totals near $122.6 billion globally in 2019, according to BCC Research (BCC Research, 2015a).

1.2 A Brief History of Antibodies

The beginning of antibody discovery dates back as far as the 18th century when Edward Jenner essentially described vaccination when he showed that inoculating a boy with material from a cowpox pustule rendered him immune to the similar, but more serious, smallpox (Riedel, 2005). The earliest reference to antibodies was in 1890 when Emil von

Behring and Shibasabura Kitasato demonstrated that serum from animals immunised against diphtheria could cure infected animals. The potential of this work for therapy in humans was immediately recognisable and von Behring was awarded the Nobel Prize in 1901 (Kantha, 1991).

In 1900 Paul Ehrlich, regarded as one of the founders of modern immunology, proposed a model for an antibody molecule in which the antibody was branched and consisted of multiple sites for binding to foreign material, known as antigen, and for the activation of the complement pathway (Davies & Chacko, 1993). In 1948 Astrid Fagraeus detailed the involvement of plasma B cells in the generation of antibodies in her doctoral thesis and later in 1957 Frank Burnet and David Talmage described their clonal selection theory, which stated that a lymphocyte produces a specific antibody molecule that is determined prior to any interaction with an antigen (Burnet, 1957). By 1959 the molecular structure of antibodies had been elucidated and independently published by Gerald Edelman and Rodney Porter; they were later jointly awarded the Nobel Prize in 1972 (Edelman, 1959; Porter, 1959).

In 1975, Kohler and Milstein described the process of fusing a mouse myeloma cell with a B lymphocyte isolated from the spleen of a mouse that had been immunized with an antigen of interest, thereby allowing the *in vitro* synthesis of antigen-specific, monoclonal antibodies (Kohler & Milstein, 1975). Overcoming the limitations of their polyclonal counterparts, monoclonal antibodies allowed for an almost limitless and reproducible source of a specific antibody for use in research, diagnostics or therapeutics. This breakthrough essentially ushered in the modern era of antibody research and discovery.

In 1976, only a year after Kohler and Milstein's monoclonal antibody generation breakthrough, Susumu Tonegawa described the cloning of the first antibody gene (Tonegawa, 1976). In the 40 years since, the field of monoclonal antibody discovery and the tools which can be incorporated into the process have progressed to the point where the generation of monoclonal antibodies is a common practice, through a variety of methods, in laboratories worldwide.

1.3 Antibody-producing cells

Monoclonal antibody production typically begins with B lymphocytes. These can be obtained from varied sources, predominantly from lymphoid organs of immunised animals or from the peripheral blood of recently infected or immunised humans. B lymphocytes produce antibody to target and destroy invading foreign particles (e.g. bacteria, viruses) and protect the host.

The naïve B cell repertoire is thought to consist of 10^{10} potential antibody sequences (i.e. based on recombination of 51 potential V (variable) segments, 23 D (diversity) and 6 J (joining) segments in the V_H and 360 different V_L (lambda or kappa) sequences) (Dobson *et al*., 2015). Following V(D)J recombination in the bone marrow, the naïve B cells enter the peripheral B-cell pool. On encountering an antigen they produce low affinity IgM, but then become activated and undergo affinity maturation, through somatic hypermutation, in the germinal centres (i.e. spleen, lymph nodes, tonsils, and Peyer's patches) prior to differentiation into memory or plasma cells (Dobson *et al*., 2015). The stages of B cell development and their location and antibody status are detailed below in Table 1.1.

Table 1.1: Details for each of the stages of B cell development.

Stage	Principal Location	Antibody Status
Pre-B cell	Bone Marrow	V(D)J recombined IgM No mutation in V-regions
Naïve B cell	Peripheral Blood	Selected for productively recombined antibodies IgM No mutations in V genes Antibody affinities 10^5-10^6 M^{-1}
Memory B cell	Peripheral Blood	Selected for antigen binding 10X higher Ig mRNA levels IgM/IgG/IgA/IgE Somatic mutation in the V-regions Antibody affinities 10^7-10^{10} M^{-1}
Plasma B cell	Spleen/Bone Marrow	Selected for antigen binding 100X higher Ig mRNA levels IgM/IgG/IgA/IgE Somatic mutation in the V-regions Antibody affinities 10^7-10^{10} M^{-1}

There are three subsets of B lymphocyte of interest for antibody production. These are the memory B cells, plasmablasts and plasma cells all of which have high affinity, somatically-mutated B cell antigen receptors (BCRs) (Tiller, 2011).

1.3.1 Memory B cells

Following the initial interaction with an antigen, naïve B cells produce antigen-specific IgM, followed by IgG and finally germinal centre (GC) formation. Memory B cells respond in a rapid and efficient manner when exposed to the same antigen, even at low levels, a subsequent time and quickly differentiate into antibody-secreting cells which produce protective, affinity matured Ig (Takemori, 2014).

Memory B cells persist for a lifetime and are consistently present in the peripheral blood of previously infected or immunised individuals at low levels (Lanzavecchia *et al*, 2007). Their frequency can be increased by repeated exposure to the antigen. Memory B cells can be stimulated to differentiate into antibody-secreting cells *ex vivo* by addition of mitogens or a cocktail of cytokines (Agematsu *et al*., 1998; Tarlinton, 2014).

1.3.2 Antibody-secreting cells

Derived from memory B cells upon activation there are two recognised subsets within the antibody-secreting cells (ASCs); these are plasmablasts and plasma cells (both long and short-lived).

1.3.2.1 Plasmablasts

Plasmablasts are proliferating, antibody-secreting B cells which have been activated by an antigen. They are short-lived cells which appear in the peripheral blood and lymphoid organs for a short window-of-time during secondary response (i.e. in response to an immunological boost). They appear in the peripheral blood approximately 7 days after injection (Tarlinton, 2014).

1.3.2.2 Plasma cells

Plasma cells are terminally-differentiated B cells which secrete antibodies at a very high rate, but are short-lived (~1-3 days *ex vivo*) (Tarlinton, 2014). They are present in the blood in high numbers 6-10 days after immunisation and then migrate to the bone marrow where they remain as long-lived plasma cells (Lanzavecchia *et al*., 2007). Plasma cells represent <1% of lymphoid cells, but are responsible for the production of the vast majority of circulating IgG (Clargo *et al*., 2014).

1.4 Production of monoclonal antibodies

1.4.1 Hybridoma Technology

The hybridoma method involves the immortalisation of antibody producing B cells from the spleens of immunised animals by fusion with a myeloma cell line, allowing for the continuous production of antibodies with the desired specificity (Kohler & Milstein, 1975).

There are three basic fusion techniques used to generate hybridomas for the purpose of monoclonal antibody production. The first, and most commonly used method, involves the use of fusogenic chemical agents such as polyethylene glycol (PEG). While the exact mechanism has not been elucidated it is thought that adjacent cell membranes are driven

together by volume exclusion (Smith & Crowe, 2015). This approach is relatively cheap and simple but suffers from a very low fusion efficiency with efficiencies estimated at approximately 1 stable hybridoma from 100,000 starting B cells (Greenfield, 2014).

The second involves the use of fusogenic viruses such as murine parainfluenza virus type 1, more commonly known as Sendai virus. The viral nucleocapsid is introduced into the cytoplasm of the host cell through insertion of the viral fusion protein (i.e. F protein) into the cell membrane (Smith & Crowe, 2015). There are commercially available kits, which provide Sendai virus for fusion purposes. One particular producer, Cosmo Bio, has shown mouse hybridomas that obtain a fusion efficiency of 100% using specific medium additives and 96 % of cells go on to produce antibody compared with PEG fusion which gives results of 38 % and 9 %, respectively (http://www.cosmobio.co.jp/). This approach is only compatible with murine cells and as such would not be suitable for generation of a human hybridoma.

The third and generally accepted as most efficient method of cell fusion, electrical cytofusion, is suitable for all cell types and has been optimized for the purpose of human hybridoma generation (Smith & Crowe, 2015) . It involves the application of high-intensity electric field pulses to cells.This causes transient membrane permeabilisation. This method requires optimization in a number of steps, such as means to bring cells into contact with each other (i.e. chemical or physical) and the pulsing conditions used.

Hybridoma technology has several appealing features, including a relatively simple protocol, low costs with the principle being that the authentic sequence and pairing of antibody DNA from a natural B cell is preserved and allows for the expression of a naturally occurring full-length human mAb (Smith & Crowe, 2015). In terms of screening, hybridoma cells can be plated without the need for feeder cells, even at one cell per well in 96-well plates. Although this can result in slightly lower cloning efficiency, the surviving hybrids are very stable and usually good producers of mAbs.

The major disadvantage of the hybridoma method is low fusion efficiency, resulting in a limited number of mAbs produced per experiment. This principal limitation in hybridoma screening had, in the past, tended to be concerned with screening throughput and as such the low efficiency of fusion was not extremely pressing. However, as techniques become available to process far greater numbers of hybridomas, a means to increase efficiency is needed (Greenfield, 2014). Stimulation of B cells substantially increases the fusion efficiency and development of mAbs. The most commonly used method is the infection and transformation of B cells by Epstein Barr Virus (EBV), the details of which will be discussed in the subsequent section.

1.4.2 Immortalization of the human B cells by Epstein-Barr virus (EBV)

The use of Epstein-Barr Virus (EBV) was invented by Steinitz *et al*. to generate a human monoclonal antibody specific to a synthetic hapten (NNP.20) (Steinitz *et al*., 1977). It utilises the supernatant of the B95-8 marmoset cell line which was established by Miller *et al*. The culture supernatant contains EBV which infects the B cells and transforms them in culture over a 1-2 week period (Miller *et al*., 1972).

Similar to the hybridoma process, transformation with EBV is inefficient and the transformed cells frequently suffer from genomic instability and loss of antibody production that could hamper antibody discovery (Kwakkenbos *et al*., 2014). Additionally the EBV-transformed cells are not cancerous cells and cannot continue to grow and produce antibodies indefinitely due to cell senescence. While this could be overcome by generating a large cell bank of the EBV-transformed cells early in the process, cells are often subjected to hybridoma fusion to produce the desired cell line for long-term culture and antibody production (Gorny, 2012).

A subsequent finding has shown that the activation of B cells with an oligodeoxynucleotide containing the CpG motif, which acts as an agonist to Toll-like receptor 9, can greatly enhance the efficiency of EBV transformation (i.e. transformation is increased from 1%–2% to 30%–100% of B lymphocytes) (Traggiai *et al*., 2004).

1.4.3 Combinatorial Libraries

The challenge of circumventing the limitations of the hybridoma technique coupled with the requirement for large quantities of high quality antibodies led to the emergence of methods for their generation recombinantly. These antibody libraries consist of a large number (i.e. up to billions) of heavy and light chains which have been generated by PCR from the mRNA of B cells. They can generated from a wide array of animals (e.g. mouse, rat, rabbit, camelid, shark and human among others) once enough molecular information is available for the given species to allow amplification of antibody regions (Berry & Popkov, 2015).They can be designated as either immune, naïve or synthetic.

1.4.3.1 Types of combinatorial library

1.4.3.1.1 Immune libraries

Immune libraries are generated from individuals who have mounted an antibody response following exposure to an immunogen of interest. Exposure may have occurred through active immunisation, as is the case with laboratory animals, or by natural means (e.g. exposure to an infectious agent, pathogen or toxin, as well as immune response to diseases such as cancer or those which affect the immune system). Immune libraries provide an

enriched source of antigen-specific, affinity-matured antibodies by harnessing the natural *in vivo* mechanisms of the host (Berry & Popkov, 2015). Immune libraries typically do, however, require the immunisation of animals ahead of time and with growing concerns into the ethics and procedures over extensive use of animals for experimentation the use of naïve and synthetic libraries, discussed below, are highly attractive (Festing *et al*., 1998; Leenaars & Hendriksen, 2005).

1.4.3.1.2 Naïve libraries

Naïve libraries are generated from B cells of individuals who have had no known prior exposure to a particular immunogen, which theoretically means an antibody to a vast range of targets could be isolated from a large (i.e. $>10^{10}$ library size), diverse 'single-pot' library without the need for immunisation and library construction for each different antigen of interest (Persson, 2009). The most common source of B cells is peripheral blood of multiple human donors, often from blood banks (Pansri *et al*., 2009), but theoretically naïve libraries could be generated from any animal if required (Berry & Popkov, 2015). Due to the lack of natural affinity maturation processes in the host, the isolated antibodies often require improvement by employing available mutagenesis strategies, some of which are discussed later in this chapter. Nevertheless, the use of naïve libraries has been shown to identify numerous novel antibodies, against varied targets, and offers a very attractive alternative to the use of immune libraries (Edwards *et al*., 2003; Pershad *et al*., 2010).

1.4.3.1.3 Synthetic libraries

Synthetic antibody libraries are based on synthetic DNA sequences and involve introduction of diversity in the CDRs of germline V-gene segments (Pande *et al*., 2010). While particularly promising in the fact that they offer the ability to strategically design very large antibody libraries with desired properties (e.g. low immunogenicity or high expression) their uptake has not been as great as their alternatives, most likely owing to the complexity involved in their design (Berry & Popkov, 2015). Several companies such as Medimmune, Genentech and Morphosys AG have generated very large synthetic libraries (i.e. up to 10^{11} library size) and isolated antibodies in the nano- and picomolar range (Knappik *et al*., 2000; Ponsel *et al*., 2011).

1.4.3.2 Methods for screening of combinatorial libraries

Commonly described as display methods, an array of approaches for generation and screening of these combinatorial libraries have now become commonplace in modern laboratories. They offer an attractive approach as modifications are easily introduced in the primary antibody sequence leading to affinity maturations, often resulting in higher affinity

for binding to the antigen, generation of fusion proteins and addition of detection and purification tags. Additionally, the use of recombinant technology allows us to overcome what is termed the "affinity ceiling" of antibodies produced *in vivo* and provide libraries of higher diversity than those generated in the natural immune response (Hearty & O'Kennedy, 2011). Display methods afford us the ability to choose those ligands which have the desired biological properties and permits synthesis of "tailor-made" antibodies for use in diagnosis, immunotherapy or for immunoassay development. The predominant processes which include phage display, yeast display, bacterial display, ribosome display and mRNA display are discussed below. A comparison of methods for the production of monoclonal antibodies, mentioned in this chapter, is represented in Figure 1.1.

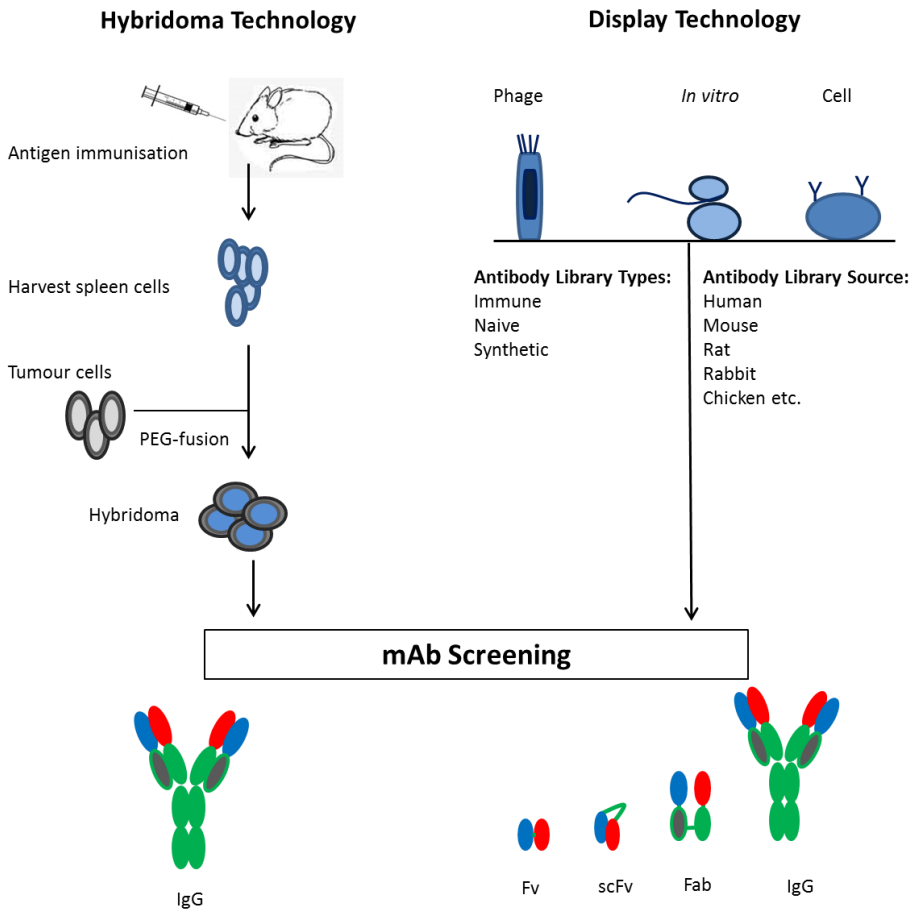

Figure 1.1: Schematic representation of the different approaches for producing monoclonal antibodies*. The traditional hybridoma technology for obtaining mouse antibodies is shown on the left. The right shows the main procedures for obtaining antibodies through display systems (phage, in vitro and cell display). With display technologies, antibodies can be developed from immune, naïve, or synthetic libraries and derived from different sources. There are also a number of different antibody fragments which can be produced. Image adapted from* (Aires da Silva *et al*., 2008).

1.4.3.2.1 Phage Display

Phage display was first described, in 1985, when George Smith demonstrated that the linkage between phenotype and genotype could be established in filamentous bacteriophage, which is illustrated in Figure 1.2 (Barbas *et al*., 2001). Following on from this discovery, in the early 1990s several laboratories described phage display systems for antibody fragments (McCafferty *et al*., 1990) (Barbas *et al*., 1991). In terms of antibody generation, antibody genes are linked to the amino terminus of the phage minor coat protein pIII. When expressed, the encoded fusion product is incorporated into the mature phage particle during normal phage biogenesis. The resulting phage particle expresses antibody on its surface and contains the antibody encoding gene (Aires da Silva *et al*., 2008).

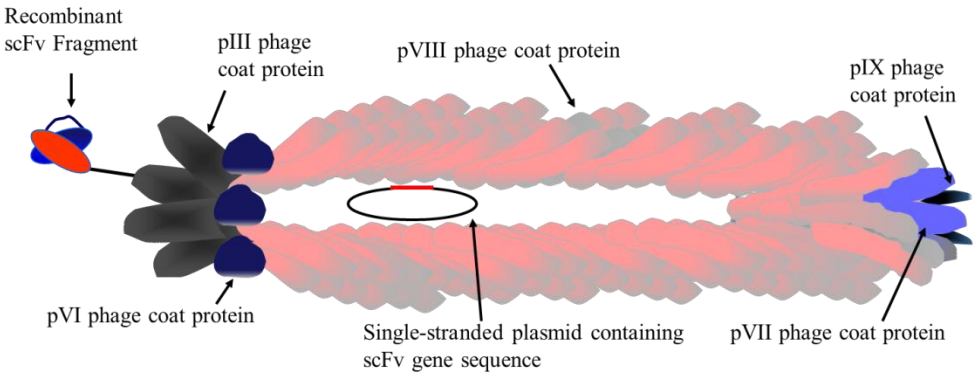

Figure 1.2: Display of a scFv antibody on a filamentous bacteriophage particle*. The location of the capsid proteins and the scFv antibody are clearly indicated by arrows. Filamentous phage particles are covered by approximately 3000 copies of the small major coat protein pVIII. Few copies of the minor coat proteins pIII and pVI are displayed at one extremity of the phage particle, while pVII and pIX proteins are located at the other extremity. The scFv is displayed as a fusion to g3p (pIII) protein at the tip of the phage. The phage particles may incorporate either pIII derived from the helper phage or from the scFv fusion protein and, therefore, in reality, the scFv is not fused to all pIII protein molecules, allowing the phage to retain its ability to infect bacteria.*

Large libraries containing millions of different antibodies can be obtained by force cloning in *Escherichia coli*. From these repertoires, phage carrying specific antibodies can be isolated by repeated cycles of selection on the antigen of interest, each of which involves binding, washing, elution and amplification (Hoogenboom, 2002). A more detailed description of considerations in selection processes is provided elsewhere by Conroy *et al*.,(2009).

1.4.3.2.2 Cell Display Methods

Cell display methods employ various cell systems, either prokaryotic or eukaryotic, for expression and selection of antibody fragments demonstrating desirable characteristics.

Prior to the introduction of phage display technologies, methods to display antibodies on bacterial cells had been demonstrated. The method was hindered by the lack of an efficient screening method but with the introduction of high-speed flow cytometry technologies, namely fluorescence-activated cell sorting (FACS) its use has grown (Aires da Silva *et al*., 2008). While Gram-positive bacteria (e.g. *Bacillus brevis* and *Bacillus subtilus*), which secrete proteins directly into the medium due to the lack of cell wall, have been exploited to produce and purify different antibody fragments, Gram-negative bacteria (e.g. *Escherichia coli*) are more commonly used (Schirrmann *et al*. 2008). Because the expression of recombinant antibodies in the cytoplasm principally results in non-functional aggregates, most are produced in the periplasm of the bacteria using N-terminal leader sequences (Schirrmann *et al*., 2008). This was enabled by the work of Skerra and Pluckthun, who described the first expression of a functional antibody fragment in *E.coli*, and observed that directing the fragment to the oxidizing environment of the periplasmic space allowed for the correct formation of disulfide bonds (Skerra & Plückthun, 1988). The expressed antibody fragment can then be isolated from the periplasmic fraction or in some cases from the culture supernatant (Schirrmann *et al*., 2008). Although bacterial display theoretically holds certain advantages over phage display (e.g. less technically demanding) a direct comparison of the methods, by Lunder *et al*., found that phage display tends to be more successful in identifying ligands of interest (Lunder *et al*., 2005). An additional disadvantage to prokaryotic expression is their inability to provide post-translational modifications such as glycosylation (Altshuler *et al*., 2011).

Using eukaryotic hosts a more sophisticated protein secretion and folding apparatus is provided, as well as the capacity for post-translational modifications, when compared to prokaryotes. While mammalian cells are still the system of choice for the production of antibodies the disadvantages associated with their use (which include a requirement for highly specialised dedicated facilities, high cost and lengthy production times) mean that other systems have been investigated. Yeast, filamentous fungi, insect cells along with transgenic plants and animals have all been used in the production of recombinant antibodies (Schirrmann *et al*., 2008). Perhaps the most widely used eukaryotic display technology is yeast display. Antibody fragments are displayed on the cell surface as part of a hybrid protein that is made up of the antibody or antibody fragment and the Aga2p agglutinin subunit, which is covalently linked by two disulfide bonds to Aga1 agglutinin in the yeast

cell wall (Altshuler *et al*., 2011). The use of yeast technology combines the ease of culture seen with prokaryotic systems with the capacity for folding and secretion of eukaryotic systems. Other benefits of the system include rapid and quantitative screening facilitated by FACS and convenient evaluation of characteristics such as affinity without the need for soluble expression and purification of each clone of interest (Gai & Wittrup, 2007).

1.4.3.2.3 '*In vitro*' display technologies

The predominant technologies described as '*In vitro*' display methods are ribosome display and mRNA display. These methods are outlined in Figure 1.3.

During ribosome display, DNA encoding the library is transcribed *in vitro*. The resulting mRNA lacks a stop codon, giving rise to linked mRNA-ribosome-protein complexes during *in vitro* translation. These can be stabilised and directly used for selection against an antigen of interest. The mRNA incorporated in bound complexes is eluted and purified. Reverse transcription polymerase chain reaction can introduce mutations and yields a DNA pool enriched for binders that can be used for the next iteration (Lipovsek & Plückthun, 2004).

In the case of mRNA display, covalent mRNA-protein complexes are created by ligation of a DNA linker with a small adaptor molecule, puromycin, to create the *in-vitro*-transcribed mRNA, which lacks a stop codon. The mRNA is translated *in vitro*, and the ribosome stalls at the RNA-DNA junction. Puromycin then binds the ribosomal A-site and the nascent polypeptide is thereby transferred to puromycin, as if it were an aminoacyl-tRNA. The resulting covalently linked mRNA-protein complex is isolated, reverse-transcribed and used in selection procedures. The DNA strand is recovered from target-bound complexes by hydrolysing the complementary mRNA at high pH and then amplified by PCR (Lipovsek & Plückthun, 2004).

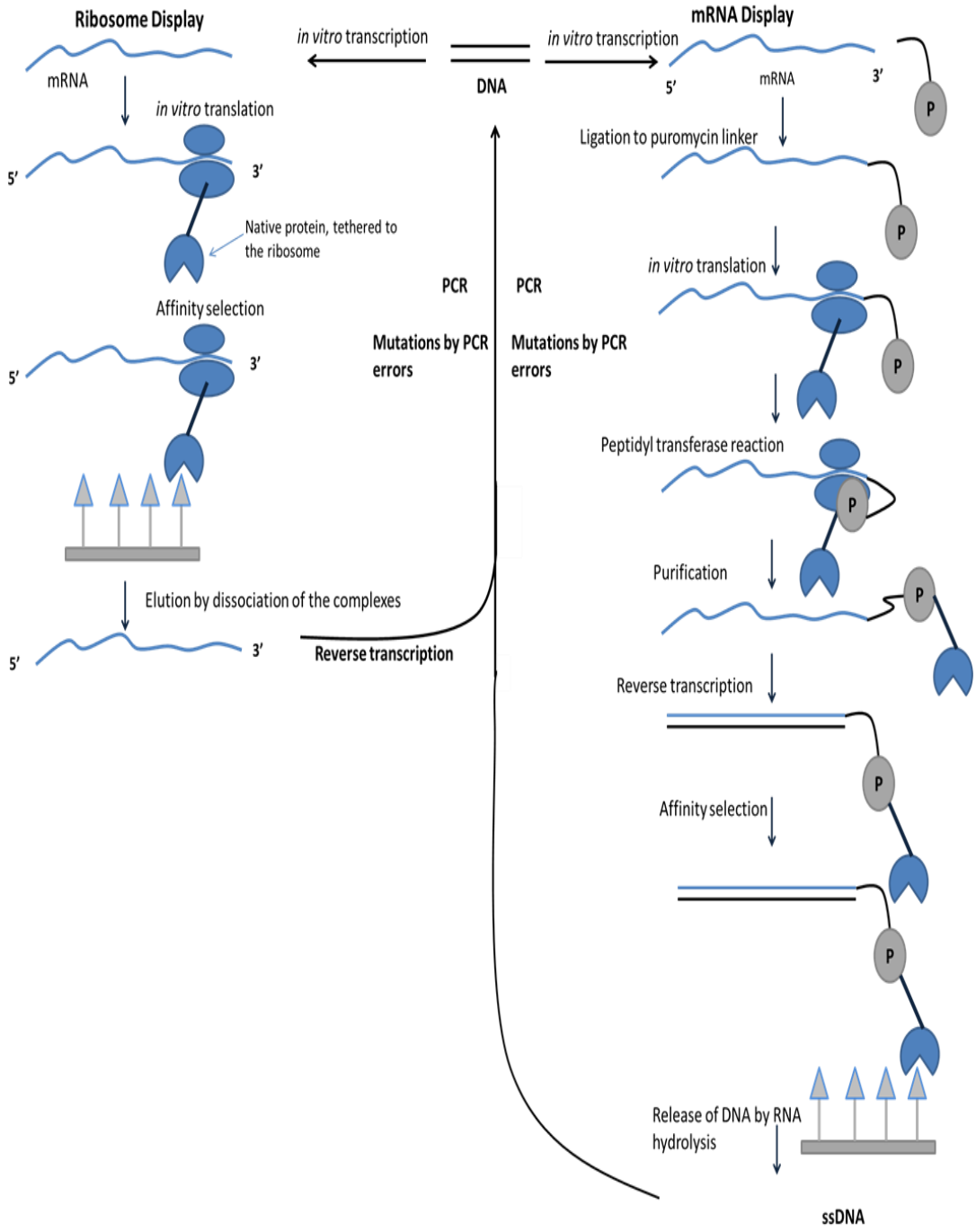

Figure 1.3: *In vitro display methods. Ribosome display and mRNA display. Image amended from* (Lipovsek & Plückthun, 2004).

Because they are performed entirely '*in vitro*' these methods hold two advantages over other competing technologies. The first depends on the fact that library size can be much larger (10^{12}-10^{14}, Aires da Silva *et al.*, 2008) as it does not depend on the transformation efficiency of competent bacteria but rather on the amount of ribosomes and different mRNA molecules present. Secondly, there is the potential to introduce random mutations after each selection

round by introducing randomization steps, using non-proofreading enzymes, introduced after RT-PCR (Amstutz *et al*., 2007).

1.5 Strategies for improving recombinant antibodies

Conventional methods of antibody production such as polyclonal and hybridoma production are severely limited in their ability for improvement or modification. Recombinant antibody technology has a clear advantage over traditional antibody production in this respect. Not only can recombinant antibodies be "tailored" through genetic modification for a particular application, they can be "retailored" and improved to keep up with changing requirements. An excellent example of this is provided by Korpimäki and colleagues, at the University of Turku, where they converted an anti-sulfonamide mAb obtained from a hybridoma and modified its specificity to bind up to 15 sulfonamide derivatives, significantly improving the capabilities of the immunoassay (Bienenmann-Ploum *et al*., 2005; Korpimäki *et al*., 2003; Korpimäki *et al*., 2002). Altering the amino acid sequence of antibodies using gene engineering allows for an improvement in characteristics such as affinity, specificity, stability and immunogenicity (Altshuler *et al*., 2011). Mutagenesis strategies can be divided into the following categories, random mutagenesis, site-directed mutagenesis and DNA recombination.

1.5.1 Random mutagenesis

Random mutagenesis involves the random change of any amino acid in the antibody gene sequence. Perhaps the most well-known method for random mutagenesis is error-prone PCR. Error-prone PCR employs low-fidelity polymerases and/or conditions which favour mis-incorporation of nucleotides (e.g. presence of Mn^{+2}, limiting amounts of dATP and dCTP) to create libraries of random mutants (Sheedy *et al*., 2007; Maynard & Georgiou, 2000). Alternatively a bacterial mutator strain, for example MutD5, can be used to introduce single base substitutions at high frequency compared to normal cells (Sheedy *et al*., 2007). These strains have an impaired mis-match repair system and can have mutation frequencies in the region of 0.2-1 substitutions per 1000 nucleotides per cycle of propagation (Maynard & Georgiou, 2000). The major advantage of random mutagenesis is that it does not require any information about the antibody structure or the interacting amino acids. However, the disadvantage lies in the fact that mutant libraries often have a large proportion of functionally inactive mutants (Altshuler *et al*., 2011).

1.5.2 Site-directed mutagenesis

Site-directed mutagenesis, in contrast to random mutagenesis, introduces mutations at chosen positions in the antibody gene sequence. Site-directed mutagenesis can be used

alongside crystallography studies to elucidate information about the active site and in this way perhaps provide an insight into antigen binding (Sheedy *et al*., 2007). The introduction of "*in silico*" techniques adds weight to this in that it could potentially allow the simulation of mutation and its consequences at specific points. This would allow for a more rational selection of those mutations, which seem to confer positive effects on antigen-antibody binding, for experimental manipulations (Altshuler *et al*., 2011).

1.5.3 DNA recombination

DNA recombination, also known as antibody shuffling, somewhat mimics somatic hypermutation and as a result is thought to be more efficient than random or directed mutagenesis approaches (Sheedy *et al*., 2007). Shuffling of antibody genes can be carried out in a number of ways. The first is chain shuffling whereby the heavy and light chains of antibodies, selected as positive binders, from an immune library, are recombined. Fitzgerald *et al*. describe a 185-fold increase in sensitivity of a halfuginone-specific scFv using chain shuffling and its subsequent incorporation into an immunoassay for detection in samples (Fitzgerald *et al*., 2011). The second, deemed DNA shuffling, involves digestion of the antibody DNA with DNAse 1 and its subsequent reassembly and amplification by PCR. A third method called staggered extension process or StEP is also reliant on PCR and involves template switching caused by shortened extension times. This shuffles portions of the parent antibody genes (Sheedy *et al*., 2007).

1.6 Strategies for reducing immunogenicity of monoclonal antibodies

Even though the first monoclonal antibody-based human therapeutic was from a murine source (i.e. muronomab-CD3 under the trade name ORTHOCLONE OKT3) concerns over their potential to be highly immunogenic in patients led to the development of strategies to minimise the non-human portion of monoclonal antibodies for therapy (Kellermann & Green, 2002). The category from which a monoclonal antibody drug has been derived can be garnered from the suffix of their generic mAb name (i.e. –omab is of murine origin, -ximab is chimeric, -zumab is humanised and –umab fully human) (Lee & Owen, 2012).

1.6.1 Chimerisation of monoclonal antibodies

Chimerisation, first described in 1984, involves the use of molecular biology to enable grafting of the antigen-specific variable domains of the selected murine antibody on to the human antibody constant domains (Ribatti, 2014). Chimeric antibodies are approximately 70% human, making them considerably less immunogenic, and have a human Fc portion which means they can interact with human effector cells and can elicit activation of

complement (Chames *et al*., 2009). A number of marketed monoclonal antibody therapeutics generated by chimerisation include abciximab (REOPRO®), rituximab (RITUXAN® or MABTHERA®) and infliximab (REMICADE®) (Zhang, 2012).

While this approach reduced the proportion of murine content in the antibody, chimeric antibodies can still be quite immunogenic (Kellermann & Green, 2002) and other strategies to further reduce the murine content of the monoclonal antibodies generated were soon developed.

1.6.2 Humanisation of monoclonal antibodies

Methods for humanisation of monoclonal antibodies, which are approximately 85-90% of human origin (Chames *et al*., 2009), can be generally divided into two approaches. The first are deemed the rational methods which employ a design cycle and aim to either minimise the non-human content or to simplify the design cycle. Examples of rational methods for humanisation include complementarity determining region (CDR) grafting, specificity determining residue (SDR) grafting, superhumanisation, resurfacing or human string content (HSC) optimisation (Lu *et al*., 2012). While advantageous because they retain the full Ig format, which can help to maintain important characteristics of the antibody, these approaches require a detailed knowledge of the sequence and structure of the selected antibody in order to be made possible.

The second are known as empirical methods, which involve the generation and screening of libraries for antibodies with desired properties, and include methods such as guided selection, humaneering, CDR repair and framework repair and shuffling. These approaches are often considered disadvantageous because the libraries generated are typically fragment libraries (i.e. scFv or Fab) and require conversion into a final Ig format (Lu *et al*., 2012).

While these approaches have been widely used and have resulted in a number of drugs reaching the market, including omalizumab (XOLAIR®), trastuzumab (HERCEPTIN®) and bevacizumab (AVASTIN®) (Zhang, 2012), the potential to alter affinity by introducing a large degree of change in the CDR regions means that these antibodies still maintain a high proportion of non-human sequence (Bernett *et al*., 2010).

1.6.3 Fully Human monoclonal antibody production

Human monoclonal antibodies are desirable because they are indistinguishable from endogenous antibodies and as such are thought to have enhanced pharmacokinetic and pharmacodynamics properties when compared to chimeric and humanised antibodies (Beerli & Rader, 2010).

Monoclonal antibodies which are designated as "fully human" typically originate from one of two different sources, transgenic mice/rats or from recombinant libraries (Bernett *et al*., 2010). Those antibodies selected from naïve recombinant libraries will often need to go through several iterations of improvement to reach the desired affinity (Lu *et al*., 2012). Nevertheless several human monoclonal drugs generated in this manner have been developed into therapeutics, e.g. adalimumab (HUMIRA®), belimumab (BENLYSTA®) (Zhang, 2012).

Transgenic rodents have been also been used to generate fully human antibodies by effectively disabling the production of the animal's own murine antibodies and replacing that function with human antibody genes (Ribatti, 2014). These mice have been shown to be capable of producing high-affinity antibodies with completely human sequences, by performing VDJ recombination and somatic hypermutation as they normally would (Maynard & Georgiou, 2000). The antibodies are generated by immunisation of these transgenic animals and subsequent generation of libraries, or by producing hybridomas.

Initially a reduced immunogenic response seen in transgenic animals meant that more animals were required to generate the diversity required for any one screening campaign (Lee & Owen, 2012) but several companies (e.g. KyMab, OMT and Ablexis among others) have responded to this shortcoming and are now generating improved transgenic strains of rodents which incorporate improvements such as retaining a greater amount of murine *cis* elements to allow more efficient B cell receptor assembly and signalling, as well as including a more complete human Ig repertoire so that responses seen are similar to those in wild-type mice (Lu *et al*., 2012). Therapeutics on the market generated in this way include ofatumumab (ARZERRA®) and panitumumab (VECTIBIX®) among others.

Of course the other potential source of fully human monoclonal antibodies would be to generate antibodies from human sources. While active immunisation of humans is obviously not possible, for ethical reasons, the use of B cells from patients recovering from a disease or those having been exposed to an infectious agent could be utilised (Aires da Silva *et al*., 2008). Moreover, it is not possible to easily access the secondary lymphoid tissues (i.e. the spleen) as would be the case when generating antibodies using immunised animals and while tonsil tissue has been utilised (Duvall *et al*., 2011), it would not typically coincide with an immune response of interest. Accordingly the main source of human B cells is peripheral blood, ideally from recently vaccinated or infected patients so that the frequency of circulating B cells is sufficient to proceed.

1.7 Rationale for moving towards human B cells as source of monoclonal antibodies

The advent of display technologies for the production of antibody fragments has greatly extended their field of application. In addition, gene engineering has allowed for the preparation of antibodies to non-immunogenic or toxic antigens which could not be generated using typical hybridoma technology. Coupled with this, the ability to evolve these antibodies by genetic means, to include more desirable traits (e.g. increased affinity, tags for detection and purification), makes recombinant antibody production an extremely powerful tool. There are several explanations given as to why antibodies generated by recombinant means have not superseded those produced by traditional hybridoma technology. These include the fact that the phage display platform was covered by a number of patents making it costly, the lack of knowledge about the technology and the apparent shortcomings in expertise and library availability and perceived issues with expression of the recombinant fragments (Bradbury *et al*., 2011). There is no universal method which can reliably provide high yields of recombinant antibody and this stems from the complexity and difference in each antibody-based molecule (Schirrmann *et al*., 2008).

The use of immunised animals in combination with the advanced technologies, discussed earlier in this chapter, has led to the successful production and employment of numerous chimeric, humanised and human monoclonal antibodies. These techniques will continue to be utilised to generate antibodies and indeed there are hundreds of new antibody candidates at various stages in clinical trials, worldwide (Zhang, 2012). Despite these successes, the fact remains that experts in the field maintain the opinion that the ideal source for monoclonal therapeutic antibodies is human B cells (Duvall & Fiorini, 2014). The immunogenicity of human and humanised antibodies have been found to be less than that of non-human and chimeric mAbs, which can elicit human anti-mouse antibody (HAMA), human anti-rat antibody (HARA) and human anti-chimeric antibody (HACA) responses. However human anti-human antibody (HAHA) responses have been observed in certain cases, mainly attributed to differences (e.g. in idiotype, allotype or glycosylation patterns) between the generated mAbs and those which would occur naturally in humans (Beerli & Rader, 2010).

A number of advantages that exist when using human B cells as the source material for the production of monoclonal antibodies include the fact that the antibodies are fully human and have minimal chance of cross reacting with self-antigens as they have been selected in the human system. Furthermore, humans can mount a powerful immune response and can direct it against the components, of the invading pathogen, that confer infection and virulence which are often not seen by immune systems of other hosts (Lanzavecchia *et al*., 2006).

Methods which have been applied to the use of human B cells, both naïve and immune, for monoclonal antibody generation include human B cell library generation (i.e. EBV immortalisation or hybridoma generation from naïve B cells or B cells of individuals who have been naturally exposed or vaccinated against a particular agent or have recovered from a disease), use of display technologies (i.e. generation of a recombinant libraries using the isolated B cells, with naïve or immune, and subsequent screening using display approaches) and the isolation and screening of single cells (Duvall & Fiorini, 2014).

The generation of human B cell libraries was initially hindered by the low efficiencies for both hybridoma fusion and EBV transformation, which have been discussed in sections 1.4.1 and 1.4.2, respectively. This level of inefficiency may be considered acceptable when dealing with the splenocytes isolated from an immunised animal (i.e. typically around 10^8 cells from personal experience) but because of the low frequency of B lymphocytes in blood (i.e. generally accepted levels in 10 mL of peripheral blood from a non-immunised donor are approximately 1×10^7 peripheral blood mononuclear cells (PBMCs) of which B lymphocytes constitute roughly 15%) (Miyahira, 2012) the inefficiencies would likely result in failure and unacceptable loss of precious cells.

Furthermore, until recently there were very few suitable fusion partners available to allow production of human hybridomas. Thankfully there are now several options available to allow successful fusion with human B cells (e.g. SHM-D3327 and HMMA 2.5 cell lines from ATCC) (Gorny, 2012). In addition, the simplest strategy for immortalisation of human B cells, EBV transformation, has recently seen the identification of methods to boost efficiency from 1-2% to 30-100%, as previously mentioned in Section 1.4.2. This will allow for B cells to be expanded *in vitro* prior to and after screening, facilitating cell banking and further characterisation (Smith & Crowe, 2015).

Although the use of display methods has proven useful in this area, the ability to maintain the natural V_HV_L pairing is highly desirable and as such has led to the development of techniques which allow for investigations of cells on an individual basis. In the next section, traditional approaches will be discussed and compared with these rapidly developing single cell techniques.

Single cell PCR, which could remove the need for generation of combinatorial libraries or long-lived cell lines, is an approach which holds a great degree of promise in the area of human mAb discovery. Issues that require attention relate to the technological issue of capturing enough genetic material from a single cell to allow amplification of both of the variable regions. Memory B cells, which persist in circulation for many years, contain very little RNA and, conversely, plasma cells which contain ample amounts of RNA appear in the

peripheral blood for a very narrow window of time (i.e. 6-10 days following exposure to a particular agent) (Clargo *et al*., 2014). The B cells can be enriched from peripheral blood by FACS or using bead-based isolation approaches, which are commercially available from numerous companies, to facilitate the process. Additionally, a number of elegant techniques which have been designed to identify antigen-specific single B cells have emerged in the field recently and they are discussed later in this chapter. By coupling these technologies with single cell PCR there is the potential to overcome all of the limitations that are associated with other methods.

1.8 Screening approaches for monoclonal antibody discovery

The continued improvement of the efficiencies of hybridoma fusion and EBV transformation and the generation of large combinatorial libraries has now become commonplace. The need for screening approaches which can comprehensively screen such a large, heterogeneous population of cells has resulted in a surge in modifications to existing technologies and also in novel antibody screening platforms.

While traditional approaches have tended to employ bulk measurements, incorporating multiple iterations of screening, before finally identifying the single cell producing the antibody of interest there has been a shift towards single cell analysis. This means that cells that may have been overgrown by fast-growing clones are now identified and not masked in the larger culture (Greenfield, 2014; Smith & Crowe, 2015).

Some of the most common approaches (e.g. microtitre plate-based screening and FACS) alongside some of the new, emerging platforms for single cell antibody discovery are described below.

1.8.1 Cloning by limiting dilution

The screening of mammalian cells in typical antibody discovery approaches (e.g. hybridoma cells, non-fused B cells or EBV transformed B cells) relies on the employment of cloning by limiting dilution. Cloning by limiting dilution is a method based on the Poisson distribution. Dilution of cells to an appropriate number per well can maximize the proportion of wells that contain one single clone (Fuller *et al*., 2001). It involves the segregation of cells in the wells of microtitre plates (96 or 384 well) and screening of the supernatants for positive binding to the required antigen, as shown in Figure 1.4. Depending on the original seeding level, several rounds of cloning may be required to attain clonality of the cells. In order to address the entire population of cells they are often plated with numerous (5-5000) cells per well. There is a balance to be met between overloading wells and the number of plates that it is feasible to screen. By adding too many cells to a well an antigen-specific cell of interest may

be overlooked due to its presence being masked by fast-growing non-productive clones. Conversely some cells do not survive well on a single cell basis for an extended period of time and may require the presence of feeder cells, growth supplements, or conditioned medium and single cell plating will result in a higher number of plates to process. Ultimately the facilities available, within a particular laboratory, may become the limiting factor and numbers screened will depend on the levels of automation available.

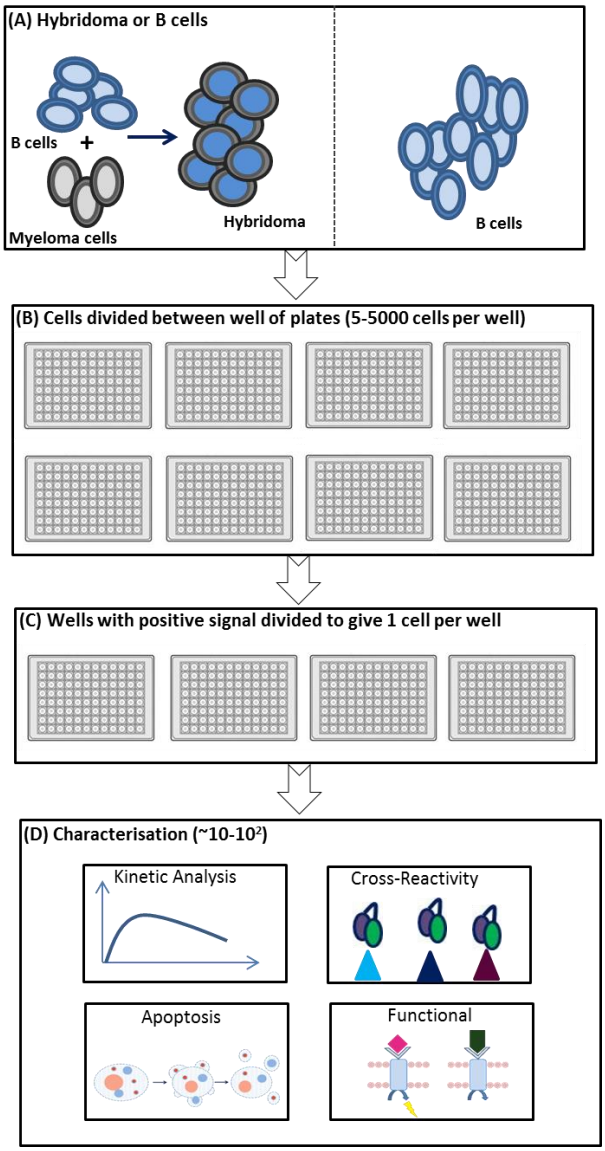

Figure 1.4: Antibody discovery process for mammalian cells. *The process of cloning by limiting dilution, for both hybridomas and B cells.* ***(A)*** *Cells are fused with a myeloma cell line (hybridoma) or remain unmodified for screening (B cells)* ***(B)*** *Cells are divided into the wells of microtitre plates at a given frequency, typically 1-5000 depending on the level of automation available. Cells are allowed to incubate for up to two weeks and then the supernatants are screened for a desired response (e.g. binding)* ***(C)*** *Wells which exhibit a positive response in initial tests are then diluted and segregated further in the wells of a microtitre plate until single cell occupancy is achieved* ***(D)*** *A number of positives are taken forward for characterisation (e.g. kinetic analysis by Biacore, cross reactivity by ELISA and functional analysis by numerous bioassays (e.g. apoptosis, agonist, antagonist).*

1.8.2 Fluorescence activated cell sorting (FACS)

Fluorescence activated cell sorting (FACS) is a modified version of flow cytometry, developed, in Stanford University by Len Herzenberg and his research group, in the 1960's (Lanier, 2014). Cells which have been stained with fluorescently-labelled antibodies, antigens or other reagents determining a property of interest are then held in suspension and the liquid broken into droplets by application of vibration. Prior to breaking into droplets the flow of liquid containing the cells passes through a measuring station where the fluorescent properties of each cell are analysed. An electrical charging ring is present at the point where the droplets are formed and a charge is assigned (i.e. positive, negative or neutral) to each droplet based on the previously determined fluorescent measurement. The droplets are then sorted and collected based on their charge as they pass through an electrostatic field, as shown in Figure 1.5 (Jahan-Tigh *et al*.,2012).

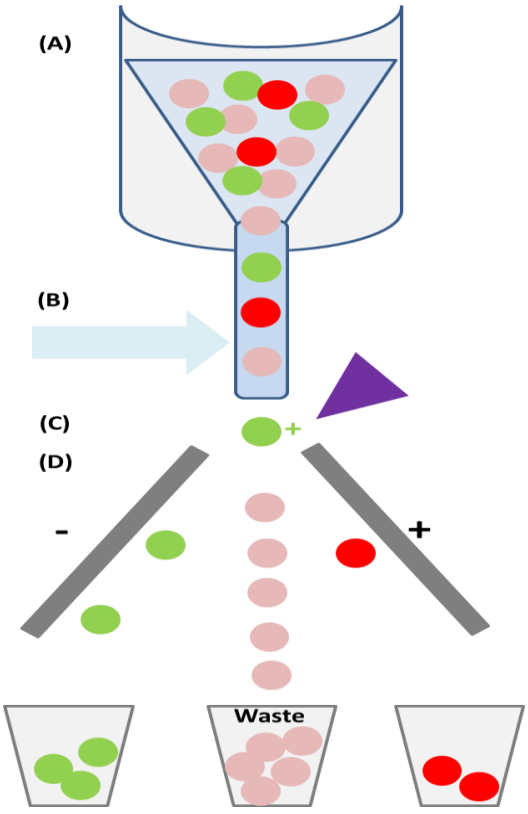

Figure 1.5: Schematic of the principle of fluorescence activated cell sorting (FACS). (A) *Hydrodynamic focusing and vibration by a transducer produces a stream that breaks into droplets, containing cells which have been stained with fluorescently-labelled antibodies* ***(B)*** *Droplets are interrogated by the laser and the signals are processed to give the "sort decision" (i.e. if the droplet contains a green fluorescent cell it will be charged and sorted accordingly)* ***(C)*** *A charge is applied, if appropriate, as the droplet breaks off.* ***(D)*** *The charged droplet containing the cell of interest is directed by the electrostatic field into the designated collection tube.*

1.8.3 Next generation sequencing

Next generation sequencing (NGS) or "deep sequencing" has been employed in the area of antibody discovery to complement, and indeed entirely eliminate, the need for traditional screening. It involves in depth sequence analysis of the variable region gene repertoires from a heterogeneous population of cells (e.g. B lymphocytes from an immunised animal or members of a combinatorial library) and the use of bioinformatics to allow pairing of the appropriate V_H and V_L regions, based on their frequency in the population (Haessler & Reddy, 2014). The antibodies can then be synthesised, expressed and tested in terms of antigen binding or other required properties.

The increase in throughput and the decrease in cost for this type of sequencing analysis that has occurred in the past number of years means that it is now possible to determine the sequence of millions of different variable regions in a short space of time (Dobson *et al*., 2015).

One drawback of this approach is that the natural V_H/V_L pairings are not definitively identified and are instead elucidated from the frequencies of each in the particular population (Mathonet & Ullman, 2013). Other limitations include the costs associated with processing of such a large number of samples and the fact that the error rate is higher than that observed in Sanger sequencing (Mathonet & Ullman, 2013).

While NGS cannot yet address the entire potential human repertoire (i.e. theoretically 10^{11}) its use in conjunction with other techniques can be used to provide a more streamlined and informed antibody discovery process. The use of NGS to perform quality control on combinatorial libraries could serve to provide a more accurate evaluation of diversity of a particular library in place of simply stating the number of transformants as is currently the case (Fischer, 2011). Furthermore its application to populations before and after each round of immunisation, or after natural infection, could lead to important insights into humoral immunity and aid in the development of more effective vaccines (Mathonet & Ullman, 2013).

As the technology is relatively new to the field, it can be expected that the limitations that currently exist for the method will be circumvented in coming years and that the utilisation of NGS as an antibody discovery process will improve the ability to identify new therapeutic candidates. A particularly interesting example of such an advancement is described by DeKosky *et al*. who demonstrate a means to maintain the V_H/V_L pairing in their screening of peripheral blood B cells of both healthy and recovering donors. By spatially addressing the

cells in microengraved microwells which contained poly(dT) magnetic beads the individual cells could be lysed and the mRNA captured on the beads and *in situ*. The variable regions were then linked by performing PCR on the cDNA generated and sequencing carried out (DeKosky *et al*., 2013).

1.8.4 Microengraved microwells

Microengraved microwells are produced using a soft-lithography approach, which results in a chip comprising up to 62,500 individual polydimethylsiloxane (PDMS) wells which can range in volume from 0.1-1.0 nL. A number of research groups, and companies, utilise this approach to analyse heterogeneous populations of cells. Detection of a secreted response can be achieved in a number of ways. The first is the so-called ISAAC (i.e. immunoassay array on a chip) method where specificity is detected as a halo of fluorescence around the well containing the cell of interest (Figure 1.6 A-C) (Jin *et al*., 2009).

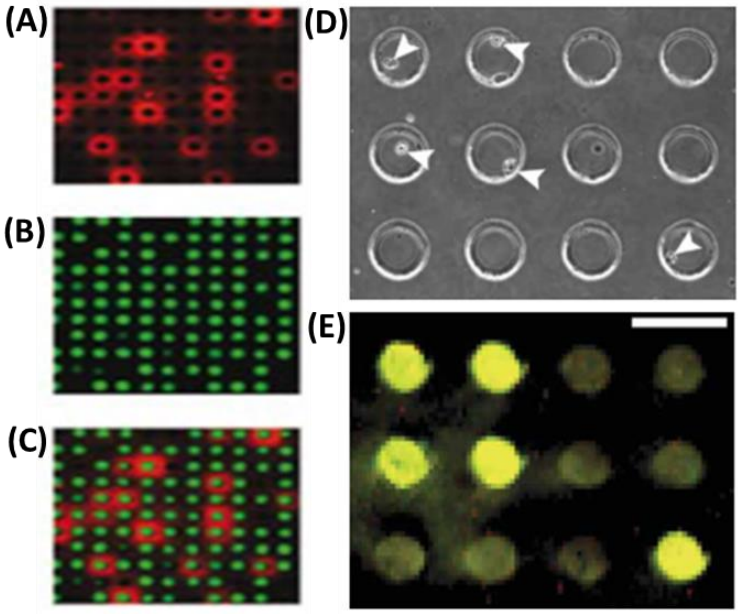

Figure 1.6: Examples of outputs from microwell-based antibody screening platforms. (A) *Signals for antigen-specific antibodies secreted from individual cells were detected and visualised as a halo of fluorescence around the well containing the cell.* ***(B)*** *The cells are then stained using Oregon Green* ***(C)*** *Antibody signals and cell signals were merged to allow for accurate retrieval of the desired cell.* ***(D)*** *Phase contrast micrograph of cells in the microwells of the microarray. The arrowheads indicate the location of cells in the wells. Scale bar, 200 mm.* ***(E)*** *Fluorescence micrograph of the corresponding region of a microarray showing conjugation of captured antibody with fluorescently-labelled antigen. Images A-C were taken from Jin et al* (Jin *et al*., 2009). *Images D-E were taken from Love et al.* (Love *et al*., 2006).

The second involves sealing a functionalised glass slide to the top of the array and generating a printed array of secreted proteins from each of the cells, which can then be probed with appropriate secondary reagents similar to commercial arrays of antibodies or proteins (Figure 1.6 D-E) (Love *et al*., 2006). They are also amenable to performance of homogenous assays in the microwell itself. Following identification of those cells exhibiting desired properties, such as secretion of an antigen-specific antibody, the cells are subsequently recovered for clonal expansion/recombinant retrieval using a micropipette or the cell is lysed, RT-PCR carried out and the V_H and V_L sequences paired using next generation sequencing (DeKosky *et al*., 2013).

1.8.5 Microfluidic microchambers

This approach employs a microfluidic chip with 'lab in a trench' like chambers where cells are captured and cultured, as shown in Figure 1.7 (Diercks *et al*., 2009). For antibody discovery applications, secreted antibodies are captured on protein beads and later detected by washing out the chambers with medium prior to the addition of labelled antigen.

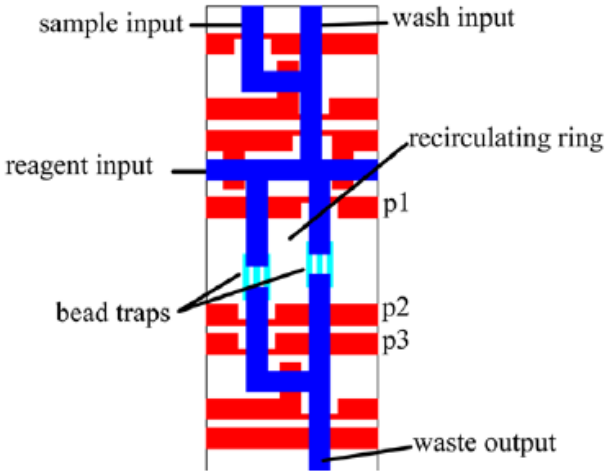

Figure 1.7: Schematic of the layout for one recirculating microchamber. *Control channels are shown in red (100 µm width), and flow channels are show in blue (13 µm height) or light blue (2.5 µm height). The three valves forming the recirculating peristaltic pump are labelled p1, p2, and p3. The total volume of the recirculating chamber is 4.7 nL. Sample is loaded into a 2.7 nL segment of this chamber. Image taken from Diercks et al.* (Diercks *et al*., 2009).

Positive antibody binders are visualised by automated microscopy of fluorescent beads in each well and cells removed by micromanipulation for further manipulations, similar to those mentioned above. Cells can be cultured and expanded in the chambers, before retrieval, if required.

Lecault *et al*. describe the production of a PDMS chip using multi-layer photolithography, which contained 1,600 individual microchambers each of 4.1 nL volume, and whose environment can be precisely controlled by the integrated micro-valves (Lecault *et al*., 2011). A researcher working in the same research group also demonstrated the use of a similar chip, with 200 pL chambers, to accurately determine the kinetic rate constants of antibodies secreted from single hybridoma cells by flushing each different concentration of antigen utilised from the chamber by way of microfluidic sieve valves (Singhal *et al*., 2010).

1.8.6 Microdroplets

The use of microdroplets as a single cell screening tool has also recently developed within the field. It involves the isolation of individual cells by capturing them in two-phase droplets which range in volume from picolitre to nanolitre (Mazutis *et al*., 2013). They can be used for single cell analysis in a range of applications from antibody and cytokine secretion to gene expression or enzyme activity (Love *et al*., 2013). Droplets are then sorted by FADS (Figure 1.8). FADS (Fluorescence activated droplet sorting) works on the same principle as FACS but overcomes the limitation of detection of secreted product as secreted product is also contained within the droplet or using microfluidic chips to interrogate each individual droplet. Interestingly, passive droplet fusion, electro-coalescence, and pico-injection have all been utilised to allow addition of reagents at a defined time point. This adds to the diversity of assays which can be carried out in droplets (Mazutis *et al*., 2013).

These platforms utilising microdroplets are employed for a vast array of single cell studies which range from viability and cytotoxicity studies utilising libraries of drug candidates (Brouzes *et al*., 2009), heterogeneous immunoassay development using magnetic beads (Sista *et al*., 2008) and monoclonal antibody discovery (El Debs *et al*., 2012; Mazutis *et al*., 2013) all in highly reproducible nano- to femto-litre droplets.

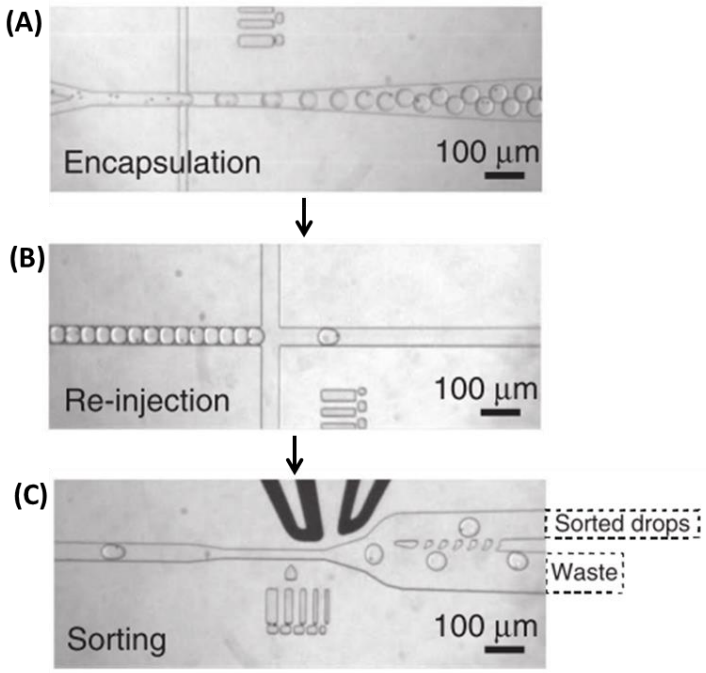

Figure 1.8: Application of microdroplet screening, using a microfluidic sorting device, for antibody discovery. (A) *The cells to be screened are encapsulated in a microdroplet with the assay reagents under laminar flow (i.e. In this case the cells are hybridoma cells secreting murine antibodies and the assay reagents comprise a goat anti-mouse-Fc capture antibody and a GFP-labelled anti-goat detection antibody). Droplets are collected and incubated off-chip for a designated amount of time to allow secretion of antibody from the cells.* ***(B)*** *The droplets are then re-injected into a second microfluidic device to be sorted* ***(C)*** *Cells are sorted based on the presence of a bright green (GFP) colour. Droplets containing no bead, no cell, a cell which does not secrete antibody, or an antibody-producing cell but no bead are discarded, whereas droplets containing an antibody-producing cell and a bead (which becomes fluorescent due to specific interaction of the components of the droplet) are collected. Image taken from Mazutis et al.* (Mazutis *et al*., 2013).

1.9 Antibody Characterisation

Following their successful generation, screening and selection the need to further characterise the chosen antibodies in a number of different ways, for a number of different reasons or applications will emerge. In typical screening approaches, the final number of candidates selected for characterisation are often reduced to a manageable number through iterative rounds of selection. The development of high-throughput, automated procedures which allow for a higher number of candidates to be characterised are becoming commonplace and the ability to carry out at least some of the characterisation on the entire population of antibodies in a particular library or cell population would be highly advantageous. The concept of screening more or all potential candidates, to help identify better monoclonal antibodies, is elaborated upon in experimental chapters 6 and 3, respectively.

Those methods predominantly used as standard, to determine key attributes of each candidate antibody, and which are most relevant to the work carried out in this thesis are the use of immunoassays, bioassays and determination of kinetic rate constants using surface plasmon resonance (SPR).

1.9.1 Characterisation using Immunoassays

Following selection, monoclonal antibodies will typically be tested for maintained recognition of the desired antigen, by employing an immunoassay. For recombinant systems, lysates of individual clones can be prepared and for mammalian systems, the supernatants are assessed. This can be a time-consuming process but is a necessary step to ensuring that only clones which exhibit true recognition of the antigen are brought forward to the next rounds of screening and characterisation. Typically this screening can represent a bottle-neck in the overall process and in many facilities automation is applied to reduce the labour and time involved. Other clever methods can serve to address this bottle-neck such as that of Guo *et al.*, who describe an 'on-plate' method which negates the need for the preparation of lysates for each bacterial clone. Instead, the method detects secreted antibody directly in the growth medium (Guo *et al*., 2010).

Following identification of the clones which exhibit maintained binding to the antigen, it is customary to then define certain characteristics of each of the antibody candidates. Criteria such as specificity for the target antigen, cross-reactivity with similar antigens, sensitivity (limits of detection (LOD) and quantitation (LOQ)) and detection range can all be determined utilising various immunoassays.

Within Chapter 6 the characterisation of a recombinant scFv fragment against C-Reactive protein, and its subsequent employment in a sandwich assay for the detection of CRP in patient serum samples is described.

1.9.2 Characterisation using Bioassays

In the development of therapeutic monoclonal antibodies, it is not sufficient that they merely bind to the target antigen for which they have selected but that they elicit the desired response in the patient. The response may include neutralisation of a particular agent or inhibition, interruption or induction of a particular cellular process as well as triggering cell death. There are three main modes of action for therapeutic antibodies which, along with examples of assays used to identify them, are briefly described below.

1.9.2.1 Binding to cell bound antigen involving Fc effector function

This first class of antibody operates by binding to their antigen, on the cell surface, which results in cell death through Fc-mediated effector functions. These include (i) antibody-dependent cell-mediated cytotoxicity (ADCC) which occurs when Fc-receptors on effector cells recognise the antibody bound to the cell and the cells are killed by cell-mediated lysis or phagocytosis, (ii) complement-dependent cytotoxicity (CDC) occurs when complement binds to Fc of the cell bound antibody, and (iii) Fc-dependent apoptosis (Harris, 2004)

Therapeutic monoclonal antibodies in this class include RITUXAN® (i.e. Rituximab induces apoptosis, ADCC and CDC and renders cells more sensitive to chemotherapy) and HERCEPTIN® (i.e. Trastuzumab induces ADCC and causes cell sensitivity to chemotherapy) (Jiang *et al*., 2011). Detection of these effects can be carried out using several commercial assays which measure release of certain compounds (e.g. Lactate dehydrogenase or LDH cytotoxicity assays such as CytoTox-ONE from Promega), monitor cell viability (e.g. Tetrazolium or Resazurin reduction cell viability assays available from multiple suppliers such as Roche and Sigma) or apoptosis (e.g. Annexin V assays or detection of caspases such as Apo-ONE from Promega) (Riss, 2005).

1.9.2.2 Binding to cell bound antigen not involving Fc effector function

The second class of antibody also involves the binding of a cell surface antigen but its effects are not mediated by the Fc region. Instead it effectively neutralises, blocks, inhibits or induces a particular process within that cell. Antibody drugs which proceed through this mechanism include REMICADE® (i.e. Infliximab neutralises the activity of TNF-α) and VECTIBIX® (i.e. Panitumumab inhibits EGRF signalling and induces apoptosis) (Jiang *et al*., 2011). Assays typically monitor effects on cell proliferation (e.g. CyQUANT from Invitrogen or WST-1 assay from Roche) or cell viability and death, as mentioned above.

1.9.2.3 Binding of soluble antigens

The final class of therapeutic class of antibodies work by binding a particular soluble ligand, thus preventing it from binding to its receptor and inducing a particular cellular pathway. They include AVASTIN® (i.e. Bevacizuab which neutralises VEGFA activity) and XOLAIR® (i.e. Omalizumab which inhibits the binding of IgE to FcεRI) (Jiang *et al*., 2011). These are again monitored as mentioned in previous sections or can be measured in serum to determine their efficacy (Sato *et al*., 2012).

Due to the complexity of the assays utilised for assessment of therapeutic effect, they are predominantly carried out at the very end of the screening and characterisation process. The

ability to apply these types of assays earlier in the overall process is becoming more and more desirable as selecting candidates on functional effect, and not merely on binding, may help to improve the rate of failure in the final stages of antibody characterisation (Smith *et al*., 2012). Within chapter 5 a proof-of-concept experiment to employ a purified anti-Fas antibody to induce apoptosis in mammalian cells (i.e. Jurkat cells which have a Fas receptor) in an ultra-high throughput manner is demonstrated.

1.9.3 Characterisation using surface plasmon resonance (SPR)

Characterisation by SPR allows a label-free real time approach to elucidate a number of different qualities of each of the antibodies to be screened. These include measurement of quality of binding, affinity and rate constant determinations as well as the ability to study aspects such as thermodynamics of the antibodies. These SPR instruments, all with slightly different specifications, are available from a number of different vendors including Reichert Technologies (e.g. SR7000DC and SR7500DC SPR systems), BioRad (e.g. Proteon XPR36), ICx Nomadics (e.g. sensiQ) and GE Healthcare (e.g. Biacore instruments 3000, T100, T200, 4000).

1.9.3.1 Principle of SPR

SPR is commonly used for the study of molecular binding interactions between free analyte in solution and probe molecules which are immobilised to the sensor surface. Instruments which employ surface plasmon resonance for the study of biomolecular interaction typically use a standard Kretschmann configuration (Kretschmann, 1971) and utilise sensor chips which consist of a glass slide coated in a thin layer of metal, usually gold. In this setup light passes through a prism, reflects off the back side of the sensor chip surface and into the detector (Figure 1.9A). At a certain incident angle, known as the resonance angle, light is absorbed by the electrons in the metal film of the sensor chip causing them to resonate. These resonating electrons, known as surface plasmons, are highly sensitive to changes in their environment. The result of the reflection on the sensor chip is an intensity loss in the reflected beam which appears as a dark band and can be seen as a dip in the SPR reflection intensity curve. When a binding event takes place the angular position of the dark band shifts and shift in the reflection curve is detected and can be converted into response units (RU) to allow assessment of the interaction between the two binding partners in a time-dependent manner (Figure 1.9B).

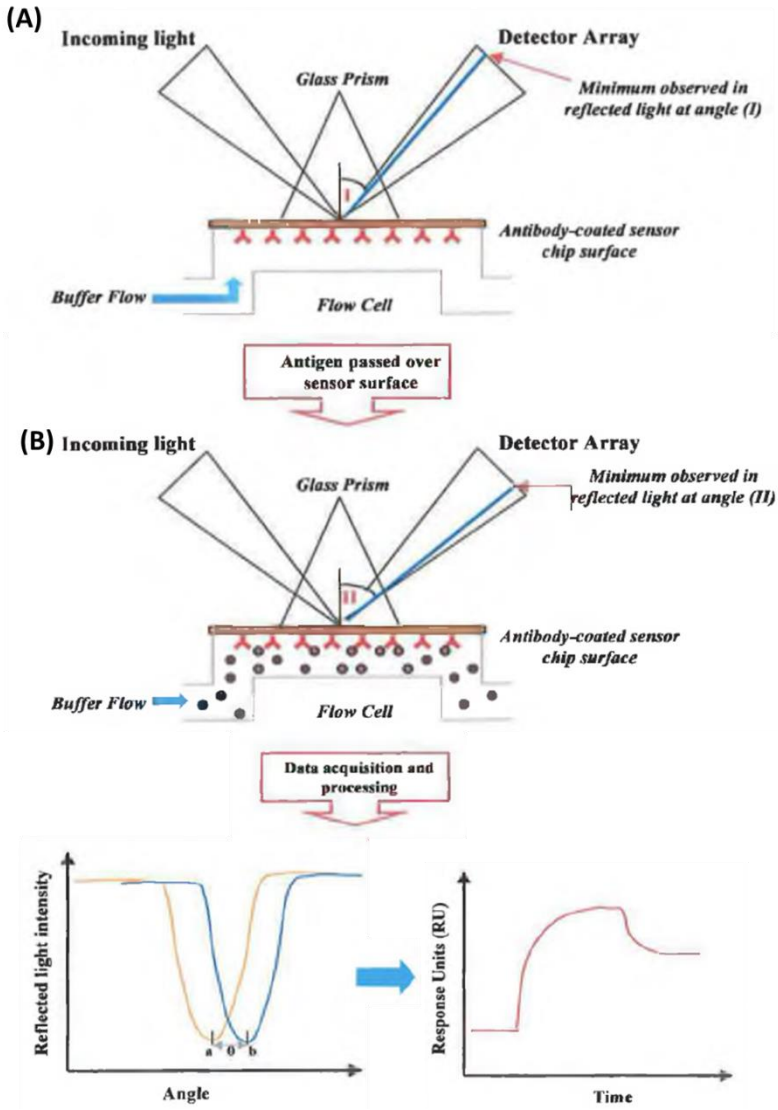

Figure 1.9: ***Basis of the detection principle in an SPR instrument. (A)*** *Monochromatic and p-polarised light is focused onto a gold chip surface immobilised with an antibody, by means of a prism under conditions of total internal reflection.* ***(B)*** *As antigen is injected over the immobilised surface, a change in refractive index near the sensor chip surface, results in a shift in the resonant angle of reflected light which is measured and recorded. The shift in reflected light is measured constantly and the results presented as a sensorgram of the change in measured response over time.*

1.9.3.2 Application of SPR to antibody screening

In a typical setup one binding partner, known as the ligand, is immobilised to the sensor surface and the other binding partner, known as the analyte, free in solution is injected over the sensor surface. In the case of a specific antibody the kinetics of the binding event can be studied by monitoring the change in the SPR response over time, which is displayed as a sensogram (Figure 1.10).

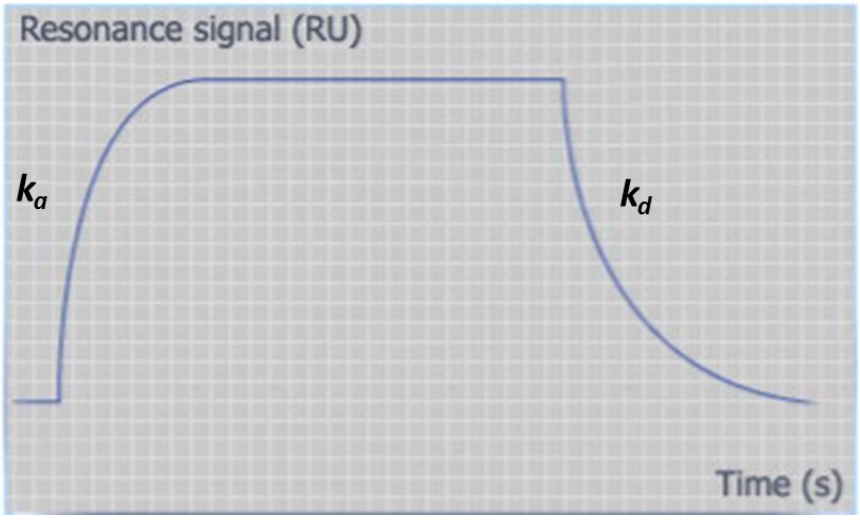

Figure 1.10: Typical sensogram output monitoring a binding interaction using SPR. *Initially there are many binding sites available resulting in rapid increase in the SPR response as the analyte binds to the ligand. The analyte continues to be delivered to the surface, it continues to bind and the number of binding sites decreases corresponding to a decrease in binding rate. As the system reaches equilibrium (i.e. the number of binding and unbinding events becomes equal) the SPR response levels off. When no more analyte is introduced the molecules will continue to unbind resulting in a decrease in SPR response. The association constant (*k_a*) can be extracted from the behaviour of the binding response and likewise the dissociation constant (*k_d*) is generated based on the unbinding response. The ratio of the two rate constants yields the affinity (*K_D*) of the system.*

Other setups can be employed to increase the efficiency of the system. In a capture format, for example, an anti-species or anti-tag antibody is immobilised on the sensor surface, followed by the antibody and sample and then the antigen of interest.

In terms of antibody screening, the employment of this type of screening provides several important traits of each of the antibody candidates. As well as confirming binding to the antigen in solution under flow conditions the ability to elucidate the rate constants (i.e. association constant or "on-rate" k_a and the dissociation constant or "off-rate" k_d) as well as the affinity (i.e. K_D) means that antibodies best suited to their final application can be identified easily.

For instance, in selecting antibodies for diagnostic point-of care applications a high affinity as well a fast association rate (i.e. binds to the antigen quickly) and a slow dissociation rate (i.e. remains bound to the antigen in a stable manner to allow accurate reading of the result) are all important features. In addition the study of the effect of ambient temperature and the thermodynamics of the interaction become of paramount importance in this type of analysis, as testing environments will vary and antibodies will need to maintain stable kinetics over a range of temperatures (Leonard *et al*., 2011).

Furthermore, when screening therapeutic antibodies the rate constants are particularly important in the effectiveness of that drug *in vivo*. A particularly striking example is that of anti-viral mAb, pavlivizumab (SYNAGIS®), which targets Respiratory syncytial virus (RSV). It was succeeded by a second generation variant, motavizumab, which had a 70 times greater affinity, a six-fold faster association rate and eleven-fold slower dissociation rate (Marasco & Sui, 2007). The increased potency of motavizumab, which shows a 83% reduction in RSV hospitalisations and 71% reduction in the number of patients needing outpatient management, is thought to be attributed to its superior association rate (Marasco & Sui, 2007).

A specific and detailed example of the application of SPR screening to a particular antibody population is discussed in Chapter 6. Using a capture format assay on a Biacore 4000, 960 scFv clones are screened, ranked and the top binders subjected to kinetic analysis in a high-throughput manner.

1.10 Thesis description and summary

This thesis will describe the development of a novel microcapillary-based microtool for the discovery of monoclonal antibodies and its application to both recombinant and mammalian antibody sources, as outlined in Figure 1.11. To demonstrate the flexibility of the new microcapillary tool, throughout the thesis the sources of monoclonal antibodies vary greatly, from commercial and custom-built recombinant libraries to native B cells from immunised animals. These antibodies are directed towards both diagnostic (e.g. cardiac biomarkers such as Troponin I, C-reactive protein, Myoglobin and Myeloperoxidase) and therapeutic targets (e.g. Intact cancer cells) covering a wide range of potential applications. Furthermore there are numerous antigens used in the screens carried out and, unlike many studies, the aim of this body of research is to demonstrate the utility of the newly developed technology rather than to identify antibodies to any one designated target.

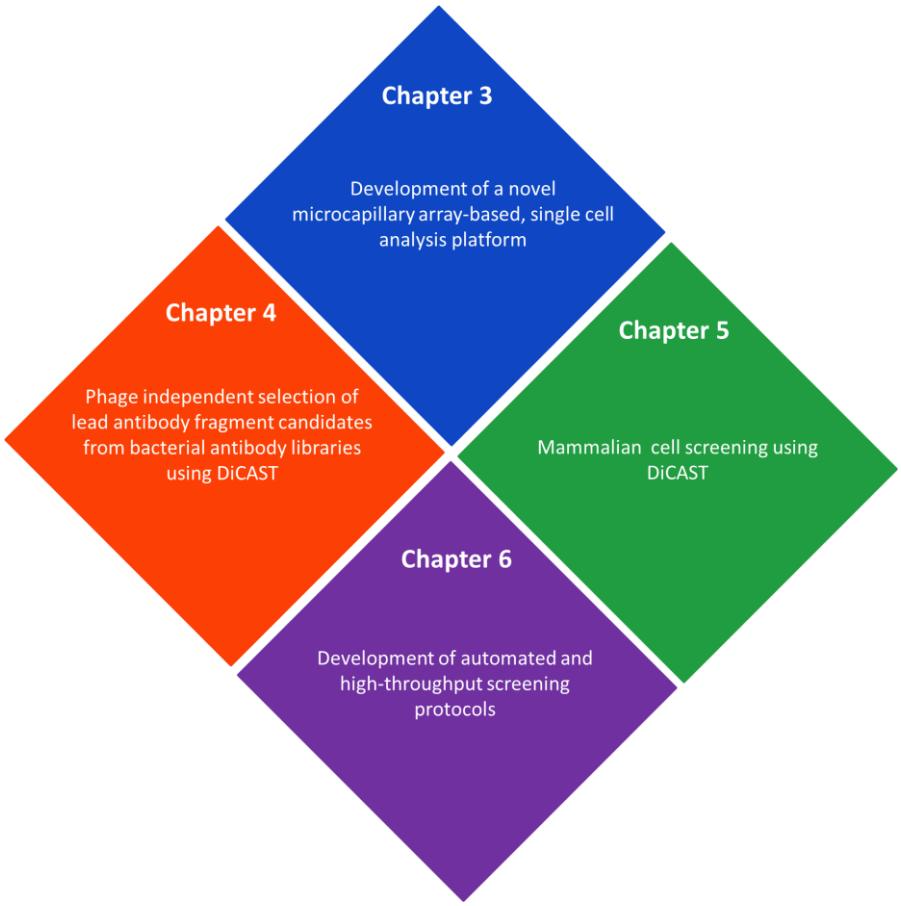

Figure 1.11: Experimental chapter designation. *Each of the experimental chapters (3-6) addresses a specific application in high-throughput antibody screening. Chapter 3 involves the development of a new microcapillary-based microtool to facilitate single cell screening. Chapter 4 and Chapter 5 describe the application of the newly developed technology to bacterial libraries and mammalian cells, respectively. Chapter 6 details the use of ancillary high-throughput techniques (i.e. SPR screening and automated immunoassay platforms) to enhance the overall screening procedure.*

The initial experimental chapter, Chapter 3, investigates the potential to utilise arrays of fused silica capillaries to spatially address millions of individual cells and to detect antibody secreted from any one cell in the population in a reliable manner. The thorough investigations carried out in this body of work detail the development of the technology from a radical idea to an actual realised entity. Areas of investigation include the ability to grow and detect secreted products from individual cells, the ability to carry out an antibody detection assay on a removable surface to identify cells of interest, the development of a procedure to allow relation of the physical array to the assay image generated as well as demonstration of a number of homogenous assay solutions as well as several additional multiplexing offerings.

The second experimental chapter, Chapter 4, describes the application of this newly developed microtool to a number of different recombinant libraries to assess its performance against the traditional approach of phage display. The first challenge involves the use of the technology in screening an anti-CRP library, which was previously developed and screened in-house, and comparison of the resultant antibodies with those obtained in the earlier campaigns. The next challenge involves the use of a commercially available naïve library to screen for antibody fragments against multiple targets. The final challenge was to undertake the screening and comparison of outputs from an externally sourced library.

The third experimental chapter, Chapter 5, details the feasibility studies carried out to determine the possibility of screening of mammalian cells using the technology. It involves the use of a mammalian cell line to optimise the process for cells of this type and then the application of these optimised protocols to allow for screening of native B cells using the developed technology. In addition to these screening campaigns, methods to capture the genetic information from cells of interest through single cell PCR and the development and demonstration of a functional bioassay (i.e. detection of apoptosis) on the microcapillary array platform are described.

The final experimental chapter, Chapter 6, details approaches which can be used to complement the type of high throughput screening approach that has been developed. By screening more cells, one may ultimately end up with more positive hits and high-throughput methods for ranking and characterisation become important. In order to address this need, a series of protocols were developed for high-throughput SPR-based ranking of antibody fragments in crude lysates. In addition, a set of procedures to utilise automated liquid handling stations to carry out ELISA-based screening and characterisation of selected antibodies were also optimised.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Purchased Reagents

All general reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

Item	Source
Series S sensor chips CM5 Amine coupling reagents 10X Stock HBS-EP+ buffer	GE Healthcare, Uppsala, Sweden.
Polyclonal (goat) anti- HA epitope tag PageRuler pre-stained protein ladder	Thermo Scientific, Waltham, MA, USA.
Monoclonal (mouse) anti-cTnI antibodies	Hytest Ltd, Turku, Finland.
HRP-labelled monoclonal (rat) anti-HA	Roche Diagnostics, Switzerland.
Overnight express auto-induction medium pET28b plasmid vector Rosetta 2 competent cells Polyclonal (rabbit) anti-C-Reactive Protein Cy5-labelled streptavidin Cy5-labelled monoclonal (goat) anti-mouse Monoclonal (mouse) anti-Fas, human activating clone CH11	Merck Millipore, Cork, Ireland.
1KB plus DNA ladder SybrSafe DNA stain PCR reagents CO_2 Independent Medium Superscript® III reverse transcriptase Trizol	Invitrogen, Dublin, Ireland.
Restriction enzymes Antarctic phosphatase T4 ligase	New England Biolabs (NEB), MA, USA.
Nickel-NTA purification resin	Qiagen, Hilden, Germany.
NucleoSpin® plasmid purification kit NucleoSpin® Extract II Purification System	Macherey Nagel, Dueren, Germany.
Vivaspin concentration columns	Sartorius Ltd., Goettingen, Germany.
XL1-Blue electro-competent cells	Agilent technologies, CA, USA.
NUNC maxisorb ninety-six well plates	Fisher Ireland, Dublin, Ireland.
Sterile ninety-six well plates	Cruinn Diagnostics, Dublin, Ireland.
(3-Aminopropyl) triethoxysilane (APTES) Bovine Serum Albumin (BSA)	Sigma Aldrich, St. Louis, MO, USA.

Foetal Bovine Serum (FBS) RPMI 1640 medium Monoclonal (mouse) anti-cMYC HRP-labelled polyclonal (goat) anti-HIS tag HRP-labelled polyclonal (goat) anti-Mouse Polyclonal (goat) anti-mouse C4-HSL 3,3', 5, 5'-Tetramethylbenzidine (TMB) Isopropyl β-D-1-thiogalactopyranoside (IPTG) Chloroform Isopropanol Ethanol DEPC Water	
Polydimethylsiloxane (PDMS)	Stockwell Elastomerics, PA, USA.
Cardiac Troponin I (cTnI) Cardiac Troponin T (cTnT)	Life Diagnostics, Westchester, PA, USA.
C-Reactive protein (CRP) Myeloperoxidase (MPO) Transferrin (TRA) CRP-depleted serum	BBI solutions, Cardiff, UK.
Monoclonal (mouse) anti-human IgE 107 Biotinylated monoclonal (mouse) anti-human IgE 182	mAbTech AB, Sweden.
Monoclonal (mouse) anti-CK18 M5 Monoclonal (mouse) anti-CK18 M6 Monoclonal (mouse) anti-CK18 M30	BioAxxess, Tewkesbury, UK.
Human Domain Library	Source Bioscience, Nottingham, UK.
Cy5-labelled monoclonal (mouse) anti-HA	Tebu Bio, Le-Perray-en-Yvelines, France.
Human IgG Cy5-labelled polyclonal (goat) anti-human IgG	Biomeda Corp., CA, USA.
Labelling Kits (Cy3/Cy5)	Thermo Fisher Scientific, IL, USA.
Polyclonal (goat) anti-HA	Bethyl Laboratories Inc., UK.
Monoclonal (mouse) anti-EpCAM Cy5-labelled monoclonal (mouse) anti-EpCAM	Biolegend Inc., CA, USA.
Briclone	National Institute of Cellular Biology, DCU, Dublin 9, Ireland.
HSL conjugates and unconjugated compounds	Supplied, for evaluation, by independent UK laboratory.
Jurkat T lymphoblastoid cell line (ATCC TIB-152) Multiple myeloma B lymphocyte cell line (ATCC TIB-196)	Kindly donated by ABG, DCU, Dublin.
FRET DNA probes	TIB MOLBIOL, Berlin, Germany
GFP reassembly vectors [pET11a-Z-NGFP (NZ), pMRBAD-Z-CGFP (CZ)]	Obtained under MTA from Yale University
pQE30-GFP vector	Kindly donated by Dr. Paul Clarke, Dublin City University, Dublin, Ireland.
Clinical samples (CRP)	Obtained from the Mater Misericordiae University Hospital, Dublin, Ireland.

2.1.2 Buffer and Media Formulations

2.1.2.1 Phosphate buffered saline (PBS)

One Dulbecco's A tablet (DA, Oxoid) was dissolved per 100 mL of distilled water according to the manufacturer's instructions. When dissolved, prepared PBS contained 0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, pH 7.4. This buffer will be referred to throughout as PBS.

2.1.2.2 PBS-Tween (PBST)

Tween 20 surfactant was added to PBS (described in 2.1.2.1 above) to a final concentration of 0.05% (v/v) and mixed.

2.1.2.3 Tris-acetic acid-EDTA buffer (TAE)

A 50 X stock solution of TAE buffer was prepared in a final volume of 1 litre by dissolving 242 g Tris, and addition of 57.1 mL glacial acetic acid followed by 100 mL of 0.5 M EDTA. pH 8.0. All agarose gels were run in 1 X TAE by diluting the stock solution 1/50 with ultra-pure water.

2.1.2.4 Luria Bertani broth (LB)

Medium was prepared by dissolving 10g Tryptone, 5g Yeast Extract and 5g NaCl, in 1L distilled water. Medium was then autoclaved for 15 minutes at 121ºC, for sterilisation. Solid medium was made by adding 15 g/L bacteriological agar.

2.1.2.5 SOC broth

Medium was prepared by dissolving 20g Tryptone, 5g Yeast Extract and 0.5g NaCl, in 1L distilled water. Medium was then autoclaved for 15 minutes at 121ºC, for sterilisation. Once cooled additional components (2.5mM KCl, 20mM $MgCl_2$, 20mM glucose) were added, by sterile means to the medium.

2.1.2.6 ZYP-5052 broth

ZYP-5052, the auto-induction medium prepared in-house and described in Studier *et al*., is a rich medium for growth with little or no induction during log phase and auto-induction of expression as the culture approaches saturation (Studier, 2005). It is prepared by adding 1 mL 1M $MgSO_4$, 20 mL 50X 5052 and 50 mL 20X NPS to approximately 928 mL of ZY medium. Medium was then autoclaved for 15 minutes at 121ºC, for sterilisation.

ZY medium was prepared by dissolving 10 g tryptone and 5 g yeast extract in 1 L of distilled water. 5052 (50X) was prepared by dissolving 25 g glycerol, 2.5 g glucose and 10 g α-lactose in 100 mL distilled H_2O. (5052 = 0.5% (v/v) glycerol, 0.05% (w/v) glucose, 0.2% (w/v) α-lactose). NPS (20X) was prepared by dissolving 6.6 g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4 and 14.2 g Na_2HPO_4 in 100 mL of distilled H_2O.

2.2 Methods-General

2.2.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The gel casting apparatus was prepared, as per manufacturer's instructions. The mixture for the 12.5% separation gel and 4.5% stacking gel were prepared as outlined in Table 2.1, without adding the TEMED as this would accelerate the polymerisation.

Table 2.1: Composition of separation and stacking gels for SDS-PAGE analysis of proteins.

Reagent	Separating Gel (12.5%)	Reagent	Stacking Gel (4.5%)
1M TrisHCl, pH 8.8	1.5 mL	1M TrisHCl, pH 6.8	300 µL
30% (v/v) Acrylagel	2.5 mL	30% (v/v) Acrylagel	375 µL
2% (v/v) Bis-Acrylagel	1.0 mL	2% (v/v) Bis-Acrylagel	150 µL
Water Deionized	934 µL	Water Deionized	1.74 mL
10% (w/v) SDS	30 µL	10% (w/v) SDS	24 µL
10% (w/v) APS	30 µL	10% (w/v) APS	24 µL
TEMED	6 µL	TEMED	2.5 µL

On addition of the TEMED to the separation gel only, the mixture was mixed thoroughly and pipetted into the space between the two glass plates of the Biorad electrophoresis apparatus. The mixture was allowed to reach approximately 2-3 cm from the top of the smaller plate. The mixture was then immediately covered gently with isopropanol to promote more rapid polymerisation of the gel. Once the gel had polymerised, the isopropanol layer was removed by inverting the apparatus. The gel was then rinsed with distilled water and blotted dry with filter paper. The TEMED was then added to the stacking gel mixture and mixed thoroughly. The stacking gel mixture was then pipetted on the surface of the separating gel. The plastic comb was gently inserted into the gap between the two glass plates and the gel was allowed to set.

Samples, at the required dilution, were prepared for loading by adding 4X loading dye (60 mM Tris-HCl (pH 6.8), 25% (w/v) glycerol, 2% (w/v) SDS, 14.4 mM 2-mercaptoethanol and 0.1% (w/v) bromophenol blue) to a final concentration of 1X and heated for 10 minutes at 98ºC. Gel apparatus was assembled according to manufacturer's instructions and 1X electrophoresis buffer (25 mM Tris, 250 mM Glycine (electrophoresis grade), pH 8.3 and 0.1% (w/v) SDS) was poured into the inner chamber until it reached the top of the plates. More of the running buffer was then gently poured into the outer chamber until it covered at least the bottom centimetre of the gel. Approximately 20 µL of sample and 6 µL of molecular weight marker (PageRuler pre-stained protein marker) were loaded onto the gel and it was run at 200 V until the tracker dye (bromophenol blue) had almost reached the

bottom of the gel. The gel was stained in Instant Blue stain for 1 hour and subsequently documented, using a BioRad GelDoc™ EZ system.

2.2.2 Small-scale antibody production

Cultures were grown in 50 mL tubes containing 10 mL of auto-induction medium, to allow for expression of desired protein, overnight at 37 ºC /200 rpm. For the pCOMB vector LB medium containing 100 µg/mL carbenicillin was utilised, while for the pET vectors LB medium containing 70 µg/mL kanamycin was used. The cultures were then centrifuged at 4,500 x g for 20 minutes, the cell pellets were re-suspended in one-tenth the original culture volume of PBS and subjected to three freeze-thaw cycles (-80ºC until frozen/37 ºC until thawed). Cell debris was removed by centrifugation at 4,500 x g for 10 minutes. Supernatant was stored at 4 ºC for immediate use or at -20 ºC for future use.

2.2.3 Large-scale antibody production

On the first day, a 10 mL culture of XL1 Blue *E.coli* expressing the desired protein were incubated overnight at 37ºC/200 rpm. For the pCOMB vector and the HSL clones, LB medium containing 100 µg/mL carbenicillin was utilised, while for the pET vectors LB medium containing 70 µg/mL kanamycin was used. On the following day a 250 mL culture, of overnight express auto-induction medium containing 100 µg/mL carbenicillin, was inoculated with 250 µL of the overnight culture. The culture was incubated at 37ºC/200 rpm overnight. Following expression of the desired protein the culture was centrifuged at 4,500 x g for 20 minutes to pellet the cells. The pellet was then re-suspended in one-tenth the original culture volume of 1X PBS and subjected to three rounds of freeze-thawing (-80ºC/37ºC) to completely lyse the cells. The cell debris was pelleted by centrifugation at 14,500 x g for 20 minutes. The supernatant was filtered through a 20 µm filter and the scFv purified by IMAC, as described in Section 2.2.4.

2.2.4 IMAC purification

The Ni-NTA mixture (2 mL) was loaded into a column (2mLs slurry gives 1 mL column) and allowed to settle. The column was equilibrated using 10mLs of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 7.5). The filtered lysate was passed through the column. The flow through was collected and passed through the column once more. The resin was then washed once with 8mLs of wash buffer A (50 mM NaH_2PO_4, 1M NaCl, 10% (v/v) glycerol, 20mM imidazole, 1% (v/v) triton X100, pH 7.5) and twice with 8 mL of wash buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 7.5. The desired proteins were eluted with 5 mL elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 7.5). A 100 µL sample of each fraction was kept for analysis on SDS-

PAGE gel. The elution fraction was buffer exchanged and concentrated into PBS by passage through a vivaspin (10 kDa MWCO) column.

2.2.5 Recombinant library information

The anti-Troponin scFv antibody library was constructed from mRNA extracted from the spleen and bone marrow of two chickens immunised with purified cardiac Troponin I, cardiac Troponin T and myoglobin. The antibody variable domain genes were cloned into the pComb3XSS phagemid vector according to protocols described by Barbas and co-workers (Barbas *et al*., 2001). The library size was 2.2×10^9. The clones analysed throughout the Biacore study were monoclonal scFvs from the final output (Round 4) of phage display screening against cTnI.

The anti-CRP and anti-MPO scFv antibody library was constructed from mRNA extracted from the spleen and bone marrow of two chickens immunised with purified CRP, MPO and FABP. The antibody variable domain genes were cloned into the pComb3XSS phagemid vector according to protocols described by Barbas and co-workers (Barbas *et al*., 2001). The library size was 3×10^7.

2.2.6 Purification of plasmid vectors

An *E. coli* colony was taken from a LB agar plate and grown overnight in 5 mL LB broth, containing appropriate antibiotic, at 37^{o}C and 200 rpm. The plasmid was then purified using the Macherey Nagel NucleoSpin® plasmid purification kit, according to the manufacturer's instructions. Briefly, the bacteria were pelleted by centrifugation at 4,500 x g. for 5 minutes and the supernatant discarded. The cell pellet was completely resuspended in 250 µL buffer A1. Following this 250 µL lysis buffer A2 was added to the cells, mixed gently by inverting the tube 6–8 times, and incubated at room temperature for up to 5 min or until lysate appears clear. Neutralisation buffer A3 (300 µL) was then added and mixed as previously described. Lysate was then transferred to a clean 2 mL micro-centrifuge tube and clarified by centrifugation for 5 min at 11,000 x g. The supernatant was decanted into a NucleoSpin® plasmid column and centrifuged for 1 min at 11,000 x g. The flow-through was discarded and the column was placed back into the collection tube. The silica membrane was then washed by addition of 600 µL ethanol-supplemented buffer A4 and centrifuged for 1 min at 11,000 x g. The flow-through was once again discarded and the column centrifuged as previously to dry the silica membrane. Pure DNA was finally eluted, into a fresh collection tube, under low ionic strength conditions with 50 µL slightly alkaline buffer AE (5 mM, Tris-HCl, pH8.5) or DEPC water.

2.2.7 PCR amplification of specific gene products.

A polymerase chain reaction (PCR) was carried out using various templates. The PCR reaction was prepared as detailed in Table 2.2. The reactions were then subjected to cycling conditions as outlined in Table 2.3. Primers used for each different PCR are listed in Table 2.4.

Table 2.2: Reaction volumes for polymerase chain reaction (PCR). *The reactions were then subjected to cycling conditions as outlined in Table 2.3. Products were then analysed on a 1% (w/v) agarose gel by electrophoresis.*

Reagent	**Volume (µL)**
Template	2
5X Reaction Buffer	10
25 mM $MgCl_2$	4
10 mM dNTPs	1
Primer 1	1
Primer 2	1
Taq Polymerase	1
DEPC Water	30 (to give 50 µL total volume)

Table 2.3: PCR cycling conditions.

Temperature	**Step**	**Time**	
95°C	Initial Denaturation	5 min	
95°C	Denaturation	30 sec	30 Cycles
56°C	Annealing	30 sec	
72°C	Extension	1 min	
72°C	Final Extension	10 min	

Table 2.4 DNA primers used for amplification

PCR	**Section**	**Primer Sequence**
Human IgE CH1 region	5.2.3	**ConE-IgE** CCA CAC AGA GCC CAC CCG TC **CE1-IgE** GCT GAA ACT AGT GTT GTC GAC
Avian V_H	5.4.4	**CSCVHo-FL (Long Linker)** GGT CAG TCC TCT AGA TCT TCC GGC GGT GGT GGC AGC TCC GGT GGT GGC GGT TCC GCC GTG ACG TTG GAC GAG **CSCG-B** CTG GCC GGC CTG GCC ACT AGT GGA GGA GAC GAT GAC TTC GGT CC
Avian V_λ	5.4.4	**CSCVK** GTG GCC CAG GCG GCC CTG ACT CAG CCG TCC TCG GTG TC **CKJo-B** GGA AGA TCT AGA GGA CTG ACC TAG GAC GGT CAG G
Avian SOE	5.4.4	**CSC-F** GAG GAG GAG GAG GAG GAG GTG GCC CAG GCG

		GCC CTG ACT CAG **CSC-B** GAG GAG GAG GAG GAG GAG GAG CTG GCC GGC CTG GCC ACT AGT GGA GG
cTnI peptides	6.2.4	**GFP-pep1-For** ACG TGG ATC CGA AAA TTA GCG CGA GCC GTA AAC TGC AGC TGA AAA CCG AGG GTG GTG GCA TGA GTA AAG GAG AAG AAC **GFP-pep2-For** ACG TGG ATC CGA ACT ATC GTG CGT ATG CGA CCG AAC CGC ATG CGA AGA AGA AAA GCA AAA TTG AGG GTG GTG GCA TGA GTA AAG GAG AAG AAC **GFP-pep3-For** ACG TGG ATC CGC AGC CGC TGG AAC TGA CCG GCC TGG GCT TTG CGG AAC TGC AGG AGG GTG GTG GCA TGA GTA AAG GAG AAG AAC **GFP-pET-Back** ACG TAA GCT TTT TGT ATA GTT CAT CCA TGC

2.2.8 Agarose gel electrophoresis for DNA characterisation

DNA was analysed by running on agarose gels (containing 1X SybrSafe for staining of DNA) in a horizontal gel apparatus. Gels were prepared by dissolving agarose (typically 1% (w/v)) in 1X TAE buffer by heating and SybrSafe added to the cooled gel prior to setting. The gels were placed in the gel apparatus and 1X TAE used as the running buffer. A tracker dye was incorporated into the sample (1 µL dye + 5 µL sample) to facilitate loading of samples. Gels were run at 100 Volts for 1 hour or until the tracker dye reached the end of the gel. Gels were then visualised on a blue light transilluminator and photographed using using a BioRad GelDoc^{TM} EZ system.

2.2.9 Gel purification of PCR products from agarose gel

DNA was purified from the agarose gel using a Macherey Nagel NucleoSpin® Extract II Purification System as per the manufacturer's instructions. Briefly, the DNA band of interest was excised using a clean, sterile scalpel blade and the agarose slice transferred to a sterile 1.5 mL microcentrifuge tube and weighed. The fragment was then dissolved in NT1 buffer (200 µL buffer NTI 100 mg of agarose gel) at 50ºC for 5-10 minutes or until the agarose had completely dissolved. Once dissolved, the mixture was applied onto the silica membrane of the NucleoSpin® Extract II columns provided, and centrifuged for 30 seconds at 11,000 x g. The flow-through was discarded the column placed back into the collection tube. Contaminations were removed by washing with 700 µL ethanolic buffer NT3 and centrifuging as previously. Pure DNA was finally eluted, into a fresh collection tube, under

low ionic strength conditions with slightly alkaline buffer NE (5 mM, Tris-HCl, pH8.5) or DEPC water.

2.2.10 Dephosphorylation of digested plasmid vector using Antarctic phosphatase.

The purified vector was de-phosphorylated using Antarctic phosphatase, which catalyses the removal of 5´ phosphate groups from DNA and RNA, according to manufacturer's instructions. Briefly, 1/10 volume of 10X Antarctic phosphatase reaction buffer was added to 1-5 µg of DNA cut with restriction endonuclease. 1 µL of Antarctic phosphatase (5 units) was added, mixed and incubated at 37°C for approximately 30 minutes, The enzyme was then heat inactivated for 5 minutes at 65°C. Since phosphatase-treated fragments lack the 5´ phosphoryl termini required by ligases, they cannot self-ligate and this property is used to decrease the vector background in cloning strategies. The DNA present in both the digested insert and the digested and de-phosphorylated vector were then quantified at 260 nm, using a Nanodrop instrument, before proceeding to ligation.

2.2.11 Transformation of ligation mixes into electro-competent cells by electroporation

A 2 µL fraction of the ligation mix was added to 50 µL of electro-competent cells (on ice) and stirred gently with a pipette tip. The mixture was added to an ice-cold electroporation cuvette which was placed in the gene pulser chamber and pulsed at 2.5 kV. The cuvette was removed from the apparatus, transferred to a sterile 20 mL tube and 3 mL of SOC medium (pre-warmed to 37°C) added. The transformation was then incubated at 250 rpm and 37°C for 1 hour. Serial dilutions were then carried out and 100 µL spread on LB agar plates, containing appropriate antibiotic, and incubated overnight at 37ºC to select for positive transformants.

2.2.12 Colony pick PCR for confirmation of inserts in transformants.

Single colonies were picked from transformation plates and resuspended in 10 µL sterile DEPC water. A 2 µL volume of this was used as template in a PCR reaction, carried out as described in 2.2.7, varying the primers used depending on the peptide concerned. The remaining solution was used for expression testing or streaked on an LB agar plate containing appropriate antibiotic and incubated overnight at 37ºC to provide a stock of each transformant for future use.

2.3 Methods- Chapter 3

2.3.1 Assessment of auto-induction versus standard induction.

Each of the media was prepared as outlined in section 2.1.2. An overnight stock of GFP expressing *E.coli* was then added to a 10mL culture of each and incubated at 37°C/200rpm. The auto-induction samples were kept under these conditions overnight while the IPTG induced culture had 1 mM IPTG added once it had reached the log phase of growth (approximately 0.6 OD_{600nm}). It was then incubated at 30°C/200 rpm overnight. On the following day the cultures were centrifuged at 4,500 x g and the pellets observed. A lysate was subsequently produced from each of the cultures and analysed by SDS-PAGE, as described in section 2.2.1. All media are detailed in section 2.1.2

2.3.2 Assessment of expression of protein and viability of cells in the microcapillary array.

A stock of GFP-expressing *E. coli*, at a concentration of 4.6 x 10^9 cells/mL, were centrifuged at 4,500 x g for 10 minutes and re-suspended in overnight express medium. A 100 µL was then added to an array, using a micropipette. The array was then sealed with a thin layer of PDMS, imaged using a fluorescent microscope (Olympus IX81 fitted with a FITC filter; 10X magnification) and subsequently incubated at 37°C overnight. The area was then imaged, on the following day, as described previously. A nitrogen gas flow was then applied to the top of the array and any contents removed were then captured on an agar plate beneath. The plate was then incubated at 37°C overnight.

2.3.3 Analysis of GFP-expressing *Escherichia coli* in the microcapillary array

A stock of GFP expressing *E.coli*, at a concentration of 4.6 x 10^9 cells/mL, was diluted in series (1/10) in overnight express medium. A 5 µL aliquot of each dilution was then added to an array. The array was then sealed with a thin layer of PDMS and incubated at 37°C overnight. After incubation, the array was imaged using a fluorescent microscope (Olympus IX81 fitted with a FITC filter; 10X magnification). In order to identify a cell number which results in single cell occupancy of the array, a cell enumeration was carried out on agar plates simultaneously.

2.3.4 Performance of a direct assay through the microcapillary array onto a PDMS surface.

The surface of the PDMS was coated with 10 µg/mL human IgG (hIgG) in PBS and incubated for 1 hour at room temperature. The hIgG was carefully removed with a pipette and the surface blocked with 3% (w/v) BSA in PBS, and incubated as before. The BSA was

removed, the surface was rinsed gently with PBS and allowed to dry. The PDMS was then degassed for 30 minutes, in a standard dessicator. A 2.5 µg/mL dilution of Cy5-labelled anti-hIgG, in 3% (w/v) BSA, was then added to the array and the array sealed to the prepared PDMS surface. The array was then incubated for 1 hour at room temperature. Following incubation the PDMS was gently rinsed with PBS, allowed to dry and imaged using either the Perkin Elmer Gx ScanArray or an Olympus IX81 fluorescent microscope.

2.3.5 Chemical modification strategies tested on polydimethylsiloxane (PDMS) surfaces.

The first two modification strategies were taken from the work of Yu *et al*. (2007) and the final three were suggested by Vladimir Gubala of the surface science group within the Biomedical Diagnostics institute. The carboxy modification strategies (1 and 2) were carried out by Dr. Gubala and are described in Coyle *et al*. (2012).

2.3.5.1 Preparation of PDMS-EDC surface

A layer of PDMS was immersed in 10% (v/v) (3-Aminopropyl)triethoxysilane (APTES) in absolute ethanol for 10 min at room temperature. The APTES-treated PDMS were rinsed by 96% (v/v) ethanol, air-dried and then heated at 80°C in a vacuum oven for 2 hours. The resulting PDMS-APTES was then modified for producing carboxyl groups by addition of succinic anhydride (SAA), at 50 µg/mL in pure water for 2 hours at room temperature. The surface was then rinsed and air-dried under nitrogen before addition of 20 µg/mL EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) mixed with coating protein (5 µg/mL human IgG) for one hour at room temperature. The surface was then rinsed with Tris-HCl buffer to stop the crosslinking reaction and the surface was blocked with 1% (w/v) BSA for 2 hours at 37°C.

2.3.5.2 Preparation of PDMS-GA surface

A layer of PDMS was immersed in 10% (v/v) APTES in absolute ethanol for 10 min at room temperature. The APTES-treated PDMS were rinsed by 96% (v/v) ethanol, air-dried and then heated at 80°C in a vacuum oven for 2 hours. The surface was then modified by 2.5 % (v/v) glutaraldehyde (GA) for 1 hour at room temperature. The surface was then rinsed with Tris-HCl and dried under nitrogen flow. Coating protein (5 µg/mL human IgG) was then added in Tris-HCl at room temperature for 2 hours and subsequently blocked with 1% (w/v) BSA for 2 hours at 37°C.

2.3.5.3 Preparation of PDMS-Carboxy (1) surface

Coatings were kindly carried out by Dr. Vladimir Gubala using a computer-controlled PECVD reactor Europlasma, model CD300 (Oudenaarde, Ghent, Belgium). Surfaces were prepared as described in Coyle *et al*. (2012) with this Carboxy 1 surface representing the tetraethyl orthosilicate (TEOS) only surface.

2.3.5.4 Preparation of PDMS-Carboxy (2) surface

Coatings were kindly carried out by Dr. Vladimir Gubala using a computer controlled PECVD reactor Europlasma, model CD300 (Oudenaarde, Ghent, Belgium). Surfaces were prepared as described in Coyle *et al*. with this Carboxy 2 surface representing the tetraethyl orthosilicate (TEOS) and acrylic acid (AA) surface.

2.3.5.5 Preparation of PDMS-APTES surface

A layer of PDMS was subjected to oxygen plasma treatment for 10 minutes and then submerged in 3% (v/v) APTES in 98% (v/v) ethanol for 1 hour at room temperature. The surface was then rinsed with 98% (v/v) ethanol and baked at 60°C overnight.

2.3.6 Determine feasibility of performing immunoassay in the array and comparison with microtitre plates.

A DiCAST screen was carried out using a sandwich FLISA on array. A thin-layer of APTES-modified PDMS was coated with 5 µg/mL anti-hIgG (un-labelled) in PBS and incubated for one hour at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for one hour at room temperature. The PDMS was then degassed for 30 minutes, in a standard dessicator. Dilutions (1/10 series) of hIgG were prepared in 1.5% (w/v) BSA and added to distinct areas of a 40 µm array starting at a dilution of 250 µg/mL and the array was sealed to the prepared PDMS. The array was then incubated for one hour at room temperature. After incubation the array was carefully removed and the PDMS surface was washed gently with PBS. A 2.5 µg/mL dilution of Cy5-labelled anti-hIgG was then added to the surface and incubated for one hour at room temperature. Plates were then washed, as previously described. The thin layer of PDMS was then air-dried and scanned using a Perkin Elmer GxArray scanner with a 633 nm laser. A microtitre plate was simultaneously prepared using the same solutions so that a direct comparison could be carried out.

2.3.7 Shaking versus static investigation assay

Wells of a microtitre plate (standard or with a layer of cured PDMS) were coated with 2 µg/mL CRP and incubated for one hour at 37°C. The wells were then blocked by addition of

3% (w/v) BSA and incubated for one hour at room temperature. A culture of avian anti-CRP scFv (F8) was diluted in auto-induction medium, added to the wells and incubated at 37°C overnight, either static or shaking (200rpm). After incubation, the plates were washed three times with PBST followed by three times with PBS. A 2.5 µg/mL dilution of Cy5-labelled anti-HA was added to the plates and incubated for one hour at 37°C. The plates were then washed as previously described, and the fluorescence read using a Tecan Safire II plate reader.

2.3.8 Expression of recombinant system and detection of expressed antibodies on array.

A thin-layer of APTES-modified PDMS was coated with 5 µg/mL C-reactive protein (CRP) in PBS and incubated for one hour at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for one hour at room temperature. The PDMS was then degassed for 30 minutes, in a standard dessicator. A stock (approx. 2 x 10^9 cfu/mL) of anti-CRP avian scFv (Clone F8) previously selected using phage display was diluted in series (1 in 10) in overnight express medium and added to distinct areas of a 40 µm microcapillary array. The array was then incubated at 37°C overnight. After incubation the array was carefully removed and the PDMS surface was washed gently with PBS. A 2.5µg/mL dilution of Cy5-labelled anti-HA was then added to the surface and incubated for one hour at room temperature. The surface was then washed as previously described. The thin layer of PDMS was then air-dried and scanned using a Perkin Elmer GxArray scanner with a 633 nm laser.

2.3.9 Multi-analyte assay development

2.3.9.1 Analysis of potential cross-talk between available fluorophores.

Initially a test to ensure that no interference from the different fluorescent proteins was trialled on the scanner used. To do this Cy3-labelled BSA and Cy5-labelled BSA were added to and array singly and mixed, sealed to PDMS and incubated for 1 hour at RT. The array was then removed and the PDMS scanned using a Perkin Elmer GxArray scanner using both the 633 nm laser and the 543 nm laser. An average fluorescence was taken from each area on the scan. There is no non-specific signal observed when analysing the proteins in a mixture. This means that it is possible to use the dyes in the same DiCAST assay without interference. Solutions prepared were also analysed in the wells of a microtitre plate using a Tecan Safire II plate reader at wavelengths for Cy3 (Excitation 550 nm/ Emission 570 nm) and Cy5 (Excitation 650 nm/ Emission 670 nm).

2.3.9.2 Assessment of the performance of a capture format assay

A thin-layer of APTES-modified PDMS was coated with 4 µg/mL anti-HA in PBS and incubated for 1 hour at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for 1 hour at room temperature. The PDMS was degassed for 30 minutes, in a standard dessicator. A 10^{-6} dilution of anti-CRP scFv expressed in *E.coli* was prepared in auto-induction medium and added to a 40 µm array. The array was sealed to the prepared PDMS and incubated at 37°C overnight. After incubation, the array was carefully removed and the PDMS surface was washed gently with PBS. A 2.5 µg/mL dilution of Cy5-labelled CRP was added to the surface and incubated for 1 hour at room temperature. The surface was then washed as previously described. The thin layer of PDMS was then air-dried and scanned using a Perkin Elmer GxArray scanner using a 633 nm laser.

2.3.9.3 Multi-analyte proof-of-concept assay

A thin-layer of APTES-modified PDMS was coated with 4 µg/mL anti-HA in PBS and incubated for 1 hour at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for 1 hour at room temperature. The PDMS was then degassed for 30 minutes, in a standard dessicator. A 10^{-6} dilution of anti-CRP scFv expressed in *E.coli* was prepared in auto-induction medium and added to 3 distinct areas of a 40 µm array and the array was sealed to the prepared PDMS. The array was then incubated at 37°C overnight. After incubation the array was carefully removed and the PDMS surface was washed gently with PBS. A 2.5 µg/mL dilution of Cy5-labelled CRP alone, Cy5-labelled CRP mixed with Cy3-labelled CRP and Cy3-labelled CRP alone was added to the surface and incubated for 1 hour at room temperature. The surface was then washed as previously described. The thin layer of PDMS was then air-dried and scanned using a Perkin Elmer GxArray scanner using both the 543 nm and 633nm lasers.

2.3.10 Double sided analysis

Two pieces of APTES-modified PDMS were coated with 5 µg/mL CRP and 5µg/mL human IgG (hIgG) and incubated for 1h at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for 1h at room temperature. The PDMS was degassed for 30 minutes, in a standard dessicator. A 10^{-6} dilution of anti-CRP scFv expressed in *E.coli* was prepared in auto-induction medium and added a 40 µm array and the array was sealed to the prepared PDMS, sequentially. The array was then incubated, in a vertical orientation, at 37°C overnight. After incubation the arrays were carefully removed and the PDMS surfaces washed gently with PBS. A 2.5 µg/mL dilution of Cy5-labelled anti-HA was added to the surface and incubated for 1h at room temperature. The surface was then washed

as previously described. The thin layers of PDMS were then air-dried and scanned using a Perkin Elmer GxArray scanner using a 633 nm laser.

2.3.11 Multi-lift analysis

APTES-modified PDMS were coated with 5 µg/mL CRP and 5 µg/mL human IgG (hIgG) and incubated for 1h at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for 1h at room temperature. The PDMS was degassed for 30 minutes, in a standard dessicator. A 10^{-6} dilution of anti-CRP scFv expressed in *E.coli* was prepared in auto-induction medium and added a 40 µm array which was sealed to the prepared PDMS. The array was then incubated at 37°C for eight hours. After incubation the arrays were carefully removed and the PDMS surfaces washed gently with PBS and a 2.5 µg/mL dilution of Cy5-labelled anti-HA was added to the surface and incubated for 1h at room temperature. The surface was then washed as previously described. The thin layers of PDMS were then air-dried and scanned using a Perkin Elmer GxArray scanner using a 633 nm laser. The array was sealed to a new, identically prepared surface and allowed to incubate overnight at 37°C. The assay was then completed, as described previously.

2.3.12 Diffusion

PDMS-APTES was coated with 5 µg/mL human IgG and incubated for 1 hour at room temperature. The surface was then blocked using 3% (w/v) BSA for 1 hour at room temperature. Following removal of the blocking solution the surface was rinsed with PBS, air-dried and degassed for 30 minutes, in a standard dessicator. Dilutions of Cy5-labelled anti-human IgG were then added to a microcapillary array in one of three ways, prior to sealing to the prepared PDMS surface:

1. Standard - A solution of 2.5 µg/mL of Cy5-labelled anti-human IgG was loaded directly into the array as per standard procedures.
2. Diffusion proof-of-concept- PBS was loaded into the array and the 2.5 µg/mL solution of Cy5-labelled anti-human IgG were added on top of the array.
3. Diffusion Control- The 2.5 µg/mL solution of Cy5-labelled anti-human IgG was added on top in a dry area of the array.

The array was allowed to incubate for three hours at room temperature then washed with PBS and imaged using a Perkin Elmer ScanArray GX microarray scanner.

2.3.13 Determination of feasibility of in-solution analysis/FRET analysis on array

The DNA probes were as follows:

DNA probe 1: 5′- **Cy5**-TTA CGG TTG GTG GCG TCT CTG

DNA probe 2: 3′- AAT GCC AAC CAC CGC AGA GAC-**Cy5.5**

In order to measure the energy transfer between the donor and acceptor in solution, 1 x 10^{-8} M solutions of the donor labelled oligonucleotide was mixed 1:1 (v/v) with hybridisation buffer alone, or with hybridisation buffer containing 1 x 10^{-8} M acceptor-labelled complementary oligonucleotide. Acceptor-labelled oligonucleotide was also mixed 1:1 (v/v) with buffer resulting in three final solutions containing donor alone, donor and acceptor mixed and acceptor alone. 100 µL of each solution was immediately added to wells of a black NUNC 96 well plate and 2 µL of each solution added to different regions of a 40 µm diameter microcapillary array. Both array and the micotitre plate were incubated at 37^{o}C for 30 min before analysis. The micotitre plate was analysed using a Tecan Sapphire II (Ex 610 nm and Em 650-750 scan) fluorescent plate reader and the array analysed using a fluorescent microscope with a filter for use with Cy5.

2.3.14 Determination of feasibility of in-solution analysis/GFP-reassembly on array

These experiments were carried out using the control vector set supplied. The first vector, pET11a-Z-NGFP (NZ), contained the N-terminal region of GFP fused to a leucine zipper peptide (anti-parallel to that on Vector 2). It was ampicillin resistant and expression was induced by IPTG addition (Lac I operon). The second vector, pMRBAD-Z-CGFP (CZ), encoded the C-terminal of GFP fused to a different leucine zipper peptide (anti-parallel to that on Vector 1). It was kanamycin resistant and expression was induced by arabinose addition (AraC operon).

Plasmids were transformed into BL21 (DE3) *E. coli* separately. A sequential transformation was then carried out, meaning that the cells transformed with one plasmid were made competent and then transformed with the second plasmid. This meant that both plasmids were contained within the same cell, denoted in this report as "*in vivo*". Positive transformants were identified by growth on screening medium which contains 1µM IPTG, 0.2% (w/v) arabinose, 35µg/mL kanamycin, 100µg/mL carbenicillin. This was carried out on solid medium and also in solution (in plate and on array). Cultures were grown at 37°C overnight, followed by 2-3 days at 15-25°C.

In addition to '*in vivo*' screening, cells containing single plasmids were mixed and co-expressed, denoted in this report as '*in vitro*'. This was done in the absence of antibiotics, as growth would have been inhibited. A 10 µL aliquot of each culture [CZ only, NZ only,

CZ+NZ '*in vivo'* (i.e. in the same cell), CZ+NZ '*in vitro'* (i.e. mixed culture) in appropriate expression medium, was added to the array and incubated for 5 days at 25°C.

2.3.15 Removal of contents of a microcapillary array using high pressure nitrogen.

A laser hole was burned through an acetate sheet using a CO_2 laser. The ablation was kindly carried out by Jose Garcia in the BDI. The size of the laser hole was observed and measured using a light microscope. The acetate was placed over a microcapillary array containing a solution of food dye and a flow of high pressure nitrogen applied to the area on the acetate which contained the ablated hole. The clearance zone in the array was then observed and measured using a light microscope using 10X magnification.

2.3.16 Investigation into volume retrieved and viability of cells following removal from the microcapillary array using the removal prototype.

The contents of a single capillary were then removed from the array and captured in the well of a microtitre plate (containing 85 µL of medium) below, six times. The contents of three of the wells were serially diluted and plated overnight at 37°C. The remaining three wells were incubated overnight at 37°C/200 rpm and plated, as before, on the subsequent day. A control was included for each condition (immediate enumeration or post 18 hour incubation enumeration) by pipetting 1.2 µL of the starting culture into 85 µL in the microtitre plate. This control would represent a 1000X greater number of cells than the standard retrieval. Bacteria were enumerated on each of the plates and the average for each condition calculated.

2.3.17 Development of a system for alignment of the array to the scanned image.

Alignment marks were introduced, by engineer Brian O'Reilly, into the array using SU8 photoresist polymer. To test the feasibility of their use a control assay was carried out. Briefly a thin-layer of APTES-modified PDMS was coated with 5 µg/mL of hIgG (un-labelled) in PBS and incubated for 1 hour at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for 1 hour at room temperature. The PDMS was then degassed for 30 minute, in a standard dessicator.. A 2.5 µg/mL solution of Cy5-labelled anti-hIgG was added to the array in the area of the alignment mark and was sealed to the prepared PDMS. The thin layer of PDMS was then air-dried and scanned using a Perkin Elmer GxArray scanner using a 633 nm laser.

2.3.18 Dual coating for alignment

APTES-modified PDMS was coated with either dual or single coating. Single coating involves coating with 5 µg/mL CRP in the assay area and 5 µg/mL human IgG (hIgG) in the alignment area. Dual coating involves 5µg/mL CRP mixed with 5 µg/mL hIgG in PBS. Both surfaces were incubated for 1h at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for 1h at room temperature. The PDMS was degassed for 30 minutes, in a standard dessicator. The control avian anti-CRP scFv (F8) was diluted in series (1 in 5) in auto-induction medium and added to two 40 µm arrays and the arrays were sealed to the prepared PDMS surfaces (Dual and singly coated). The arrays were then incubated at 37°C overnight. After incubation the arrays were carefully removed and the PDMS surfaces washed gently with PBS. A 2.5 µg/mL dilution of Cy5-labelled anti-HA was added to the surface and incubated for 1h at room temperature. The surface was then washed as previously described. The thin layer of PDMS was then air-dried and scanned using a Perkin Elmer GxArray scanner using a 633 nm laser.

2.4 Methods-Chapter 4

2.4.1 Microcapillary array assay

APTES-modified PDMS was coated with 5 µg/mL antigen (various, detailed in Table 2.5) and 5 µg/mL human IgG (alignment assay) in PBS and incubated for 1h at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for 1h at room temperature. The PDMS was degassed for 30 minutes, in a standard dessicator. The cells to be screened were diluted to the required level, in appropriate medium, loaded on to a 40µm array and the array was sealed to the prepared PDMS. Cell number loaded to the array is on the basis of limiting dilution. A solution of Cy5-labelled anti-human IgG (1 µg/mL) was also added in the designated alignment areas, prior to sealing. The array was then incubated at 37°C overnight for bacteria and 2-3 days for mammalian cells. After incubation the array was carefully removed and the PDMS surface was washed gently with PBS. A secondary detection agent (various, detailed in Table 2.5) was added to the surface and incubated for 1h at room temperature. The surface was washed, as previously described, air-dried and scanned using a Perkin Elmer GxArray scanner with a 533nm (Cy3) or 633nm laser (Cy5).

Details for microcapillary array assays, coating and detection reagents, are detailed in Table 2.5. Each of the coating reagents also had 5 µg/mL human IgG included, to allow for alignment. For bacterial assays, cells were diluted in auto-induction medium. Mammalian cells were diluted in CO_2 independent medium with 10% (v/v) FCS and 5% (v/v) Briclone.

Table 2.5: Array assay reagent details.

Assay	Section	Coating Reagent	Detection Reagent
CRP Library	4.1.2	anti-HA (4 µg/mL)	Cy5-labelled CRP (2.5 µg/mL)
Multi-Analyte	4.1.4	anti-HA (4 µg/mL)	Cy5-labelled CRP (2.5 µg/mL) Cy3-labelled MPO (2.5 µg/mL)
Double-sided	4.1.4	Side 1:MPO (5 µg/mL) Side 2:CRP (5 µg/mL)	Cy5-labelled anti-HA (5 µg/mL)
dAb Library (Protein)	4.2.3	Transferrin, cTnI or cTnT (5 µg/mL)	Anti-cMYC (5 µg/mL) Cy5-labelled anti-Mouse (2 µg/mL)
dAb Library (Cells)	4.2.3	Anti-cMYC (5 µg/mL)	Jurkat cells (1 x 10^6 cells/mL) stained with anti-EpCAM (5 µg/mL)
HSL (Antigen)	4.3.2 4.3.3	HSL-BSA conjugate (16 µg/mL)	Anti-cMYC (5 µg/mL) Cy5-labelled anti-Mouse (2 µg/mL)
HSL (Capture)	4.3.2	Anti-cMYC (5 µg/mL)	Cy5-labelled HSL-BSA conjugate (16 µg/mL)

2.4.2 Bacterial ELISA confirmation (On-plate assay)

From the positive clones identified, those retrieved from the microcapillary array were grown overnight in LB medium at 37°C/200rpm. Cultures were plated out twice and single colonies selected for each retrieval, to ensure monoclonality. A confirmatory ELISA was then carried out on these clones, using an on-plate method previously described in Guo *et al*., (2010). Briefly, plates were coated with 2 µg/mL of the selected antigen (Table 2.6) for 1 hour at 37°C. Wells were then blocked with 3% (w/v) BSA for 1 hour at 37°C. Clones were then expressed (1 per well) in the prepared wells in auto-induction medium overnight at 37°C/200 rpm. After incubation wells were washed three times with PBST and three times with PBS. Secondary detection was carried out using the appropriate secondary antibody, as detailed in Table 2.6, and incubated at 37°C for 1 hour. Plates were then washed as previously described and TMB added to the wells and incubated for 20 minutes at room temperature. Colour development was stopped using 10% (v/v) HCl and plates were read at 450 nm using a Tecan Safire II plate reader.

Assay details, coating and detection reagents, are given in Table 2.6. Cells were added to the plate in auto-induction medium.

Table 2.6: On-plate assay reagent details.

Assay	Section	Coating Reagent	Detection Reagent
CRP Library	4.1.2	CRP (2 µg/mL)	HRP-labelled anti-HA (50 ng/mL)
Multi-Analyte	4.1.4	CRP (2 µg/mL) MPO (1 µg/mL)	HRP-labelled anti-HA (50 ng/mL)
Double-sided	4.1.4	CRP (2 µg/mL) MPO (1 µg/mL)	HRP-labelled anti-HA (50 ng/mL)
dAb Library (Protein)	4.2.3	Transferrin, cTnI or cTnT (2 µg/mL)	Anti-cMYC (5 µg/mL) HRP-labelled anti-Mouse (1 µg/mL)
dAb Library (Cells)	4.2.3	Jurkat cells (1 x 10^6 cells/mL)	Anti-cMYC (5 µg/mL) HRP-labelled anti-Mouse (1 µg/mL)
HSL (Antigen)	4.3.2 4.3.3	HSL-BSA conjugate (8 µg/mL)	Anti-cMYC (5 µg/mL) HRP-labelled anti-Mouse (1 µg/mL)
HSL (Capture)	4.3.2	Anti-cMYC (5 µg/mL)	HSL-BSA conjugate (8µg/mL) HRP-labelled anti-BSA (2.0µg/mL)

2.4.3 Non-competitive ELISA for determination of antibody lysate titer.

Microtitre plates were coated with 2 µg/mL antigen, detailed in Table 2.7, for 1 hour at 37°C. The plates were then blocked with 200 µL of a 3% (w/v) BSA and incubated at 37°C for 1 hour. Dilutions (1in 5 series) of lysate were prepared in 1% (w/v) BSA and each dilution added to the plates in triplicate. The plates were then incubated at 37°C for 1 hour, followed by 3 washes with PBS-Tween and 3 washes with PBS. The secondary detection reagent (Table 2.7) was prepared in 1% (w/v) BSA, added to the plates and incubated at 37°C for 1 hour. Wells were then washed, as before and TMB was added and incubated for 20 minutes at room temperature. Colour development was stopped using 10% (v/v) HCl and plates were read using a Tecan Safire II plate reader at 450 nm.

Table 2.7: Titer ELISA Reagent details

Assay	Section	Coating reagent	Dilution Series	Detection reagent
CRP	4.1.3	2 µg/mL CRP	1 in 5	HRP-labelled anti-HA (50 ng/mL)
MPO	4.1.4	1 µg/mL MPO	1 in 5	HRP-labelled anti-HA (50 ng/mL)
HSL (All)	4.3.4	4 µg/mL HSL-BSA	1 in 5	HRP-labelled anti-HA (50 ng/mL)

2.4.4 Competitive ELISA for determination of antibody performance.

The ELISA plates were coated with antigen, detailed in Table 2.8, and blocked with 3% (w/v) BSA. Dilutions of antigen, as shown in Table 2.8, were prepared in 1% (w/v) BSA and mixed with an equal volume of each antibody. The dilution of each individual scFv lysate was determined from the titer determined as described in section 2.4.3. This mixture was added to the coated and blocked plates and incubated at 37°C for 1 hour. Plates were washed

three times with PBS-Tween and 3 times with PBS. Secondary detection was again carried out using selected detection reagent (Table 2.8) in 1% (w/v) BSA, incubated at 37°C for 1 hour. Wells were then washed, as before and TMB was added and incubated for 20 minutes at room temperature. Colour development was stopped using 10% (v/v) HCl and absorbances were read using a Tecan Safire II plate reader at 450 nm.

Table 2.8: Competitive ELISA Reagent details

Assay	**Section**	**Coating reagent**	**Antigen Conc. Dilution series**	**Detection reagent**
CRP	4.1.3	2 µg/mL CRP	100 µg/mL 1 in 5 series	HRP-labelled anti-HA (50 ng/mL)
MPO	4.1.4	1 µg/mL MPO	100 µg/mL 1 in 5 series	HRP-labelled anti-HA (50 ng/mL)
HSL (3OXO)	4.3.4	4 µg/mL 3OXO-BSA	250 µM 1 in 5 series	HRP-labelled anti-HA (50 ng/mL)
HSL (3OH)	4.3.4	4 µg/mL 3OH-BSA	250 µM 1 in 5 series	HRP-labelled anti-HA (50 ng/mL)
HSL (NAC)	4.3.4	4 µg/mL NAC-BSA	250 µM 1 in 5 series	HRP-labelled anti-HIS (1 µg/mL)
HSL (C4)	4.3.4	4 µg/mL NAC-BSA	25 mM 1 in 5 series	HRP-labelled anti-HIS (1 µg/mL)

2.4.5 ELISA testing of purification fractions for final anti-HSL scFv clones.

Microtitre plates were coated with 4 µg/mL 3OXO-BSA for 1 hour at 37°C. The plates were then blocked with 200 µL of a 3% (w/v) BSA and incubated at 37°C for 1 hour. Each of the purification fractions were added to the plates for each of the clones. The plates were then incubated at 37°C for 1 hour, followed by 3 washes with PBS-Tween and 3 washes with PBS. The secondary detection reagent, HRP-labelled anti-HIS (1 µg/mL) was prepared in 1% (w/v) BSA, added to the plates and incubated at 37°C for 1 hour. Wells were then washed, as before and TMB was added and incubated for 20 minutes at room temperature. Colour development was stopped using 10% (v/v) HCl and plates were read using a Tecan Safire II plate reader at 450 nm.

2.5 Methods-Chapter 5

2.5.1 Sandwich ELISA for the detection of human IgE (hIgE).

A 96 well microtitre plate was coated with 2 µg/mL anti-hIgE mAb 107 in PBS and incubated for 1 hour at 37°C. The wells were emptied and blocked by addition of 3% (w/v) BSA and incubated for 1 hour at 37°C. Supernatant from hIgE secreting multiple myeloma cells was added to the plates which were then incubated at 37°C for 1 hour. Wells were washed 3 times with PBST and 3 times with PBS. A 0.33 µg/mL dilution of anti-hIgE mAb

182-biotin was added to the wells and incubated for 1 hour at 37°C. Plates were washed as before and 2 µg/mL of HRP-labelled streptavidin was added to each well and incubated for 1 hour at 37°C. Plates were then washed as previously described and absorbance was read using a Tecan Safire plate-reader at 450 nm.

2.5.2 Detection of human IgE secreted from multiple myeloma cells which have been incubated on the microcapillary array.

Cells were pelleted by centrifugation at 4,500 x g for 3 minutes and re-suspended in fresh RPMI 1640 medium supplemented with 10% (v/v) FBS. The cells were enumerated and a dilution of 7.2 x 10^4 cells/mL was added to a 40 µm array and incubated for 2 days at 37°C and 5% CO_2. Following incubation, the cells were removed from the array using nitrogen gas and collected. The cells were the pelleted as previously described and the supernatant analysed by ELISA for hIgE, as detailed in section 2.5.1.

2.5.3 Establishment of appropriate incubation time for immortalised B-cells (U266 multiple myeloma cell line) to detect secreted antibody.

A time course was carried out on hIgE-secreting U266 cells to determine the optimum incubation time to allow detectable levels of hIgE to accumulate. The medium used for all cell manipulations was RPMI 1640 with 10% (v/v) foetal bovine serum (FBS). A sandwich FLISA was carried out on each day of the time course as described in section 2.5.1, with the HRP-labelled streptavidin replaced with 2.5 µg/mL of Cy5-labelled streptavidin. Fluorescence was read using a Tecan Safire plate-reader at 650 nm excitation/670 nm emission with a bandwidth of 10 nm.

2.5.4 Growth of multiple myeloma cells in the array and subsequent detection of secreted human IgE.

A DiCAST screen was carried out using a sandwich FLISA on array. A thin-layer of APTES-modified PDMS was coated with 2 µg/mL anti-hIgE mAb 107 in PBS and incubated for 1 hour at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for 1 hour at room temperature. The PDMS was then degassed for 30 minutes, in a standard dessicator. Cell dilutions were added to distinct areas of a 40 µm array starting at a dilution of 5 x 10^5 cells/mL and diluted in series (1/10) and the array was sealed to the prepared PDMS. The array was then incubated at 37°C and 5% CO_2 for 4 days. After incubation cell the array was carefully removed and the PDMS surface was washed gently with PBS. A 0.33 µg/mL dilution of anti-hIgE mAb 182-biotin was added to the PDMS and incubated for 1 hour at room temperature. The PDMS was then washed as before and 2.5 µg/mL of Cy5-labelled streptavidin was added to the surface and incubated for 1 hour at

37°C. Plates were then washed as previously described. The thin layer of PDMS was then air-dried and scanned using a Perkin Elmer GxArray scanner using a 633 nm laser.

2.5.5 Determination of possibility of passing mammalian cells through the capillaries of an array.

Newly passaged U266 multiple myeloma cells were enumerated and added to an area of a 40 µm microcapillary array at a cell number of 7.2 x 10^4 cells/mL. They were then removed from the array using high pressure nitrogen gas and collected in a microtitre plate. The cells were subsequently enumerated to estimate the loss of cells on passage through the capillaries of the array. In addition the size of the cells was determined using a Millipore Sceptor device.

2.5.6 Removal of mammalian cells from the capillaries of an array using the DiCAST prototypes laser removal approach.

Multiple myeloma cells were added to a 40 µm array at a concentration of approximately 2 x 10^6 cells/mL. The array was then loaded on to the prototype and a standard retrieval was carried out. Cells were captured in 384 well plates containing 50 µL/well of RPMI 1640 medium supplemented with 10 % (v/v) FBS. Intact cells, in target wells, were observed using a light microscope at 4X and 10X magnifications. For subsequent retrievals, following observations made using HRP into TMB the volume per well was increased to 85 µL/well.

2.5.7 Detection of mouse IgG from naïve B cells isolated from a murine spleen.

In order to determine the feasibility of screening murine splenic cells directly after sacrifice a sandwich FLISA, against mouse IgG, was carried out using DiCAST. A thin-layer of APTES-modified PDMS was coated with 2 µg/mL anti-hIgE mAb 107 in PBS and incubated for 1 hour at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for 1 hour at room temperature. The PDMS was then degassed for 30 minutes, in a standard dessicator. A mouse was sacrificed and the spleen removed. The spleen was then added to 1mL RPI 1640 medium with 10% (v/v) FBS. The B cells were collected by rinsing the medium through the spleen. Cells were centrifuged for 5 minutes at 4,500 x g. The pelleted cells were then re-suspended in fresh medium and enumerated. The cell count was 3 x 10^7 cells/mL. Cells were diluted in series (1/10) and loaded onto a 40 µm array in distinct areas. The array was then incubated for 4 days at 37°C and 5% CO_2. Following incubation the array was removed from the PDMS and washed gently with PBS. A 2.5 µg/mL of Cy5-labelled anti-mouse IgG was prepared, added to the PDMS and incubated for 1 hour at 37°C. The PDMS was then washed gently with PBS, air-dried and scanned using a Perkin Elmer GxArray scanner using a 633 nm laser.

2.5.8 Multiplexed screening of murine B cells from a mouse immunised with three different proteins.

Two pieces of thin-layer APTES-modified PDMS were coated, with either 5 µg/mL Myoglobin or 5 µg/mL anti-mouse IgG in PBS, and incubated for 1 hour at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated for 1 hour at room temperature. The PDMS was degassed for 30 minutes, in a standard dessicator. A mouse previously immunised with CRP, Transferrin and Myoglobin was sacrificed and the spleen removed. The spleen was then added to 1 mL CO_2 independent medium with 10% (v/v) FBS and 5% (v/v) Briclone. The B cells were collected by rinsing the medium through the spleen. Cells were centrifuged for 5 minutes at 4,500 x g. The pelleted cells were then re-suspended in fresh medium, enumerated and diluted to a suitable loading density, in this case 1 x 10^6 cells/mL. The array was then sealed to each surface, sequentially, and incubated for 3 days at 37°C and 5% CO_2. Following incubation the array was removed from the PDMS and stored and the PDMS surfaces were washed gently with PBS. A 2.5 µg/mL dilution of Cy5-labelled anti-mouse IgG was prepared, added to the PDMS surface for Side 1 (Myoglobin coated). A 2.5 µg/mL dilution of Cy3-labelled CRP and Cy5-labelled Transferrin was prepared and added to the surface for Side 2 (anti-mouse IgG coated). Both were incubated for 1 hour at room temperature. The PDMS was then washed gently with PBS, air-dried and scanned using a Perkin Elmer GxArray scanner with a 633nm laser (Cy5) and a 533nm laser (Cy3).

2.5.9 B cell confirmatory ELISA

The contents of capillaries of interest were captured in the wells of 384-well plates containing DMEM with 10% (v/v) FCS and 5% (v/v) Briclone and incubated at 37°C and 5% CO_2. Subsequent to appropriate incubation supernatants were analysed by direct binding ELISA. Briefly, ELISA plates were coated with 2 µg/mL of the selected antigen for 1 hour at 37°C. Wells were blocked with 3% (w/v) BSA for 1 hour at 37°C. Supernatants from B cells were then added to the wells of the plate and incubated for 1 hour at 37°C. After incubation wells were washed three times with PBST and three times with PBS. A solution of HRP-labelled anti-mouse IgG was added to the wells and incubated at 37°C for 1 hour. Plates were then washed as previously described and TMB added to the wells and incubated for 20 minutes at room temperature. Colour development was stopped using 10% (v/v) HCl and absorbances were read at 450 nm using a Tecan Safire II plate reader.

2.5.10 Labelling of Jurkat cells as antigen

Jurkat cells were centrifuged for 3 minutes at 4,500 x g and resuspended in 3 % (w/v) BSA containing 5 µg/mL Cy5-labelled anti-EpCAM and incubated at 37°C for 1 hour. The cells

were washed three times by centrifuging as described previously and resuspending in PBS. Cells were finally resuspended in 3 % (w/v) BSA and used for screening.

2.5.11 Isolation of mRNA from cells

Multiple myeloma cells were enumerated, diluted in series (1/10) and pelleted by centrifugation at 4,500 x g for 3 minutes. The pellet was then resuspended in 1 mL Trizol and incubated at room temperature for 5 minutes. Following incubation 250 µL of Chloroform was added, the tube was shaken vigorously for 15 seconds and then centrifuged at 12,000 x g for 5 minutes. At this point there were three layers visible in the tube (Top Layer-Aqueous; Middle Layer/Interphase-White precipitated DNA; Bottom Layer-Pink Organic phase). The aqueous layer was carefully pipetted into a fresh 1.5 mL Eppendorf and 550 µL of isopropanol was added to the aqueous phase, mixed gently and incubated at room temperature for 5 minutes. The sample was then centrifuged at maximal speed (~14,500 x g) for 20 minutes. The isopropanol was carefully removed from the pellet and 1 mL of 75% (v/v) ethanol (in DEPC-treated water) added to the tube, mixed gently and centrifuged as before. The ethanol was then poured off and the pellets allowed to air dry. The pellet was then resuspended in 20 µL of DEPC-treated water, quantified using a Nanodrop 2000 and stored at -80°C until further use.

2.5.12 Reverse transcription of mRNA from cells to cDNA using Superscript® III reverse transcriptase

The mRNA was reverse-transcribed to produce cDNA using Superscript® III reverse transcriptase, as per the manufacturer's instructions, which are outlined below in Table 2.9.

***Table 2.9: Procedure and reagent preparation for reverse transcription of mRNA using Superscript® III reverse transcriptase*.**

Reagent	**Volume (µL)**
MIX 1	
RNA	5
Oligo dT Primer	1
10mM dNTPs	1
DEPC Water	3 (to give 10 µL total volume)
Mix thoroughly and incubate at 65°C for 5 minutes. Place on ice for > 1 minute.	
MIX 2	
10X RT Buffer	2
25 mM $MgCl_2$	4
0.1mM DTT	2
RNAOut	1
Superscript RT	1
Add 10 µL of Mix 2 to Mix 1. Mix thoroughly. Incubate at 50°C for 50 minutes, followed by 85°C for 5 minutes. Place on ice. Add 1 µL of RNase H and incubate at 37°C for 20 minutes. Store at -20°C for further analysis.	

2.5.13 Amplification of variable regions for single cell cloning

The cDNA generated from RNA obtained from single cells (avian splenic cells producing anti-Jurkat antibodies) was used as a template to amplify the variable regions of the antibodies using PCR, as described in section 2.2.7. Splicing by overlap extension (SOE) was generated from the variable regions to generate a scFv fragment. All primers are listed in Table 2.4. The scFv fragment for each cell was then cloned into a phagemid vector as described in section 2.5.14.

2.5.14 Restriction digestion of cloning fragments for single cell cloning

Digestion of 10 µg of both the purified PCR product and vector backbone (pCOMB3XSS) was carried out using NEB restriction enzyme Sfi I (36 U/µg DNA for insert and 6 U/µg DNA for vector) for 5 hours at 50°C, as per the Cold Spring Harbour, Phage display laboratory manual (Barbas *et al*., 2001). Digested fragments were then run on a 1% (w/v) agarose gel (section 2.2.8) and purified as described in section 2.2.9. The digested and purified vector was then dephosphorylated using antarctic phosphatase as detailed in section 2.2.10 and used in a ligation reaction, as described in section 2.5.15.

2.5.15 Ligation of cloning fragments for single cell cloning

The ligation reaction was set up as follows and incubated at room temperature overnight

Sfi I digested pCOMB 3XSS	(700 ng/rxn)	17	µL
Sfi I digested scFv insert	(350 ng/rxn)	23.6	µL
Ligase		2	µL
Ligase Buffer (10X)		10	µL
DEPC sterile water		47.4	µL (to give 100 µL reaction volume)

Following the incubation, the ligation mix was transformed into XL1-Blue electro-competent cells as described in section 2.2.11. A colony pick PCR was carried out on single colonies from transformations plates to confirm presence of the scFv insert was carried out, as described in section 2.2.12. Colonies positive for insert were then expressed and lysates tested for maintained binding as detailed in section 2.5.16.

2.5.16 Assessment of binding of cloned scFv fragments to intact Jurkat cells

Freshly passaged Jurkat cells were washed in PBS, resuspended in lysate of each different clone to be tested and incubated for 1 hour at 37°C. The cells were washed three times by centrifuging for 3 minutes at 4,500 x g and resuspending in PBS. Following the final wash,

the cells were transferred to a fresh tube containing 2.5 µg/mL biotinylated anti-HA and incubated for 1 hour at 37°C. The cells were then washed as previously described and resuspended in 5 µg/mL Cy5-labelled streptavidin in a fresh tube. Following the incubation cells were washed as previously described and visualised using a fluorescent microscope with a Cy5 filter at 10X and 20X magnifications.

2.5.17 Functional Bioassay proof of concept

PDMS was coated with 1 µg/mL M6 (anti-CK18) antibody for 1 hour at room temperature. The PDMS was then blocked by addition of 3% (w/v) BSA and incubated at room temperature for 1 hour. Jurkat cells (ATCC) diluted to 10^5 cells/mL were treated with a final volume of 20 µg/mL anti-Fas antibody, added to the array and incubated at 37°C/5% CO_2 for 24 hours. Following incubation, the array was removed carefully and the PDMS surface was washed gently with PBS. Secondary detection was carried out using a mixture of 1 µg/mL Cy3-labelled M5 (anti-CK18) and 1 µg/mL Cy5-labelled M30 (anti-caspase cleaved CK18), which was labelled in-house, and incubated for 1 hour at room temperature. The surface was then washed as previously described, air-dried and scanned using a Perkin Elmer GxArray scanner with a 533nm (Cy3) and a 633nm laser (Cy5).

2.6 Methods-Chapter 6

2.6.1 Biomek Usage

Use of the Beckman Coulter Biomek 2000 laboratory automation workstation was carried out as per the manufacturer's instructions. Programmes written were designed for use in coating, blocking, filling, carrying out dilution series and washing plates for all assay work.

2.6.2 Checkerboard Sandwich ELISA for determination of optimal coating concentration

Plates were coated with a range of dilutions of the polyclonal anti-CRP antibody (20/4/0.8/0.16/0 µg/mL) for 1 hour at 37°C. Wells were then blocked with 3% (w/v) BSA for 1 hour at 37°C. Dilutions of CRP (15 µg/mL; diluted in series 1/5) were prepared, added to the wells of the plate and incubated at 37°C for 1 hour. After incubation wells were washed three times with PBST and three times with PBS. The anti-CRP scFv A6 was then added to the wells at a 1 in 500 dilution (~1 µg/mL). Plates were washed as previously described and a 50 ng/mL dilution of HRP-labelled anti-HA was then added to the wells and incubated at 37°C for 1 hour. Plates were then washed as previously described and TMB added to the wells and incubated for 20 minutes at room temperature. Colour development was stopped using 10% (v/v) HCl and plates were read at 450nm using a Tecan Safire II plate reader.

2.6.3 Checkerboard Sandwich ELISA for determination of primary antibody (A6) concentration.

Plates were coated with a 4 µg/mL polyclonal anti-CRP antibody for 1 hour at 37°C. Wells were then blocked with 3% (w/v) BSA for 1 hour at 37°C. Dilutions of CRP (15 µg/mL; diluted in series 1/5) were added to the well of the plate, in triplicate, and incubated at 37°C for 1 hour. After incubation wells were washed three times with PBST and three times with PBS. The anti-CRP scFv A6 was then added to the wells at 1 in 100 (~5 µg/mL), 1 in 250 (~2 µg/mL) or 1 in 500 (~1 µg/mL) dilution. Plates were washed as previously described and a 50 ng/mL dilution of HRP-labelled anti-HA was then added to the wells and incubated at 37°C for 1 hour. Plates were then washed as previously described and TMB added to the wells and incubated for 20 minutes at room temperature. Colour development was stopped using 10% (v/v) HCl and absorbances were read at 450 nm using a Tecan Safire II plate reader.

2.6.4 Optimised anti-CRP sandwich ELISA

Plates were coated with 4 µg/mL, as determined to be optimal in section 2.6.2, of the polyclonal anti-CRP antibody for 1 hour at 37°C. Wells were then blocked with 3% (w/v) BSA for 1 hour at 37°C. Dilutions of CRP (1 µg/mL; diluted in series 1/2.5) were added to the well of the plate, in triplicate, and incubated at 37°C for 1 hour. After incubation wells were washed three times with PBST and three times with PBS. The anti-CRP scFv A6 was then added to the wells at a 1 in 500 dilution (~1 µg/mL). Plates were washed as previously described and a 50 ng/mL dilution of HRP-labelled anti-HA was then added to the wells and incubated at 37°C for 1 hour. Plates were then washed as previously described and TMB added to the wells and incubated for 20 minutes at room temperature. Colour development was stopped using 10% (v/v) HCl and absorbances were read at 450 nm using a Tecan Safire II plate reader.

2.6.5 Determination of CRP levels in spiked samples using anti-CRP sandwich ELISA

A standard curve was generated as described in section 2.6.4, with each standard determination carried out in triplicate. The spiked samples were obtained by preparing ten separate samples containing 0.001 µg/mL CRP. For samples designated buffer both standard curve and "unknown" samples were prepared in PBS containing 1.5% (w/v) BSA. For samples labelled serum, both standard curve and "unknown" samples were prepared in CRP depleted serum. The standard curve was generated using the BIAevaluation software and a four parameter equation fitted to the data. Sample concentrations were then calculated based on the standard curve obtained.

2.6.6 Application of anti-CRP sandwich ELISA to clinical samples

Plates were coated with 4 µg/mL, as determined to be optimal in section 2.6.2, of the polyclonal anti-CRP antibody for 1 hour at 37°C. Wells were then blocked with 3% (w/v) BSA for 1 hour at 37°C. Dilutions of CRP in serum (1 µg/mL; diluted in series 1/2.5) were added to the well of the plate, in triplicate, and incubated at 37°C for 1 hour. Each of the clinical samples was diluted 1 in 2 and then 1 in 5 series and added to the plate. After incubation wells were washed three times with PBST and three times with PBS. The anti-CRP scFv A6 was then added to the wells at a 1 in 500 dilution (~1 µg/mL). Plates were washed as previously described and a 50 ng/mL dilution of HRP-labelled anti-HA was then added to the wells and incubated at 37°C for 1 hour. Plates were washed as previously described and TMB added to the wells and incubated for 20 minutes at room temperature. Colour development was stopped using 10% (v/v) HCl and absorbances were read at 450nm using a Tecan Safire II plate reader. The standard curve was generated using the BIAevaluation software and a four parameter equation fitted to the data. Sample concentrations were then calculated based on the standard curve obtained.

2.6.7 Determination of CRP levels in clinical serum samples using anti-CRP sandwich ELISA

Assays were carried out, as per section 2.6.6. In place of a dilution series for each of the clinical samples, ten samples at a 1 in 2000 dilution were assayed for each of the samples. The standard curve was generated using the BIAevaluation software and a four parameter equation fitted to the data. Sample concentrations were then calculated based on the standard curve obtained.

2.6.8 Antibody lysate preparation for Biacore 4000 analysis

Cultures were grown in sterile 96-well plates in auto-induction medium, to allow for expression of scFvs, overnight at 37 ºC /200 rpm. The plates were then centrifuged at 4,500 x g for 20 minutes, the cell pellets were re-suspended in one-tenth the original culture volume of HBS-EP+ running buffer and subjected to three freeze-thaw cycles (-80ºC/37 ºC). Cell debris was removed by centrifugation at 4,500 x g for 10 minutes. Supernatant was diluted 1 in 4 in HBS-EP+ running buffer and used for analysis.

2.6.9 Antibody immobilisation and antigen screening for high-throughput screen

The anti-HA tag antibody was immobilised onto spots 1, 2, 4 and 5 of each flow cell, through amine coupling to a series S CM5 chip, by a 10 minute injection of 15 µg/mL antibody in 10 mM sodium acetate buffer, pH 4.2. Crude antibody-containing bacterial

lysates, diluted one in four in HBS-EP+ running buffer, were subsequently injected over spots 1 and 5 of each flow cell resulting in 8 different antibodies captured in every analysis cycle. The analyte (cTnI), at a concentration of 50nM, was passed over all five analysis spots simultaneously at a flow rate of 30 µL/minute. Association and dissociation times were 1 and 5 minutes, respectively, and the regeneration consisted of a 60 second injection of 20 mM NaOH. A total of 960 avian antibodies were analysed in this high-throughput screen, at an analysis temperature of 25ºC.

Data analysis was performed using the dedicated Biacore 4000 software and the antibody/antigen interaction was analysed by calculating the complex stability (% left) by expressing 'stability late' (calculated from the average response from 295 to 300 s after antigen injection) as a percentage of 'stability early' (calculated from the average response from 5 s after antigen injection).

2.6.10 Antibody immobilisation and antigen screening for '2 over 2' Kinetics

The anti-HA antibody was immobilised onto spots 1, 2, 4 and 5 of each flow cell, through amine coupling to a series S CM5 chip, by a 10 minute injection of 7.5 µg/mL antibody in 10mM sodium acetate buffer, pH 4.2. The immobilisation was carried out using separate injections for each spot on the flow cell, meaning that different quantities of ligand were immobilised on each of the spots. This allowed for the two ligand densities required for the '2 over 2' screen. The ligand was also immobilised on spot 3 to provide an appropriate reference and to negate the effect of non-specific binding of cTnI to the dextran surface of the chip. Each antibody-containing bacterial lysate, diluted one in four in HBS-EP+ running buffer, was injected over a pair of spots (1&2 or 4&5) of each flow cell. This resulted in 8 different antibodies being captured, on two different density surfaces, in every analysis cycle. Two separate injections of analyte, at concentrations of 100 nM and 25 nM, were passed over all five analysis spots at a flow rate of 30 µL/min. A zero injection was also included to allow for adequate referencing. Association and dissociation times were 1 and 10 minutes, respectively, and the surface was regenerated with a 60 second injection of 20 mM NaOH. Ninety-six antibody fragments, the top 10% chosen based on their complex stability, were analysed in total. Analysis temperatures of 25ºC and 37ºC were compared. The response data was fitted globally to a 1:1 interaction model using the Biacore 4000 evaluation software.

2.6.11 Full Kinetics

The anti-HA tag antibody was immobilised onto spots 1, 2, 4 and 5 of each flow cell, through amine coupling to a series S CM5 chip, by a 10 minute injection of 7.5 µg/mL antibody in 10mM sodium acetate buffer pH 4.2. Crude antibody-containing bacterial lysates, diluted one in four in HBS-EP+ running buffer, were subsequently injected over spots 1 and 5 of each flow cell. The analyte (cTnI), at a range of concentrations (100 nM to 6.25 nM; diluted in series (1/2)) was passed over all five analysis spots simultaneously at a flow rate of 30 µl/min. Association and dissociation times were 1 and 10 minutes, respectively, and the regeneration consisted of a 60 second injection of 20mM NaOH. The analysis temperature was 25 ºC. The response data was fitted globally to a 1:1 interaction model using the Biacore 4000 evaluation software.

2.6.12 Cloning of cTnI peptides as GFP fusion into pET28b vector

All conceptual and design elements of this cloning section were carried out by Dr. Paul Leonard. The cloning strategy involved the cloning of the gene encoding green fluorescence protein (GFP) into pET28b using *Bam*HI and *Hind*III. A plasmid prep of pQE30 with eGFP insert was used as template DNA for the reaction, detailed in section 2.2.7. The cTnI peptides (1, 2 and 3) were incorporated into the 5' primer to yield 6xHis-T7 tag-Peptide-GFP-6xHis-STOP. The same back primer was used for all peptides with the forward primer (encoding peptide) changing for each peptide. Primers sequences are listed in Table 2.4.

2.6.12.1 Restriction digestion of cloning fragments

Digestion of 10 µg of both the purified PCR product and vector backbone (pET 28b) was carried out using NEB restriction enzymes BamHI and HindIII (1 U enzyme/µg DNA). Compatibility of the enzymes was assessed by using NEB guidelines and it was determined that a sequential digest was required with the two enzymes concerned. The restriction digestion with HindIII was carried out first for 1 hour at 37ºC, after which the enzyme was inactivated by treatment at 80ºC for 20 minutes. This was followed by digestion with BamHI for 1 hour at 37ºC. Digested fragments were then run on a 1% (w/v) agarose gel (section 2.2.8) and purified as described in section 2.2.9. The purified vector (pET28b) was then de-phosphorylated using Antarctic phosphatase as detailed in section 2.2.10 before proceeding to ligation as described in section 2.6.12.2.

2.6.12.2 Ligation of cloning fragments

The ligation reaction was set up as follows and incubated at room temperature overnight

Hind III /BamHI digested pET28b (300 ng/rxn)	12	µL
Hind III/BamHI digested gene insert (300 ng/rxn)	7	µL
Ligase	1	µL
Ligase Buffer (10X)	3	µL
DEPC sterile water	7	µL (to give 30 µL reaction volume)

Following the incubation, the ligation mix was transformed into Rosetta 2 electro-competent cells as described in section 2.2.11. A colony pick PCR was carried out as detailed in section 2.2.12. A small scale expression culture was produced for each of the GFP-peptides to ensure correct expression. Following confirmation of expression, large-scale cultures were prepared as detailed in 2.2.3 and purified by IMAC (section 2.2.4) for use in Biacore epitope mapping experiments (section 2.6.13).

2.6.13 Epitope mapping of recombinant scFv fragments on Biacore 4000.

Each of the purified peptides was immobilised on a spot of each of the four flow cells through amine coupling to a series S CM5 chip, by a 10 minute injection of 7.5 µg/mL antibody in 10mM sodium acetate buffer, pH 4.2. Peptide 1 was immobilised to Spot 1, peptide 2 was immobilised to Spot 2, peptide 3 was immobilised to Spot 4 and peptide 4 was immobilised to Spot 5. Spot 3 remained unmodified to serve as a reference. Crude antibody-containing bacterial lysates, diluted one in four in HBS-EP+ running buffer, were subsequently injected over all spots of each flow cell. Association and dissociation times were 1 and 5 minutes, respectively, and the regeneration consisted of a 60 second injection of 20 mM NaOH. The analysis temperature was 25ºC. The binding response for each clone was then obtained, for each different analyte, using the Biacore 4000 evaluation software.

Chapter 3

Development of a novel microcapillary array-based, single cell analysis platform.

Hypothesis

Currently antibody screening approaches make use of successive rounds of screening, with each round containing fewer candidate antibodies, until a manageable number of potential clones has been achieved for further characterisation. By spatially addressing antibody-secreting cells (ASCs) in the capillaries of a microcapillary array, all cells could be subjected to screening. In analysing the entire population on a single cell basis, with no bias towards fast-growing clones, the chance of finding rare and unique clones could hopefully be improved. This approach would provide a high-throughput, non-biased and streamlined antibody screening process for novel antibody discovery.

Rationale

The intrinsic specificities of monoclonal antibodies for their target antigen are fundamental for their application as research tools, in the field of diagnostics and for therapy. By exploiting the capabilities of the immune system to generate billions of potential antibodies that can specifically target practically any molecules they encounter, it is possible to generate mAbs for therapy from large combinatorial libraries expressed in bacteria (Sblattero & Bradbury, 2000), yeast (Gai & Wittrup, 2007) and mammalian cells (Wurm, 2004) and even by *in vitro* amplification techniques such as mRNA/ribosome display (Lipovsek & Plückthun, 2004).

The ability to generate these libraries brings with it the problem of interrogating them efficiently, leaving researchers with the task of finding the one or few particular antibodies with desired characteristics from a highly diverse population of candidates. Depending on the starting source of antibodies current solutions involve selection by displaying the antibody on the surface of a phage particle (Bradbury *et al*., 2011) or cell (Bowers *et al*., 2014) and selection by affinity-based biopanning (Ehrlich *et al*.,2000) or FACS (de Kruif *et al*., 1995). This is followed by further testing on a single cell basis in microtitre plate-type assays (Barbas *et al*., 2001; Guo *et al*., 2010; Lee *et al*., 2007). Searching for antigen-specific ASCs from large populations of hybridomas or native B cells normally involves spatially addressing cell populations into microtitre plates for analysis. This is followed by cloning by limiting dilution of positive wells to obtain monoclonality (Staszewski, 1984).

This chapter investigates the feasibility of utilising porous, glass filters (Figure 3.1) as an ultra-high throughput screening tool for antibody discovery. The glass filters are manufactured commercially by fusing bundles of capillaries, of a certain diameter (5-100 µm), and cutting them into slices (1mm thick or less). By spatially addressing antibody-secreting cells (ASCs) in the capillaries, it was hoped that a powerful new tool to interrogate cells on a single cell basis could be achieved.

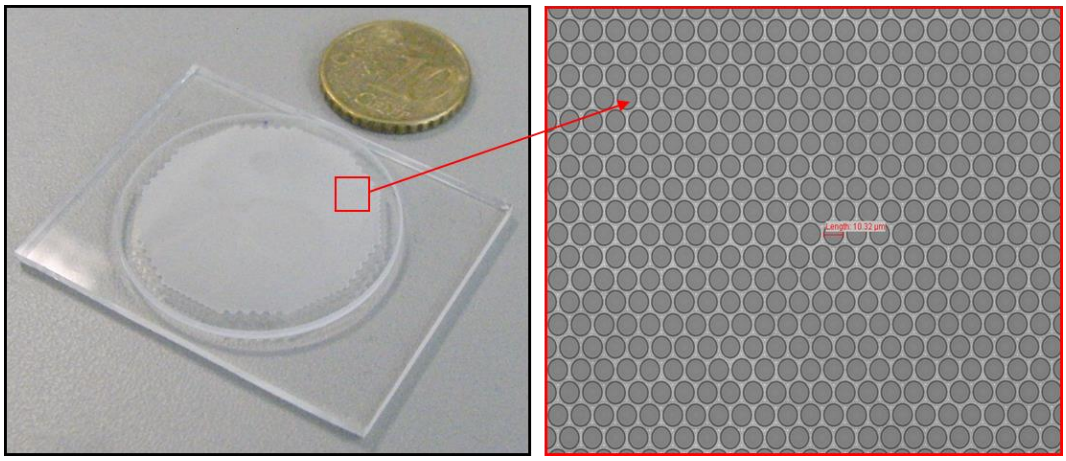

Figure 3.1: Microcaplliary array consisting of bundled, fused capillaries. *The array used was composed of capillaries with a 10 µm diameter and the total number of capillaries present, for this particular array, was approximately 4.5 million.*

The envisioned procedure, illustrated in Figure 3.2, would begin by filling the capillaries with culture medium containing antibody-secreting cells. The cells would grow and express antibodies into the medium which could bind to antigen immobilised onto a PDMS surface reversibly sealed to the array. The PDMS was degassed prior to sealing to provide a means of pulling the medium, in the capillaries, into contact with the assay surface and also to obtain a tighter seal. Upon removal of the porous glass array, the bound antibody would be detected by the addition of secondary fluorescent reagents (e.g. fluorescently-labelled antibodies or antigens). Positive signals, represented by a fluorescent spot, would correspond to capillaries containing cells with antigen-specific antibody that could be recovered from the porous glass array for further analysis.

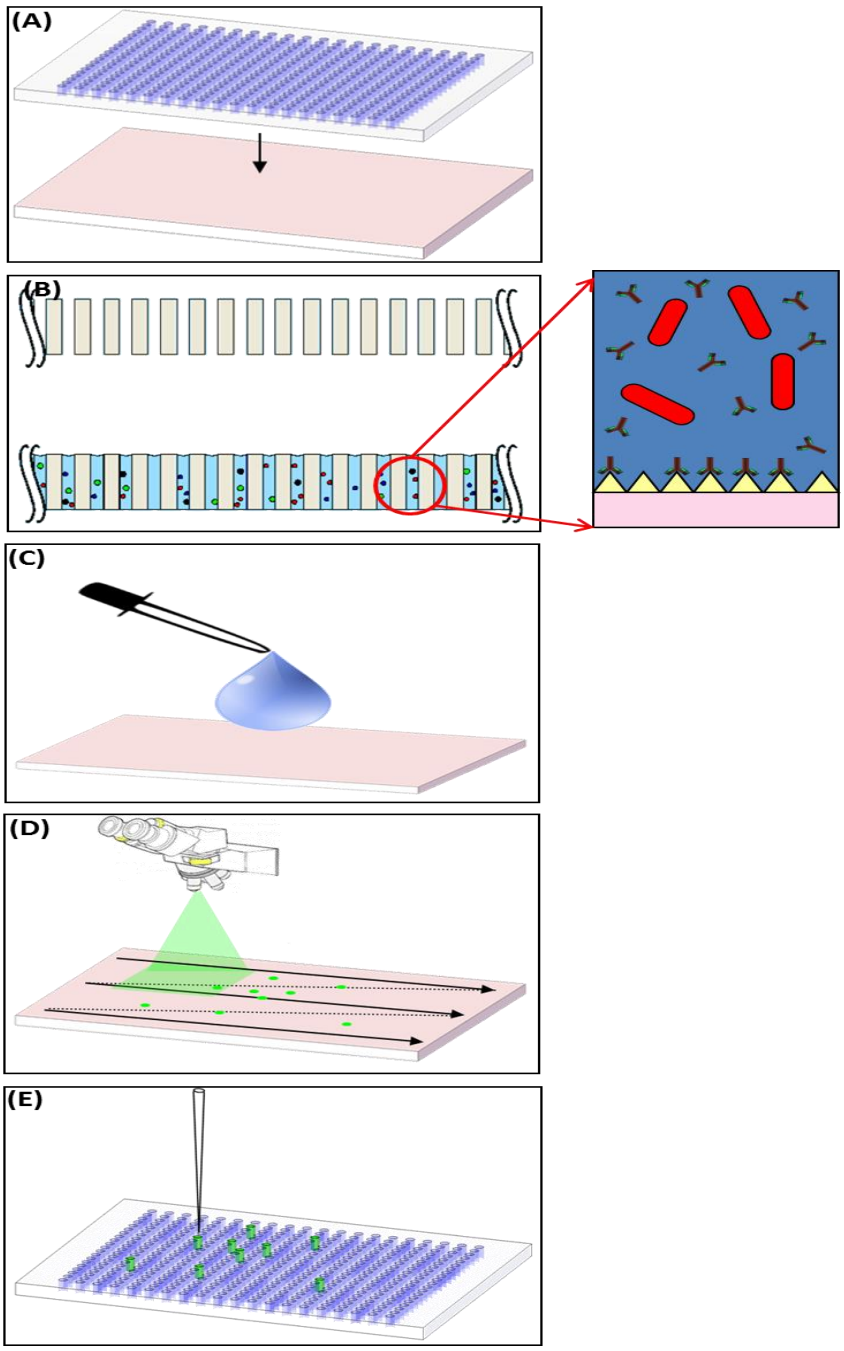

Figure 3.2: Proposed steps to allow for single cell analysis in the capillaries of a manufactured microcapillary array. (A) *The microcapillary array is sealed to the antigen-coated PDMS surface.* ***(B)*** *The antigen secreting cells are loaded into the array and begin to grow and express protein which, if specific to the antigen, will bind to the PDMS surface.* ***(C)*** *The array is removed and the PDMS surface subjected to several washing steps and fluorescence detection carried out.* ***(D)*** *The surface is visualised under a fluorescent microscope to identify any positive spots corresponding to antigen-specific antibodies.* ***(E)*** *The capillary containing the cell secreting the antibody of interest is located in the array and the contents removed.*

The proposed technology would need to meet several demanding criteria prior to being declared fit for purpose. These include:

- The ability to grow and observe detectable levels of expressed protein from the cells in the capillaries of the array.
- The ability to carry out an assay, determining one or more characteristics of the cell/antibody expressed, within the capillary or on a removable surface.
- The ability to retrieve the cell of interest, once identified, from the capillary for further analysis and cell banking.

Each of these challenges, illustrated in Figure 3.3, were investigated initially in terms of their feasibility and then further developed and modified to allow for integration into a functional screening system.

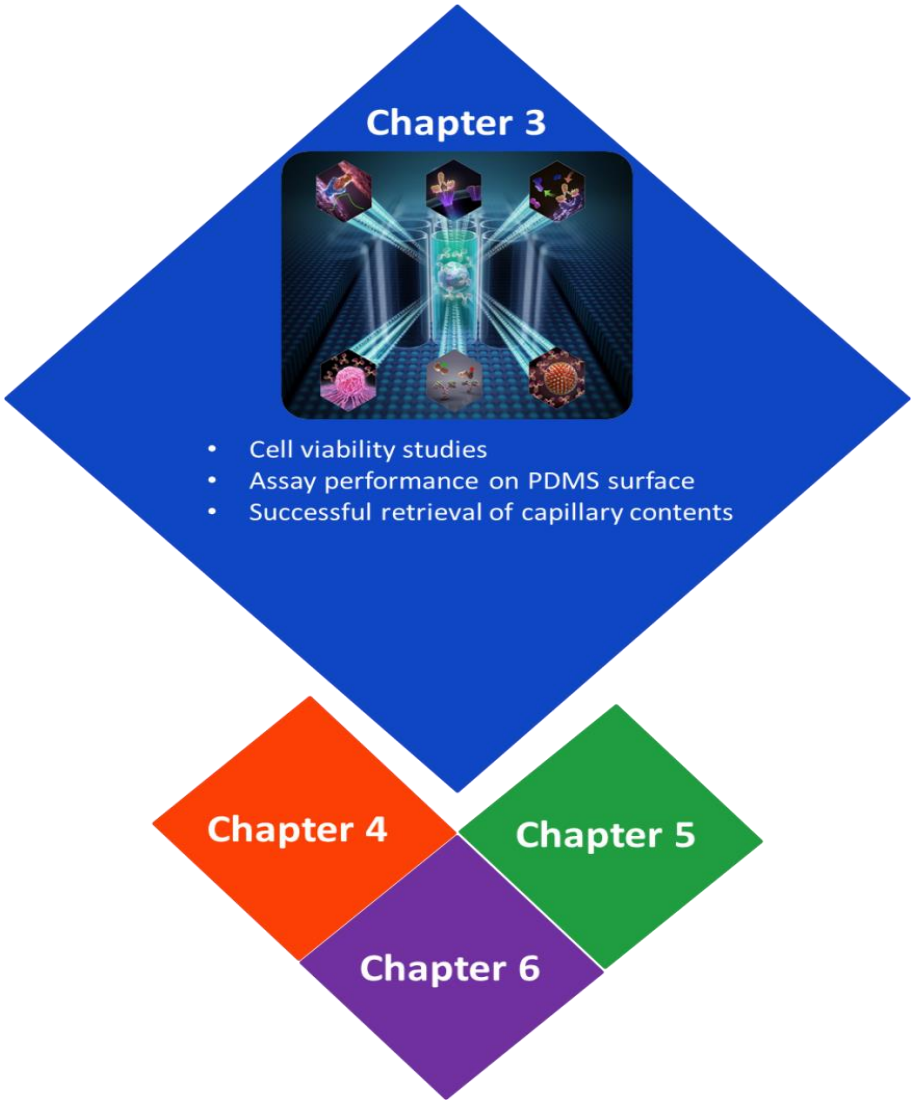

Figure 3.3: Schematic overview of thesis layout. *Chapter 3 demonstrates the development of the single cell analysis technology from a concept into a fully integrated system.*

Key Challenges

Key Challenge 3.1 Assessment of the possibility of growing bacterial cells in the array

3.1.1 Experimental Approach

The ability of the bacterial cells to grow, remain viable and express protein which can subsequently be detected on an assay surface in the proposed process is investigated in this section. Initially a method to express the desired protein, without the need for addition of an induction agent at a later stage, was assessed. Following the identification of an appropriate method to auto-induce protein expression, the ability of cells to grow and express protein in the nanolitre volume capillaries was investigated.

3.1.2 Comparison of auto-induction media with standard expression procedures.

Typically, using recombinant systems, the induction of antibody production would be initiated using the compound Isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG inactivates the *lac* repressor and induces synthesis of beta-galactosidase, an enzyme that promotes lactose utilization. It is used to induce expression of cloned genes under control of the *lac* operon (Sambrook & Russell, 2001).

As the bacteria will be loaded into the microcapillary array and sealed at the beginning of each experiment the use of an auto-induction system would be advantageous and would remove the need, and the uncertainty associated, with the addition of a reagent to the array. Auto-induction systems are based on the findings of Studier, who described the development of auto-induction medium, based on the fact that lactose can be responsible for target protein production but is hindered by certain compounds which become depleted during growth (Studier, 2005). On this basis, detailed medium formulations include components (e.g. glucose, amino acids and glycerol) which are differentially metabolised and allow for high density growth and auto-induction (Grabski *et al*., 2005).

The performance of two different auto-induction systems, one prepared ‘in-house’ and one commercial, were compared with a standard IPTG-induced culture, as described in section 2.3.1. The cultures were expressed overnight and then centrifuged to collect the cells at the base of the tube. The amount of cell material was observed as well as the amount of protein (GFP) expressed upon visualisation of the pelleted cells (Figure 3.4). In direct comparison the ZY and IPTG induced culture are similar in terms of cell density with overnight express medium producing slightly less. When the level of fluorescence is compared, as a means of assessing protein expression, the auto-induction media appear to outperform the traditional approach. Both of them produced pellets with uniform and high fluorescence, while the

IPTG-induced culture was not particularly uniform and was lower overall than the other cultures.

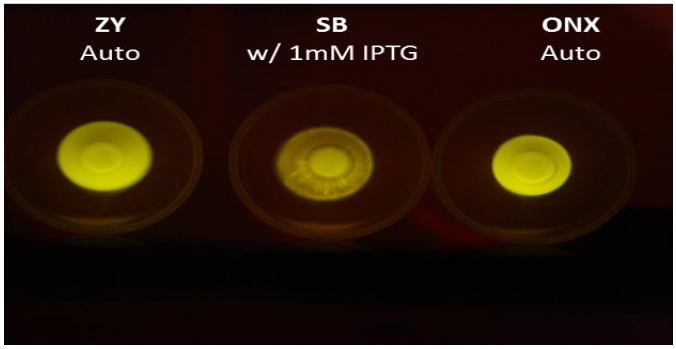

Figure 3.4: Comparison of growth and expression in various media types. *The cultures were grown in identical culture volumes overnight and the expressed cultures centrifuged and compared for each of the media types. ZY and ONX are auto-induction media systems and they were compared with the standard procedure, which involves the addition of IPTG once the culture has reached the logarithmic growth phase.*

In addition to visualisation of the cells the level of protein present in the lysate was analysed by SDS-PAGE as described in section 2.2.1. The result, shown in Figure 3.5, demonstrates that the methods all produce the specific protein, with the ZY producing the largest amount. Overnight express seems to have the least amount of non-target protein present in the lysate, with a strong band present for the target protein (Figure 3.5).

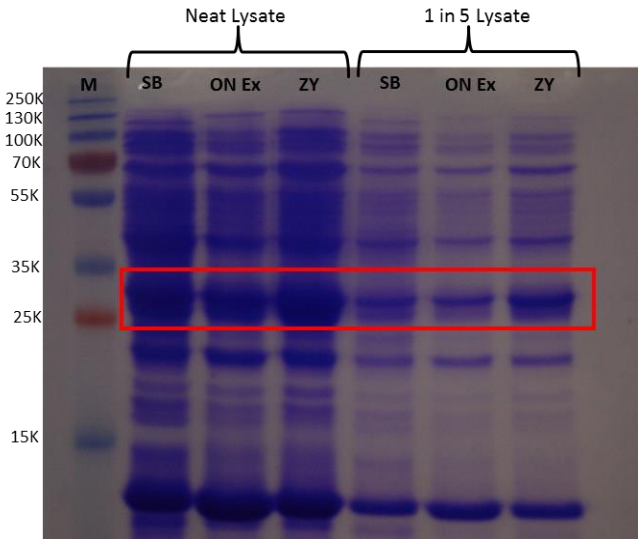

Figure 3.5: SDS-PAGE analysis of target protein produced using various media types. *Lysate was prepared for each of the expression cultures and analysed on SDS-PAGE to compare the levels of target protein expressed in each of the different media. A neat and a 1 in 5 dilution were analysed, for each sample, to allow for accurate visualisation of the samples. The expected size is approximately 27 kDa for the target protein.*

Based on the results obtained it appeared that the use of auto-induction media for array assays was a viable and desirable option. It allows each cell to analysed without introducing any bias based on their growth rate.

3.1.3 Investigation into protein expression and survival of bacteria in the microcapillary array.

In order to determine whether cells can reproduce, express protein and survive in the microcapillary array *E. coli* expressing GFP were added to the array in auto-induction medium and allowed to incubate overnight at 37°C, as described in section 2.3.2.

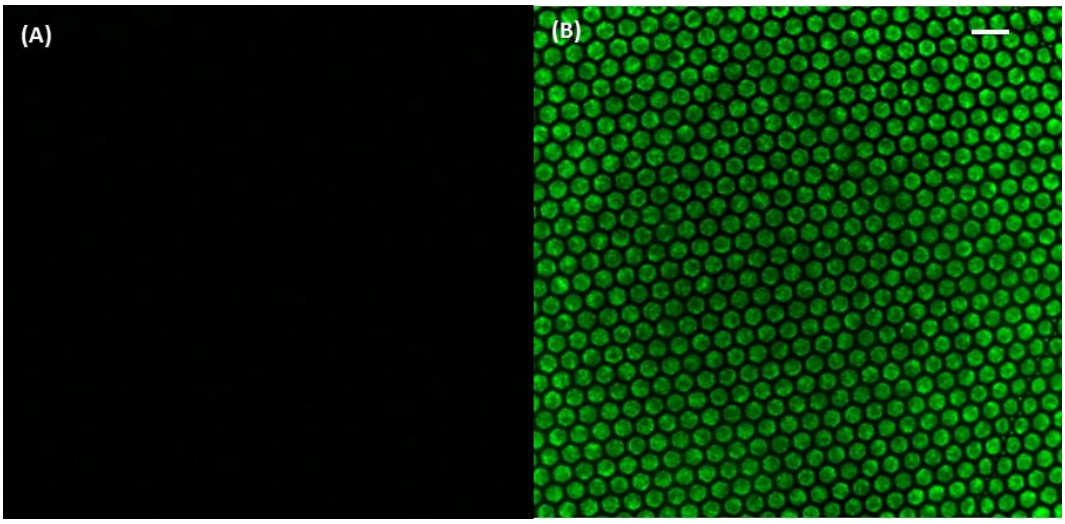

Figure 3.6: Expression of GFP by E. coli in the capillaries of the microcapillary array. *The area of the array which contained cells was imaged, using a fluorescent microscope with FITC filter, at* ***(A)*** *0 hours and* ***(B)*** *18 hours. Scale bar is 100 µm.*

The ability of the cells to replicate and express protein in the array was then confirmed by direct visualisation of expressed GFP after 18 hours (Figure 3.6B). When compared with an image taken at 0 hours (Figure 3.6A) a distinct green colour can be seen in the capillaries indicating the expression of the protein by *E. coli*.

In addition to visualisation of protein expression in the cells, a nitrogen flow was applied to a discrete portion of the array and the contents of several capillaries captured on an agar plate beneath. The plate was then incubated overnight to assess the viability of the cells following incubation in and removal from the microcapillary array. Bacterial growth was evident, following overnight incubation at 37 °C, as shown below in Figure 3.7.

Figure 3.7: Growth of bacterial cells following removal from the microcapillary array. *Cells were removed from the array under a stream of nitrogen and captured on the agar plate beneath. Subsequent to incubation, bacterial growth was evident, indicating that the cells can remain viable throughout the process.*

3.1.4 Determine feasibility of single capillary analysis

A key consideration when developing the microcapillary assay system proposed is the ability to address cells on a single cell basis, thus allowing for a non-biased screening approach. To determine the ability to screen recombinant systems at single cell level, *E. coli* which express a GFP protein were diluted in series (1in 10) and loaded into the array in discrete areas, as detailed in section 2.3.3. After overnight incubation, each area of the array was observed and recorded, as shown in Figure 3.8. On visualisation of the array, a decrease in fluorescence could be seen down to the point where single capillaries were clearly visible. Based on a Poisson distribution, this indicates that by diluting the cells added to a suitable level, single cell occupancy of capillaries can be obtained and detected.

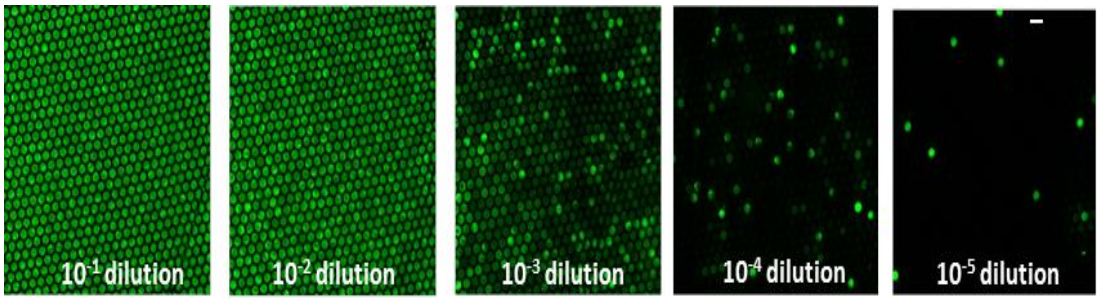

Figure 3.8: Visualisation of GFP-expressing E. coli in the capillaries of the array. *Dilutions of the culture were observed until single capillaries could be identified. The cell count from plates was 4.6 x 10^9 cfu/mL. Scale bar is 100 µm.*

Approximately 1 x 10^6 cfu/mL should theoretically equate to 1 cell per capillary, based on the 40 µm capillary diameter (1.2 nL volume). Based on Figure 3.8, this appears to be accurate with the 10^{-3} dilution representing 1 cell per capillary, 10^{-4} representing 1 cell in every ten capillaries and 10^{-5} showing 1 cell in every hundred capillaries. The ability to carry out single-cell analysis has been shown by observing expression of varying dilutions of a GFP-expressing *E. coli* bacteria. The dilution and cell number is largely important to achieving this, and will need to be determined for each recombinant system analysed.

Summary for key challenge 3.1

The ultimate aim for this particular section was to determine the possibility of growing cells, in addition to expressing and detecting target protein, in the capillaries of a microcapillary array. On investigation, the cells were proven to grow, express and remain viable in the array throughout incubation and retrieval. Furthermore, an auto-induction system was found to be comparable with traditional expression approaches.

Key Challenge 3.2 Investigation into performance of assays on a polydimethylsiloxane (PDMS) surface.

3.2.1 Experimental approach

Following confirmation of cell growth in the capillaries of the array, the ability to detect the proteins expressed was investigated. Initial investigations into performing a direct assay on the PDMS surface and observing the resultant pattern were completed, as described in 2.3.4. The introduction of required washing and secondary detection steps identified instability in the coated surface and meant that assay performance was negatively affected (e.g. loss of coated protein resulted in loss of detectable reagent and an increase in non-specific binding as the blocking solution was removed). The discovery of this instability then required the development of a method to modify the PDMS to provide a more stable, reliable and

uniform surface. The surface was then compared with standard microtitre plates, under a number of conditions to verify its performance and suitability for future use. Assay development was then undertaken to enhance the offering in terms of information garnered about each single cell analysed.

3.2.2 Feasibility of carrying out an assay on a PDMS surface.

PDMS was identified as the ideal assay surface as it could reversibly seal to the array surface. In order to determine if protein could be patterned through the array and detected on the surface, human IgG was coated on the PDMS and fluorescently-labelled anti-IgG was loaded into the array. The array was sealed to the PDMS, as described in section 2.3.4, and following incubation the surface was imaged using a microarray scanner and a fluorescent microscope.

Based on the images obtained (Figure 3.9), it was concluded that not only could protein be passed through the array and detected on PDMS surface but that the bonding between the PDMS and the array was sufficient to allow each capillary to remain sealed (i.e. no bleeding of protein between capillaries) throughout the process. This is evidenced by the clear black rings surrounding each capillary which corresponds to the capillary wall, as shown in Figure 3.9B.

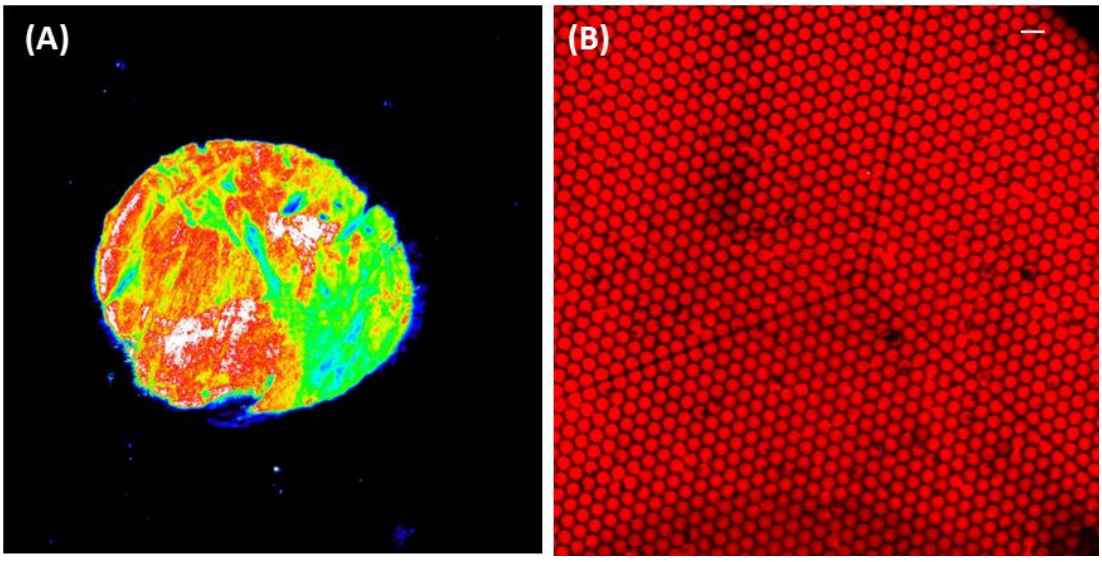

Figure 3.9: Detection of fluorescent protein on the PDMS surface. *The PDMS surface was coated with 10 µg/mL of human IgG. Fluorescently labelled anti-human IgG was then added to the capillaries of a microcapillary array with capillaries of 10 µm diameter, sealed to the PDMS and allowed to incubate. Following incubation, the array was removed and the surface imaged using* ***(A)*** *a Perkin Elmer Gx ScanArray microarray scanner (5 µm resolution) and* ***(B)*** *an Olympus IX81 fluorescent microscope (20X magnification). Scale bar is 100 µm.*

The resolution limit (5 µm) of the microarray scanner meant that individual capillary patterns were not visible using the capillaries with a 10 µm diameter, as the distance between capillaries was only 1 µm. The use of the microarray scanner was the desired approach as it would remove several of the limitations of using the standard fluorescent microscope which included the need for image stitching, extended imaging times and low sensitivity. In response to this, the process was tested with a microcapillary array with 40 µm diameter capillaries (10 µm distance between capillaries) and the patterning process repeated, as above. The resulting image (Figure 3.10) showed clear definition between capillaries when scanned in the microarray scanner at a 5 µm resolution. While the increase in capillary diameter would represent a loss in overall throughput (approximately 10-fold reduction), the benefits of using the microarray scanner were considered more important during development stages of the technology. As a result, the remaining development was predominantly carried out using arrays with 40 µm diameter capillaries and imaged using the microarray scanner.

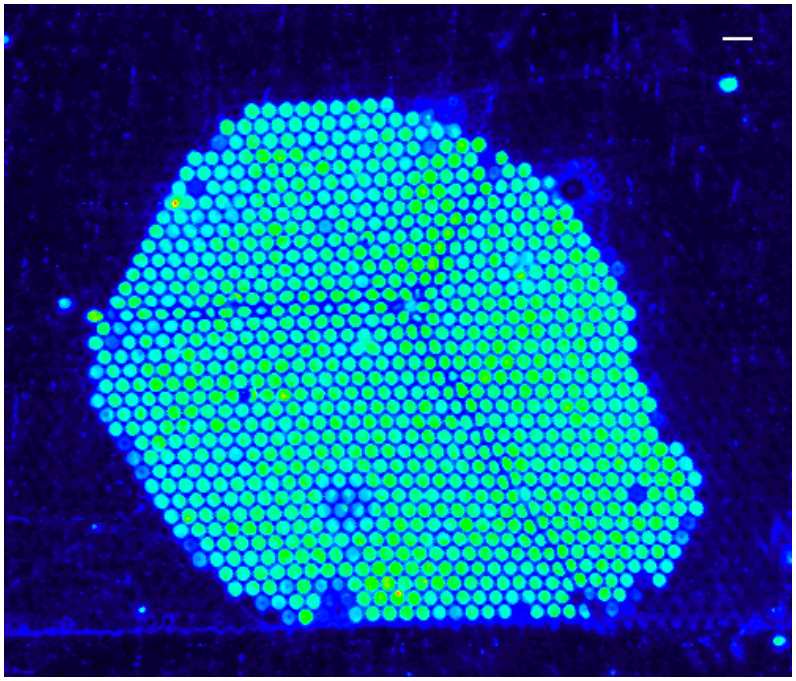

Figure 3.10: Pattern of fluorescent protein on a PDMS surface using a microcapillary array with 40 µm diameter capillaries. *The fluorescently labelled protein was added to the capillaries of an array, sealed to the PDMS and then allowed to incubate. Following incubation, the array was removed and the surface imaged using a Perkin Elmer Gx ScanArray microarray scanner at a 5 µm resolution. Scale bar is 100 µm.*

3.2.3 Identification of an appropriate functionalisation approach to allow for assays to be reliably performed on PDMS surface.

The assay surface, PDMS, was selected for use in the process as it allowed for complete but reversible seal to the microcapillary array. PDMS is naturally hydrophobic and chemically inert which lowers the possibility of immobilising biomolecules on its surface. It often has a tendency towards bio-fouling, which causes non-specific binding to the surface (Heyries, Marquette, & Blum, 2007). While straightforward assays (e.g. direct human IgG assays) performed well on non-treated or "native" PDMS, shown in section 3.2.2, problems became evident when performing expression assays and including washing and secondary detection steps. Based on these observations, and because of the importance of having a reproducible and uniform surface which remained stable during extended incubation of bacterial cultures, surface modification was undertaken. Several different methods to chemically modify the surface of the PDMS were tested, using a number of important criteria, with a view to enhancing the reliability of the assay performance.

Each of the modification approaches, detailed in section 2.3.5, were subjected to numerous different challenges which would determine if they were compatible for use with the process (Table 3.1). In order to simplify the initial testing process, PDMS was cast and cured in the wells of microtitre places and the modifications and assays performed in the wells. In assays utilising purified antibodies in buffer all surfaces performed in a satisfactory manner, even surfaces which were untreated. In addition, none of the modifications inhibited sealing between the assay surface and array. However, several of the surface modifications performed poorly when they were tested in an expression on-plate assay. It appeared that they were not stable in extended incubations with culture as the background fluorescence was very high, indicating that the blocking carried out did not remain intact and secondary antibody bound non-specifically to the exposed surface.

Table 3.1: Summary of performance for each of the chemical modification strategies tested. *EDC is a (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) surface; GA is a glutaraldehyde surface; The Carboxy A surface is a tetraethyl orthosilicate (TEOS) surface; Carboxy B surface is a tetraethyl orthosilicate (TEOS) and acrylic acid (AA) surface and APTES is a (3-Aminopropyl)triethoxysilane surface.*

Method	None	EDC	GA	Carboxy A	Carboxy B	APTES
Performance (Purified antibodies)	✓	✓	✓	✓	✓	✓
Sealing	✓	✓	✓	✓	✓	✓
Performance (Expressed antibodies)	✗	✗	✗	✓	✓	✓
Performance (On array)				✗	✗	✓

The three modification strategies which performed satisfactorily in the 'on-plate' expression tests were then taken forward for further investigation. A thin layer of PDMS was modified with each of the different strategies and the quality of the surface provided visualised by adding 2.5 µg/mL of Cy5-labelled anti-human IgG to the surface for one hour. The surface was then washed, air-dried and scanned using the Perkin Elmer microarray scanner (Figure 3.11).

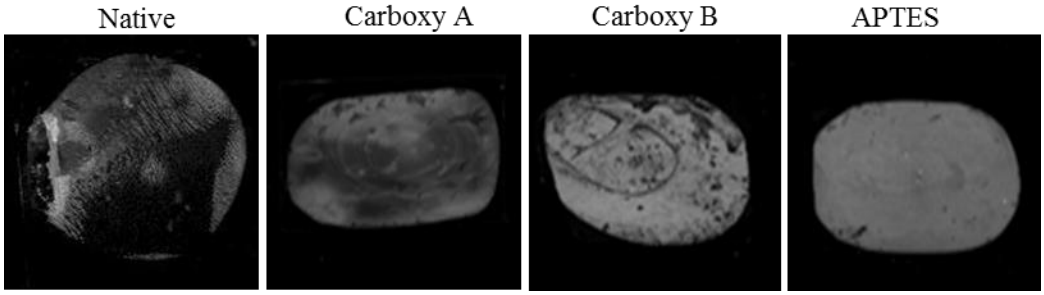

Figure 3.11: Visualisation of surface quality obtained with several surface modification strategies. *A square of PDMS, modified by each of the different methods, was incubated with a 2.5 µg/mL dilution of Cy5 labelled anti-hIgG for 1 hour. The surface was subsequently washed, air-dried and imaged in a microarray scanner to allow visualisation of the level and quality of protein binding on each of the surfaces.*

Based on the outcome of this investigation, shown in Figure 3.11, it was evident that the APTES approach gave the best and most uniform coverage. The uniformity is hugely important when comparing fluorescent signals, representing different antibody candidates, across the entire surface. It was selected as the most appropriate method to modify the PDMS surfaces used in the process. Previous studies had shown that modifications of this nature, carried out on PDMS surfaces were often time-limited due to hydrophobic recovery

caused by migration of buried PDMS (Heyries *et al*., 2007). In light of this fact, APTES surfaces used in assays were as fresh as possible throughout all development phases.

3.2.4 Direct comparison between an immunoassay performed in a microcapillary array and in a microtitre plate.

This particular experiment was carried out to ascertain whether it was possible to carry out an immunoassay, with comparable sensitivity to those obtained using a microtitre plate, on the array. A direct comparison of a sandwich assay, using the same reagent preparations and conditions, was carried out on a microtitre plate and on a 40µm array, as described in section 2.3.6. The results obtained were plotted and compared, as shown in Figure 3.12. The result obtained shows that detection levels and background values are comparable. This means that it is possible to carry out an immunoassay through the array, onto the coated and blocked PDMS surface.

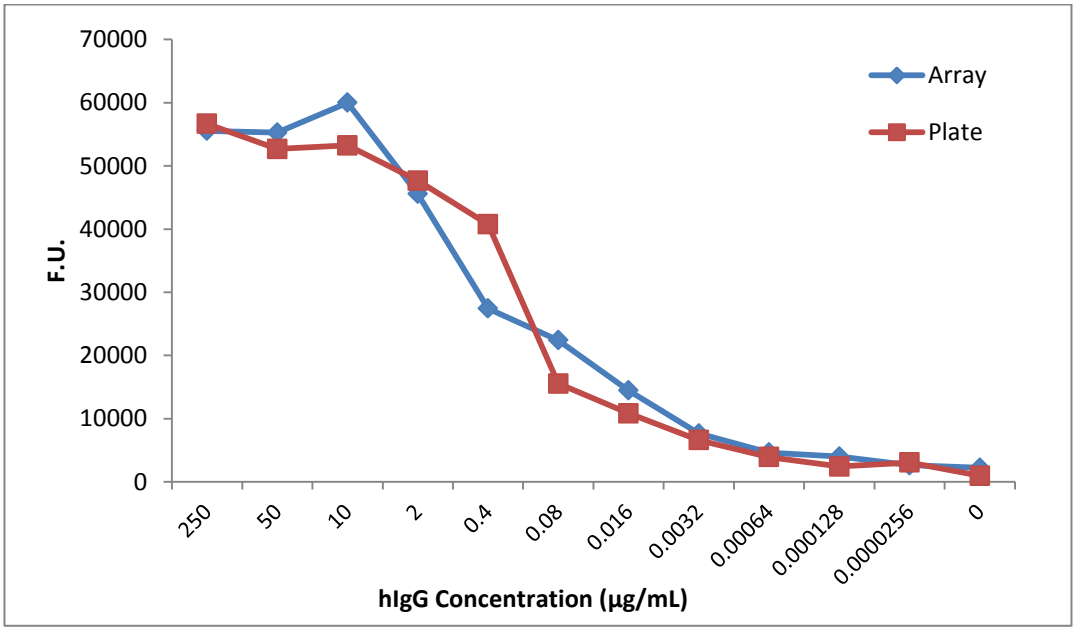

Figure 3.12: Comparison of immunoassay performance carried out on a plate and on array. *The immunoassay carried out was an anti-human IgG sandwich FLISA. Human IgG concentration was varied to assess detection range. Readings obtained from the array analysis were based on the grey value obtained from a 1mm circular area for each of the assay dilutions. F.U. denotes fluorescent units. Fluorescence was read at Cy5 wavelengths (Excitation 650 nm; emission 670 nm) on the plate reader and using the 633 nm laser on the microarray scanner.*

The assay response obtained is comparable with that from an assay carried out on a standard microtitre plate. The detection range is almost identical and background levels are also similar. The slight differences in curve shape between methods may be attributed to the variations in machines used for fluorescence detection. In addition, the 1mm^2 area selected

(roughly 20 capillaries) on the array image encompasses the small dark area surrounding every capillary, which will alter the grey value reading and hence the smoothness of the graph generated.

3.2.5 Shaking Vs Static expression

The development of the on-plate expression method, described in Guo *et al*. (2012) demonstrated that it was possible to grow, express and detect antibodies in culture simultaneously. This is key in the development of the proposed microcapillary array assays, as antibody-producing cells would be incubated in the array and the antibody detected on a removable surface sealed to the array. The use of array assays in place of standard practices (microtitre plates) would require for antibody expression and detection to occur on a surface PDMS surface without shaking. As such, the levels of antibody expressed in both shaking and static experiments, on PDMS and a standard microtitre plate were compared, as detailed in section 2.3.7 and shown in Figure 3.13.

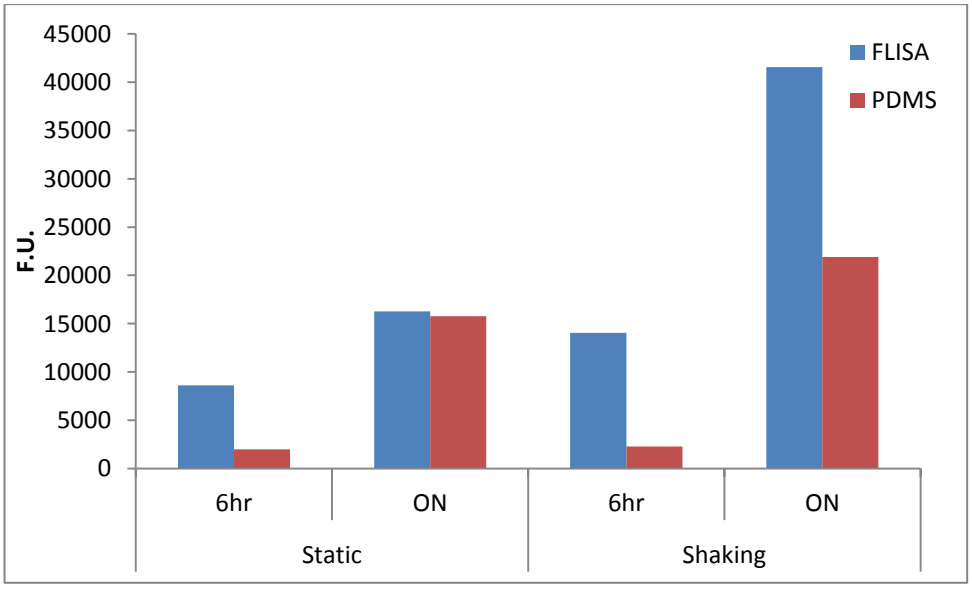

Figure 3.13: Comparison of antibody production in static and agitated systems on-plate. *The level of specific antibody was determined in shaking and static embodiments on both a standard microtitre plate format and also on a PDMS surface. The assay was performed at two time-points [6 hours and overnight (ON)]. F.U. denotes fluorescent units. Fluorescence was read at Cy5 wavelengths (Excitation 650 nm; emission 670 nm).*

The absorbance levels were higher in all cases for the overnight time point, which was expected. Additionally, as expected, the cultures which were shaken at 200rpm showed higher levels of antibody than the static equivalents. Overall the absorbance values on the PDMS surface, while sufficient, were lower than those observed on the microtitre plate. This was most likely due to inefficient modification of PDMS surface in the microtitre plate (i.e.

oxygen plasma treatment may not have efficiently accessed the surface. This problem should not arise when carrying out the proposed assays on flat PDMS pieces. When comparing the overnight, static data it could be observed that the PDMS surface and microtitre plate performed very similarly.

3.2.6 Expression of recombinant antibodies in the microcapillary array and subsequent detection on a PDMS surface.

Ultimately, the aim of this section was to develop a method whereby the antibodies secreted from growing cells could be reliably detected on a removable PDMS surface. Following identification of appropriate medium, conditions and substantial investigations into the modification of the PDMS surface this was deemed possible. The experiment used an anti-CRP scFv, developed in the BDI, to demonstrate the principle. The direct format assay used is described in section 2.3.8 and illustrated in Figure 3.14. A range of cell dilutions was added to the array and analysed, and the resulting images are shown in Figure 3.15.

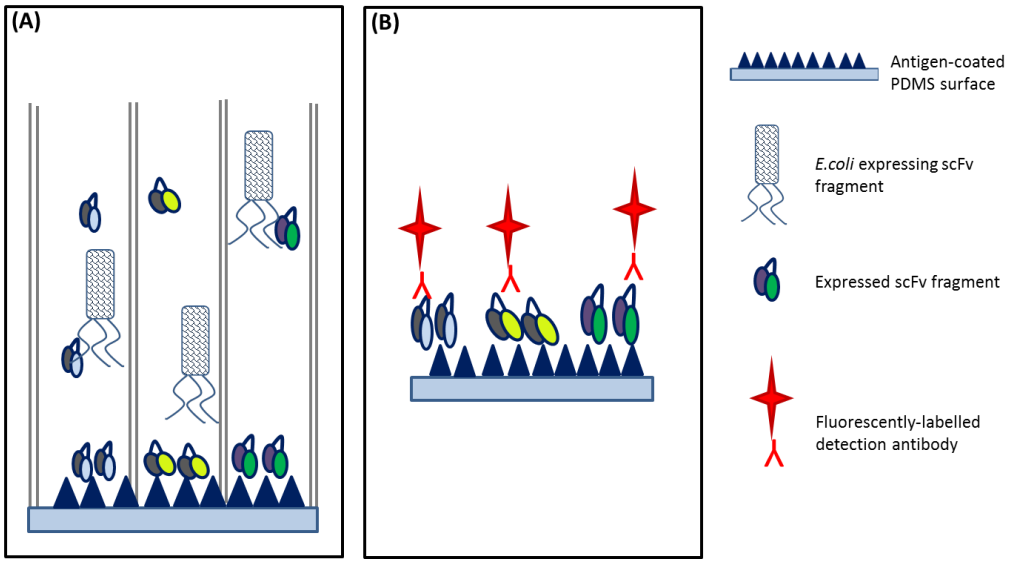

Figure 3.14: Schematic of the format used for typical array assay. (A) *Bacterial cells grow and express scFv fragments which, if antigen-specific, will bind to the antigen-coated PDMS surface.* ***(B)*** *Following incubation the array is removed and stored and secondary detection is carried out using a fluorescently labelled anti-tag antibody, before washing and scanning during image analysis.*

To successfully use the proposed technology to screen large antibody libraries, cells must be able to survive and express protein as previously investigated in this chapter. In addition, an assay on the PDMS surface must be carried out on the modified PDMS surface to allow for accurate identification of capillaries which contain a cell expressing an antibody of interest (e.g. antigen-specific).

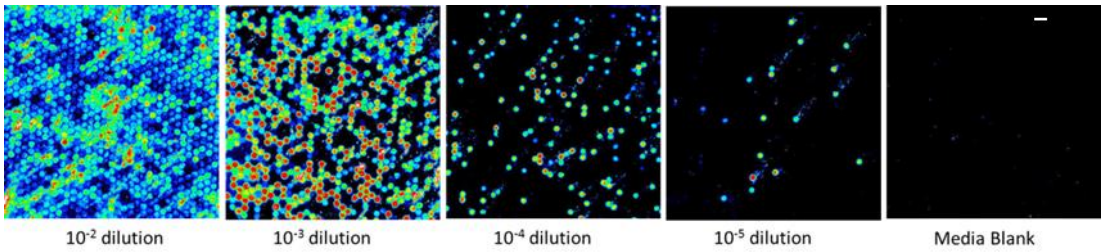

Figure 3.15: Detection of anti-CRP avian scFv released from cells in the capillaries of a microcapillary array. *Dilutions (1 in 10 series) of the scFv-expressing E. coli were added to the array and allowed to incubate overnight. Capillaries containing a cell producing an antigen-specific scFv were detected through the use of a fluorescently-labelled secondary anti-HA antibody. All steps are described in section 2.3.8. Scale bar is 100 µm.*

Here it was demonstrated that it is possible to detect the presence of an antigen-specific, recombinantly-expressed antibody on a modified PDMS surface. Each fluorescent spot on the image relates to a capillary which contains a cell secreting an antigen-specific scFv. The use of the microcapillary array in combination with this surface allows for the compartmentalisation of each cell and thus allows for the high-density, low volume, single cell analysis that was required.

3.2.7 Assay format investigations

Following on from the development of a successful recombinant assay in the microcapillary array (Section 3.2.6), a number of assay format changes that would allow for an increased functionality in the employment of the proposed technology for high-throughput single cell screening were identified. Each of these format changes are discussed, developed and the final optimised system demonstrated within this section.

3.2.7.1 Multi-Analyte analysis

The proposed multi-analyte assay would utilise a common coating element on the surface and probe the captured antibodies with two (or more) differentially labelled secondary reagents. This could allow for easier comparison between the antibodies, in terms of expression level or affinity, by enabling ranking based on the fluorescent intensity of the spot representing each antibody.

In order to facilitate this approach the assay is required to use a "capture format" where all antibodies are captured (in this case by recombinant tag anti-HA) and, following incubation and expression, are probed with fluorescently labelled antigens, of interest. In this section the ability to use a capture format, as well as the ability to probe with two alternately labelled antigens simultaneously, illustrated in Figure 3.16, was investigated as detailed in section 2.3.9.

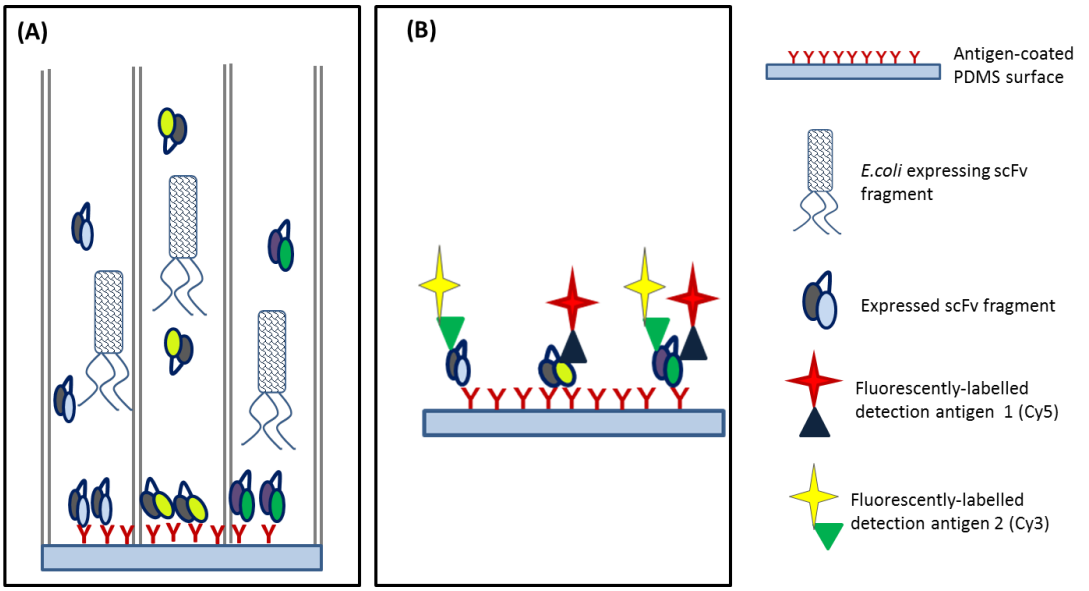

Figure 3.16: ***Schematic of the format used for a multi-analyte array assay. (A)*** *Bacterial cells grow and express scFv fragments which will all bind to the anti-tag antibody-coated on the PDMS surface.* ***(B)*** *Following incubation the array is removed and stored and secondary detection is carried out using a mixture of two different antigens labelled with alternate fluorescent dyes (Cy3/Cy5) before washing and scanning during image analysis. Outputs for each fluorophore can be overlaid and cells which bind one or both of the antigens in same screen can be identified.*

An anti-HA "capture" approach was tested to confirm that the alternative assay format would not negatively affect the performance of the system. The result obtained is shown in Figure 3.17. The assay performed very well using the new format and could be implemented for use in multi-analyte detection assays as planned.

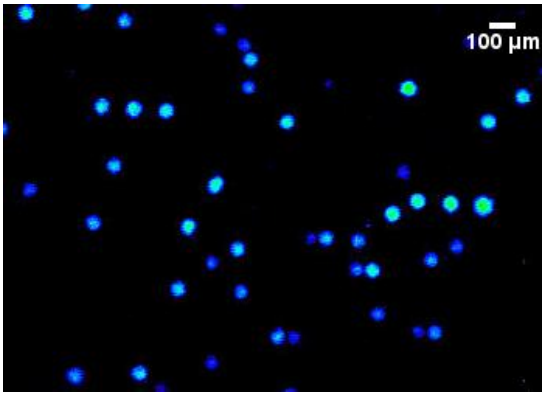

Figure 3.17: Demonstration of assay using an alternative capture approach. *All antibodies are captured and are detected with fluorescently labelled antigen. Single capillaries (40µm spots) are clearly evident at the designated dilution. Scale bar is 100 µm.*

Following confirmation of the proposed capture format, a series of experiments to ascertain whether it was feasible to read Cy3 and Cy5 simultaneously were carried out. Using both

the Tecan Safire plate reader and the Perkin Elmer scanner, tests to identify any interference between dyes, were carried out using control proteins. Both dyes were analysed separately and then mixed and analysed, to determine any cross-talk that may be evident, as detailed in section 2.3.9.1. For each analysis type, average fluorescence was recorded and the results obtained are shown in Figure 3.18 and Figure 3.19.

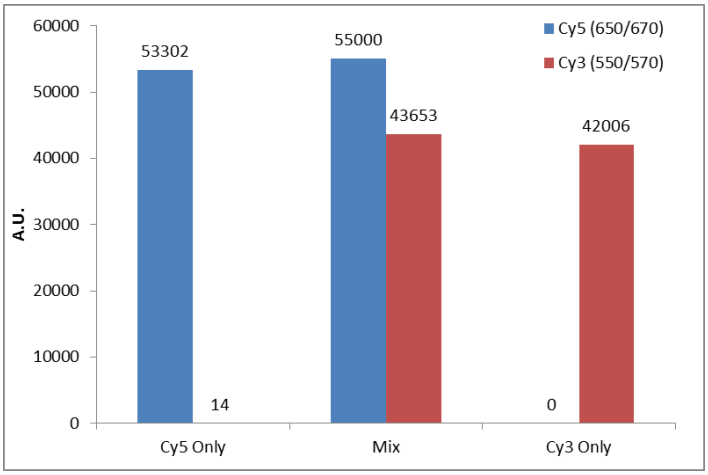

Figure 3.18: Testing of any interference obtained between Cy3 and Cy5 samples using microtitre plate reader. *No non-specific fluorescence is seen in the case of either protein. The Cy3 and Cy5 samples are diluted 1 in 2,000 in PBS while the mix samples is a 1:1 mix of each protein diluted 1 in 1,000 in PBS. Cy 3 has an excitation at 550nm and emission at 570nm, while Cy5 has an excitation at 650nm and emission at 670nm.*

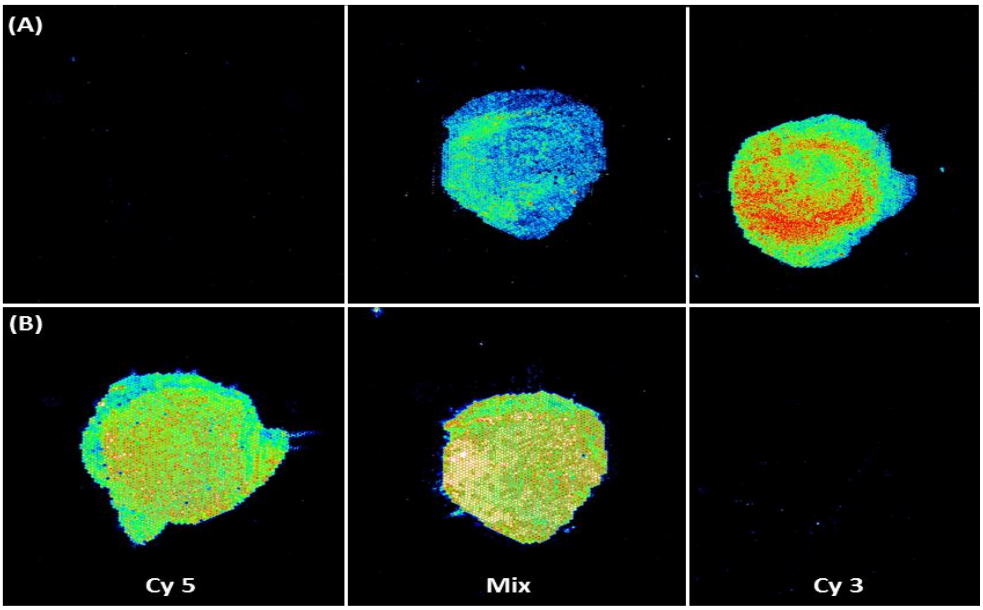

Figure 3.19: Fluorescence pattern obtained from dyes passed through the array, singly and mixed. *No non-specific fluorescence or cross over between fluorophores was seen in the case of either protein. The Cy3 and Cy5 samples are diluted 1 in 2,000 in PBS while the mix sample is a 1:1 mix of each protein diluted 1 in 1,000 in PBS.*

No level of cross-talk or non-specific fluorescence is seen between the samples (Cy3 and Cy5) using either a plate reader or scanner. Once the format and scanning conditions were confirmed to be compatible with a multiplex approach, an assay (described in section 2.3.9.3) was carried out on array to test the performance. The result obtained is shown in Figure 3.20.

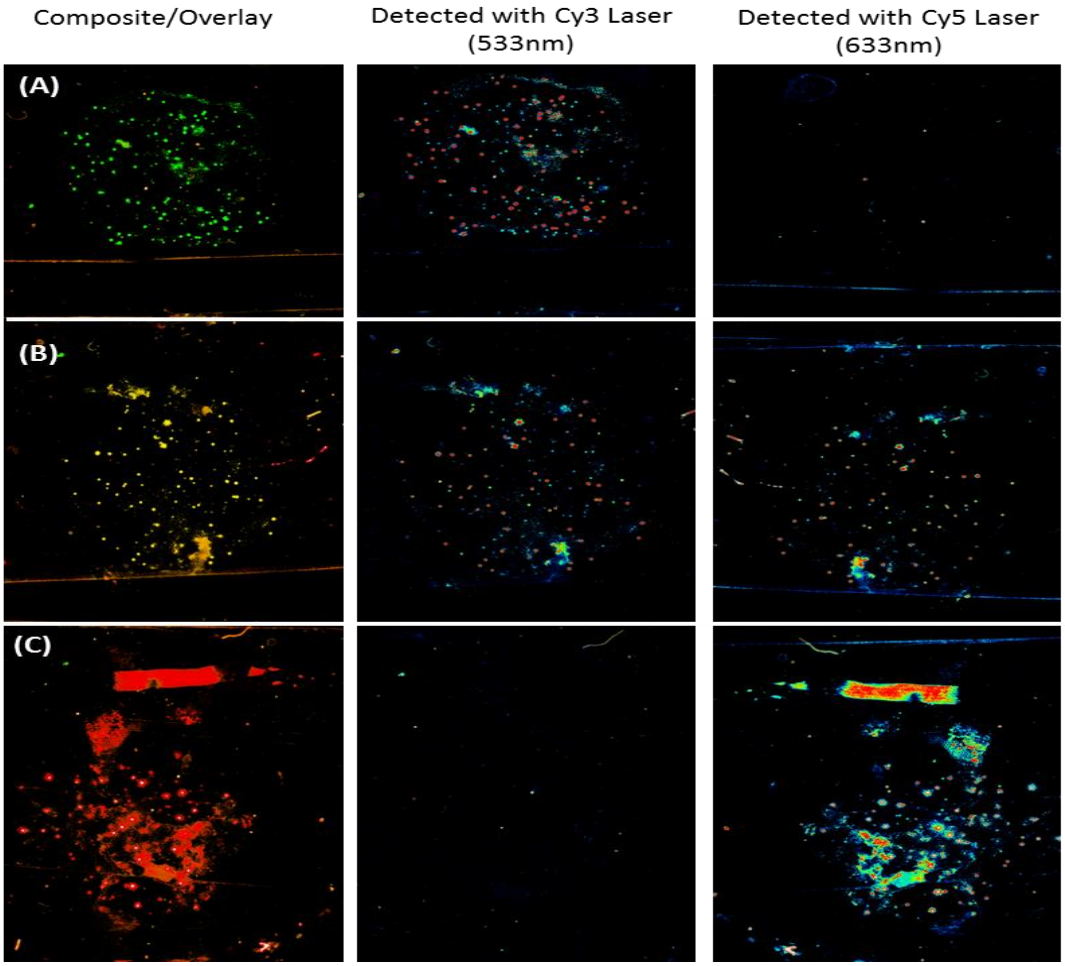

Figure 3.20: Multi-analyte analysis feasibility assay. *The same clone (anti-CRP scFv F8) was captured on an anti-HA tag-coated surface and probed with* ***(A)*** *CRP labelled with Cy3,* ***(C)*** *CRP labelled with Cy5 or* ***(B)*** *a mixture of the two. Each was scanned with the Cy3 laser (543nm) and the Cy5 laser (633nm).*

From the results obtained in this study it can be seen that there is no non-specific fluorescence in the single analyte samples. It can also be concluded that it is possible to use several fluorophores, depending on their excitation and emission profiles, on the same surface simultaneously to garner additional cross-reactivity information from an assay. This will be of particular interest when carrying out library screens. The ability to probe a library for antibodies against several different antigens (labelled with different fluorophores)

simultaneously allows for a great deal more information about potential candidates to be identified from a single assay, saving time and reagents and streamlining the discovery process. While the present analysis was limited to two fluorophores (Cy 3 and Cy 5) based on the lasers available in the instrument, the analysis methodology could be greatly enhanced by the introduction of additional lasers to the detection instrument.

3.2.7.2 Double-sided analysis

The straw-like nature of the microcapillary array means that there are two open surfaces available for analysis. While the multi-analyte approach, demonstrated in section 3.2.6.1, relies on a common coating element a double-sided approach would not; this point is explained in Figure 3.21. It offers the benefit of carrying out two very different assays on the same population of cells simultaneously, ultimately increasing overall throughout and the amount of information obtained in each screen.

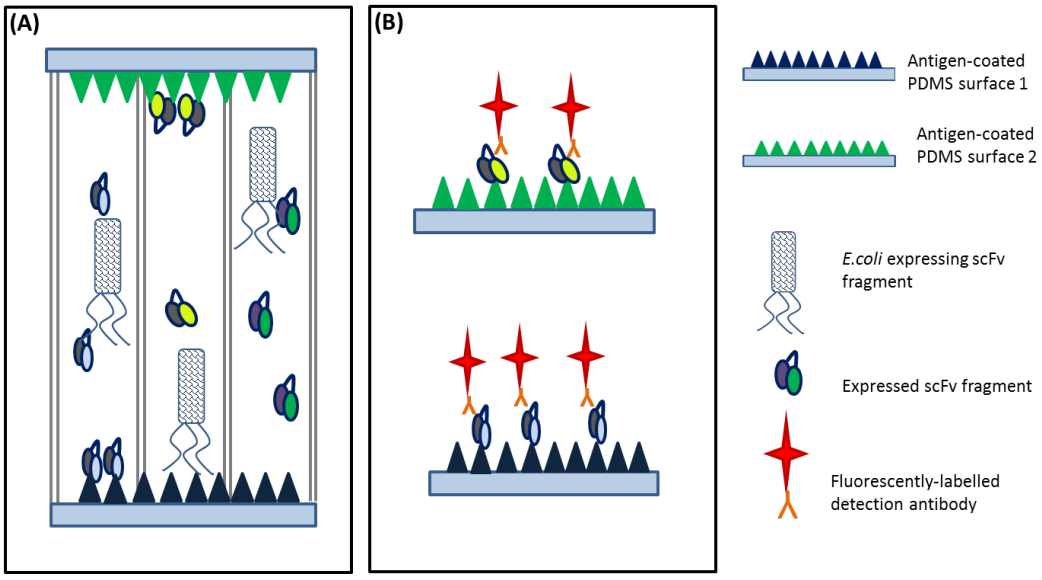

Figure 3.21: ***Schematic of the format used for a double sided array assay. (A)*** *Bacterial cells grow and express scFv fragments which, if specific to either antigen/antibody on either of the surfaces, will bind to the prepared PDMS surface.* ***(B)*** *Following incubation the array is removed and stored and secondary detection is carried out using an appropriate detection element for each separate assay surface. Following this the surfaces were washed and scanned and detailed image analysis carried out to relate the two images to each other.*

Development of double-sided analysis, detailed in section 2.3.10, went through several iterations before performing successfully. The optimisation involved numerous sealing and incubation procedures. Sealing that was found to be optimal was sequential, with one side being sealed at a time. Typically the assay components would be incubated in a horizontal orientation, with the array on top of the PDMS. This means that the degas power pulls the

liquid to the assay surface, which is normally advantageous. Due to the fact that horizontal incubation could introduce a bias towards the lower assay surface, either through the degas power or through natural sedimentation, vertical incubation was found to provide the best coverage on both sides of the assay. A sample of a successful double-sided assay carried out using these optimised conditions is shown in Figure 3.22.

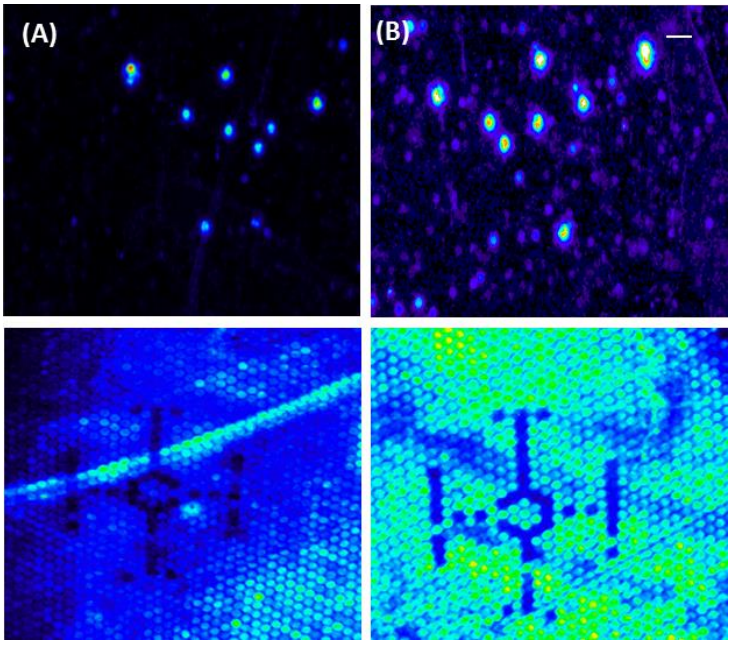

Figure 3.22: Double sided assay feasibility. *The cell portion of the assay, depicted in the top of the image, shows single cells expressing and detected on each side. The alignment marks obtained on each side of the assay are shown and compared in the bottom part of the image. The first side of the assay* ***(A)*** *provides a mirror image of the second side of the assay* ***(B).*** *A specific area of the total image was selected for illustration purposes. Scale bar is 100 µm.*

The double-sided analysis provides two scanned assay surfaces which are in fact mirror images of each other. Specialised software, developed by Brian Manning, can relate the images to each other and allows for each of the screening criteria to be combined for each individual clone. It offers a very powerful method to ask distinct questions of each clone, in each screen.

3.2.7.3 Multi-lift analysis

An additional approach, to increase the throughput of the process, is deemed 'Multi-lift'. This approach involves removing the array from its original PDMS surface and sealing it to a subsequently prepared assay surface, as illustrated in Figure 3.23. Much like the multi-analyte and the double-sided assay approaches this expands the level and quantity of information that can be obtained simultaneously, for each individual antibody-secreting cell analysed.

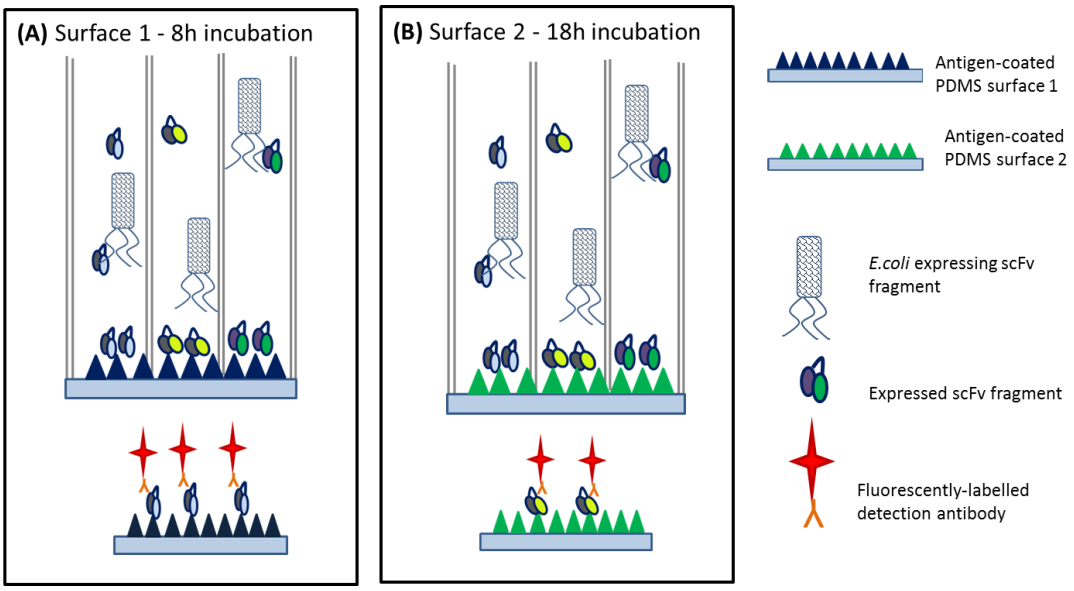

Figure 3.23: ***Schematic of the format used for a multi-lift array assay. (A)*** *Bacterial cells grow and express scFv fragments which, if specific to either antigen/antibody on the surface, will bind to the prepared PDMS surface.* ***(B)*** *Following the initial incubation (8h) the array is removed, sealed to another freshly prepared assay and incubated again (18h). Post-incubation, both surfaces have secondary detection carried out using an appropriate detection element. Following this the surfaces were washed and scanned and detailed image analysis carried out to relate the two images to each other.*

The multi-lift assay, described in section 2.3.11 and demonstrated in Figure 3.24, shows two identical assay images which represent two separate incubations on different assay surfaces.

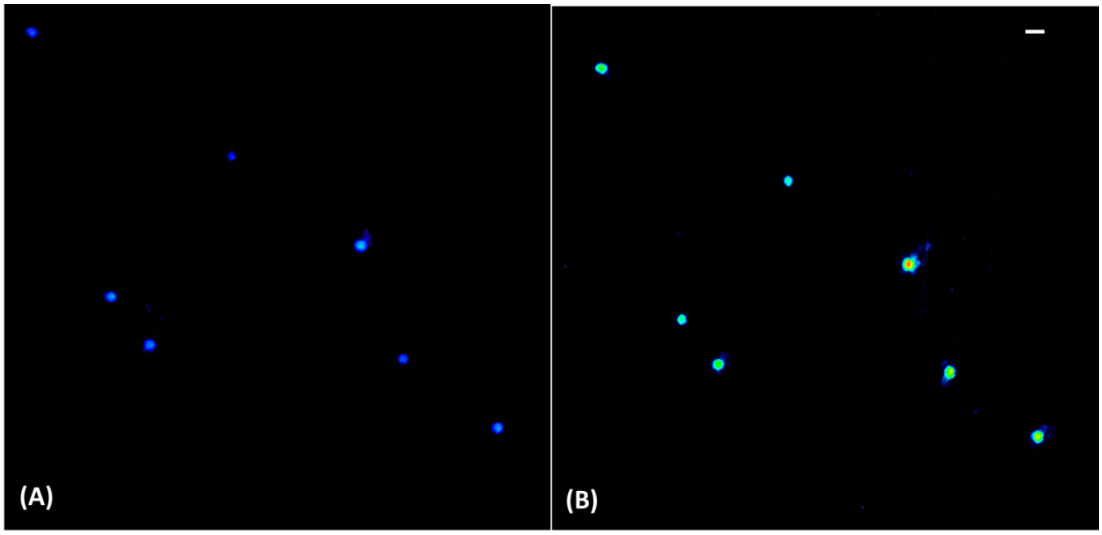

Figure 3.24: Feasibility for multi-lift approach using microcapillary array assay. *A standard assay was carried out, as described in section 2.3.11. The first assay* ***(A)*** *was allowed to incubate for 8 hours before removal and completion of the assay. The array was then sealed to a second surface and incubated overnight and completed on the following day* ***(B)****. A specific area of the total image was selected for illustration purposes. Scale bar is 100 µm.*

In developing this approach two very important factors for success became evident. The first was the requirement for each assay surface to be as fresh as possible prior to sealing. If a prepared surface was not required for a substantial amount of time (e.g. overnight) it would be best to either leave it in blocking solution or prepare it just before it was required. The second observation was that additional alignment solution should be added prior to each sealing. If this is not carried out the quality of alignment marks is poor in assays which are subsequent to the initial assay. This is most likely due to the evaporation of the solution during incubation.

3.2.7.4 Assessment of diffusion through the capillaries.

The purpose of this investigation was to determine whether diffusion through the array occurs. This would be advantageous in the development of functional bioassays which may require a secondary reagent added later on in the process. The ability to add an additional reagent to a microcapillary array which has already been incubating for a defined length of time could add a higher level of functionality to the assays carried out using the technology (e.g. Roche Diagnostics WST-1 assay for analysis of cell proliferation requires addition of a secondary reagent to growing cells after 24-48 hours incubation). The ability to have these secondary agents efficiently diffuse through the medium or buffer present means that reagents which would hinder the interaction to be studied, if present from the beginning of assay, can be added at a later point. The experiment was carried out, as described in section 2.3.12 and illustrated in Figure 3.25.

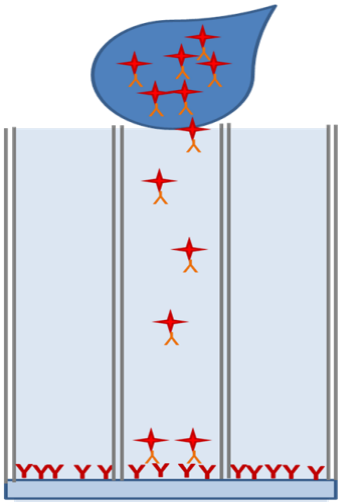

Figure 3.25: Schematic demonstrating the principle of diffusion of reagents through a buffering solution in the capillaries of the array. *The secondary reagent is added to the top of the array, allowed to diffuse through the buffer and bind to the prepared surface sealing the opposite side of the array.*

The result obtained, shown in Figure 3.26, confirmed that diffusion through the array is possible. This is indicated by the presence of fluorescence bound to the human IgG-coated surface which was sealed to the opposite side of the array. The fluorescence seen in the diffusion sample is lower than the standard sealing (values are present in the image) but this can most likely be attributed to the dilution effect from the PBS, in the array, which the secondary antibody diffused through. The diffusion control lacked the PBS in the array and there was very little binding seen on the PDMS in these areas. While an outline can be seen, this can be explained as a drying effect artefact.

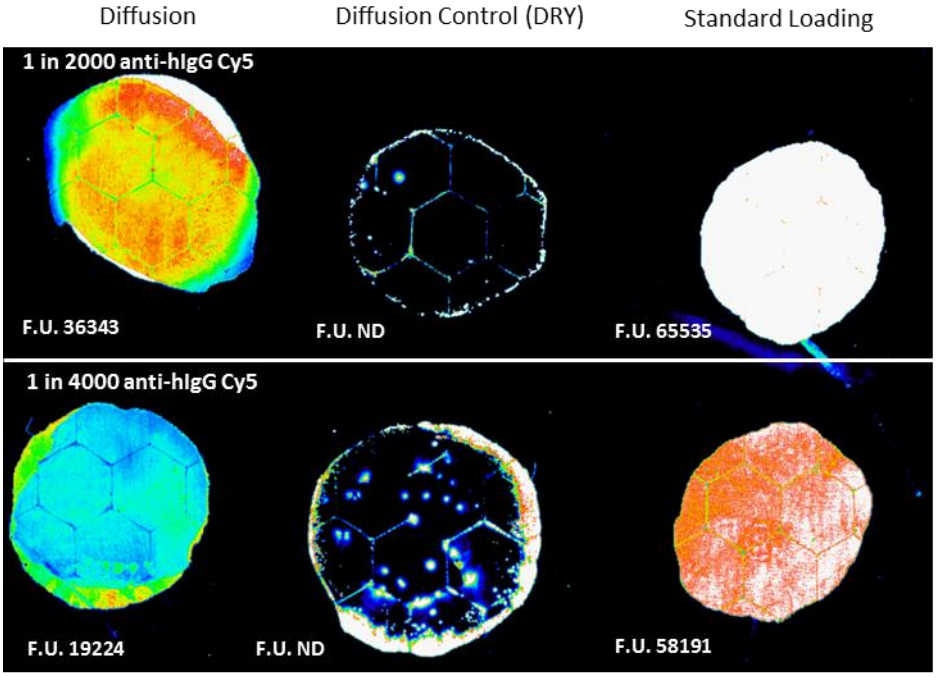

Figure 3.26: Demonstration of diffusion through a microcapillary array. *Varying dilutions of Cy5-labelled anti-human IgG were added through the array in either a standard or diffusion format. The resulting image showed that there was diffusion of the secondary antibody through the PBS loaded in the array, indicated by its binding to the human IgG coated surface sealed to the opposite side of the array. F.U. denotes 'Fluorescent Units' obtained from the microarray scanner software. ND denotes 'Not Determined'.*

Based on these proof-of-concept investigations, it has been shown that diffusion through another liquid, in the capillaries of the array, is entirely possible. If this were to be used in a working assay format, a consideration should be made to the concentration of secondary agent that is added to the top of the array, as the liquid in the array will dilute that actual concentration by a certain factor.

3.2.8 Determine feasibility of in-solution analysis

Homogeneous or in-solution assays hold the promise of delivering simplicity to end users by including fewer addition or reagent-transfer steps (Walters & Namchuk, 2003). It makes them highly amenable to automation and miniaturisation. The advantages for their application on the platform are obvious. They would reduce the need for additional assay steps and could possibly remove the need for a surface entirely. This would mean that retrieval of the desired cell from a capillary would be greatly simplified. This proof-of-concept data was also included in the patent filing for the proposed technology, demonstrating that surface independent analysis was possible (Leonard *et al*., 2012).

3.2.8.1 Determination of feasibility of in-solution analysis/FRET analysis on array

The first in solution approach that was tested in the array was based on the detection of Förster or Fluorescence Resonance Energy Transfer (FRET), as detailed in section 2.3.13. FRET occurs based on the binding of two complementary DNA probes labelled with Cy5 and Cy5.5. Once DNA hybridisation occurs, the Cy5 and Cy5.5 dyes are brought into close proximity of each other so that FRET can occur between the Cy5 donor and Cy5.5 acceptor. Due to the transfer of energy, the intensity of emitted light from Cy5 is reduced in the presence of Cy5.5 held in close proximity by the bound DNA. In that way specific binding interactions could potentially be monitored on-array by measuring a shift in the level of fluorescence at a particular wavelength.

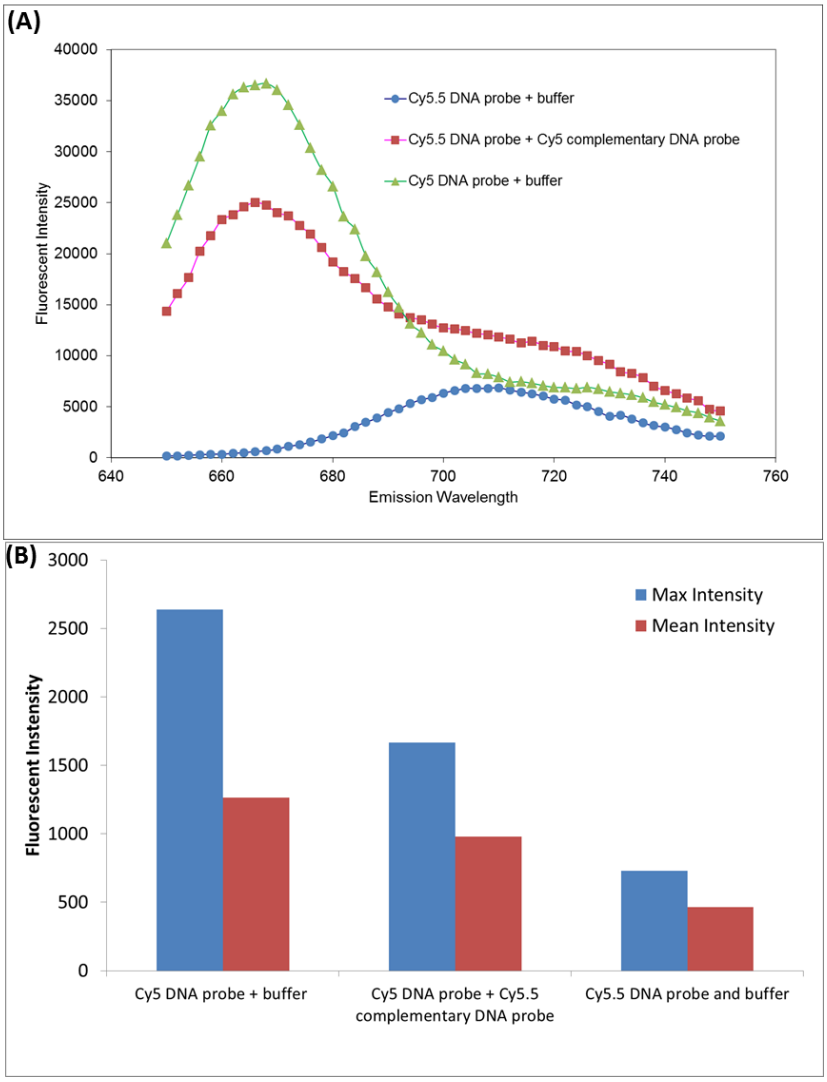

Figure 3.27: Demonstration of FRET between two complementary DNA probes. *Analysis was carried out in a 96-well microtitre plate and read using a Tecan Sapphire II* ***(A)*** *as well as in the microcapillary array, using a fluorescent microscope* ***(B)***.

DNA-FRET was successfully demonstrated using fluorescence intensity measurements from spectral analysis in a microtitre plate (Figure 3.27A) and in a 40 µm diameter microcapillary array (Figure 3.27B). Binding of the two complementary DNA probes is detected by a clear decrease in Cy5 emission due to fluorescent energy transfer with the Cy5.5 acceptor. While it is possible to carry out FRET analysis on the array, the ability to use a time-lapse option or to have an automated scanner would greatly improve the feasibility of using this as a method for detection of binding on the array in future.

3.2.8.2 Determination of feasibility of in-solution analysis/GFP-reassembly on array

An additional demonstration of a cell-based in-solution binding assay in the array was carried out, using the GFP reassembly vectors described by Magliery *et al*. (2005). This method identifies the interaction between binding partners based on reassembly of dissected

fragments of GFP, fused to antiparallel leucine zipper peptides. In the event of a positive interaction the "bait" and "prey" would bind to each other, the GFP fragments (N-terminal and C-terminal) will be brought together and a green fluorescence would be emitted. The potential application would involve fusion of one of these dissected fragments to a recombinant antibody library construct and attachment of the other fragment to the antigen of interest. The observation of fluorescent signal within the capillary would indicate a specific interaction between the binding partners (i.e. expressed antibody and antigen), as illustrated in Figure 3.28.

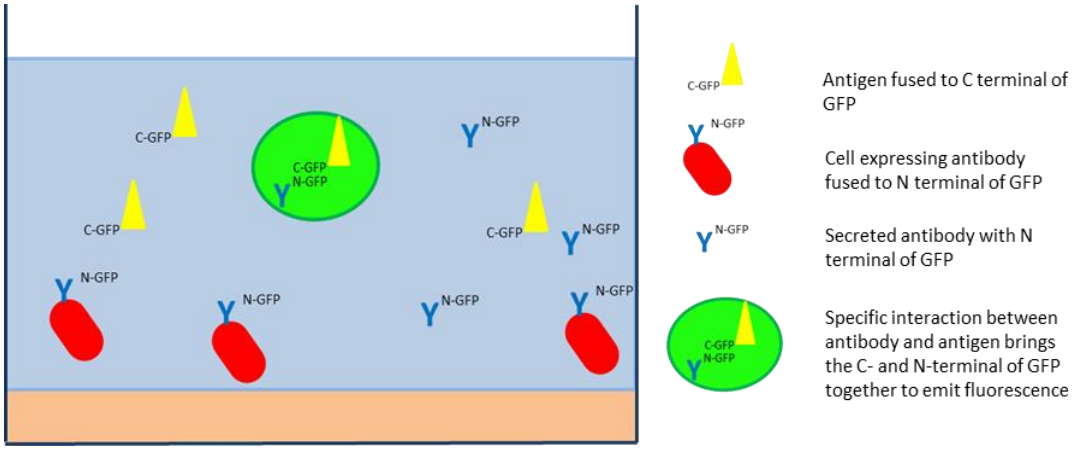

Figure 3.28: Illustration of the proposed GFP Reassembly concept. *As the two complementary proteins are brought together the dissected GFP is reassembled and emits a fluorescent signal. In the proposed screening format the antibody library would be loaded into the well along with the antigen, and on expression of a specific antibody, the interaction would be evidenced by the emission of fluorescence from the particular capillary.*

In order to demonstrate the potential of using this approach in array assays, the set of control vectors were obtained under MTA from the authors in Yale University. Initially the principle was confirmed to work by sequentially transforming both of the control vectors into the same cell. Throughout this study this embodiment, where both plasmids were contained within the one cell, was called '*in vivo'*. A number of the transformants were plated on screening media (solid and liquid), as described in section 2.3.14. Following incubation, the fluorescence present in the cells was observed on a transilluminator at an approximate wavelength of 365nm, as shown in Figure 3.29. Those cells containing both vectors fluoresced with a strong green colour and confirmed that the system was functional.

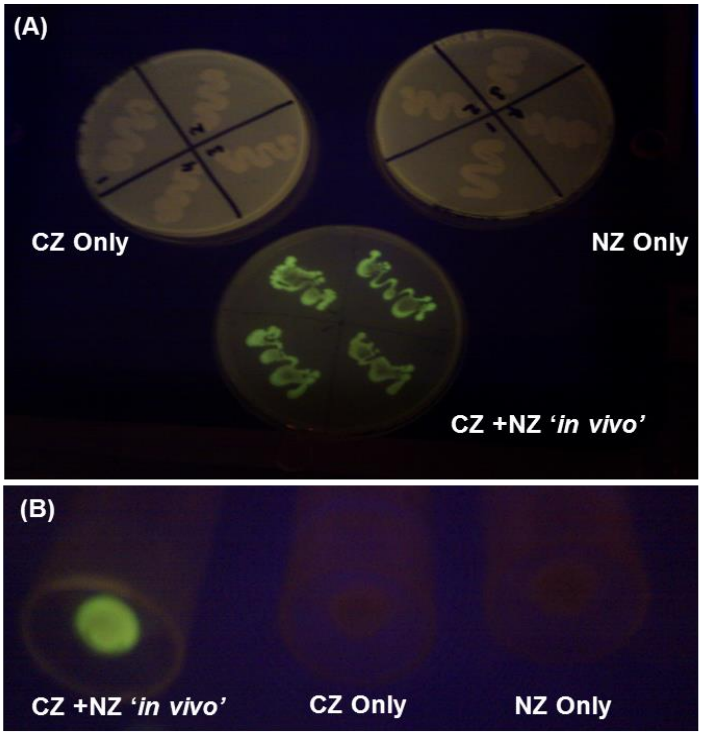

Figure 3.29: Demonstration of reassembly of GFP 'in vivo'. *The 'in vivo' samples had both plasmids (NZ and CZ) sequentially transformed into the same cell, allowing for GFP reassembly. The GFP reassembly was visualised on* ***(A)*** *Solid expression medium and in* ***(B)*** *liquid medium. Selection medium was LB containing 10 µM IPTG, 0.2% (w/v) arabinose, 35µg/mL Kanamycin, 100µg/mL Carbenicillin. Cells have been pelleted by centrifugation at 4,500 x g for 10mins and the supernatant removed.*

The vector set was then trialled, in what was deemed an '*in vitro'* approach. This involved the co-incubation of the two control vectors, expressed singly in different cells, described in section 2.3.14. This would be akin to the final proposed screening format for use on the platform and it was hoped that as the cells grew and died, the dissected fragments would be released into the medium and would interact. The cells were loaded, in screening medium without antibiotic, into the microcapillary array and following two days incubation at 37°C the level of fluorescence was observed using a fluorescent microscope.

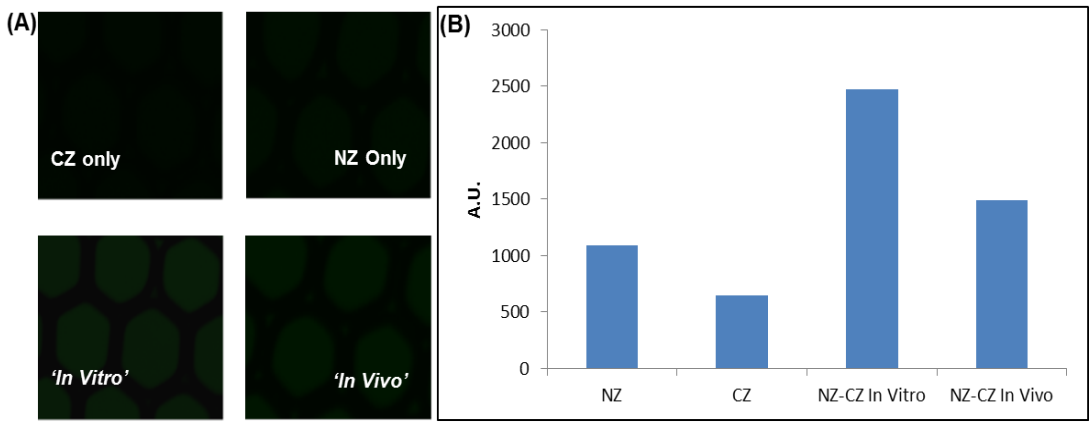

Figure 3.30: Determination of feasibility for GFP reassembly detection on array. *Both 'in vitro' and 'in vivo' embodiments were loaded on to the array in discreet areas and visualised following incubation.* ***(A)*** *Images obtained of microcapillary array with E. coli cultures harbouring split GFP fragments using a fluorescent microscope* ***(B)*** *Graphical representation of the fluorescent intensity values obtained images in Figure 3.30A.*

The level of fluorescence in each of the embodiments (CZ only, NZ only, '*In vivo'* and '*In vitro*') was compared, following observation under a fluorescent microscope. Based on the result obtained, shown in Figure 3.30, it appears that both the *In vivo'* and '*In vitro*' samples have performed as expected, with a level of fluorescence above the negative controls (CZ only and NZ only). The level of fluorescence is low and the implications of this, in terms of assay sensitivity, would be something to consider if moving forward with the approach.

3.2.9 Summary for key challenge 3.2

The purpose of this section was to develop a means to carry out assays using the microcapillary arrays. A chemical modification strategy, which will allow for assays to be performed in a reliable and robust manner on a PDMS surface, was identified. The use of the chemically modified surfaces means that assays with washing and secondary steps can be utilised in the process without non-specific binding or unfavourable signal-to-noise ratios. Standard assays carried out on the selected modified surface perform similarly to those carried out in microtitre plates. A screening assay was then successfully carried out on the modified surface, allowing single cell analysis of the control recombinant antibody. Building on this the expansion of the functionality and performance of the microcapillary array assay process, through implementation of various assay embodiments, was explored. For each of the different assay formats, a proof-of concept experiment was optimised and demonstrated.

The possibility of carrying out 'in-solution' analysis on the array appears feasible based on the demonstration of GFP reassembly and the success of the FRET method in the array. This will mean that the need for an assay on a separate surface would not necessarily be required. This would serve to greatly reduce the overall time needed to carry out a screen and remove

any complications anticipated with alignment of the assay image to the physical array. Unfortunately it brings with it its own limitations in that only certain types of assay (i.e. homogenous formats with no washing steps), which may not be broadly applicable, can be performed.

Key Challenge 3.3 Development of a method to accurately retrieve the contents of a target capillary.

3.3.1 Experimental approach

In order for the technology to be complete, a means to retrieve the cell producing the antibody of interest, for further analysis, is required. This portion of the technology development is concerned with the identification of an appropriate way to remove the cell of interest efficiently and reliably. It involves the design and building of a prototype device to allow single capillary contents to be retrieved. This required the integration of hardware and software elements, which were developed within the dedicated team of biologists (i.e. assay support and test reagents provided) and engineers. A means to relate the assay image to the location of the desired capillary in the physical microcapillary array was also developed and verified for use.

3.3.2 Feasibility investigations for the removal of contents of capillaries using high pressure nitrogen.

Initial investigations involved an experiment to determine if it was possible to remove the contents of a specific area of an array, without disturbing the surrounding areas, with high pressure nitrogen. The experiment, described in section 2.3.15, involved the removal of the contents from an exposed area of the array. A hole was burned in a piece of acetate and placed over the array, which was filled with liquid food dye. Nitrogen gas was then applied to the area exposed by the laser hole, and the array observed using a light microscope. The resulting image is shown in Figure 3.31.

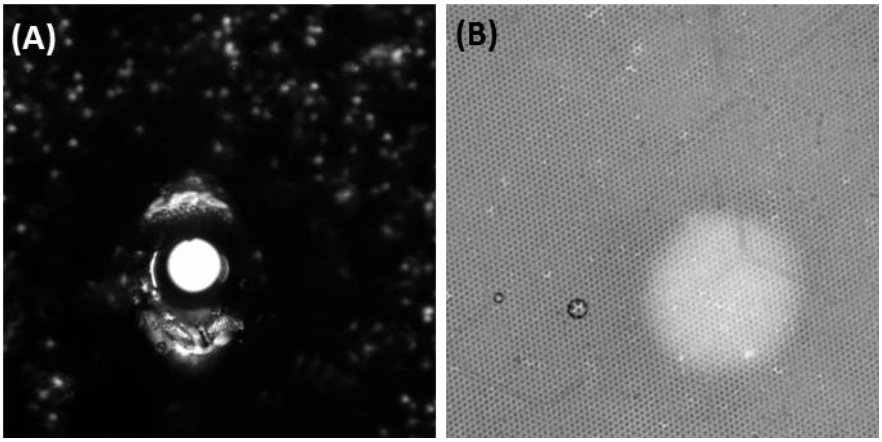

Figure 3.31: Removal of the contents of an exposed portion of the microcapillary array. *The contents of an area, comprising numerous capillaries, were blown out of the array using high-pressure nitrogen as described in section 2.3.15.* ***(A)*** *A laser was used to ablate an area of an acetate sheet. The hole in the acetate measured approximately 100 µm.* ***(B)*** *The acetate was placed over an array containing coloured liquid and air pressure was applied to remove the contents from those capillaries exposed by the ablated area. The clearance zone in the array was approximately 300 µm. Removal of the liquid was verified by observation of colour on the tissue paper which had been placed underneath.*

The image showed that it was entirely possible to remove the contents of designated area of the array. The area which was cleared of its contents was approximately three times larger than the laser hole (i.e. the laser hole measured 100 µm while the area of emptied capillaries had an approximate diameter of 300 µm). The reason for this was mostly the highly crude nature of the experiment, with the acetate not sealed to the array and the nitrogen blast applied by hand through a wide nozzle. The experiment did show that it was possible to remove contents of capillaries in a directed manner; however, the low level of accuracy meant that the development of a dedicated retrieval system was required. This work was undertaken by mechanical engineer Barry O'Donnell at the BDI and the working prototype developed is described in section 3.3.3.

3.3.3 Design and production of a dedicated prototype device for removal of the contents of single capillaries in the microcapillary array.

In order to achieve efficient single capillary removal, the development of a prototype for removal of the contents of a single capillary was undertaken by mechanical engineer Barry O' Donnell. The prototype, shown in Figure 3.32, consisted of high precision XY stages for capillary alignment and a high powered laser for ablation of sealant above target capillaries and subsequent retrieval of capillary contents under a flow of high pressure nitrogen (Figure 3.32B).

The design of the DiCAST prototype was continually improved during testing to optimise the performance of the system. A wide variety of modifications were made to the system

including the introduction of a beam expander to reduce the laser spot size, inversion of the top XY stage to increase freedom of movement, the addition of an image system to allow in-line viewing of the array and the introduction of a Z stage to enable focusing of the laser and image system. These improvements to the design of the prototype resulted in a reduction of the spot size of the laser from 100 µm down to 10 µm. The addition of an in-line viewing system allowed real-time observation of the laser ablations and extractions and allowed for accurate alignment of the system and these improvements helped to increase the reliability of recoveries and the overall efficiency of the system.

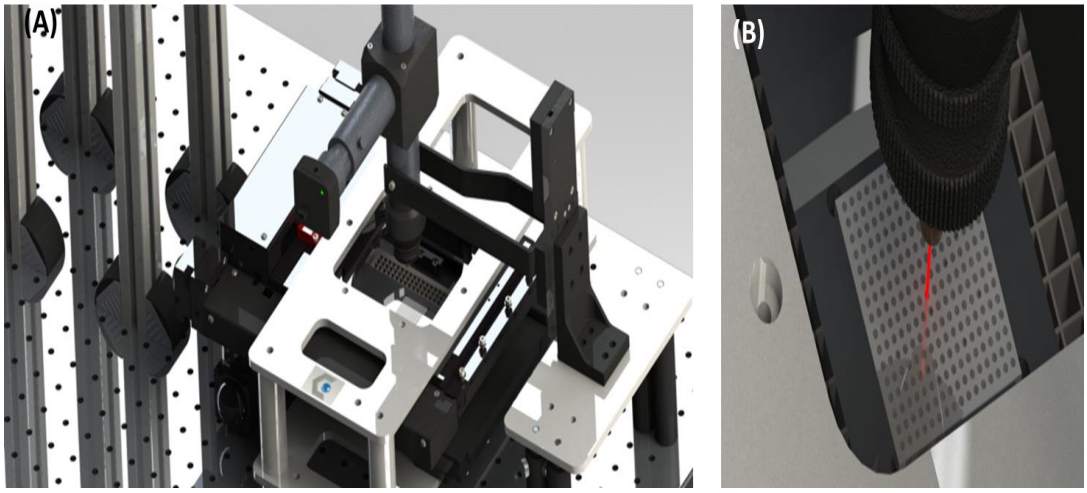

Figure 3.32: DiCAST prototype system for cell retrieval. (A) *Drawing of the setup with laser and dual high precision XY stages for array alignment.* ***(B)*** *The laser nozzle with nitrogen supply is placed close to the array for cell removal. Contents are collected in a 384 well plate housed below the array.*

The prototype system was fully optimised to allow the removal of the contents of an isolated targeted capillary, as shown in Figure 3.33. The system is controlled by LabVIEW based software, custom written and implemented by electronic engineer Brian Manning. The software is further discussed in section 3.3.6.

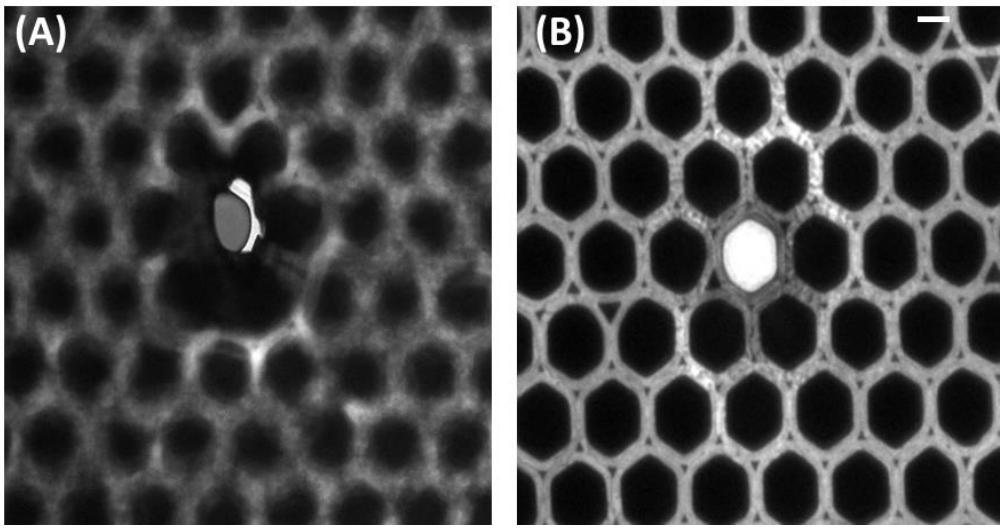

Figure 3.33: Demonstration of single capillary removal. ***(A)*** *Using the high powered laser, a ~30 µm hole was ablated in scotch tape (55 micron in thickness) which has been placed over a 40 micron array containing E. coli cells. The scotch tape seals the array and prevents removal of cells by air pressure.* ***(B)*** *Image of the array once the scotch tape has been removed. The dark capillaries represent those containing E. coli cells while the lighter capillary represents the one from which the contents were retrieved due to the laser ablation of the scotch tape. Scale bar is 25 µm.*

The viability of cells after incubation on and retrieval from the array has also been confirmed, as described in section 2.3.16. This was ascertained by removal of cells from the array, using the retrieval prototype, and their subsequent incubation overnight (37°C/200rpm).

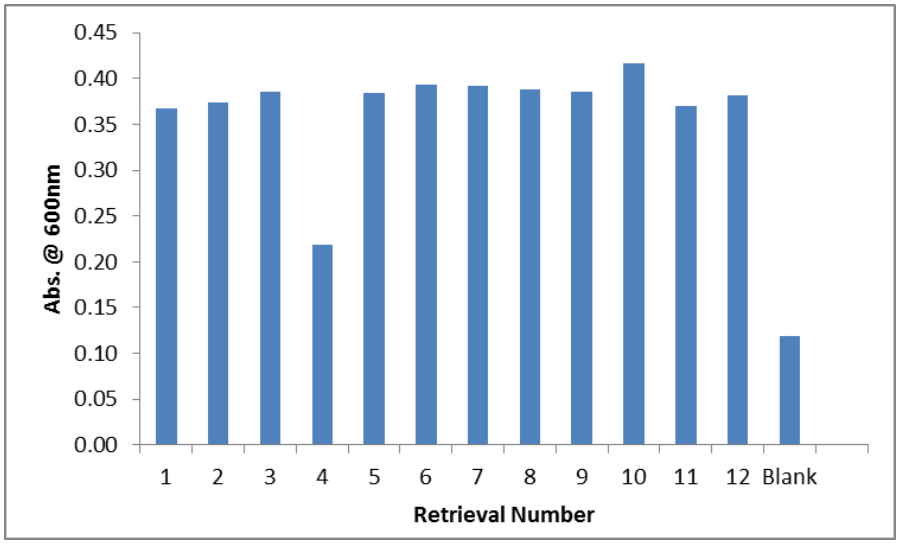

Figure 3.34: Bacterial growth recorded following removal from the microcapillary array. *Following 18 hours incubation, cells were blown out of the array and captured in a microtitre plate beneath. The plate was then incubated at 37°C/200rpm overnight and read, using a spectrophotometer to determine cell density, on the following day. Blank is medium only.*

Cell growth was recorded by spectrophotometer (OD_{600nm}) for twelve separate retrievals, on the following day, and is displayed in Figure 3.34. In addition to observing growth post-removal from the array, the cells present were also enumerated to ascertain the portion of the capillary contents which are captured. In order to determine this, a solution of bacteria (9 x 10^8 cfu/mL) was added to the array and the contents of an individual capillary recovered into six different wells of a microtitre plate, containing 85 µL medium. Three were plated out immediately and three were allowed to grow overnight at 37°C/200rpm. A pipetted control, 1.2 µL into 85 µL medium was also enumerated. Each capillary has a volume of 1.2 nL so the control would represent 1000-fold greater cell count.

Table 3.2: Enumeration of bacteria following removal from the microcapillary array. Each of the cell counts represents an average of three different retrievals.

Condition	Cell count-0 hours	Cell count-18 hours
Stock	9 x 10^8 cfu/mL	N/A
Removal (single capillary)	1 x 10^2 cfu/mL	4 x 10^8 cfu/mL
Pipetted control (1000X)	1 x 10^7 cfu/mL	5 x 10^8 cfu/mL

If the entire contents of the capillary had been retrieved then one would expect in the region of 1 x 10^3 cfu/mL, allowing for the dilution factor of retrieving 1.2 nL into 85 µL of medium. The pipetted control performed as expected with the dilution factor imposed by retrieval (approximately 1 in 70) reflected in the overall numbers. The results, shown in Table 3.2, indicate that approximately one tenth of the total volume of the targeted capillary is retrieved and that following an eighteen hour incubation, the number of cells has reached saturation. From the results obtained it has been determined that the cells can grow, express a protein of interest and be retrieved from the array in a viable state. While some of the culture is lost on removal from the array, allowing the retrieval to grow overnight allows a stock to be obtained for further use.

3.3.4 Development of a system for alignment of the array to the scanned image.

In order for the software to accurately locate a capillary corresponding to a fluorescent spot on the assay image, there would need to be reference points present in each (i.e. the physical array and the image). A method to relate the position of a positive signal on the assay image to the corresponding capillary in the physical array was therefore devised. This was carried out by introducing a unique mark into the array and carrying out a control assay in this area to generate a pattern on the scanned assay image, as described in section 2.3.17. The unique mark was introduced using SU-8 photoresist polymer, by biomedical engineer Brian

O'Reilly. SU-8 is a viscous polymer, typically used in photolithography for the manufacture of microstructures. It remains in liquid form until exposed to light of a designated wavelength (i.e. UV at 365 nm) and subsequent curing. The SU-8 was added to the array in a defined pattern and the cross-linking and baking applied so that a permanent blockage of certain capillaries was achieved. By performing a control assay in this area, blocked capillaries would hopefully appear as dark areas (i.e. where the fluorescent protein could not pass through) and the surrounding area would consist of the regularly observed pattern.

The scanned images obtained were compared with the patterns in the array to determine if the pattern can be used to accurately pinpoint the exact location of a selected capillary in the physical array. Images obtained show excellent correlation between the mark in the array and the corresponding fluorescent pattern, as demonstrated in Figure 3.35.

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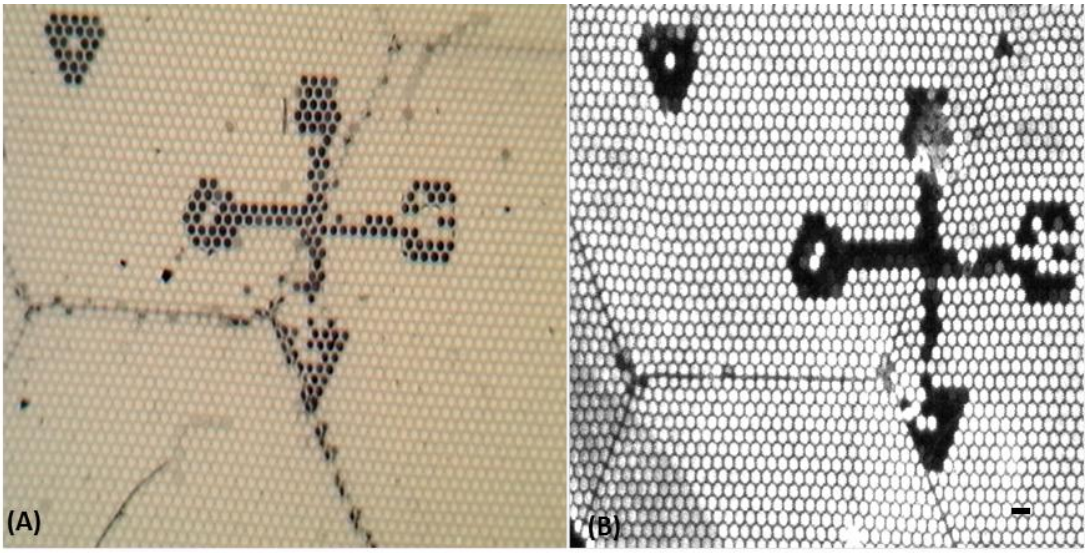

Figure 3.35: Strategy for alignment of physical array to the assay image obtained. (A) *A unique mark was introduced into the array by sealing capillaries in a specific pattern with SU-8 polymer.* ***(B)*** *A pattern was then carried out by passing 2.5 µg/mL Cy5-labelled anti-human IgG through the array in the area of the blocked capillaries and subsequently observing the image obtained. Scale bar is 100 µm.*

Based on these images it was shown that the alignment marks, introduced by blocking capillaries in a distinct pattern in discreet areas of the array, can be successfully used to assign unique coordinates to the spots of interest on the scanned image.

3.3.5 Dual assay for alignment

In order for the full process to work, it is necessary to correctly identify the exact location of the capillary on the microcapillary array which relates back to the fluorescent spot on the image obtained after scanning. In order to make this possible an alignment system was devised. As shown in section 3.3.4, it involves the introduction of a physical feature (i.e. blocked capillaries in a highly defined pattern) in the microcapillary array which will also be present on the scanned image by implementation of a control fluorescent assay. The aim of this section is to ascertain whether it is possible to dual coat the surface with the reagents for the alignment assay along with the reagents for the screening assay without any negative effect on either assay's performance, as described in section 2.3.18.

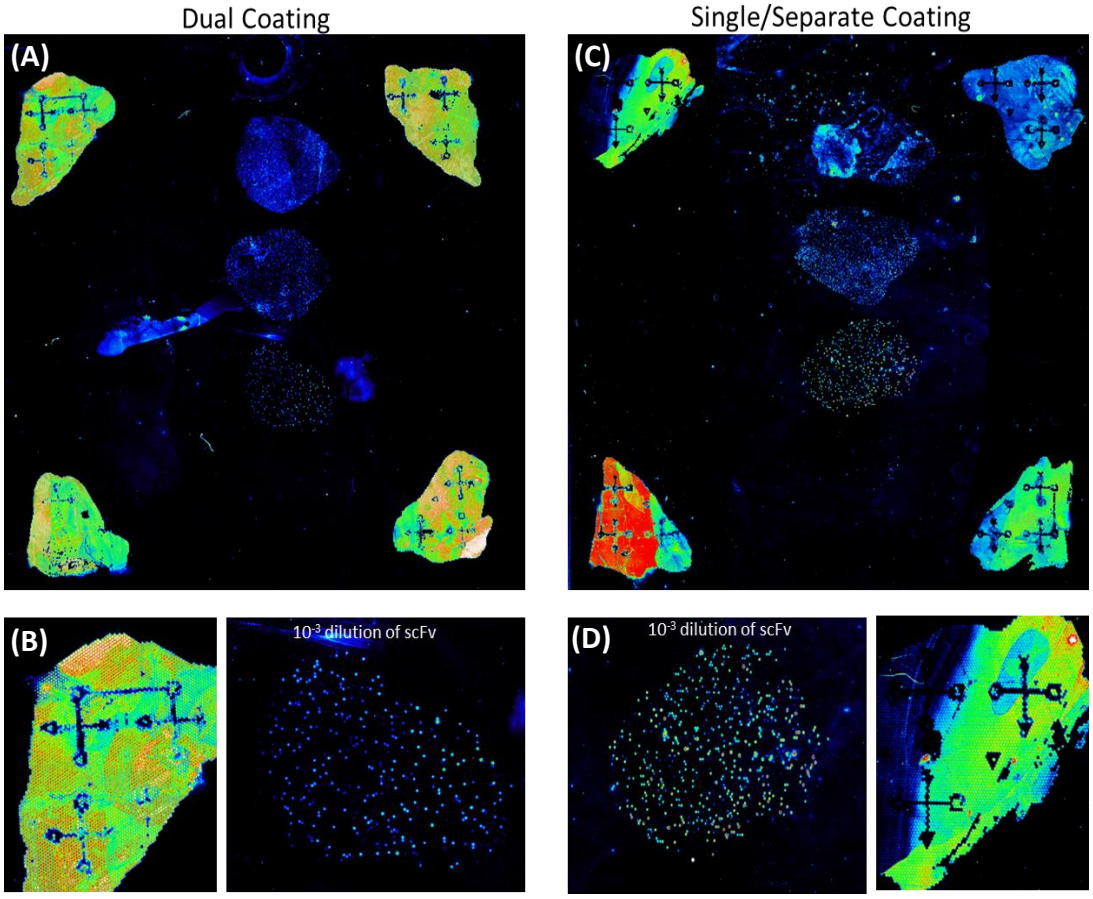

Figure 3.36: Comparison of performance for single and dual coating systems on PDMS assay surfaces. *The image demonstrates a dual coating system (A and B) in direct comparison with a single/separate coating system (C and D). The assay for alignment (corners) is a control anti-human IgG assay and the assay for screening (centre) is expressed anti-CRP scFv F8 detected by a fluorescently labelled HA-tag antibody.*

Both assay formats, compared in Figure 3.36, performed well in terms of the alignment and screening assays. While the single coating surface seems to have a clearer definition of the fluorescent spots the pattern obtained on the dual coated surface is acceptable for analysis by

the recognition software developed in-house by Brian Manning. There is a lower fluorescent signal seen on the screening assay. This is probably as a result of a lower level of CRP antigen being immobilised to the PDMS surface when in competition with the hIgG from the alignment assay. This can be overcome by altering the ratio of screening and alignment reagents in the coating solution.

By directly comparing the single and dual coating system it can be concluded that the performance is not negatively affected by the more desirable dual coating approach. Overall the use of a dual assay will make the process more streamlined by removing the need for a segregated surface and step-wise coating. In addition, the throughput for the final screens will be greatly improved as a greater area of the coated surface will be available for screening.

3.3.6 Design and implementation of process control software.

Dedicated LabVIEW software has been designed and developed by Brian Manning that allows for image analysis, alignment of the assay image to the physical array and instrument control. Additional functionality allows for the automated selection of lead clones depending on user selection criteria such as signal intensity and dual signal data (positive/negative etc.). Multi-dye filters are incorporated allowing the user to design custom screening applications on the platform. The software has been tested thoroughly to ensure that the targeting and retrieval of capillaries is highly accurate and reproducible, as demonstrated in Figure 3.37 which shows the targeting efficiency of the software controlled prototype through 800 iterations.

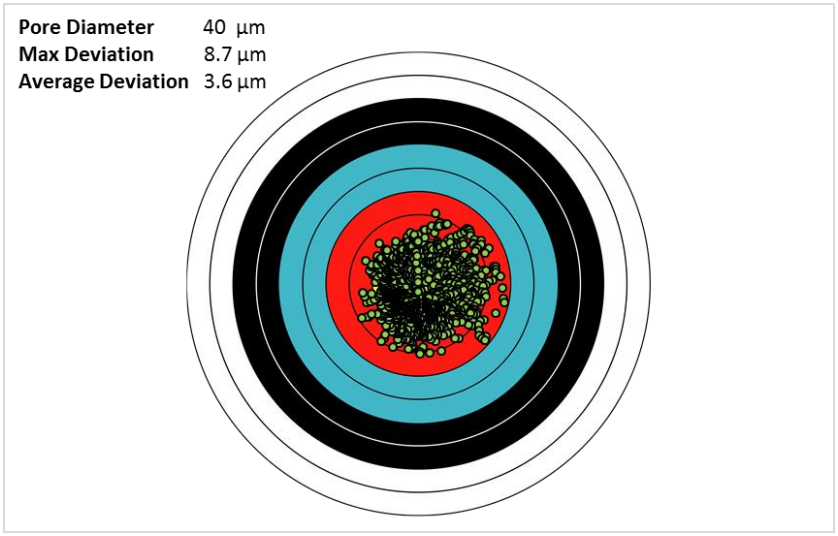

Figure 3.37: Demonstration of the accuracy of the DiCAST data analysis and recovery system. *A total of 800 targets were analysed by the software, coordinates calculated and specific regions targeted with the recovery system. The average deviation from the centre was 3.6 µm and maximum deviation was only 8.6 µm from the centre.*

3.3.7 Summary for key challenge 3.3

This key challenge was concerned with the development of a means to allow for accurate retrieval of the contents of a desired capillary following screening. In order to achieve this, the dedicated team manufactured a retrieval prototype and designed several software applications which could both control the prototype and analyse the image to locate the capillary of interest. Furthermore the introduction of distinct patterns in the physical array was carried out with a view to providing a means by which each image obtained could be related to the microcapillary array. Without such a means of alignment, it would be impossible to identify and retrieve the contents of capillaries of interest (i.e. those containing a cell expressing an antibody with the desired characteristics). By relating the two patterns to each other, a mapping of the entire assay surface is possible and each capillary can be located accurately on the physical array and its contents removed for future use.

Conclusion and Significance:

In this chapter the development of a single cell screening technology, now deemed the **D**irect **C**lone **A**nalysis and **S**election **T**echnology (DiCAST), was brought from concept through to practice.

DiCAST provides a miniaturised, ultra-high throughput method to screen millions of single cells in parallel without the need for enrichment, or without introducing bias towards fast growing or high expressing clones, typically seen in many traditional approaches. The technology is a novel approach for the identification of antigen-specific mAbs from millions of individual bacterial and mammalian cells using densely packed microcapillary arrays. By utilising dual detection surfaces, antigen-specific mAbs to multiple targets can be obtained in one single experiment. DiCAST incorporates the spatial addressing of single cells in individual microcapillaries with multiplex testing and automated data analysis and recovery in one system (Figure 3.38).

The process involves analysing the supernatant from ASCs captured and cultured in microcapillaries ranging in volume from 80 pL to 1.2 nL per individual capillary. The capillaries are sealed at one or both ends with an antigen-coated and blocked surface that essentially forms a miniature removable assay surface for each capillary. Once removed the surfaces are washed and specific antibody, if present, is detected with fluorescently-labelled secondary antibody. Scanning the assay surface using a standard microarray scanner provides an image map of fluorescent spots corresponding to a positive assay signal. The image pixel coordinates are then interrogated to provide the location of the capillary containing the single cell producing the antibody to the target of interest. Using custom written software (coded by Brian Manning) for image analysis, alignment and instrument control, individual cells are recovered into 384 well microtitre plates using a high precision alignment setup. During this process, the densely packed capillary arrays containing the cells are sealed and loaded into the recovery instrument. The single capillary of interest is automatically located using the software and the contents of the capillary recovered by ablating a hole in the sealant to allow a flow of nitrogen to simply blow the contents in to a well of a 384 well plate positioned 1mm below the capillary array. Since all the coordinates are automatically calculated by the software, including full instrument control, one cell can be recovered every 3 seconds.

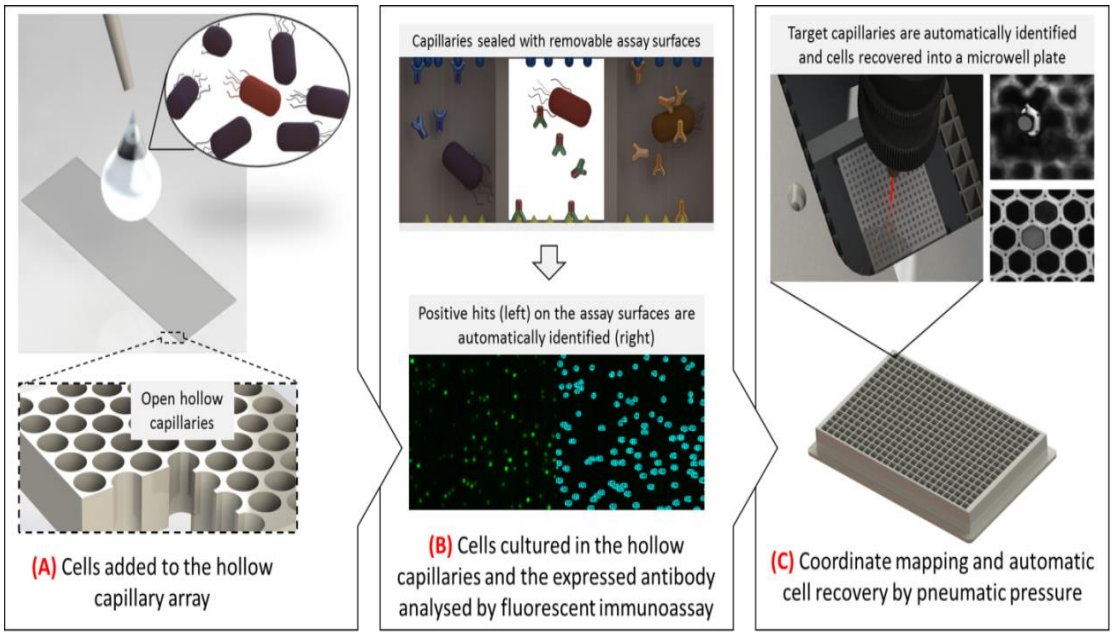

***Figure 3.38: Schematic of DiCAST process**. During the DiCAST process, single cells are captured in densely packed microcapillary arrays **(A)** and sealed reversibly to pre-coated assay surfaces to detect antibodies of interest. Cells which produce an antigen-specific antibody will be represented on the assay surface as a fluorescent spot when imaged, which can then be related back to the exact capillary containing the desired cell **(B)**. A high resolution image of a fluorescent assay is generated and subjected to image analysis and processing **(C)**. The coordinates for each spot on the image can then be used to find the corresponding capillary location on the physical array when loaded into the DiCAST instrument. Instrument control, image analysis, coordinate mapping and data processing are all controlled within the same user interface. Retrieval can be carried out with the custom recovery instrument to micron scale precision.*

In essence, the DiCAST process involves the integration of three key stages, Assay, Scan and Align/Recovery, as shown in Figure 3.38. In addition to complex integration challenges such as software and mechanical design, each individual process step presented important developmental hurdles that needed to be addressed before the technology could be employed for its final purpose.

The primary portion of the chapter was concerned with the ability to incubate, grow and express a target protein from bacterial cells contained within the capillaries of the array. In addition because of the proposed embodiment for the DiCAST assays there was a requirement that the cells would remain viable throughout the incubation and removal processes. The employment of an auto-induction system was investigated as it would, if successful and comparable to traditional methods, allow for efficient expression and remove the need for addition of a secondary component to the array. The two auto-induction media formulations tested not only compared with but outperformed the standard IPTG induction

method usually employed within the laboratory (Figure 3.4 and Figure 3.5). Bacterial cells were visualised growing and expressing protein (GFP) in the capillaries of the arrays (Figure 3.6) and viability of the cells, subsequent to retrieval from the array, was also confirmed (Figure 3.7). Another area of interest in this section was the ability to dilute cells to allow for single cell analysis. This was carried out by analysing dilutions of GFP expressing *E. coli* in the capillaries of the array. It was evident from this analysis (Figure 3.8) that it is possible to dilute the cells to allow for single cell occupancy, based on a Poisson distribution. To accurately know the number of cells per capillary in a particular screen, the cells in the culture loaded onto the array would need to be enumerated and the culture diluted accordingly. More than one cell can be loaded into each capillary but on retrieval, additional processing steps would be required to ensure monoclonality of the cells.

Following on from these investigations, the ability to carry out an immunoassay on the proposed assay surface (PDMS) was investigated. When it came to carrying out more complex assays that required extended incubation with culture, washing steps or multiple secondary assay steps the surface was not stable and could not be used. In light of this a campaign was undertaken to identify a suitable and reliable chemical modification strategy for the PDMS to allow assays on the surface. Testing of each modification was carried out to assess its potential, under conditions such as effect on sealing and performance with purified antibodies and expressed antibodies (Table 3.1). From the numerous strategies tested the APTES modification performed the best overall, as shown in Figure 3.11, and was selected for use going forward. Following this modification it was possible to obtain similar results, when directly comparing a modified PDMS surface and a microtitre plate, using purified antibodies in a sandwich format (Figure 3.12).

Once the technology had been brought to a stage where an expressing assay could be carried out on the PDMS surface (Figure 3.15), a number of different assay formats were developed to improve the diversity within the DiCAST offering.

Initially, the ability to carry out a multi-analyte assay was investigated. This approach involved the verification of two different criteria. The first was the ability to capture antibodies on the surface through an epitope tag (in this case the HA tag) using an antibody coated on the surface. The presence of specific antibody was then detected using fluorescently-labelled antigen. This essentially turns the assay, previously described, upside-down and potentially means that responses to several antigens can be gathered simultaneously. The capture approach was shown to be possible (Figure 3.17) and the second requirement for multi-analyte, multiple fluorophore reading, was carried out. The Perkin Elmer microarray scanner has lasers for Cy3 and Cy5 detection installed. The excitation and

emission of these two fluorophores are well separated and although cross-talk was not expected its absence was confirmed by analysis on plate (Figure 3.18) and on array (Figure 3.19). As final proof-of-concept for this approach an assay using an anti-CRP scFv was carried out, using the capture approach, and probed with CRP labelled with Cy 3 and Cy5 (either singly or mixed) to observe response. The result (Figure 3.20) showed that it was possible to probe, and differentiate responses, with a mixture of alternately labelled antigens on the same assay surface.

Following the development of the multi-analyte approach, two additional multiplexing approaches were tested. The first of these was the employment of a double-sided assay, which utilised the two open surfaces of the microcapillary array to carry out two different assays, on the same population of cells, simultaneously (Figure 3.22). The second involved the demonstration of a multi-lift approach, which allowed several assays to be carried out by sealing the array to multiple prepared surfaces sequentially (Figure 3.24). These additional multiplex approaches could be used as an alternative to the multi-analyte approach which is reliant on a common capture element. They could also be used in combination with the multi-analyte approach and each other to increase the number of "questions" asked of each antibody screened in the same iteration.

The final assay format that was studied in this chapter was the possibility of applying a secondary solution to the top of the sealed array, and observing diffusion through the contents of the capillary. The benefit of this approach would allow for more complex assay systems, such as functional bio-assays which require addition of reagents after accumulation of antibody, to be performed on the array. The diffusion of a fluorescent-labelled antibody, through buffer loaded into the capillaries, was observed and shown to give excellent coverage over the area to which it was added (Figure 3.26).

In addition to developing a stable surface approach, as described previously, several methods to analyse interaction in the actual capillaries were assessed. These methods, if successful, would offer the ability to carry out the assays on the array without the need for an external assay surface. This would remove the need to align the image to the physical array and would perhaps streamline and save time in the overall process. Both the FRET (Figure 3.27) and GFP reassembly (Figure 3.29 and Figure 3.30) approaches trialled proved to be amenable to use on the array, but it was thought that the low signals generated in both systems may affect assay sensitivity. This could likely be overcome by further optimisation of the assay and documentation systems employed.

In the final section of this chapter the issue of retrieval was investigated. If the cell of interest cannot be accurately retrieved from the capillary in which it is housed, then the entire

DiCAST screening process becomes futile. The development of a system to reliably and accurately retrieve the contents of a desired capillary was undertaken by the DiCAST team, consisting of biologists and mechanical and electronic engineers. A custom built retrieval instrument (Figure 3.32) was designed and produced along with several software packages responsible for instrument control, image analysis and alignment of the assay image to the physical array.

In terms of assay development within this area a method to relate the assay image to the physical array was devised. It involved the performance of an alignment assay in areas where distinct marks had been introduced into the array by blocking certain capillaries in a specific pattern (Figure 3.35). In this way the exact location of certain capillaries (those in the blocked pattern) was known, and could be extrapolated to identify the location of capillaries which contain antibodies of interest. It also involved carrying out an assay with dual coating, to allow for the alignment assay described above to be carried out in combination with the screening assay. If dual coating of the surface was not possible it would mean that sequential coating of different solutions in discrete areas of the assay surfaces would be required. This would make the entire process more complicated and most likely reduce the surface available for screening, neither of which would be ideal. Fortunately the dual coating process proved successful and when compared with single coating performed well, as shown in Figure 3.36.

In all, this chapter has served to verify conditions and to develop and optimise procedures that allow the DiCAST technology to meet its full potential. The fully optimised technology will be employed and compared with traditional screening approaches in several campaigns, both bacterial and mammalian, in subsequent thesis Chapters 4 and 5, respectively. A detailed comparison of DiCAST and its advantages over existing technologies is provided in Chapter 7.

Chapter 4

Phage-independent selection of lead antibody fragment candidates from bacterial antibody libraries using DiCAST

Hypothesis

Presently, the major problem facing the antibody discovery space is not the ability to generate vast repertoires of antibodies but the capacity to test their potential and identify those with the unique characteristics required. Recent innovations in automation and miniaturisation of this process have resulted in the emergence of elegant techniques such as microengraving of miniature wells (Love *et al*., 2006; Ogunniyi *et al*., 2009; Sendra *et al*., 2013), homogeneous cell spot assays (Jin *et al*., 2009) and microfluidic cell sorting (El Debs *et al*., 2012; Ryan *et al*., 2011; Wheeler *et al*., 2003). This chapter demonstrates the advantages of DiCAST for direct clone analysis and also provides a comparison between the newly developed DiCAST technology and the gold standard screening approach of phage display.

Rationale

Despite being the gold standard technology for screening bacterial antibody libraries for the last 30 years, phage display is not without limitations. Typically screening by phage display involves several rounds of bio-panning, as illustrated in Figure 4.1, gradually enriching the pool with high-affinity candidates. It is a laborious technique, when compared with the DiCAST screening process, and commonly identifies high numbers of false positives, that can be attributed to non-specific binding of phage to reagents or consumables (e.g. polystyrene) and their subsequent propagation throughout each iteration (Hoen *et al*., 2012). The frequency of non-binders can often be reduced by employing multiple rounds of selection; unfortunately, this strategy can also result in the loss of rare or desirable clones (Buckler *et al*., 2008).

Other complications that can occur when using phage display include poor or non-existent soluble expression subsequent to selection. This is often attributed to the presence of what are deemed "truncated" clones, with frame shifts or internal amber stop codons, which fail to bind to the antigen following initial selection (Goldman *et al*., 2000; Shinohara & Fukuda, 2002). In addition the reliance of display-based screening on competitive selection based on target affinity can result in the oversight of protein molecules with properties that are often incompatible with high affinity, such as agonists, partial agonists and antagonists, and modulators of target function (Mao *et al*., 2010).

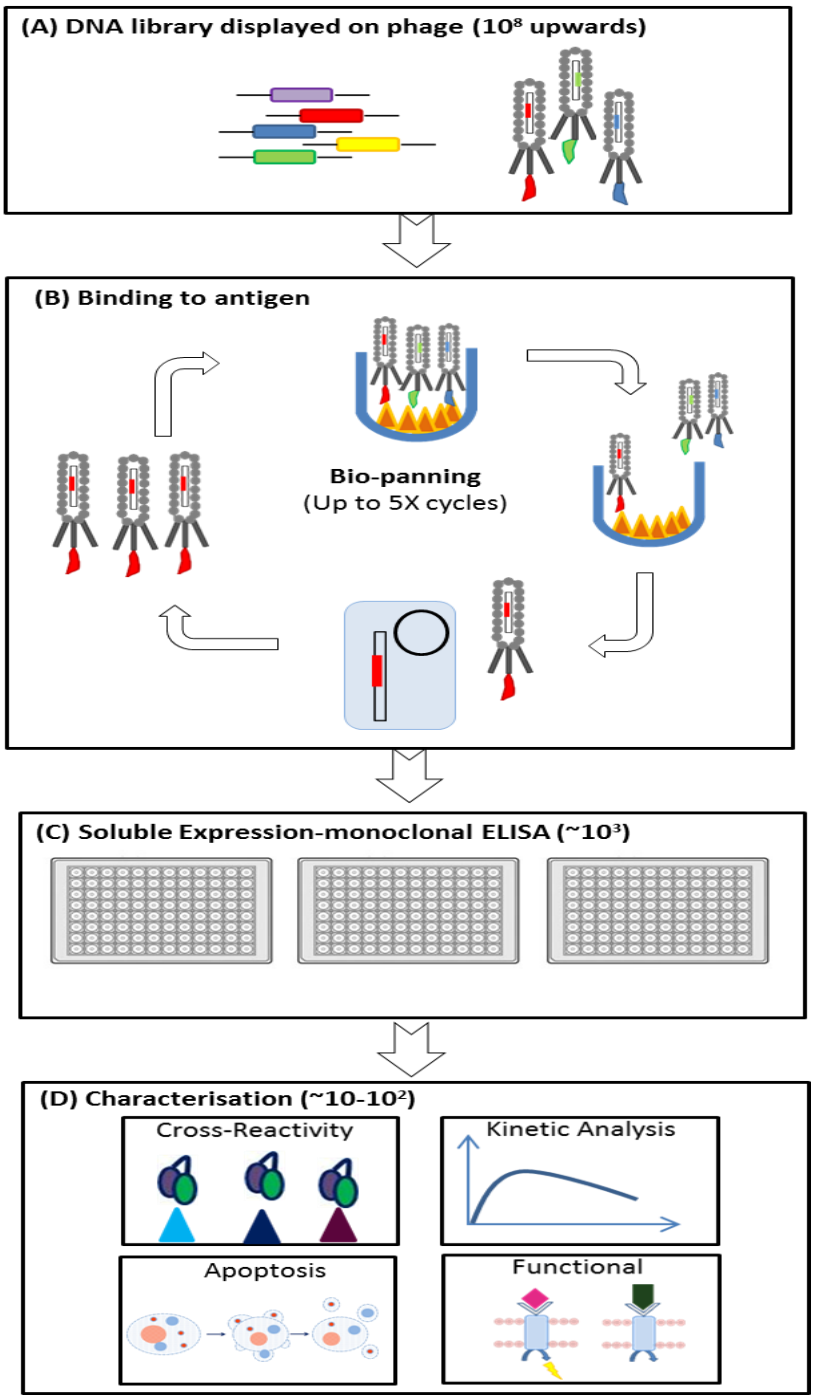

Figure 4.1: Antibody Discovery Process using phage display. ***(A)*** *A library of variant DNA sequences encoding antibody fragments are cloned and displayed on the surface of phage particles as fusions to the phage coat protein.* ***(B)*** *Bio-panning involves exposure of the phage library to the antigen of interest. Non-binding phage are washed away and the bound phage are eluted and infected into bacterial cells, allowing enrichment within the population of variants which bind the target antigen. Several rounds of bio-panning are typically carried out.* ***(C)*** *Following bio-panning monoclonal soluble ELISAs are carried out to test binding of variants from the output of the final round.* ***(D)*** *Those clones which exhibit binding, or a smaller subset depending on facility capacity, are taken forward for further characterisation studies that are relevant to the antibody fragments' final use (e.g. specificity, affinity, functional effect).*

As discussed in Chapter 3, DiCAST is a new spatial addressing technique (each cell is physically separated from each other in a microcapillary array) that allows the simultaneous analysis and selection of millions of individual cells in parallel. DiCAST can be used to identify ASCs from both bacterial and mammalian cells, reducing the time and resources required with current methods. As the cells are spatially addressed in individual microcapillary chambers, any bias or competition due to growth and expression levels is eliminated, preserving slow-growing or rare clones. Using DiCAST, heterogeneous populations of cells can be analysed to determine which cells are producing antibodies of interest. Once identified, the cells harbouring the desired antibody can be recovered from the array for further characterisation and use.

When compared to phage display, DiCAST not only saves on time and costs but also circumvents the drawbacks of display systems by removing the competitive element from the selection as well screening fragments in soluble form (i.e. not on the surface of phage), enabling the discovery of novel and fit-for-purpose antibodies It also allows for multiple tests to be carried out, on the entire population of variants, simultaneously (Figure 4.2).

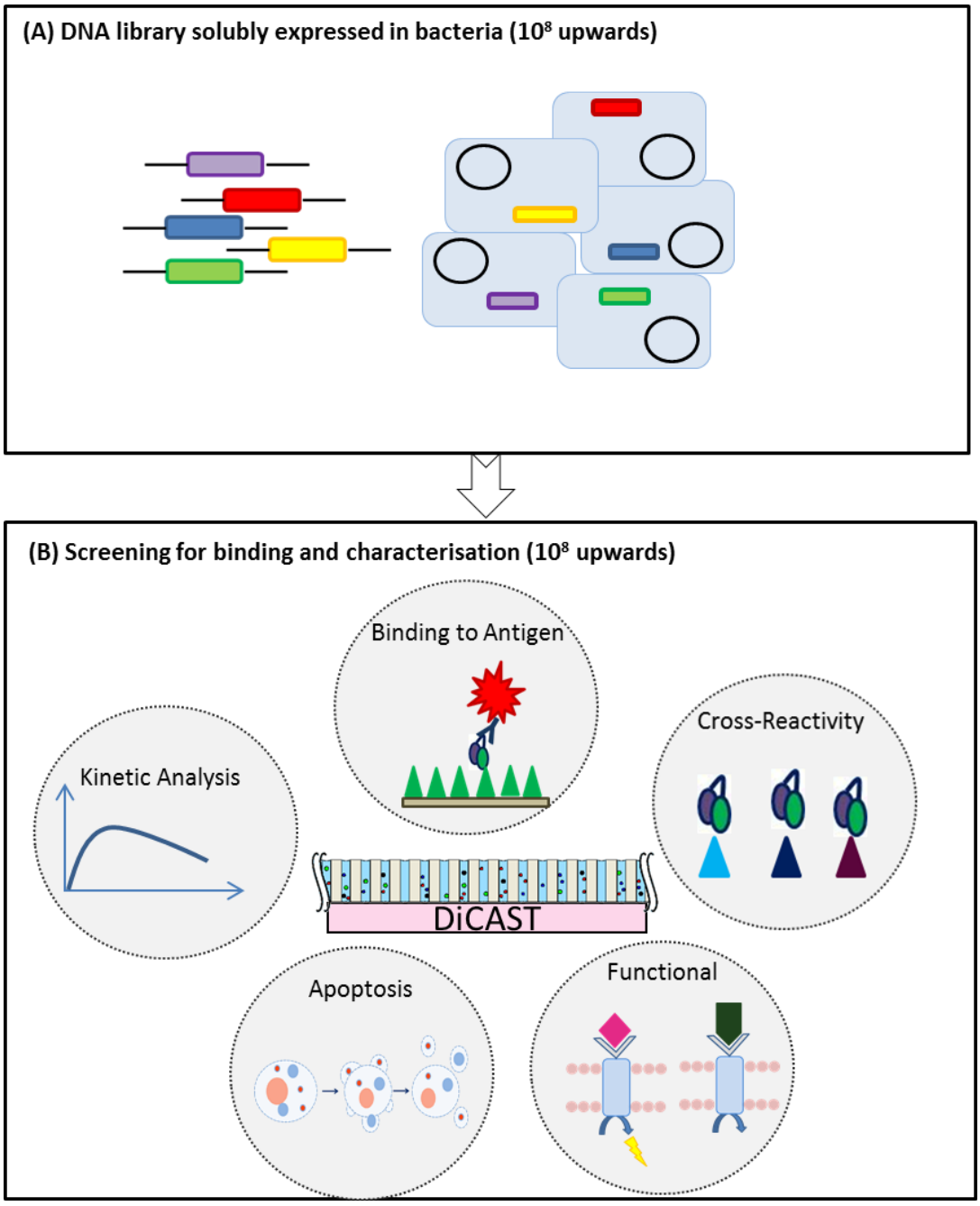

Figure 4.2: Antibody Discovery Process using DiCAST. (A) *A library of variant DNA sequences encoding antibody fragments are cloned and expressed in soluble form in bacterial cells.* ***(B)****The library is diluted to achieve the equivalent of 1 cell per capillary and analysed using DiCAST. By utilising the multiplexing capabilities of the technology, discussed in Chapter 3, it is possible to carry out multiple, diverse tests on the entire library of variants simultaneously.*

In this chapter a series of bacterial screening campaigns utilising DiCAST, which was fully developed at DCU and the development of which is described in detail in Chapter 3, are discussed (Figure 4.3)

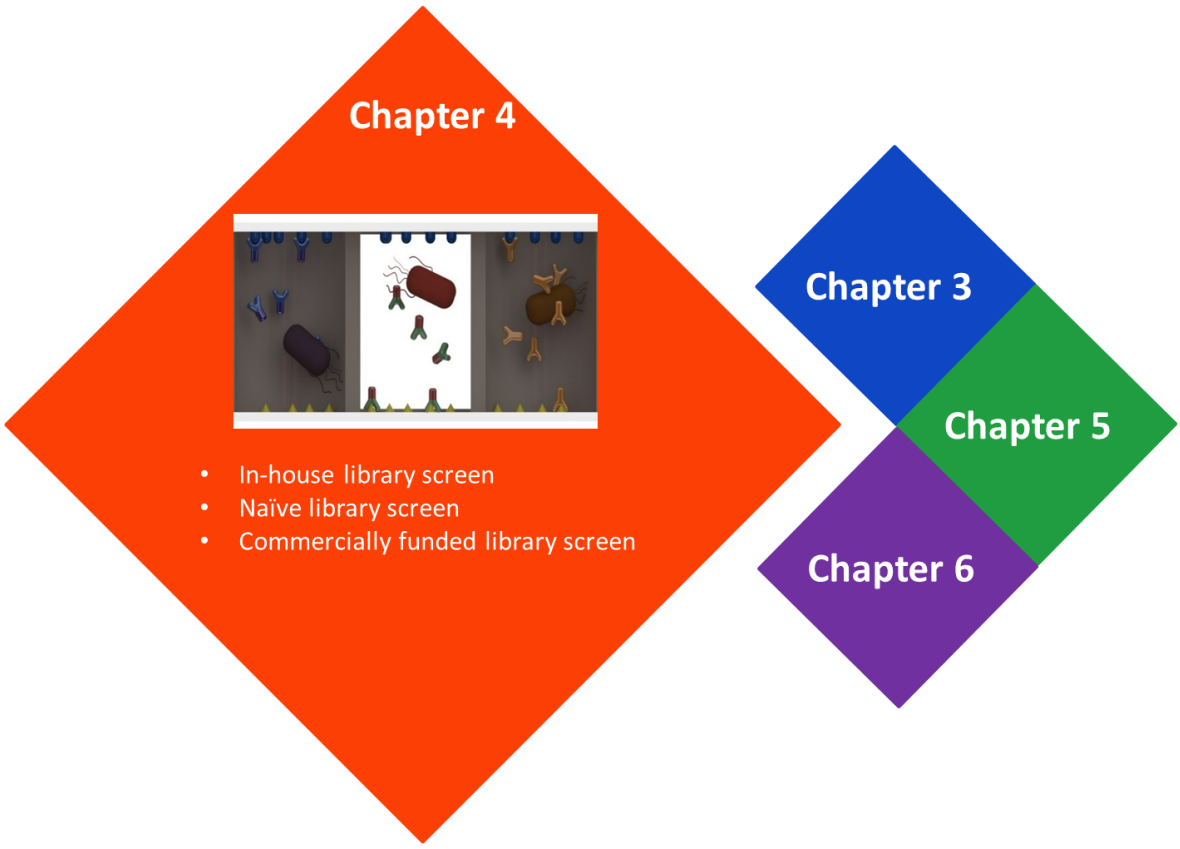

Figure 4.3: Schematic overview of thesis layout. *Chapter 4 is concerned with demonstration of phage-independent selection of lead antibody fragment candidates from bacterial antibody libraries using DiCAST.*

To demonstrate the utility of the DiCAST system, over 1 million bacterial cells from an immune avian scFv library were directly analysed, in one experiment on a single cell basis, without enrichment by phage display. In addition to the standard screen multiplexing, experiments were carried out with the same immune library to illustrate the enhanced capabilities of the screening approach. To emphasise DiCAST's utility further, antigen-specific antibodies were also selected from a commercial naïve human single domain antibody library. Additionally a commercial evaluation of the technology was commissioned by a UK company, where a previously mined external library was screened using DiCAST and outputs returned and characterised in a blind test.

Key Challenges

Key Challenge 4.1 C-Reactive Protein (CRP) in-house produced library- complete screen and comparison with phage display.

4.1.1 Experimental approach

CRP is a sensitive marker of inflammation and has been investigated as a prognostic marker for myocardial infarction (Ridker *et al*.,1998). Here the ability to select antibody fragments from a bacterial library without the need for traditional approaches of phage display and bio-panning, illustrated in Figure 4.1, is demonstrated.

4.1.2 Selection of scFv antibodies from *E. coli* without enrichment

An immune avian scFv library, previously produced and screened against C-reactive protein (CRP) in the Biomedical Diagnostics Institute in DCU, was analysed for antigen-specific antibodies using the DiCAST platform. This library was previously well characterised by Biacore and generated a number of excellent lead antibodies making it a very good library to evaluate the potential of DiCAST. In fact, two of the best clones obtained from the library were licensed to two companies, one in the UK (Integrated Magnetics) and one in Portugal (BioSurfit), highlighting the quality of the library and the challenge was to see if selection could be pushed further with DiCAST.

A total of 1.2 million clones from the original un-panned library were screened, for antigen (CRP) binding, on a single cell basis in a microcapillary array as described in 2.4.1. A selection (288 of 1631) of positive clones (Figure 4.4) were retrieved and confirmed as binding specifically to the antigen, as described in section 2.4.2 and shown in Figure 4.5. In total 98% of those retrieved were positive by subsequent ELISA analysis. A sub-set of those were then sequenced and unique sequences further investigated by ELISA, as described in section 4.1.3.

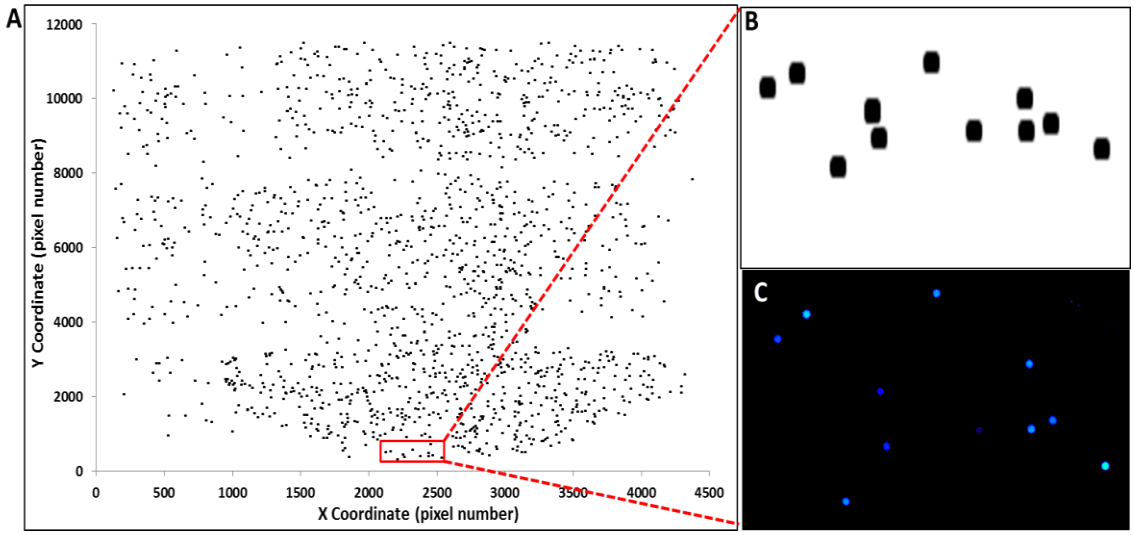

Figure 4.4: Selection of antigen-specific recombinant antibodies. *An immune avian scFv library was screened to identify antibody fragments of defined specificity.* ***(A)*** *Scatter plot representing the precise location of the 1631 CRP-specific clones identified.* ***(B)*** *Enhanced portion from the scatter plot showing a particular set of spots each of which corresponds to a capillary containing an antigen-specific (CRP) clone.* ***(C)*** *Area of scanned image, corresponding to the highlighted portion of the scatterplot shown in Figure 4.4B, mirroring the exact pattern observed in Figure 4.4A and B.*

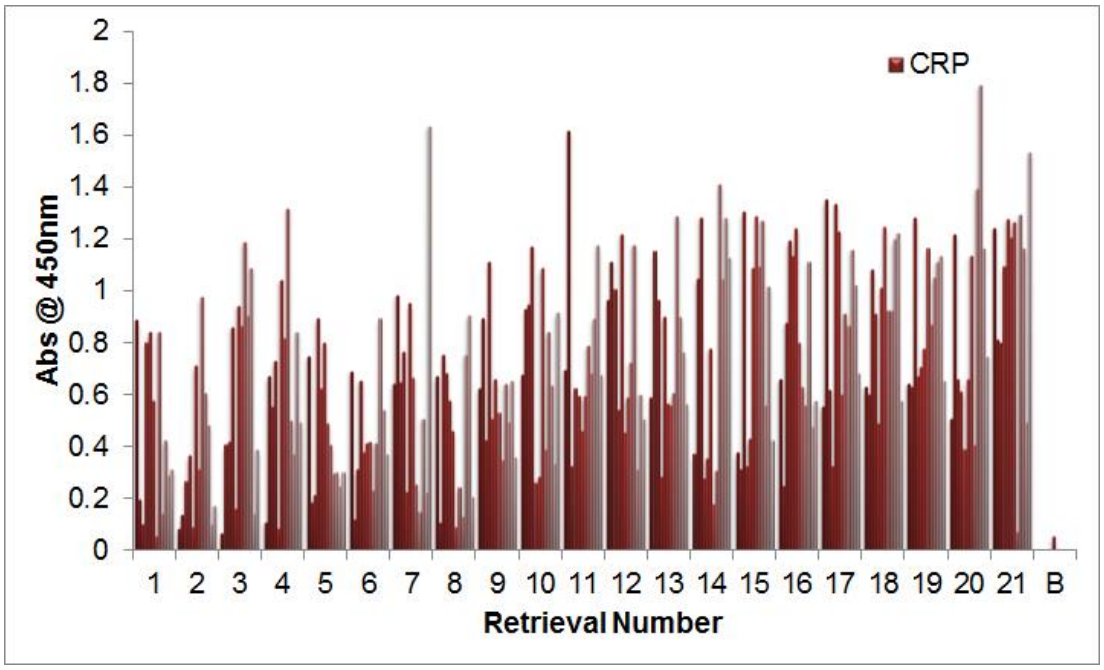

Figure 4.5: Confirmatory binding ELISA (CRP) of 288 clones selected and retrieved from analysis of avian anti-CRP scFv library on DiCAST. *The 288 clones were randomly selected from the 1631 positives observed on the entire scan, and assayed using the direct on-plate methods described in section 2.4.2. A total of 98% (283 out of 288) clones were positive binders subsequent to retrieval. The sample designated "B" represents a negative control consisting of medium only in place of expressed culture.*

4.1.3 Characterisation of clones selected from the library using DiCAST.

Following confirmation of binding to the antigen, as shown in Figure 4.5, a randomly selected sub-set (96) were sequenced, analysed and compared for assay sensitivity in competitive ELISA as detailed in section 2.4.4. Sequencing of the clones allowed for the diversity of those clones retrieved to be assessed. In all there were 27 unique sequences identified following sequencing of 96 individual clones, shown in Figure 4.6 and Table 4.1.

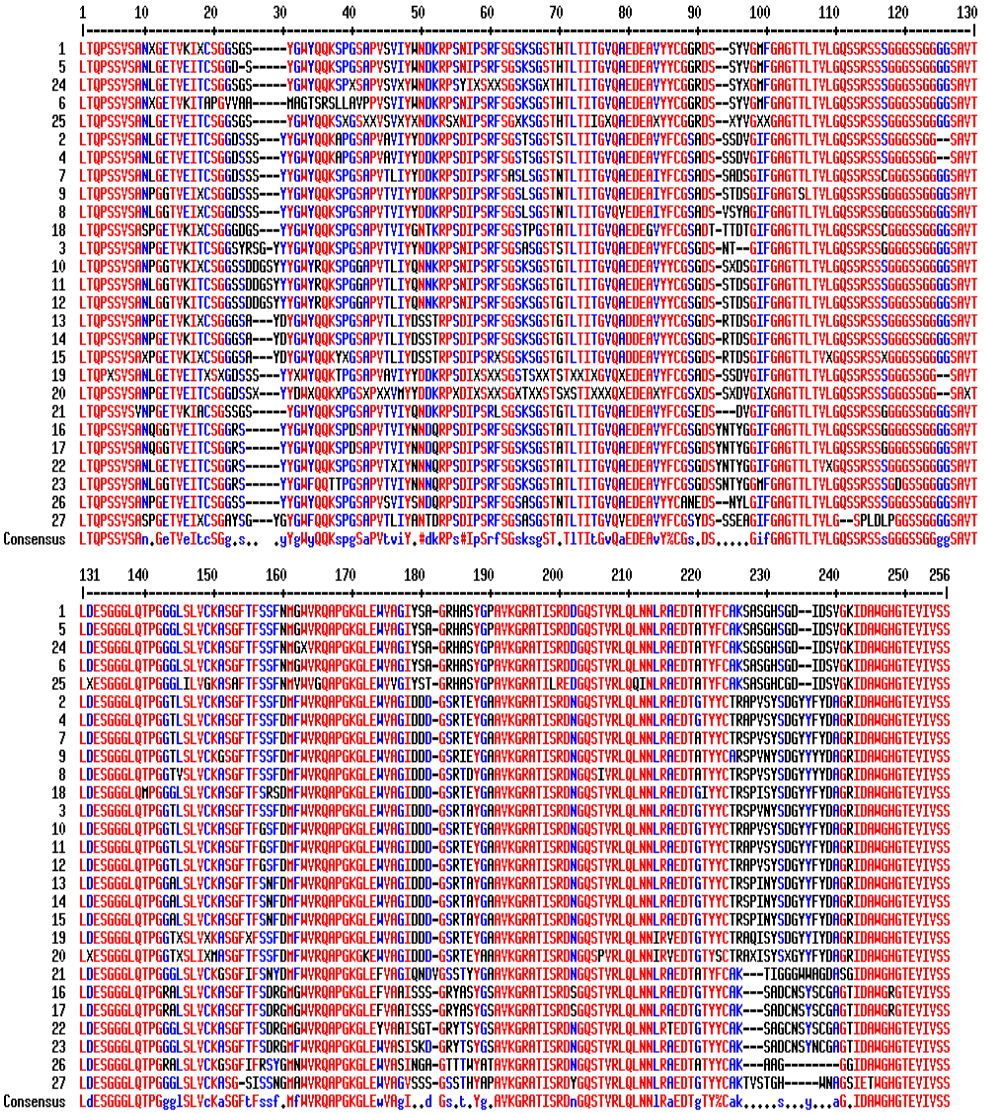

Figure 4.6: Sequence data for a selection of clones retrieved from the CRP library screen*. A subset of the positive clones identified in Figure 4.4 and confirmed in Figure 4.5 were randomly selected (90/288) for sequencing analysis. In total 27 unique sequences were observed. Sanger sequencing was carried out by Source Bioscience. Sequences were translated to amino acids using the ExPASy translate tool (http://web.expasy.org/translate/) and aligned for similarities using multiple sequence alignment tool MultAlin (http://multalin.toulouse.inra.fr/multalin)*(Corpet, 1988).

There was one predominant sequence, which had accounted for 45 of the clones as well as 21 unique sequences among those sent for sequencing. The top four, highest affinity clones (F6, F8, H2 and G5) previously screened and selected using phage display and Biacore 4000, described in Leonard *et al*. (2007), are also represented within the groups (Table 4.1).

Table 4.1: Sequencing diversity observed from 96 randomly selected clones from the anti-CRP library screen.

Group	Number of Clones
Group 1	45
Group 2 (incl. H2)	8
Group 3 (incl. F6)	4
Group 4	3
Group 5 (incl. F8)	8
Group 6 (incl. G5)	7
Unique	21

Each of the unique clones (27 in total) were then titered, as described in section 2.4.3, to identify an appropriate dilution for competitive analysis (detailed in section 2.4.4). Assays were carried out for all clones and the data fitted with a 4-parameter equation using BIAevaluation software. Based on competitive ELISA analysis (Fig. 4.5) of the selected clones a range of antibody fragments were identified, the best of which (clone A6) showed a 55-fold improvement in IC_{50} value when compared to the best (i.e. in terms of affinity ranking results) perfoming clone selected previously using phage display (clone H2) (Leonard *et al*., 2007).

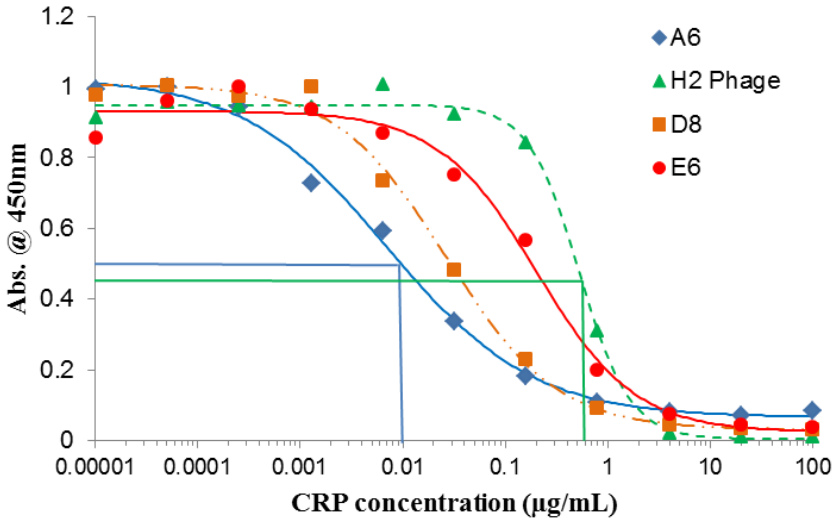

Figure 4.7: Competitive ELISA analysis of a selection of CRP clones selected using DiCAST versus H2; the highest affinity clone obtained previously using phage display. *Based on inhibitory concentrations of 50% (IC_{50}) values, clone-A6 selected by DiCAST shows a 55-fold improvement over H2. IC_{50} values were calculated based on 4 parameter logistics equation, used to fit the data, and were determined to be H2-0.55 µg/mL, E6-0.23 µg/mL, D8-0.03 µg/mL and A6-0.01 µg/mL.*

4.1.4 Multiplexed analysis and selection of antigen-specific scFv.

The ability to 'multiplex' offers huge advantages in the antibody screening process, as it both enables one to obtain more information in the same amount of time but to select antibodies which do not, or indeed do, cross-react with a particular antigen. In terms of closely related targets this can be invaluable when introduced at the early screening stage.

Multiplex analysis was performed on an immune avian scFv library, previously produced from chickens immunized with C-reactive protein (CRP) and Myeloperoxidase (MPO), on the DiCAST platform. MPO, like CRP, is a biomarker for use in the detection of cardiovascular disease (Zhang *et al.*, 2001). By use of a capture format, antibodies from 3 million antibody-secreting cells were analysed and then probed with multiple, alternately labelled antigens simultaneously. In this way three populations of antibodies could be identified. The first solely bound CRP while the second bound only to MPO. The third population were cross-reactive and bound both antigens. This was visualised as a 'traffic-light-like' system, as shown in Figure 4.8, with each population identified by colour: CRP-specific (Red/Cy5), MPO-specific (Green/Cy3) and cross-reactive (Yellow).

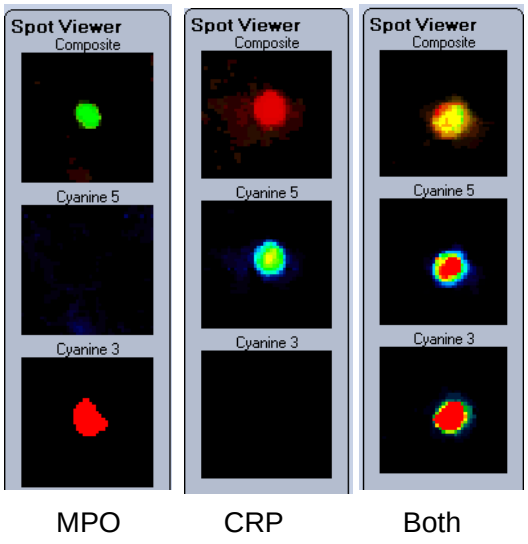

Figure 4.8: Multiplexed analysis and selection of antigen-specific scFv. *These include the performance of multi-analyte detection on the same surface using alternately labelled antigens. The surface was coated with a capture element (in this case an anti-HA antibody), all antibodies expressed in the microcapillaries were captured and secondary detection was carried out using two antigens with different fluorescent labels (Cy5-labelled CRP and Cy3-labelled MPO). The number of antigen-fluorophore combinations employed is dependent on the scanner used. Analysis of the multi-analyte surface allowed identification of three different sets of antibodies: Those which bound CRP, those which bound MPO and those which cross-reacted with both proteins. In "Spot-viewer" mode, those spots which appeared green (Cy3) in the composite image represent an MPO-specific antibody and those which appeared red (Cy5) are specific to CRP, while those which appeared yellow in the composite view are capable of binding to both proteins.*

A sub-set of each population was retrieved using the DiCAST recovery system and further characterised by ELISA to confirm binding and specificity (Figure 4.9). Those clones which were labelled cross-reactive were streaked out and single colonies tested in on-plate ELISAs to ensure that the dual specificity was not a result of two different cells present in the capillary during the screen.

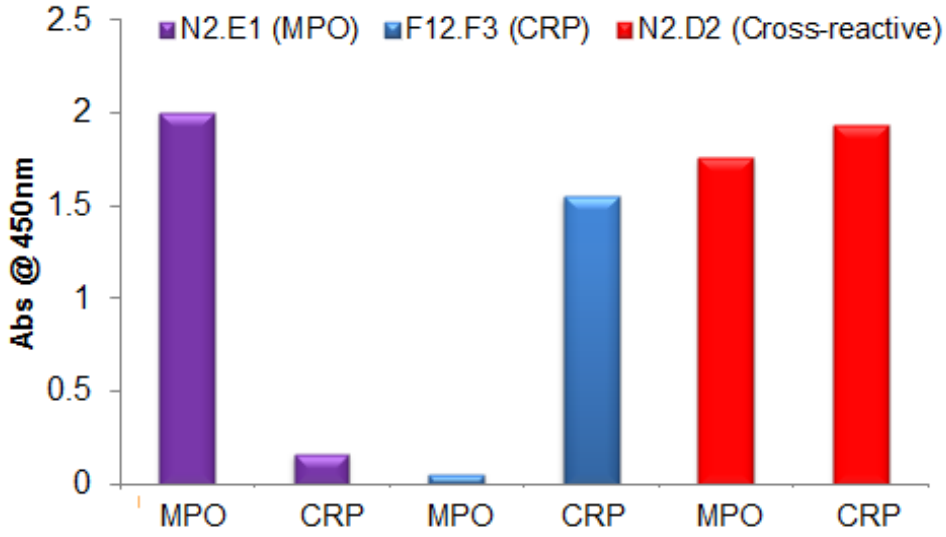

Figure 4.9: Direct binding ELISA for a representative clone from each of the three sets of antibodies evident in the screen. *Each clone was screened against both antigens. Clone N2-E2 binds MPO only, clone F12-F3 binds CRP only and clone N2-E1 binds both MPO and CRP.*

Furthermore, the nature of the microcapillary array used in the DiCAST process very easily lends itself to the implementation of a 'double-sided' analysis. In this approach both sides of the array are sealed to an assay surface (Figure 4.10). Using the double sided approach it is possible to ask two distinct questions of a particular candidate. It offers an advantage over the 'multiplex' format in the fact that a common capture element is not required. In this way a large amount of information can be obtained for each antibody screened. For example, a library could be screened for both binding to a particular target and also for functional effect at the same time. The double-sided element of the microcapillary arrays used in the DiCAST screening process gives the technology a unique advantage over many other high-throughput screening approaches.

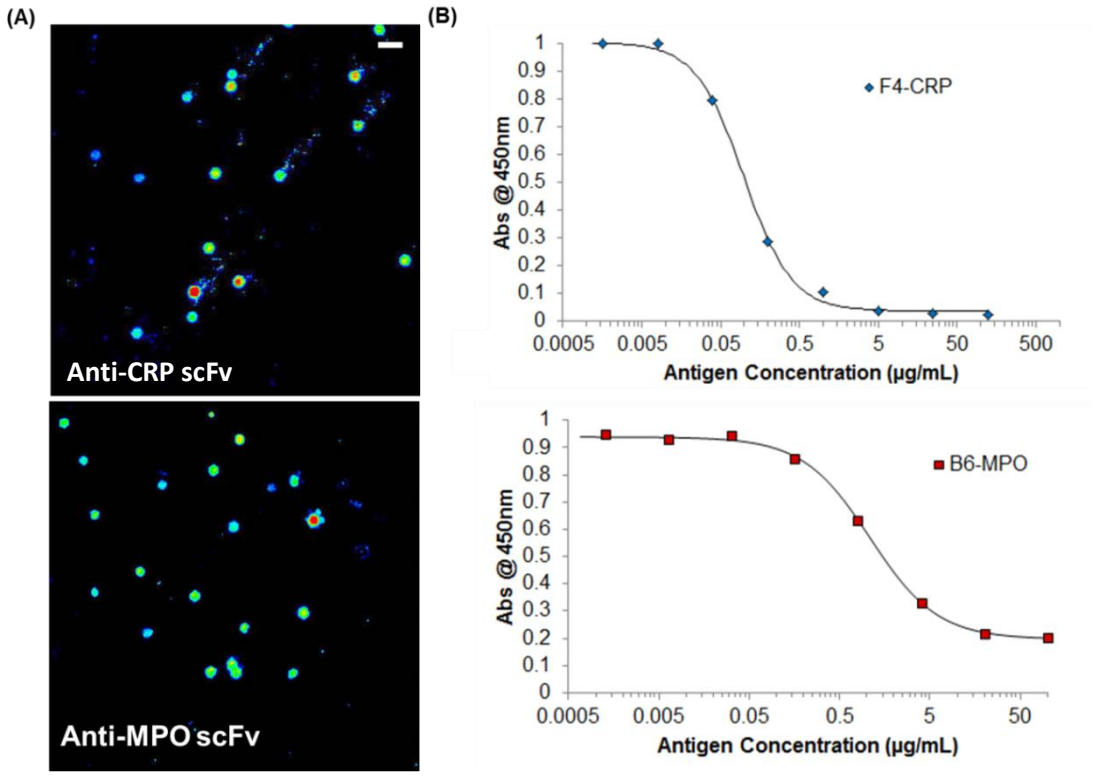

Figure 4.10: Double-sided assay using DiCAST. *This can be used to carry out two separate assays on the same population of cells simultaneously. Two surfaces are coated with different antigens (MPO and CRP) and the array is sealed to each sequentially. Subsequent to incubation two distinct assays are performed and binders to each of the antigens identified and recovered.* ***(A)*** *A section of a scan for each antigen targeted in the screening process. The specific spots (40µm diameter), clearly visible, correspond to a capillary which contains a cell secreting an antigen –specific antibody in the physical array. Scale bar is 100µm.* ***(B)*** *Competitive ELISA analysis of a typical clone from each antigen set selected.*

4.1.5 Summary for key challenge 4.1

After a thorough interrogation of this recombinant library, in various embodiments (single antigen and multiplex), the advantages and performance quality of the DiCAST technology were evident. In a direct comparison with phage display, antibodies with enhanced sensitivity were identified by using DiCAST. In addition the ability to perform multiplex assays offers the opportunity to screen for several different traits of each antibody (e.g. cross reactivity) in one single experiment and gives the user access to a more powerful screening approach. It can also offer the benefit of streamlining the antibody discovery process and interrogating a library for several different antigens in a single iteration. This not only saves time but also has inherent savings on costs associated.

The goal of this section was to demonstrate a direct comparison with phage display using the CRP library as a model library and to identify unique clones that were missed by phage display. A large number of positives were identified within the library and a number of

clones with improved binding could be observed. If there was a need to identify a higher sensitivity antibody more of the library and hits could have been screened and characterised, as necessary. Planned future work includes the incorporation of a method to affinity rank the clones detected, similar to strategies employed by MorphoSys (Poetz *et al*., 2005) or as discussed in Watkins *et al*. (1998). This will allow for enhanced information to be obtained for each clone versus direct binding screening.

Key Challenge 4.2 Screening of commercially available naïve domain antibody (dAb) library

4.2.1 Experimental approach

As clones can be biased towards the target in immune library analysis, to further challenge the DiCAST process a commercially available, naïve, human domain (dAb) library (Lee *et al*., 2007) was screened using a number of different antigens (Transferrin, cTnI, cTnT and intact Jurkat T lymphocytes). Model antigens available in the lab were chosen to provide a panel of protein and cell targets for the screening campaign.

Transferrin is a plasma protein involved in the metabolism of iron and, monitoring of serum levels can be indicative of anaemia, iron-overload conditions and diabetes mellitus among other conditions (Craig *et al*., 2001)·(Mabayoje *et al*., 2010). Cardiac troponins (cTnI and cTnT) are well established, standard biomarkers for the detection of myocardial damage and for the assessment of suspected cases of acute coronary syndrome (Apple & Collinson, 2012). The cell screening, using Jurkat T lymphocytes, was included as a demonstration of the flexibility of the DiCAST system to use whole cells as a potential antigen. This is a desirable trait of any screening system, allowing the identification of antibodies which bind cell receptors, in their native form, as they are presented on the cell surface.

4.2.2 Verification of the screening system using positive controls.

Prior to screening of the library, the proposed assay format was tested using the supplied positive control (anti-β galactosidase dAb). There is a cMYC tag present on the expressed fragments for detection, and a monoclonal anti-cMYC antibody was used as the capture reagent. The positive control was expressed in small scale culture, as described in section 2.2.2, and the supernatant (S/N) and lysate fractions tested by ELISA. The clone was also tested using the on-plate method described in section 2.4.2 (Guo *et al*., 2010). Finally once the clone was confirmed to work in ELISA (Figure 4.11A), it was tested in an array assay and performed well (Figure 4.11B).

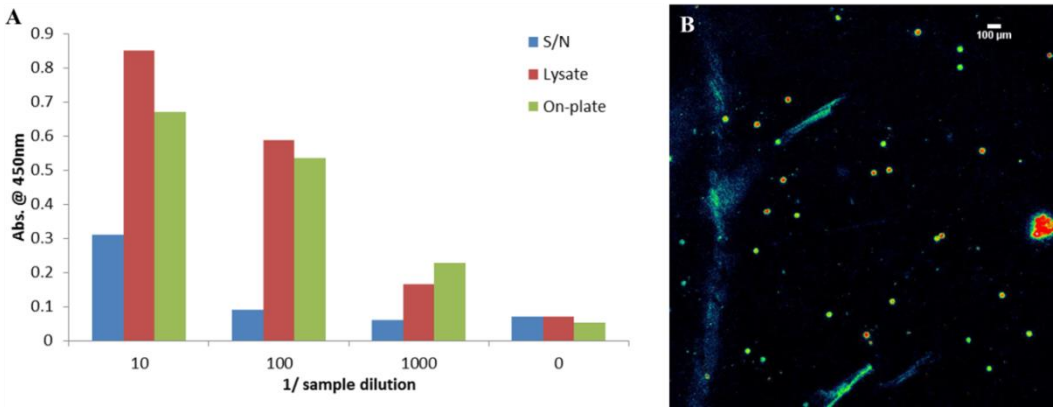

Figure 4.11: Verification of library positive controls through ELISA and using the DiCAST platform. *Control anti-β galactosidase dAb is provided with the library when purchased. The assay format and performance, in both ELISA (A) and using DiCAST array assay (B), was assessed prior to library screening. Capillaries measure 40µm in diameter. Scale bar represents 100µm.*

4.2.3 Screening of the domain library against multiple targets.

Following the verification of the assay system for analysis, using the positive control supplied as described in section 4.2.2 and demonstrated in Figure 4.13, the library was screened. Without any prior exposure to any of the antigens 3 million clones were analysed for each one, this time at 10 cells per capillary, allowing a greater portion of the library to be analysed in each experiment.

While positive responses were seen for all of the antigens, as shown in Figure 4.12, the binding response post-retrieval for the cTnI and cTnT and Jurkat screens was very low. The transferrin screen produced the highest number of positives and despite only sampling a fraction (0.1%) of the initial library a high number of transferrin-specific fragments were identified through the screen.

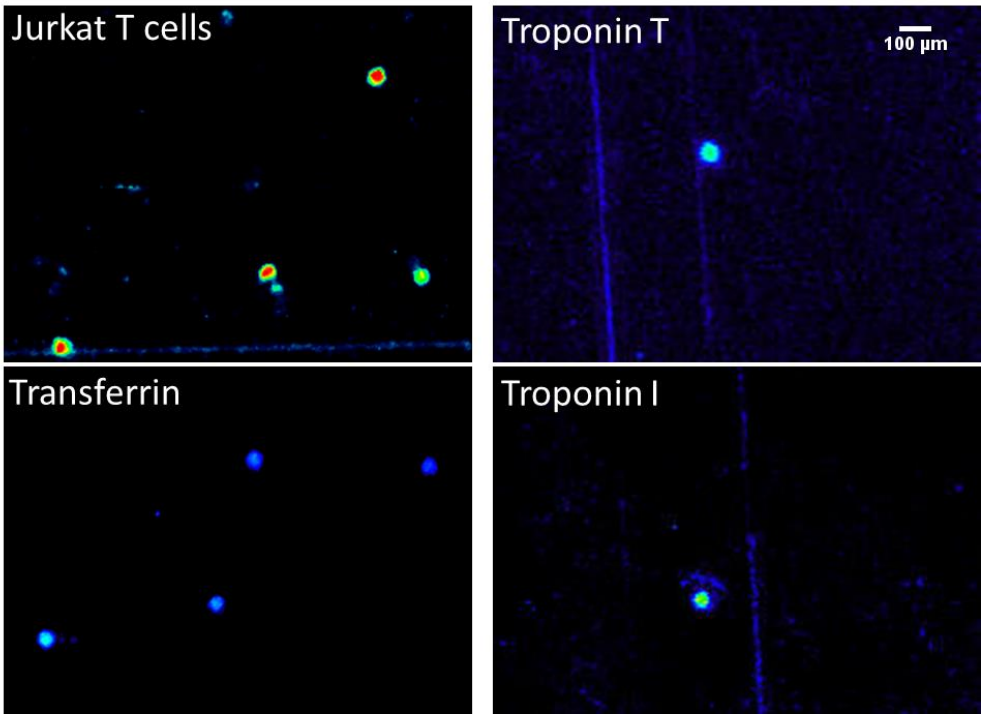

Figure 4.12: Scanned images from multiple screens carried out on the naïve dAb library. *The library was screened with various different antigens including Transferrin, cTnI, cTnT and also intact Jurkat T lymphocytes (Clone E6-1 ATCC TIB-152) which were labelled with Cy5-labelled anti-EpCAM to allow visualisation. Capillaries measure 40µm in diameter. Scale bar is 100µm.*

A selection of positives (48) were subsequently retrieved from the microcapillary array, streaked out to obtain single colonies and further confirmed as antigen-specific by ELISA analysis (Figure 4.13). All fragments recovered maintained a certain level of antigen specificity post-retrieval, but appeared to have a degree of non-specific interaction with the blocking buffer (BSA). Attempts to express the clones exhibiting a desired binding profile, in large-scale culture, were not successful and lysates produced failed to bind the antigen in subsequent ELISAs. When sequenced the clones produced very poor reads, despite originating from a single colony.

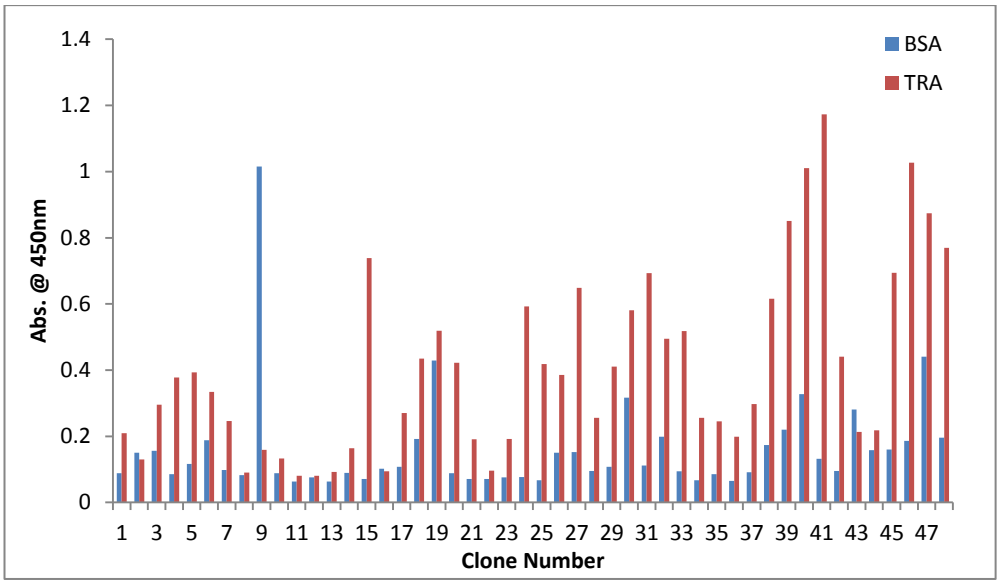

Figure 4.13: Confirmation of binding post-retrieval of anti- transferrin dAb fragments. *This analysis was carried out using the on-plate expression method described in section 2.4.2. All clones were streaked out twice and single colonies analysed against the antigen (Transferrin) and unrelated protein (BSA).*

4.2.4 Summary for key challenge 4.2

Overall, while thought to be a very useful tool to assess the power of the DiCAST platform the quality of the commercial dAb library (i.e. predominance of poly-reactive clones and lack of evident expression post-retrieval) did not allow for extensive screening to be carried out. Previous reports on single domain fragments have indicated a propensity for poor solubility and low levels of expression in certain cases and this may have been a contributing factor in this analysis (Holt *et al*., 2003). Additionally, during personal communication with Daniel Christ (who was part of the team that generated the library) the poor quality of the library, due to inadequate storage conditions, was mentioned.

Key Challenge 4.3 Commercial evaluation of DiCAST (anti-homoserine lactone library from an independent UK based laboratory)

4.3.1 Experimental approach

In order to assess and verify the performance of the DiCAST technology, a 100% industry funded evaluation of an externally sourced recombinant library was undertaken by the DiCAST team in DCU. The library, directed against a mixture of homoserine lactone (HSL) compounds (3-oxo-C12-HSL, 3-OH-C12-HSL & N-acyl-C12-HSL), had previously been generated and screened by phage display, as described in Palliyil *et al*. (2014). Homoserine lactones are used by a number of bacteria (e.g. *Pseudomonas aeruginosa*), to initiate cell-to-cell communication, as quorum sensing signalling compounds. Antibodies which block this communication could offer a possible method to control infection of certain bacteria (Palliyil *et al*., 2014). An ideal antibody would cross react with the three compounds mentioned above, as well as an additional compound C4-HSL which is thought to potentially provide additional therapeutic effect (Palliyil *et al*., 2014).

To be considered successful the evaluation of this external library would need to culminate in the identification of antibodies which bound to each of the three HSL compounds of interest at same, or better, affinity as was achieved by the original laboratory. In addition, the ability to cross react with C4-HSL was considered a point of interest. The screening conjugates (3-oxo-C12-HSL-BSA, 3-OH-C12-HSL-BSA and N-acyl-C12-HSL-BSA), the library and the positive controls (HSL2 and HSL4) were supplied by the independent UK laboratory. Unconjugated compounds were purchased to allow for functional sensitivity of antibodies to be ascertained by competitive analysis.

4.3.2 Control Verification (On-plate and array)

The proposed assay format for this screening campaign was a capture approach, using either the HIS tag or the c-MYC tag available on the fragments. Unfortunately the approach did not function as expected and no signal was seen using a capture approach (Figure 4.14 B and C) when compared to the use of antigen coated on the surface (Figure 4.14A). It was also noted that a much higher concentration of the antigen was required to obtain a positive signal in both ELISA and on array (4 µg/mL and 16 µg/mL, respectively). This was most likely due to the fact that in the case of this antigen (HSL-BSA conjugate) the predominant protein contributing to concentration reading was the carrier, BSA.

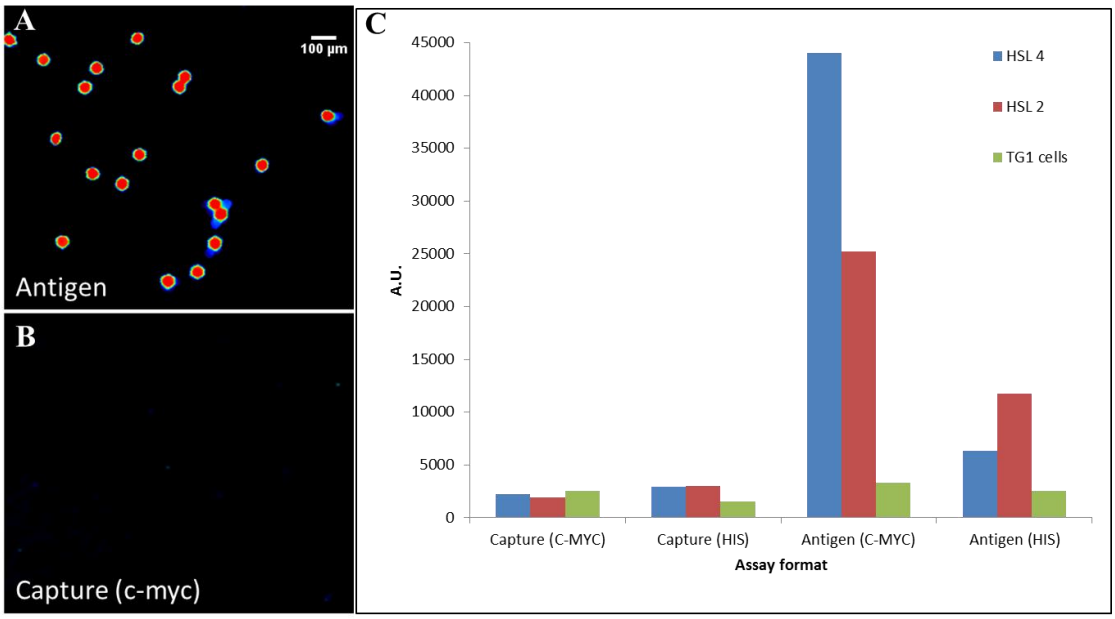

Figure 4.14: Verification of assay format on array, using control scFv (HSL4), on array and on plate. *Two different screening assays (i.e. immobilised antigen Vs capture approach) were tested for performance using the anti-HSL scFv control prior to commencing interrogation of the library. The immobilised antigen approach coats the antigen, in this case one of the HSL conjugates, on the surface and uses an anti-tag antibody (anti-cMYC or anti-HIS) for detection. The capture approach uses the reverse approach by coating the anti-tag or capture antibody on the surface and detecting presence of a specific antibody with fluorescently labelled antigen. Both formats were tested on array* ***(A)*** *and in ELISA* ***(B)*** *and their performance compared. TG1 cells, which did not contain any plasmid, were used as a negative control to assess any level of non-specific binding.*

4.3.3 Library Screening

On identification and optimisation of the assay format to be implemented, using the positive controls, screening of the library was begun. In all, over 2.5 million scFv fragments were screened on a single cell basis throughout the study. There was a low number of positives (typically ~ 1 in 100,000 clones analysed were positive), and screens were carried out against each of the three compounds (3-oxo-C12-HSL-BSA, 3-OH-C12-HSL-BSA & N-acyl-C12-HSL-BSA) to identify binders (Figure 4.15A). Post-retrieval each selected clone was tested for binding to each of the compounds (and the carrier protein, BSA) to ascertain cross-reactivity, as shown in Figure 4.15B.

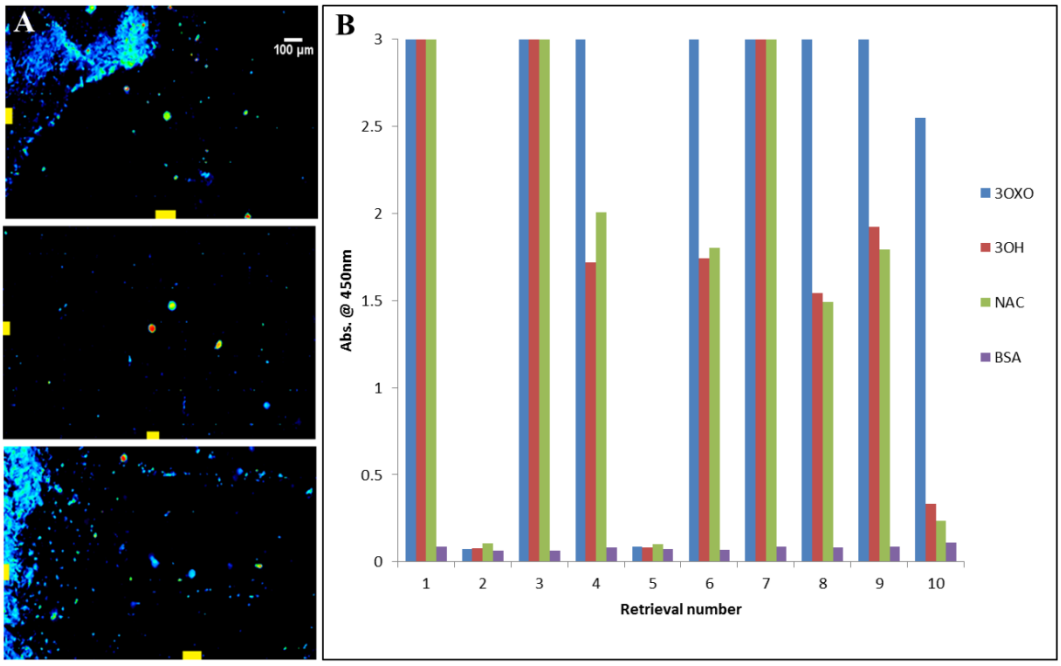

Figure 4.15: Sample post-retrieval confirmatory ELISA for clones against all conjugates. ***(A)*** *A selection of chosen positive hits from library screens carried out throughout the project. The horizontal and vertical yellow bar intersect at the spot where a candidate antibody was chosen* ***(B)*** *Each of the retrieved scFv fragments was tested for binding against each of the HSL conjugates (3OXO, 3OH, NAC and negative control BSA) following removal from the microcapillary array. Their binding was confirmed using the on-plate method, described in section 2.4.2* (Guo *et al*., 2010). *All clones were also tested against the carrier protein (BSA). This graph represents a subset of clones (10) retrieved during several experiments.*

4.3.4 Characterisation of final selected clones

Upon extensive screening of the library and further cross-reactivity testing against each of the three HSL compounds and the carrier protein, a final selection of twelve clones were chosen for assessment by the team in the UK-based laboratory. Each of the clones was expressed in large-scale culture (section 2.2.3), purified by IMAC (section 2.2.4) and compared against the two positive controls by competitive ELISA (section 2.4.4).

Owing to the large number of clones, only the final eluent was visualised by SDS-PAGE analysis as described in section 2.2.1 and shown in Figure 4.17. In order to assess the level of specific antibody in each of the purification fractions, an ELISA was carried out, as described in section 2.4.5, and shown in Figure 4.16. For the purpose of illustration, Figure 4.16 compares two of the purification profiles obtained. The purifications were successful and the eluent fractions for each were pooled and used in further characterisation experiments.

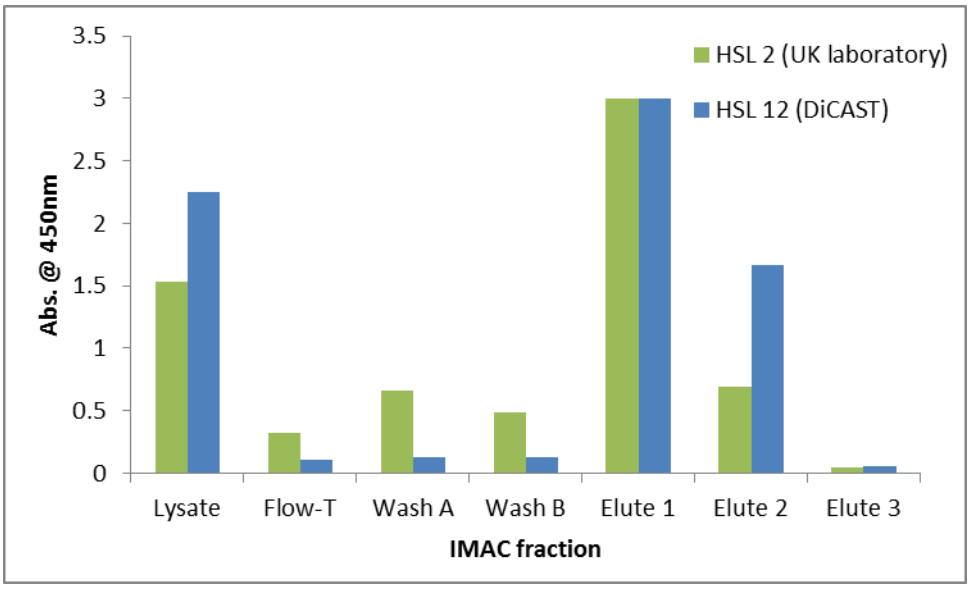

Figure 4.16: Confirmation of presence of antigen-specific scFv in each fraction of the IMAC purification. *A qualitative ELISA was performed to identify the fractions which contained the antibody of interest. For the purpose of illustration only two of the final clones were compared in terms of purification profile. The purification was successful with only small amounts of the antibody lost in flow-through and wash fractions. The first two elution fractions were pooled, buffer exchanged for use in Biacore and competitive ELISAs. The purity of the final eluent was also assessed using SDS-PAGE (Figure 4.17).*

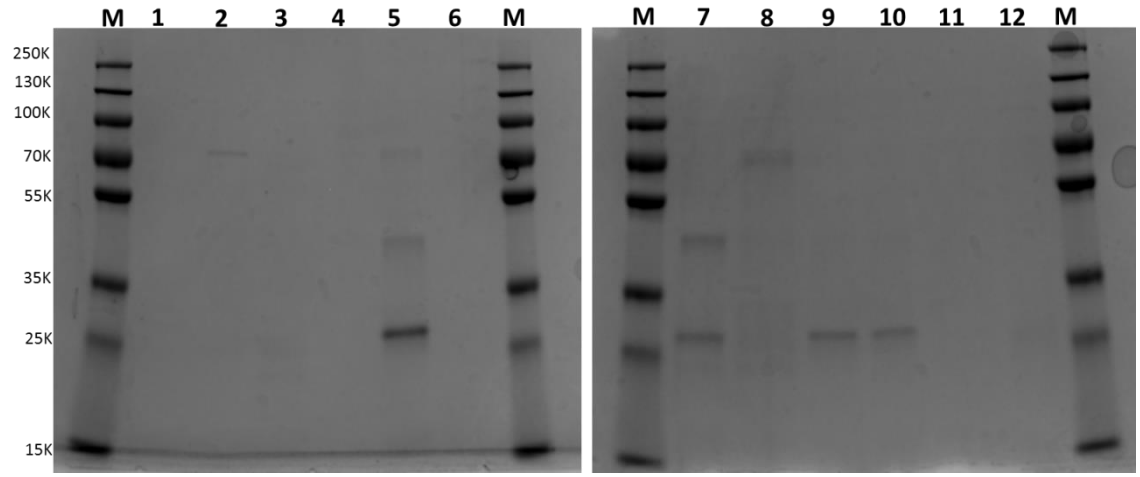

Figure 4.17: SDS-PAGE analysis of elution fraction for the final candidate clones. *Each of the HSL clones were purified by IMAC, as described in section 2.2.1, and the final eluent visualised on a SDS-PAGE gel. The first gel (Lanes 1-6) contains HSL 1-6 in a consecutive manner. The second gel (Lanes 7-12) contains HSL 7-12 in a consecutive manner. The lanes designated 'M' contain the protein marker (PageRuler plus pre-stained protein ladder, sizes for each of the bands are indicated to the left of the gel picture). The expected size is approximately 27 kDa for each scFv.*

On observation of the final eluent for each of the scFvs, it can be observed that some of the fractions would require concentration of the purified fraction in order to be visible on the gel. In the case of others there is clearly evident bands of greater size than was expected (typical scFv is approximately 27 kDa). The larger bands could indicate dimer- or trimerisation of the purified scFvs.

It is important to note, at this point, that the stability of the HSL conjugates used throughout this study was a cause for concern. While all of the final selected antibody fragments bound to all three compounds in initial confirmation ELISAs, there are cases where they do not appear to bind some of the compounds in further investigations. Nevertheless, the functional sensitivity of each of the final clones was tested against each of the compounds (non-conjugated), as described in section 4.3.4, prior to shipment of samples. The resulting data is displayed in Figure 4.18. Each of the HSL conjugates was coated on the plates and competition for binding against the free unconjugated compound assessed. In all, the analysis using 3-oxo-C12-HSL, shown in Figure 4.18A, was the most complete data set with all clones represented. The analysis for the remaining two compounds (3-OH-C12-HSL & N-acyl-C12-HSL), shown in Figure 4.18B and 4.18C respectively, was not complete and could only offer information on a subset of the clones. It appeared based on the 3-oxo-C12-HSL that a clone selected using DiCAST (Clone 4) offered superior functional sensitivity over the previously designated "best" clone, selected by phage display in the original screening campaign (HSL4).

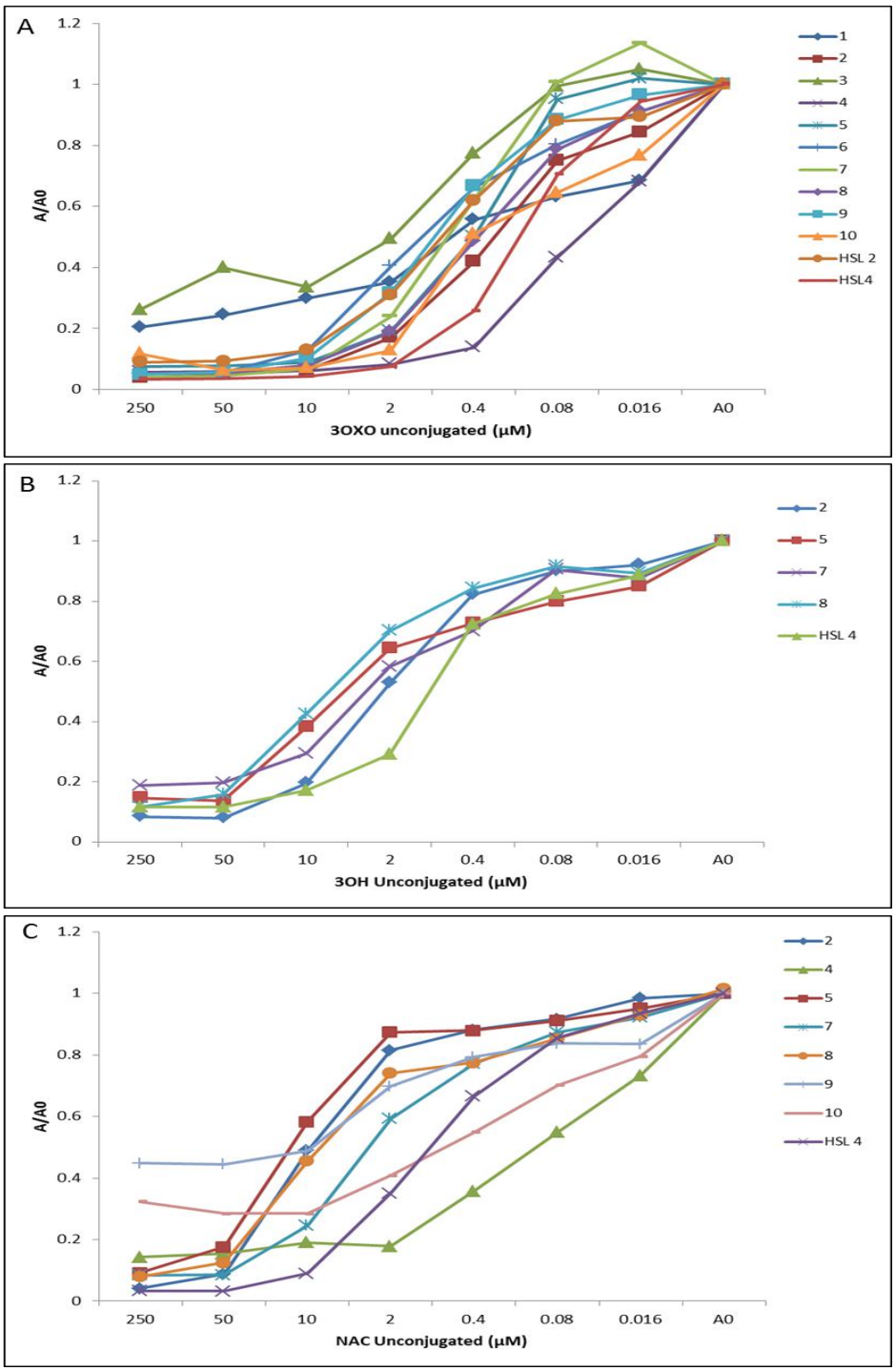

Figure 4.18: Competitive analysis of final HSL clones against the three screening compounds. *The graph shows the performance of DiCAST selected clones against the two controls provided by an independent UK based laboratory (HSL 2 and HSL 4), for each of the three screening compounds (3-oxo-C12-HSL, 3-OH-C12-HSL and N-acyl-C12-HSL). Each assay involved competition between the bound conjugate and free un-conjugated compound in solution. Outcomes following phage display of this library, reported in* Palliyil *et al*. (2014), *HSL4 was described as the best antibody identified. From competitive analysis of the DiCAST selected clones it appears that clone 4 outperforms HSL4 in functional sensitivity.*

The final clones were also tested for functionality against an additional HSL compound C4-HSL, as cross reactivity against this compound was considered to be of interest in the initial investigations of the library. This particular compound was not supplied as a screening conjugate as part of the evaluation; however, the unconjugated compound was purchased and tested in competitive analysis, using N-acyl-C12-HSL as the coating conjugate. Several of the final clones were functional, with lower sensitivity than other compounds, against this additional compound and once again DiCAST selected clone 4 demonstrated superior performance when compared to the controls (Figure 4.19).

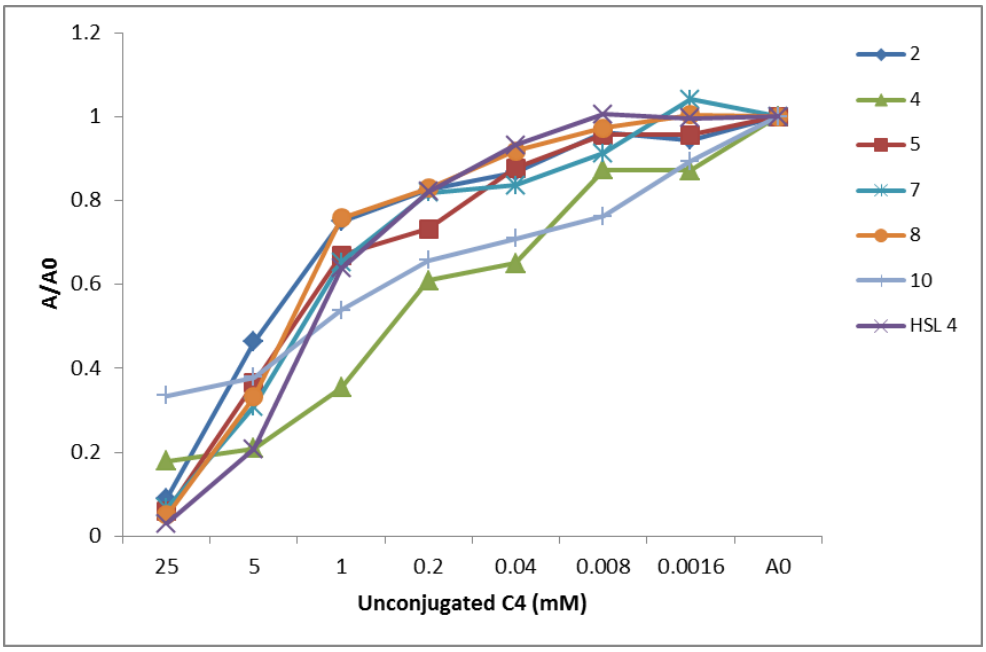

Figure 4.19: Competitive analysis of final HSL clones against additional compound C4-HSL. *The graph shows the performance of DiCAST selected clones against the two controls provided by an independent UK based laboratory (HSL 2 and HSL 4). The assay involved competition between the bound N-acyl-C12-HSL conjugate and free un-conjugated C4-HSL compound in solution. Outcomes following phage display of this library, are reported in* Palliyil *et al*. (2014), *HSL4 was described as the best antibody identified. From competitive analysis of the DiCAST selected clones it appears that clone 4 outperforms HSL4 in functional sensitivity.*

Upon sequencing of the twelve clones (ten DiCAST selected and two controls) it was found that DiCAST clone 4 matched the sequence of control HSL4 exactly. In all there was one predominant group which accounted for 6 of the final clones, the match to HSL 4 (DiCAST clone 4) and the remaining DiCAST clones were unique in sequence. The control HSL2 was not represented in the final selection. Biacore analysis was also carried out on the antibodies but, once again, due to instability of the screening conjugates the results added very little to the profile for each antibody. Finally, all selected purified antibodies were randomly

numbered and shipped to the independent UK based laboratory for blind testing. Following their analysis they delivered a positive response, describing several of the DiCAST selected clones as superior in performance to those they had selected previously, shown in Table 4.2.This demonstrates that the DiCAST technology performs desirably in comparison with traditional methods and can identify specific antibody fragments from recombinant libraries without the need for enrichment.

Table 4.2 Comparison of IC50 values obtained for DiCAST selected clones versus those selected using phage display. All analysis was carried out in a blind test by the original, independent UK-based laboratory.

Clone	3-oxo-C12-HSL	3-OH-C12-HSL	N-acyl-C12-HSL
DiCAST Clone 2	4 nM	0.8 µM	40 nM
DiCAST Clone 4	2 nM	0.8 µM	20 nM
DiCAST Clone 5	275 nM	Not determined	90 nM
HSL 2 (scAb format)	3 nM	1.8 µM	500 nM
HSL 4 (scAb format)	1.5 nM	0.8 µM	600 nM

4.3.5 Summary for key challenge 4.3

The ultimate aim of the evaluation undertaken in this section was to screen the library, supplied from an independent laboratory, and find the same (or better) clones than those that were selected using phage display. This particular aim was fulfilled with the identification of the best antibody, previously described (HSL 4). As such, the evaluation of the DiCAST was deemed successful by the UK-based company which had undertaken it.

Conclusion and Significance

In this study, three separate bacterial antibody fragment libraries from immune and naïve sources were screened. Furthermore, the libraries were derived from avian (CRP), human (Dab) and sheep (HSL) highlighting the flexibility of the DiCAST platform. In the first study approximately 1.2 million single bacterial cells from a previously characterised avian immune single chain variable fragment library (Leonard *et al*., 2007) were analysed without any enrichment by phage display. Despite only sampling ~3% of the total library diversity, thousands of positive signals were observed (Figure 4.4), of which 288 were automatically selected by image analysis software and recovered for further testing using the prototype cell removal system. Using extremely high precision XY stages to identify microcapillaries containing target cells, 98% of the recovered cells gave a signal of twice the background or higher by direct ELISA. A selection of the recovered clones were sequenced and those found to be unique were tested in a competitive immunoassay to determine their sensitivity to C-reactive protein (Figure 4.7). The best clone (clone A6), selected using DiCAST, had a 55-fold improvement in assay sensitivity when compared to the best clone (clone H2) previously selected by phage display. The ability to screen significantly more cells on a single cell basis increases the probability of recovering a rare clone with unique characteristics that may be lost due to low expression on phage, diversity loss by panning or simply by not screening enough clones post phage selection. Any of these possibilities could account for clone A6 being missed when previously selected by phage display. While with a very large library it might be necessary to enrich the library before screening, DiCAST would reduce the selection steps required and allow a much larger number of clones to be analysed.

To further demonstrate the flexibility and utility of the DiCAST method in selecting antigen-specific mAbs to multiple different target antigens is one experiment, 3 million bacterial clones from an avian immune library generated from chickens immunised with C-reactive protein and myeloperoxidase were analysed. In this assay format, expressed scFv antibody fragments were captured on the anti-HA tag monoclonal antibody coated surface via a fused HA tag which is encoded on the pComb3X phagemid used to clone the variable heavy and light chain genes. A mixture of alternately labelled antigens was then added to the captured scFv surface to identify antibody fragments that bound CRP only, MPO only or cross reacted with both CRP and MPO (Figures 4.8 and 4.9). While these results show the use of two different fluorescent labels, the ability to screen for additional analytes is entirely possible based on alternative wavelengths becoming available in the scanner used and the selection of appropriate differential labels.

In addition to using multiple labels, DiCAST has two analysis surfaces, as demonstrated in Figure 4.10, which can be removed and replaced with additional surfaces allowing the analysis for multiple screening criteria all in one experiment. Ultimately the screening of this library produced antibodies which exhibited enhanced performance over those selected using the gold standard method of phage display. It also demonstrated the ability to identify antibodies specific to very distinct antigens simultaneously.

With a view to increasing productivity in screening campaigns the testing of a naïve commercial library was also undertaken. The ability to use a non-immune library which can be used in all screening campaigns is highly advantageous as it removes the need for immunisations and subsequent library building, for each new target (Siegel, 2002). While screening using DiCAST was determined to be feasible (Figure 4.11 and 4.12), unfortunately the quality of the selected clones was not as expected and meant that a full assessment was not possible.

Finally, in this chapter, an evaluation of the technology was commissioned by a UK-based multinational company. Utilising DiCAST a recombinant scFv fragment library, generated and previously screened in an independent laboratory, was analysed and the resultant outputs sent for blind testing in the original laboratory. Screening was hampered by two key factors, the first being that the library was not amenable to a capture-based screening approach, demonstrated in Figure 4.14, and as such limited the ability to identify and rank binders for retrieval. Alternative antibodies, surfaces and tags could have been evaluated but due to the extremely tight project deadlines imposed by the industry partner, a direct binding format was utilised, albeit not optimal for affinity screening. Due to the low number of positive clones present in each screen, this did not impact greatly on the final outcome because all positive hits were retrieved and ranking was not required. The second issue that arose was the instability of the conjugates supplied. This led to a number of variable results when using the same antibodies on consecutive days and resulted in an incomplete set of data following characterisation.

Overall, approximately 2.5 million individual antibody-secreting cells were investigated for their binding status on a single cell basis. All were assessed for the desirable cross-reactivity profile and a final ten clones were selected as representative clones for external testing. Each of these ten clones, was purified and characterised by ELISA and Biacore in Dublin before shipping to the UK. All work, from receipt of library to final shipment of purified clones, was completed in six weeks. Using the traditional approach, the original lab had undertaken

a six month screening campaign to identify their best binder (HSL 4) which was also identified using DiCAST.

Based on the studies discussed in this chapter, it can be concluded that the DiCAST technology offers a very attractive alternative to the traditional approaches applied to the mining of recombinant libraries, namely phage display. Due to the fact that the cells are spatially addressed in individual microcapillary chambers any bias or competition due to growth and expression levels is eliminated, preserving slow-growing or rare clones. It also offers the ability to multiplex which adds functionality, saves time and cost of reagents and provides the possibility of carrying out several antibody discovery campaigns simultaneously.

Recent investigations into the process of phage display suggest that a reduction in the number of rounds of bio-panning might be advantageous in retaining diversity and allowing identification of clones which would otherwise have been lost (Buckler *et al*., 2008). They suggest that high-throughput automated systems or next generation sequencing be introduced to allow for screening of larger numbers of clones than would typically be analysed.

One particular study, carried out by Hoen *et al*., emphasises the potential impact that the DiCAST technology could have when employed for selection from recombinant libraries. The authors carried out a deep sequencing campaign, which analysed the phage pool after one round of bio-panning on a naïve library (Hoen *et al*., 2012). From their findings, the authors suggest that 10 million sequencing reads would allow full characterisation of the round one output for this particular library. Due to this number being considered too large, they suggest that 1 million sequencing reads should represent the library sufficiently (Hoen *et al*., 2012). By employing DiCAST the reduction in clones analysed to 1 million would not be required. All clones could potentially be analysed, not only in an antigen binding screen but with multiplexing options available. The technology provides a very promising alternative to currently used recombinant library screening approaches, both traditional (e.g. phage display) and more recent (e.g. next generation sequencing).

Chapter 5

Mammalian cell screening using DiCAST

Hypothesis

The screening of mammalian cells in typical antibody discovery approaches (e.g. Hybridoma cells, non-fused B cells or EBV transformed B cells) relies on the employment of cloning by limiting dilution. This involves spatially addressing cells in the wells of microtitre plates and monitoring the supernatant for specific antibody at several time intervals. In order to address the entire population of cells they are often plated with numerous (5-5000) cells per well. This can result in the potential loss of important antibody candidates due to overgrowth by fast-growing, non-specific cells. This chapter investigates the potential of using the newly developed DiCAST technology to screen mammalian cells, without the use of cloning by limiting dilution, for a non-biased, streamlined antibody discovery approach

Rationale

The development of the hybridoma technique by Kohler and Milstein, almost 40 years ago, revolutionised the antibody discovery space and remains today a commonly used method to generate monoclonal antibodies (Kohler & Milstein, 1975). The process which involves the fusion of a cell from an immunised source with a myeloma cell line has many desirable outcomes. These include the relatively simple procedure, the continuous production of the desired antibody and the antibody remaining in its native structure (Gorny, 2012). The method is not, however, without its limitations as the fusion efficiency can be very low (i.e. PEG fusions can produce only one viable hybridoma from 10^5 starting cells (Greenfield, 2014) inferring that valuable B cells producing antibodies of interest could be missed. This inefficiency has led to the emergence of additional techniques such as EBV transformation, electro-fusion and single cell PCR, to overcome the loss associated with the traditional approach (Gorny, 2012).

Regardless of the means in which the cells are treated (i.e. Hybridoma fusion, EBV transformation or native B cell) they are typically screened in the same way, i.e. by cloning by limiting dilution which was discussed in greater detail in Chapter 1. In order to overcome the limitations of traditional techniques a range of single cell analysis technologies have emerged in recent years. These include microengraving of miniature wells (Love *et al*., 2006; Ogunniyi *et al*., 2009; Sendra *et al*., 2013), etched fibres (Biran & Walt, 2002), homogeneous cell spot assays (Jin *et al*., 2009) and microfluidic cell sorting (El Debs *et al*., 2012; Diercks *et al*., 2009; Ryan *et al*., 2011; Singhal *et al*., 2010; Wheeler *et al*., 2003).
DiCAST offers an attractive alternative to the traditional screening approaches typically employed by allowing all cells to be screened, on a single cell basis, in a very short time-scale (i.e. antibody signal can be observed in as little as six hours but assays are typically incubated for up to 3 days to compensate for slow-growing or low expression clones). The

utilisation of the multiplexing capabilities of the DiCAST technology could reduce the overall time to carry out a mammalian antibody screening campaign significantly, as illustrated in Figure 5.1, in addition to potentially finding superior antibody candidates. DiCAST also offers an enhanced option when compared with the other microtools currently under development as the throughput is much greater than many of them, which are typically limited to numbers close to 10^5 single cells. Furthermore, the multiplexing options available are greater when using DiCAST and the retrieval of the target cell does not require the use of a micromanipulator which is often time-consuming and laborious.

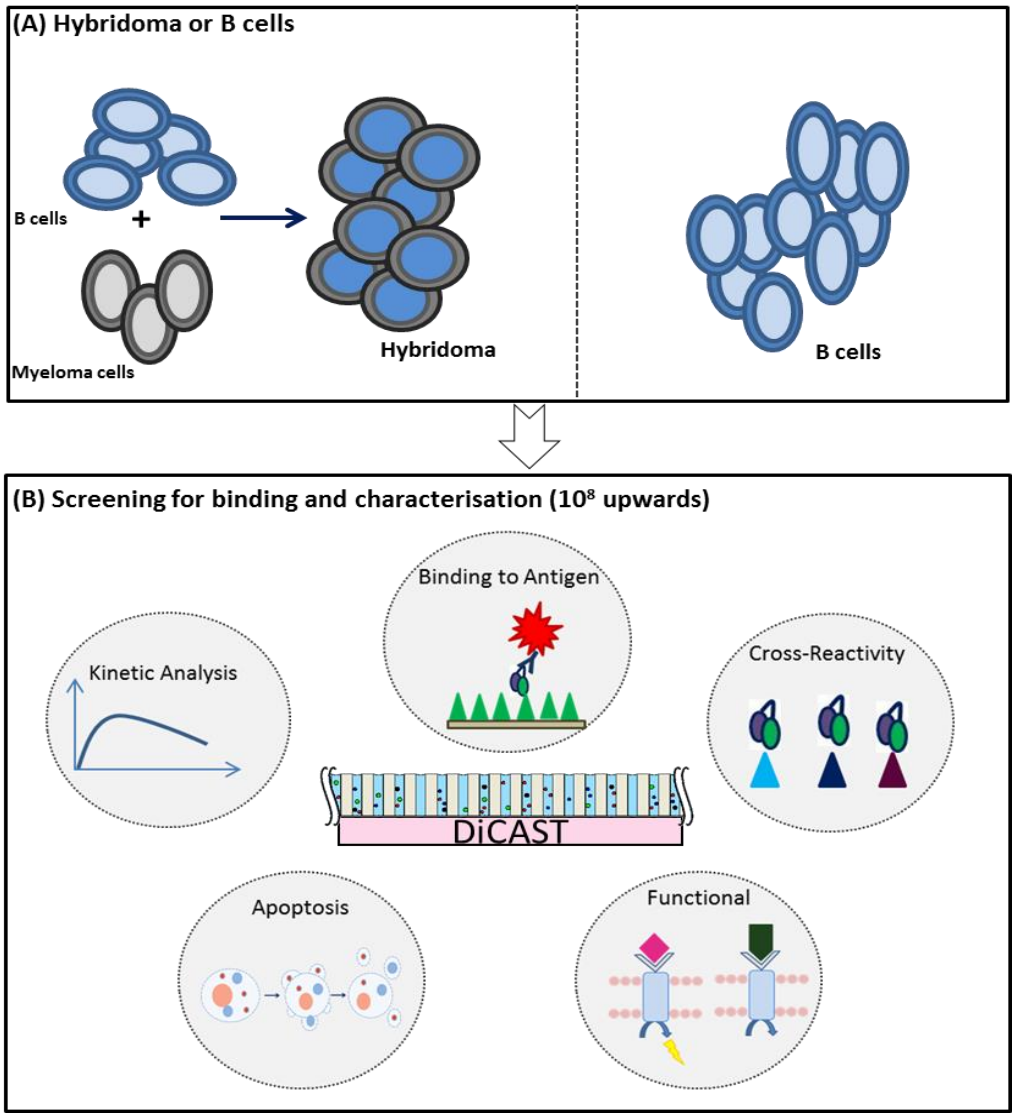

Figure 5.1: Antibody discovery process for mammalian cells using DiCAST. *DiCAST process for both hybridomas and B cells.* ***(A)*** *Cells are fused with a myeloma cell line (hybridoma) or remain unmodified for screening (B cells)* ***(B)*** *The cells are diluted to achieve the equivalent of 1 cell per capillary and analysed using DiCAST. By utilising the multiplexing capabilities of the technology, discussed in Chapter 3, it is possible to carry out multiple, diverse tests on the entire cell population of variants, on a single-cell basis, simultaneously.*

In this chapter the use of DiCAST to interrogate mammalian cells was investigated, as illustrated in Figure 5.2. Initial experiments determined the ability to detect antibody secreted from mammalian cells in the capillaries and their subsequent removal from the array. A means to capture the genetic information from each of the single B cells screened was developed to allow for recombinant cloning of the variable regions from cells showing desired binding or performance characteristics. Following on from that, a number of screening campaigns were carried out on single, non-immortalised B cells. As part of one of these screening campaigns, a single B cell was retrieved and the variable regions amplified, cloned and expressed as a scFv fragment. In addition to carrying out screening and retrieval of the genetic material from single B cells, a proof-of concept experiment for a functional bio-assay was also designed and executed on the array.

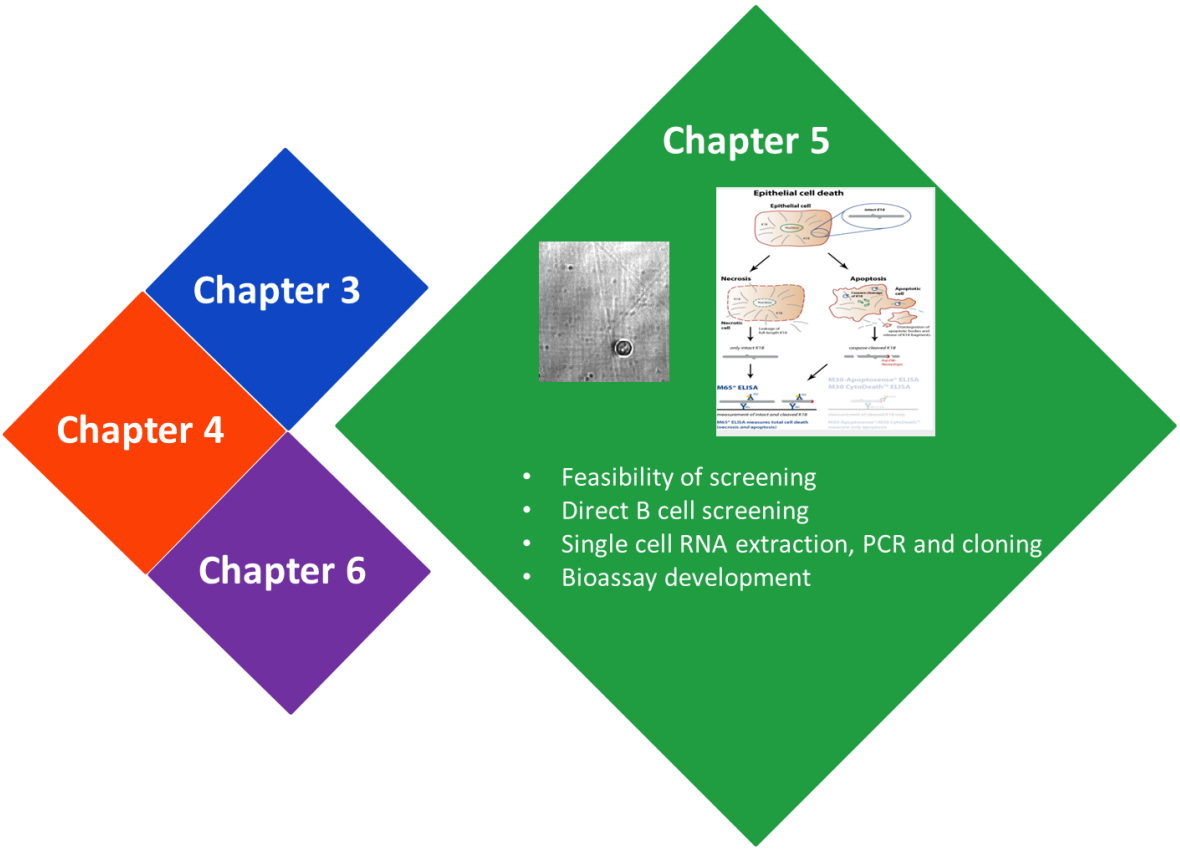

Figure 5.2: Schematic overview of thesis layout. *Chapter 5 is concerned with determining the feasibility of analysing mammalian cells using DiCAST.*

Key Challenge 5.1 Determination of the feasibility of screening mammalian cells using DiCAST.

5.1.1 Experimental Approach

Due to the nature of the microcapillary arrays used in the DiCAST technology, they are not limited to the use of any one antibody-producing technology. In this section the ability to screen and detect antibodies secreted from mammalian cells was investigated. Firstly, the ability to specifically detect the antibody secreted from mammalian cells was confirmed, followed by the identification of an appropriate incubation time for cells of this type. Lastly a test screen was carried out using DiCAST. All initial experiments utilised a multiple myeloma, human IgE (hIgE)-secreting cell line to determine the criteria required to allow mammalian cell screening.

5.1.2 Detection of IgE secreted from multiple myeloma cells which have been incubated on the microcapillary array.

To determine whether mammalian cells can express protein in the capillaries of the array, the supernatant obtained from cells incubated on array was tested, via a sandwich ELISA, for detectable hIgE. The assay was carried out, as described in sections 2.5.1 and 2.5.2. Briefly cells were added to an area microcapillary array (i.e. 100 µL equals approximately 84,000 capillaries), allowed to incubate for 2 days and then contents of the capillaries removed under nitrogen flow and assayed.

From the ELISA result, it could be seen that there was hIgE present in the supernatant of cells removed from the array after 2 days incubation. When compared, as shown in Figure 5.3, to supernatant from cells which were cultured under standard conditions the level of hIgE was low but this could be expected when the difference in culture volumes (i.e. 50 mL tissue culture flask versus 1.2 nL/capillary on array) in question are taken into account.

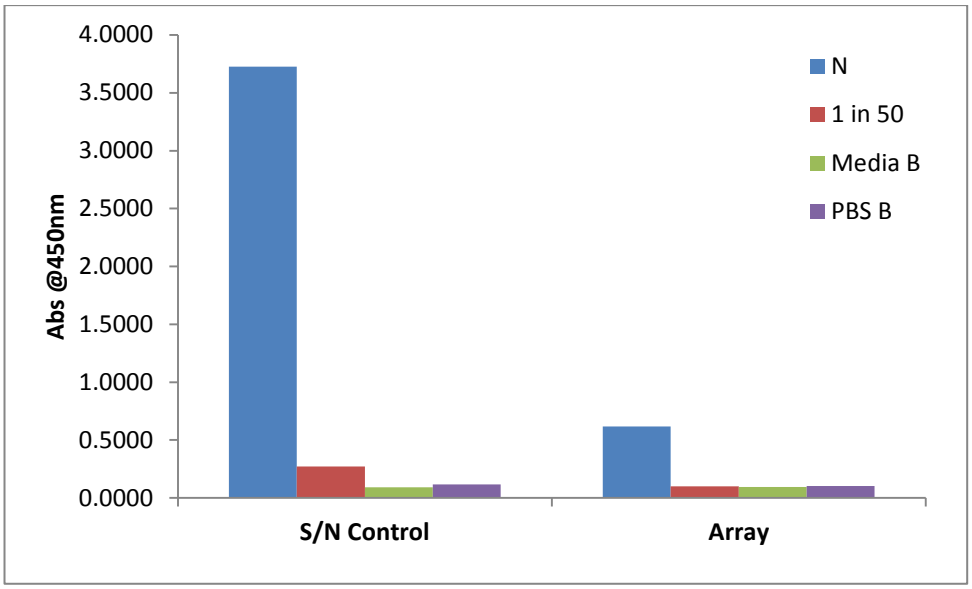

Figure 5.3: Sandwich ELISA analysis detecting levels of hIgE in the supernatant of multiple myeloma cells*. The levels of hIgE present in the supernatant, Neat (N) and 1 in 50 dilution, taken from cells cultured under regular conditions and from cells cultured on array were assayed and compared. A medium blank (Media B) and a PBS blank (PBS B) were also included in the analysis to establish the level of background.*

Based on the analysis of supernatant taken from cells cultured on the array, the cells are capable of living and producing antibody in the capillaries of the array. The level of hIgE detected from the on-array sample is lower when compared to the cells cultured in a 50 mL tissue culture flask. This is most likely due to the significant decrease in medium volume when culturing (i.e. 50 mL culture Vs 100 µL in the array) as well as the loss of a portion of the supernatant on removal from the array under nitrogen.

5.1.3 Establishment of appropriate incubation time for immortalised B-cells (multiple myeloma cell line) to detect secreted antibody.

This experiment was used to identify a suitable incubation period for the cell line to produce detectable levels of hIgE, in a microtitre plate format, before transfer to the DiCAST array system. This was carried out on plate as outlined in section 2.5.3.

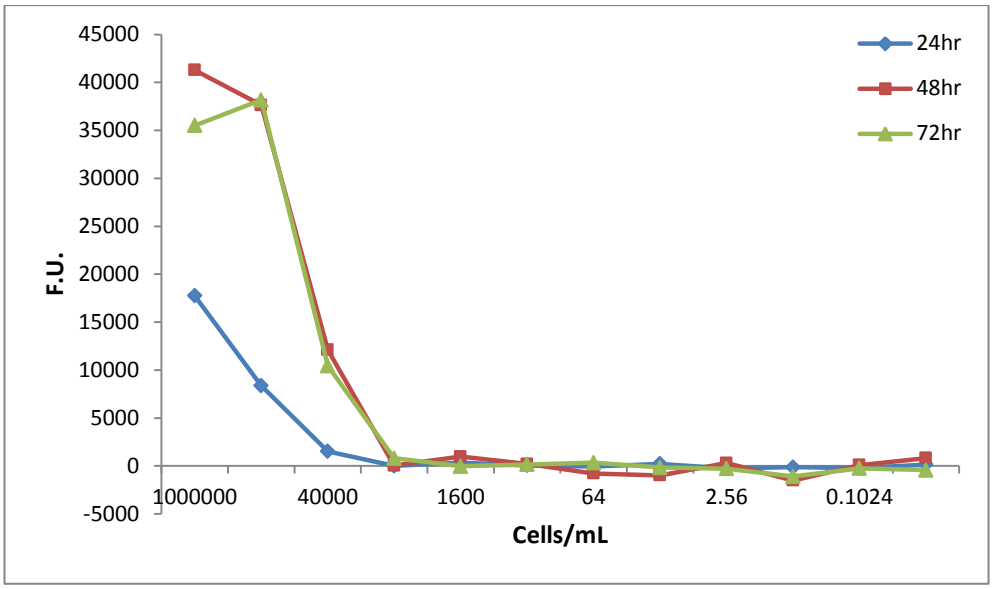

Figure 5.4: Time course using A 96 well plate FLISA for release of hIgE from multiple myeloma cells. *Cells were enumerated and diluted before addition to the plate. The level of hIgE was detected using a sandwich FLISA on consecutive days, for three days.*

The FLISA results displayed in Figure 5.4 show that expression reached a maximum at 48 hours and remained the same at 72 hours. In addition to this finding the lowest cell number from which hIgE could be reliably detected, above background, on standard microtitre plates was approximately 40,000 cells/mL. While this may appear slightly less sensitive than expected, the volume per capillary using DiCAST would be 1.2 nL, meaning that the sensitivity achieved is more than adequate (i.e. 40,000 cells/mL is equivalent to 0.05 cells/1.2nL). As such, using the DiCAST platform to screen cells on a single cell level with the small culture volume of each capillary would allow for saturation of the supernatant, with secreted hIgE quite rapidly. Thus, a single cell assay of the hIgE secreting multiple myeloma cells was attempted and is discussed in section 5.1.4 below.

5.1.4 Single cell screening of multiple myeloma cells in capillaries of the microcapillary array.

In order to assess the feasibility of screening mammalian cells using DiCAST, cells were incubated on array and detected using a sandwich anti-hIgE FLISA on an APTES treated layer of PDMS, in place of a microtitre plate, as described in section 2.5.4.

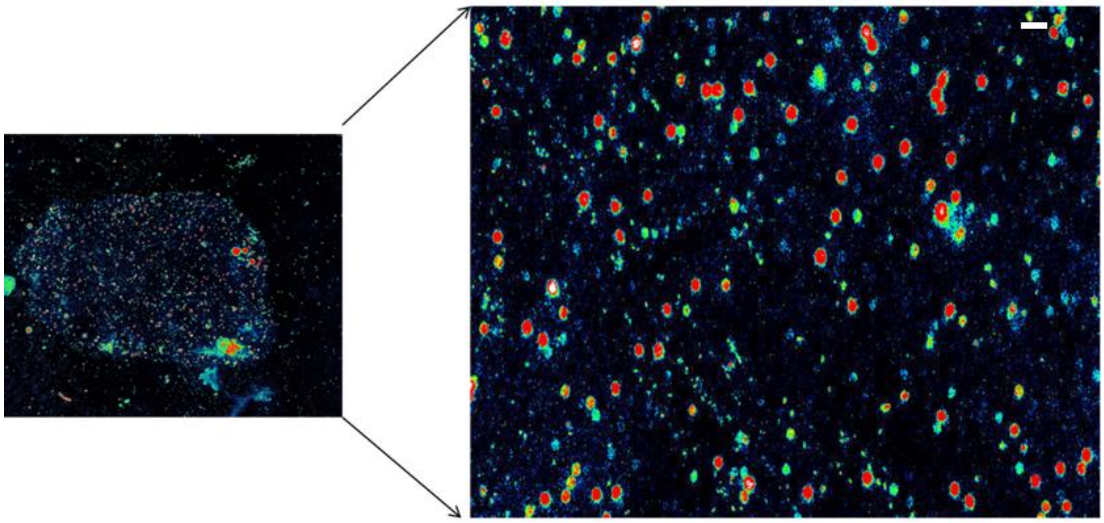

Figure 5.5: Screening of hIgE-secreting U266 cells using DiCAST. *Image showing expression and detection of hIgE secreted from multiple myeloma cells in individual capillaries. Cells (5 x 10^4 cells/mL) were added to a 40 µm capillary diameter array, cultured for 4 days in CO_2independent medium and expressed antibody analysed by sandwich FLISA. The bright (red) dots represent the antibody patterned PDMS from single capillaries that contained cells expressing antibody. Scale bar is 100 µm.*

At a dilution of 5 x 10^4 cells/mL, which would equate to a cell present in every twenty capillaries, a pattern of individual capillaries (40 µm) could be observed, as shown in Figure 5.5. While some background is obtained from the APTES-modified PDMS, probably due to the extended incubation period in the humidified CO_2 incubator, the capillaries which contain hIgE-secreting cells can be easily distinguished. This demonstrates the ability of DiCAST to screen and detect mammalian cells which secrete a protein of interest.

5.1.5 Summary for key challenge 5.1

The purpose of this section was to determine if mammalian cells could produce detectable levels of secreted protein in the capillaries of the microcapillary array. While it was proven possible to detect secreted protein from an immortalised multiple myeloma cell line, each new mammalian cell to be screened should be similarly assessed to ensure correct incubation durations are employed to allow for reliable detection. In addition, investigations into a supplemented medium (i.e. addition of cytokines, feeder cells etc.) could allow for enhanced survival and antibody production by the single cells in the capillaries.

Key Challenge 5.2 Establishment of a method for retrieval of mammalian cells from the capillaries of the array.

5.2.1 Experimental approach

Similar to the investigations carried out in Chapter 3, it is important to have the ability to retrieve the antibody-secreting cell of interest from the capillary in which it is housed subsequent to its identification. In bacterial experiments recovery of approximately 10 % of the capillary contents was achieved upon removal with the DiCAST prototype. Mammalian cells are larger, more fragile and do not replicate as quickly as bacterial cells and, as such, their successful retrieval from the array is complex.

In this section, ways in which to best capture the contents of a desired capillary are investigated. In the first instance cells were removed from the array crudely under nitrogen flow and then retrieved from the capillaries using the DiCAST prototype. Finally, a means to capture the genetic information from cells once retrieved was devised and tested using the multiple myeloma cell line.

5.2.2 Removal of intact cells from the microcapillary array.

Due to the larger size of mammalian cells the ability to successfully pass them through the 40µm capillaries of an array was tested, as described in section 2.5.5. A dilution (7.2×10^4 cells/mL) was added to the array and then removed using high pressure nitrogen gas. On enumeration of the cells subsequent to passage through the capillaries of the array very little loss in cell number was observed, with a cell number of 6.8×10^4 cells/mL obtained upon collection of the cells. On visualisation the cells were present in high numbers and appeared healthy, as shown in Figure 5.6. The average size of the newly passaged cells, as determined by Millipore Sceptor, was between 12 to 18 µm in diameter.

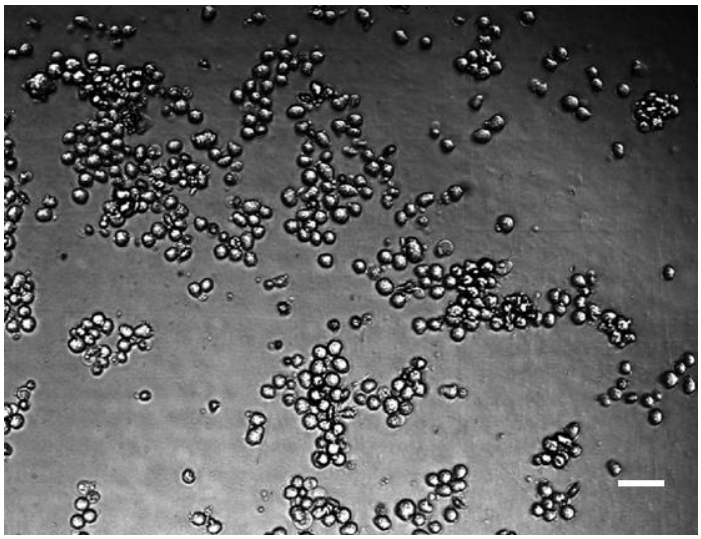

***Figure 5.6**: **Cells retrieved from the microcapillary array using nitrogen flow.** A cell dilution of 7.2 x 10^4 cells/mL was added to the array and cells, from several capillaries, were collected in the well of a 384-well microtitre plate. The image was captured using a light microscope at 20X magnification. Scale bar is 50 µm.*

Based on the success of this experiment, the prototype device was then used to carry out a retrieval of the cells loaded on the array, as detailed in section 2.5.6. On observation of the 384-well plates, no cells were observed in target wells. The system parameters were then tested and improved using colorimetric tests (TMB and HRP) so that optimum settings were used for live cells retrievals. It was found that an increase of medium (50 µL increased to 85 µL) in the 384 well plate allowed for a more accurate retrieval process using the colorimetric tests. The retrieval of mammalian cells was then repeated, as previously described.

Subsequent to retrieval, using the optimised conditions, cells were observed in some of the target wells, as shown in Figure 5.7. Cells were not observed in all target wells and viability after retrieval was poor.

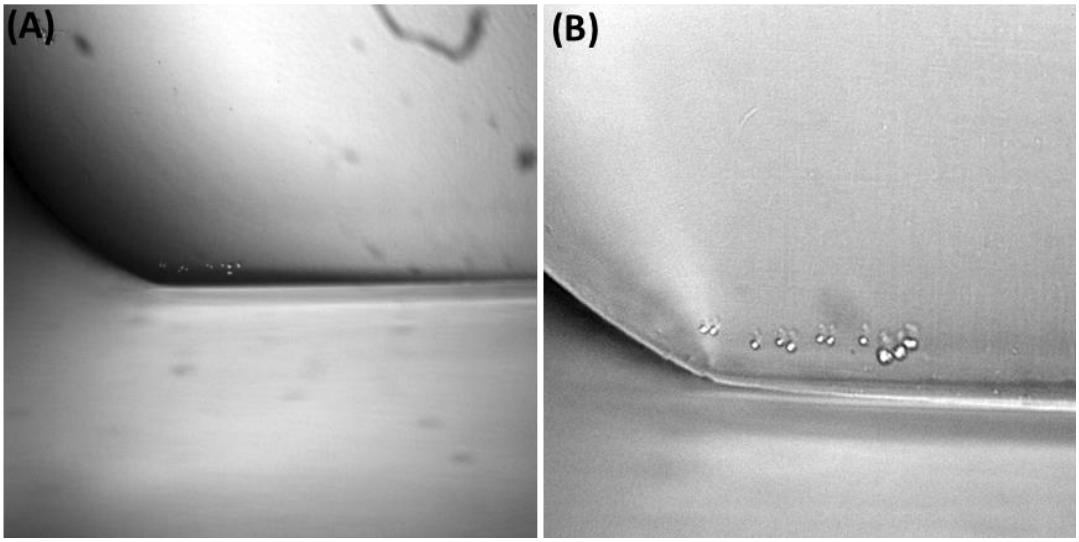

Figure 5.7: Visualisation of multiple myeloma cells retrieved into 384 well plates using the DiCAST prototype. Intact cells can be seen at **(A)** 10X magnification and **(B)** 20X magnification.

While it is possible to retrieve intact mammalian cells using the prototype, there are several issues with the current set-up. Firstly, there is not a high level of accuracy, with cells seen in only 2 out of 40 target wells. This issue will be exacerbated when working at a single cell per capillary level. Secondly, the cells do not remain viable in the plates overnight. Options available to overcome this include (i) use of an enriched medium and/or companion cells to aid proliferation of the captured cells or, alternatively, (ii) the amplification of antibody genes from the target cell, allowing antibodies to be produced recombinantly.

5.2.3 Amplification of genetic material from cells

The success of the DiCAST process relies heavily on the successful removal of the cell of interest from the array. While it has proven possible to remove intact cells from the array, successful culturing of the cells post-retrieval may prove difficult. This concern is based predominantly on the fact that mammalian cells are rarely cultured at single cell level, due to their poor performance in the absence of other cells. Because of this an alternative method, which involves the amplification of antibody genes from the cell of interest, has been investigated as detailed in sections 2.5.11, 2.5.12 and 2.2.7. On observation of the gel a specific band could be seen at the correct size (350-400 bp), for dilutions ranging from 10^7 to 10^1 cells/mL, as shown in Figure 5.8.

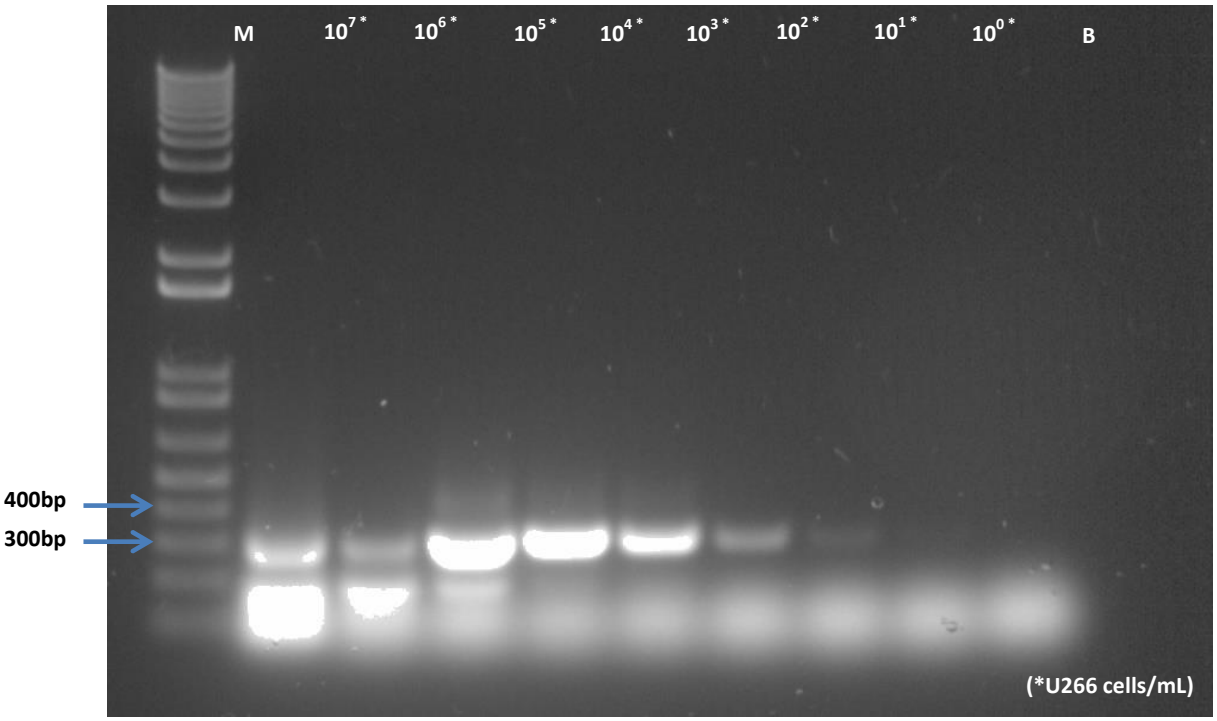

Figure 5.8: Analysis of PCR products, for the C_H1 domain of anti-hIgE antibody, obtained from cDNA generated from multiple myeloma cells. *Cell dilutions were analysed and the lowest cell dilution where detection is achievable is 10 cells/mL. Control reactions to ensure the product was not obtained from genomic DNA (PCR on the cell preparations, without the cDNA synthesis reaction) were carried out and did not contain a specific band. DNA Ladder is 1kb Plus Ladder from Life Diagnostics, CA, USA.*

Based on this result it can be concluded that it is possible to amplify antibody fragments, directly from cell preparations, with a low cell number (~10 cells/mL) as template. With such a sensitive detection rate it means that, theoretically, at a single cell per capillary level (1 cell per 1.2 nL) a PCR product can be obtained. This will mean that if cells cannot be retrieved and cultured directly that retrieval of the genetic information from a specific antibody of interest is a viable alternative.

5.2.4 Summary for key challenge 5.2

In this portion of the chapter the ability to remove the cell of interest from the capillaries of the array was determined. Unfortunately the process is highly inefficient and will need additional optimisation of the prototype to allow screens on mammalian cells carried out on DiCAST to be fully worthwhile. As the retrieval of intact, viable cells was not very efficient and an alternative approach was also investigated in this section. The ability to produce the antibody of interest by recombinant means was tested using dilutions of control cells. Based on this proof-of-concept data, it was thought feasible to amplify the genetic material from single cells removed from the array. A significant amount of development would be needed to produce a fully working system compatible with mammalian cells. However with a view

to confirming the utility of DiCAST for mammalian cell screening the ability to carry out direct screening of B cells, circumventing the need for the fusion process, was attempted and is discussed in Section 5.3.

Key challenge 5.3 Native B cell screening, from non-immunised and immunised sources, using DiCAST.

5.3.1 Experimental approach

The ability to directly screen the B cell repertoire is of great interest in the development of human therapeutics, as the naturally produced/occurring antibody is thought to be more potent and, additionally, if produced from human B cells, can potentially have reduced adverse effects.

In this section the ability to screen native B cells without any prior manipulation or immortalisation strategy, using DiCAST, will be investigated. In the first proof-of-concept experiment, B cells from the spleen of a non-immunised mouse were analysed for IgG secretion. Following that, a screen was carried out on the B cells of a mouse immunised with three different proteins in a multiplex format which allowed for responses for all three proteins to be analysed simultaneously.

5.3.2 Screening of Naïve B cells from a non-immunised mouse.

The initial investigation into screening B cells using DiCAST was carried out, using splenic B cells from a non-immunised mouse, to determine if individual B cells could secrete a detectable amount of antibody in the capillaries of the array. An assay for the detection of mouse IgG was carried out on the microcapillary array, as described in section 2.5.7. A specific response, to mouse IgG, can be observed in all dilutions. At higher concentrations of cells, all capillaries appear positive (Figure 5.9A).

At a lower dilution (Figure 5.9B) where, theoretically, the density is less than 1 cell per capillary, single capillaries which contain B-cells expressing an antibody of interest (in this case, mouse IgG) can be differentiated.

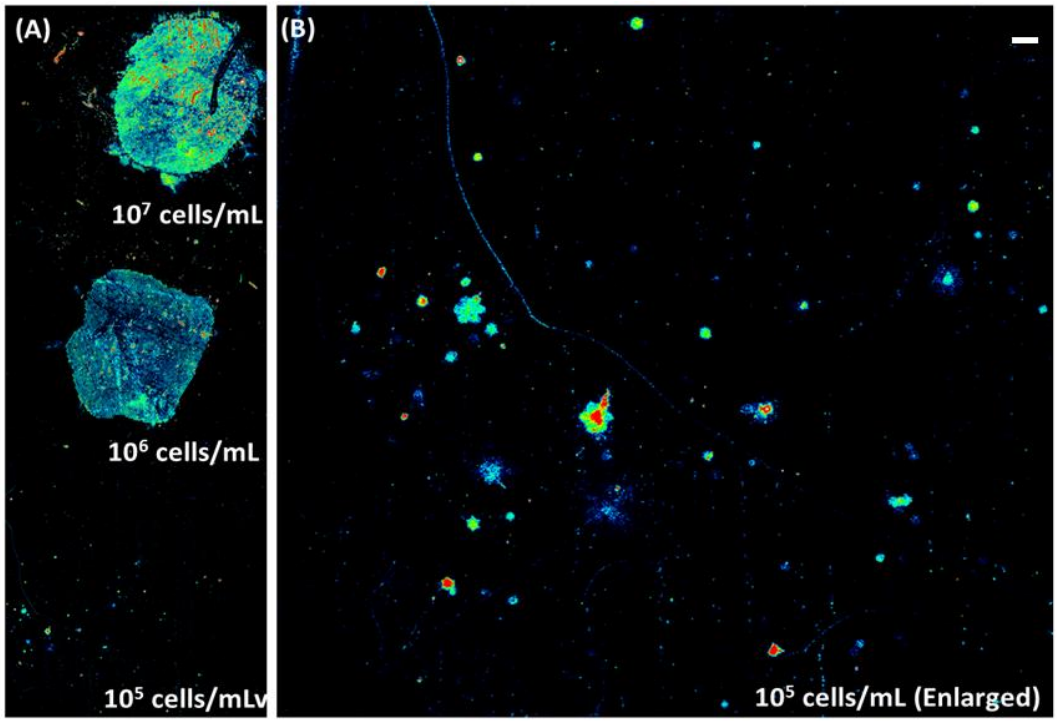

Figure 5.9: Screening of murine splenic cells for mouse IgG. (A) *Reponses obtained from serial dilutions of cells. When cells are loaded at too high a density no single capillaries can be differentiated. At 10^5 cells/mL single capillaries containing specific cells can be identified.* ***(B)*** *Enhanced image of 10^5 cells/mL dilution, showing single capillaries of 40µm diameter. Scale bar is 100 µm.*

This initial experiment served to prove that it was possible to detect antibody (IgG) secreted from individual B cells extracted directly from a non-immunised mouse spleen, without any prior manipulation.

5.3.3 Screening of B cells from a mouse immunised with three different antigens.

Following on from the successful analysis of naïve B cells in section 5.3.2, murine splenic B cells from a mouse immunised with 3 different antigens were analysed directly with DiCAST. Individual B cell responses to all 3 antigens were observed in one single experiment by multiplexing through dual surface and fluorescent label analysis.

The assay format, as shown in Figure 5.10, involved a double sided assay which also incorporated a multiplex assay on one side. The analysis incorporated a direct antigen capture on the first surface for identification of the myoglobin-specific antibodies and an anti-mouse capture approach to allow multi-analyte (Transferrin and C-reactive protein-specific antibodies) detection on the second surface of the assay.

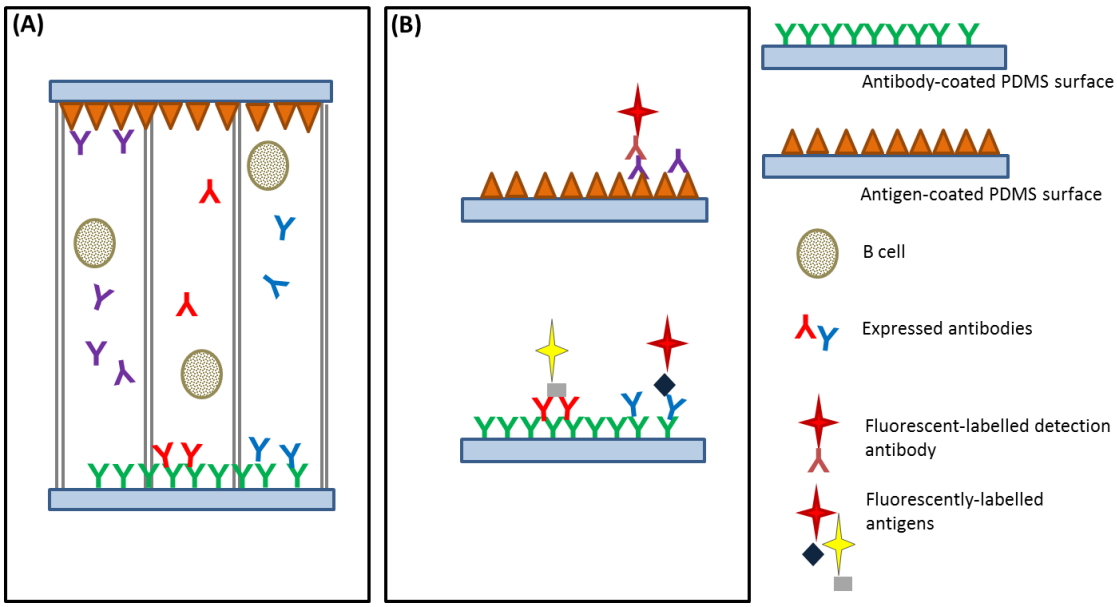

Figure 5.10: Schematic of multiplex assay approach used for B cell screening. *The assay performed involved a double-sided assay, in which one of the sides was used for multi-analyte detection.* ***(A)*** *B cells isolated from the spleen of an immunised mouse were allowed to incubate and secrete IgG which were captured on each of the prepared PDMS surface.* ***(B)*** *Following incubation the array is removed and stored and secondary detection is carried out using fluorescently labelled detection reagents. Myoglobin specific antibodies were detected on Side 1, using an antigen coated surface. Transferrin and C-reactive protein antibodies were selected from Side 2, which employed an anti-mouse IgG capture approach and was probed with Cy5-labelled Transferrin and Cy3-labelled CRP.*

Capillaries which contained antigen-specific B cells could readily be identified on observation of the scan, shown in Figure 5.11A. When analysing the multi-analyte detection surface, separate images were obtained for each of the fluorophores and these were then overlaid to obtain a composite image, using the DiCAST software. A standard output from a multiplex format can be seen in Figure 5.11B, where those spots appearing green in the composite image represent a CRP-specific antibody and those appearing red are transferrin-specific.

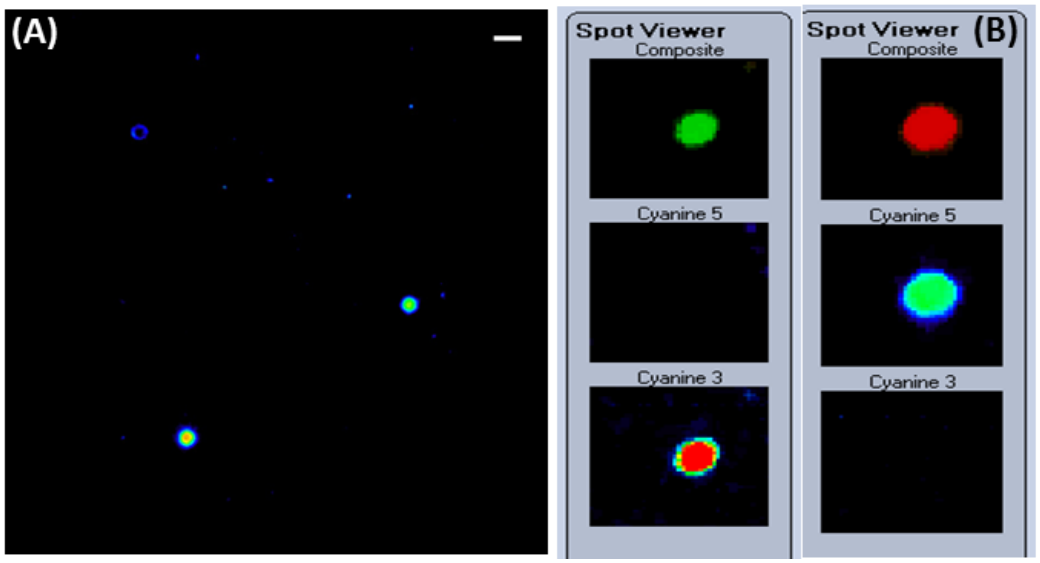

Figure 5.11: Output from multiplex B cell screening assay. (A) *An example of a scan for one of three antigens targeted in the screening process. The specific spots (40 µm diameter) correspond to a capillary which contains a cell secreting an antigen-specific antibody in the physical array. Scale bar is 100 µm.* ***(B)*** *Multiplex surface 2 of the double-sided assay allows identification of capillaries (40 µm) which contain a cell secreting an antigen-specific antibody. In "Spot-viewer" mode, those spots which appear green (Cy3) in the composite image represent a CRP-specific antibody and those which appear red (Cy5) are specific to Transferrin.*

The contents of selected capillaries were then retrieved to allow for further analysis of the cells of interest. Cells were captured in a 384 well plate and incubated for 5 days at 37°C and 5% CO_2 before an ELISA analysis (Figure 5.12) of the supernatant was carried out.

The 1.2 nL capillary volume containing the single murine B cell producing antibody to the target of interest was recovered into 85 µL of medium in a 384 well plate and, despite the large dilution factor, indicative binding signals could be observed 5 days later by ELISA (Figure 5.12).

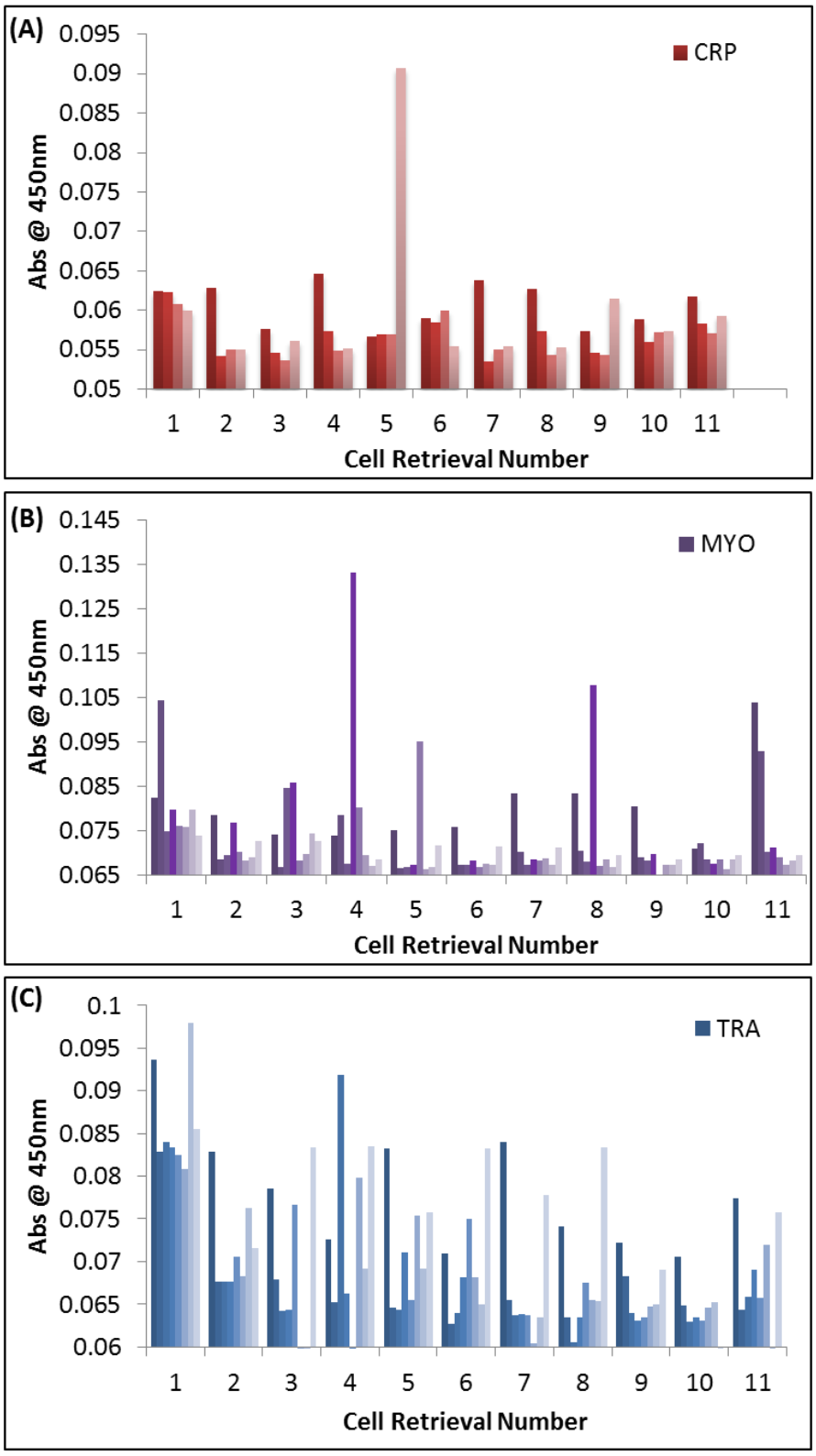

Figure 5.12: ELISA results confirming antigen specificity of antibodies secreted from the retrieved cells. *Supernatant from retrieved cells was assayed subsequent to 5 days post-retrieval incubation at 37°C/5% CO_2. For each of the three antigens screened* ***(A)*** *C-Reactive protein* ***(B)*** *Myoglobin and* ***(C)*** *Transferrin there are a selection of cells, secreting antibody of interest, available for further characterisation.*

5.3.4 Summary for key challenge 5.3

The possibility of detecting target specific antibody producing cells against the three protein targets in a single iteration, using DiCAST has been demonstrated. While not carried out in this screen the recovered cells could have been immortalised by a variety of means such as PCR cloning (Yoshioka *et al*., 2012)(Yoshioka *et al*., 2011)(X. Jiang *et al*., 2006), EBV immortalisation (if human cells) (Steinitz *et al*., 1977) or by more recent improved methods (Traggiai *et al*., 2004). Additionally while this example demonstrates determination of binding for three antigens, the flexible nature of the technology can allow for a wide-range of assays to be carried out in parallel and thus determine a large amount of desired and relevant information for each of the cells analysed.

Key Challenge 5.4 Determination of feasibility of whole cell screening (i.e. using whole cells as antigen).

5.4.1 Experimental approach

The ability to use cells as an antigen would allow for antibodies which bind an intact cell to be detected. This is an attractive approach in the development of monoclonal antibodies, for both therapeutic and diagnostic applications, where antibodies can be screened for binding to a particular cell based antigen in a relatively native state as opposed to a purified or recombinant version.

In order to determine whether this approach was feasible for use with DiCAST, a number of experiments were designed and carried out using whole cells as antigens. Initially the ability of cells to bind specifically to the PDMS surface was ascertained, followed by DiCAST screening of B cells of a chicken which had been immunised with Jurkat cells.

5.4.2 Confirmation of specific binding of cells to the PDMS surface.

Before carrying out a screening campaign, the feasibility of using cells as an antigen in the DiCAST process was assessed. In order to determine whether cells could bind specifically to the PDMS surface, cells were captured on an anti-EpCAM-coated PDMS. Cells were added to areas of PDMS which had either been coated and blocked or blocked only, and following incubation and washing, the areas were imaged using a light microscope.

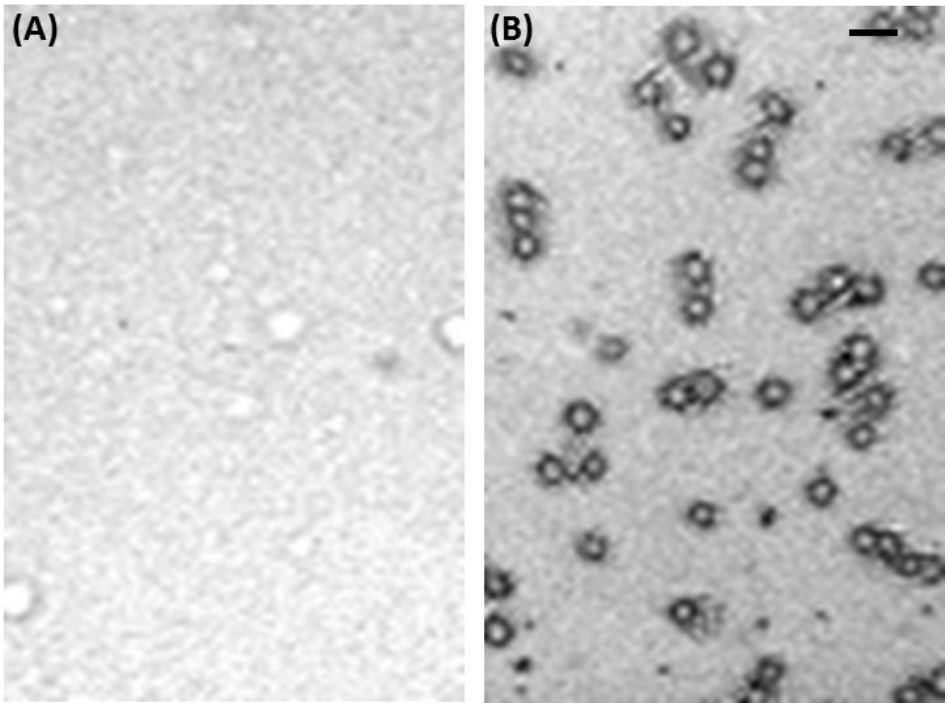

Figure 5.13: Capture of mammalian cells on an anti-EpCAM-coated PDMS surface. *The PDMS surface was observed under a light microscope (10X magnification) to ascertain whether cells had bound to* ***(A)*** *An unmodified (blocked only) PDMS APTES surface and* ***(B)*** *A PDMS APTES surface coated with anti-EpCAM monoclonal antibody. Scale bar is 25 µm.*

Upon visualisation of the different areas it was evident that cells, as seen in Figure 5.13, were captured on the region where the anti-EpCAM antibody was coated and not in the area where only blocking solution had been applied. This indicated that it was possible to specifically capture cells on the surface of the PDMS.

5.4.3 Screening campaign using whole Jurkat cells as the antigen

Based on the results obtained in section 5.4.2, a screening campaign was undertaken which involved the screening of B cells taken from the spleen of a chicken immunised with intact Jurkat cells. The screen was carried out as illustrated in Figure 5.14 and involved the capture of antibodies secreted by the B cells on the PDMS surface followed by probing with fluorescently labelled Jurkat cells, as described in 2.5.10.

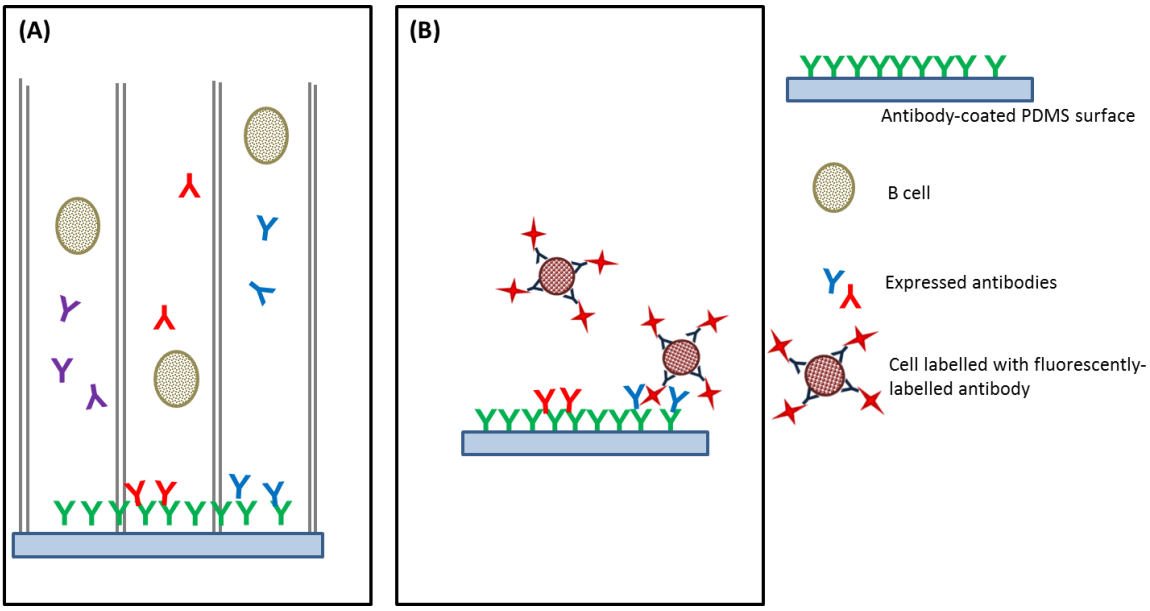

Figure 5.14: Schematic of the format used for the identification of cell-specific antibodies. ***(A)*** *B cells isolated from the spleen of an immunised chicken were allowed to incubate and secrete IgG which were captured on the prepared PDMS surface.* ***(B)*** *Following incubation the array is removed and stored and secondary detection is carried out using intact Jurkat cells which had been fluorescently labelled with an Cy5-labelled anti-EpCAM antibody. Following this the surfaces were washed and scanned and detailed image analysis carried out to identify wells which contained a B cell which secreted Jurkat cell-specific antibodies.*

The purpose of this experiment was to determine if anti-cell antibodies could be screened on the DiCAST system. By analysing the images obtained, shown in Figure 5.15, the possibility to identify antibodies which were specific to the cells with which the animal had been immunised was determined.

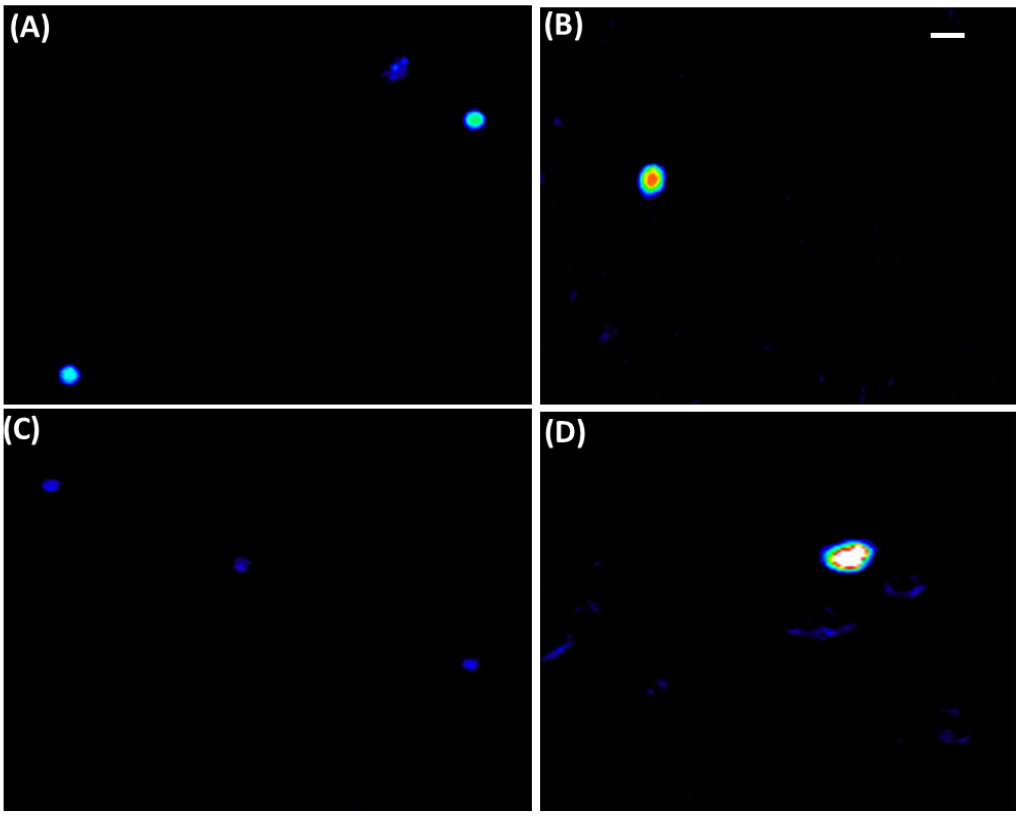

Figure 5.15: Detection of Jurkat cell-specific antibodies from B cells of an immunised chicken using the DiCAST process. *Capillaries in the microcapillary array which contain a cell secreting an antibody of interest are indicated by a spot of fluorescence. Fluorescent signal should correspond to a specific antibody but a range of responses were observed from typical output* ***(A)*** *to those which appeared larger than the capillary diameter* ***(B)*** *and* ***(D)*** *and those which appeared smaller than the capillary diameter* ***(C)****. Scale bar is 100 µm.*

Due to the complex nature of the antigen (i.e. intact cells) it was difficult to confidently identify positive hits, as the antigen solution was not a homogenous protein (as had been used in all other DiCAST screens). As a result, a range of signals were observed (Figure 5.15) that differed in size and shape. This could be attributed to multiple cells binding to the same area and causing a larger, irregular shaped signal to be observed. In order for screening of intact cells to be reliable, the use of a scanner with the limited resolution demonstrated here would not be advised. A system that used a microscope set-up would greatly enhance the ability to select positive antibodies by allowing visualisation of the fluorescent cell at a much higher resolution.

Despite the limitations of the scanner used a selection of potential positives were retrieved (250) using the DiCAST prototype. Plates were incubated overnight and single cell PCR was carried out on several of the wells, as described in section 5.4.4 below, on the following day.

5.4.4 Cloning and expression from a single B cell.

From the 250 potential positives, mentioned in section 5.4.3, only 27 wells contained positives on the following day. While retrieval rates are typically poor with mammalian screening the low retrieval rate could also have been due to a number of factors, namely the uncertainty involved in identifying positives in the initial screen and the delay in processing.

The positive wells were subjected to RNA extraction and cDNA synthesis, as detailed in section 2.5.11 and 2.5.12, respectively. The variable regions of the antibody were then amplified from the cDNA, purified and then combined by splicing overlap extension as described in section 2.5.13. An example amplification output is shown in Figure 5.16.

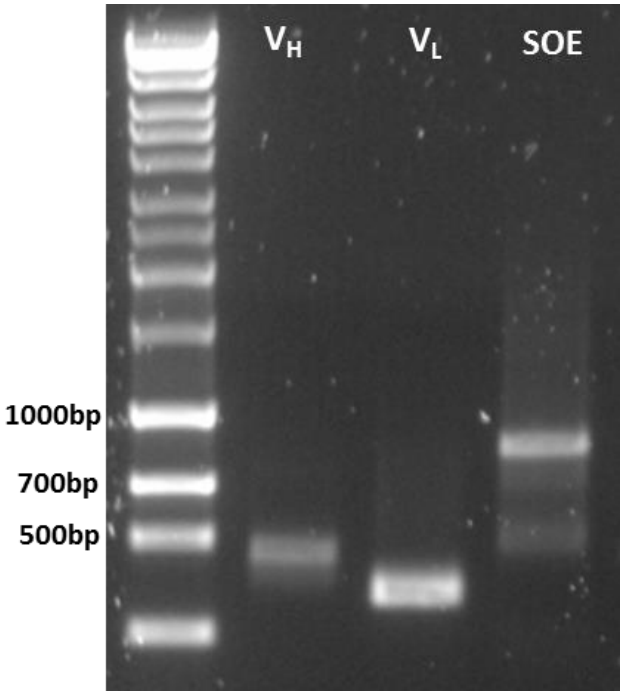

Figure 5.16: Amplification of variable regions from single B cells. *RNA extraction was carried out subsequent to retrieval. cDNA synthesis was carried out and variable regions (V_H and V_L) amplified by PCR. A splicing overlap extension (SOE) was then carried out for cloning. DNA ladder is HyperLadder 1kb Plus from Bioline.*

Of the 27 single cell RNA extractions carried out, first round PCR products were obtained from only 6. This could be attributed to low quality of the starting RNA. The samples which gave the greatest yields of product in first and second round PCRs were taken forward and the scFv fragments cloned into a pCOMB 3XSS vector, typically used for phage display, as detailed in section 2.5.14 and 2.5.15.

Following cloning, single colonies for each were expressed in small-scale culture to identify if the cloned scFv was present. The lysates were visualised by SDS-PAGE as described in section 2.2.1 and shown in Figure 5.17. Each of the lysates had a representative band, of the expected size (i.e. 27 kDa), present on observation of the gel.

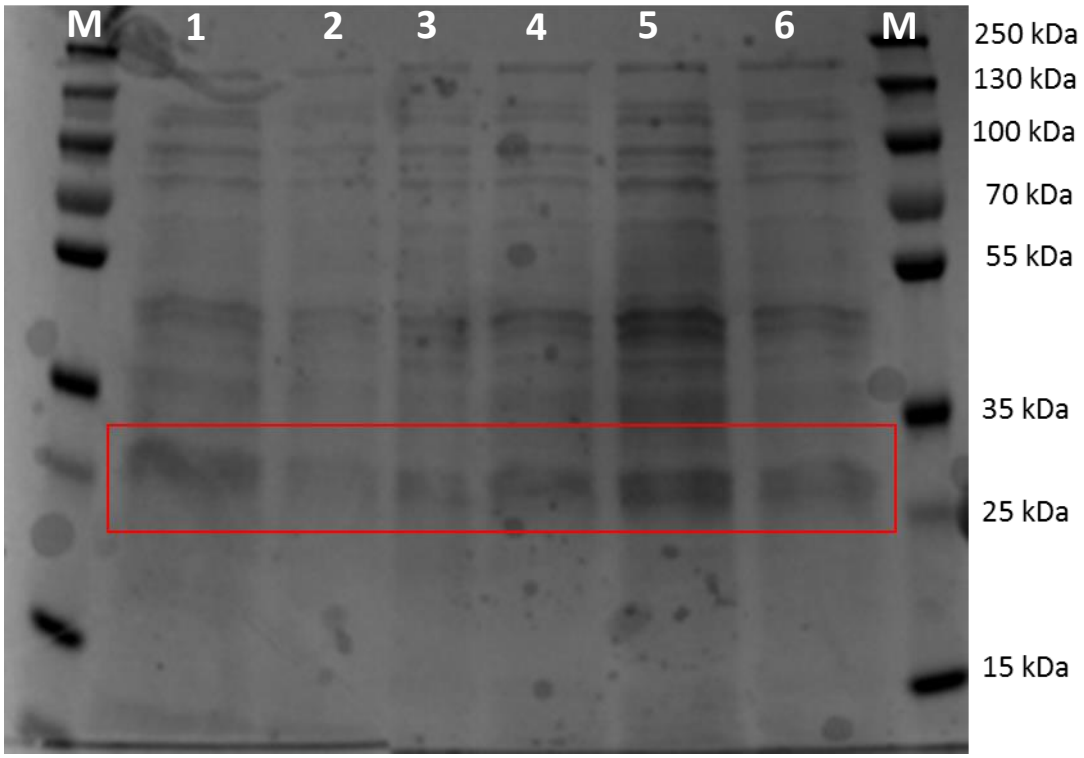

Figure 5.17: SDS-PAGE analysis of the expressed scFv fragments. *Single colonies obtained from transformation plates were expressed in small-scale culture and the lysate fraction analysed by SDS-PAGE. Lanes 1-6 contain lysates expressed from six different single colonies, each representing a different B cell extraction, from the cloning transformation plates. The lanes designated 'M' contain the protein markers (PageRuler plus pre-stained protein ladder; sizes for each of the bands are indicated to the right of the gel picture). The expected size is approximately 27 kDa for each scFv.*

Following confirmation of expression of the scFv fragments, they were tested for maintained binding against the original target antigen (i.e. Intact Jurkat cells). This was carried out as described in section 2.5.16 and the resultant images are shown in Figure 5.18.

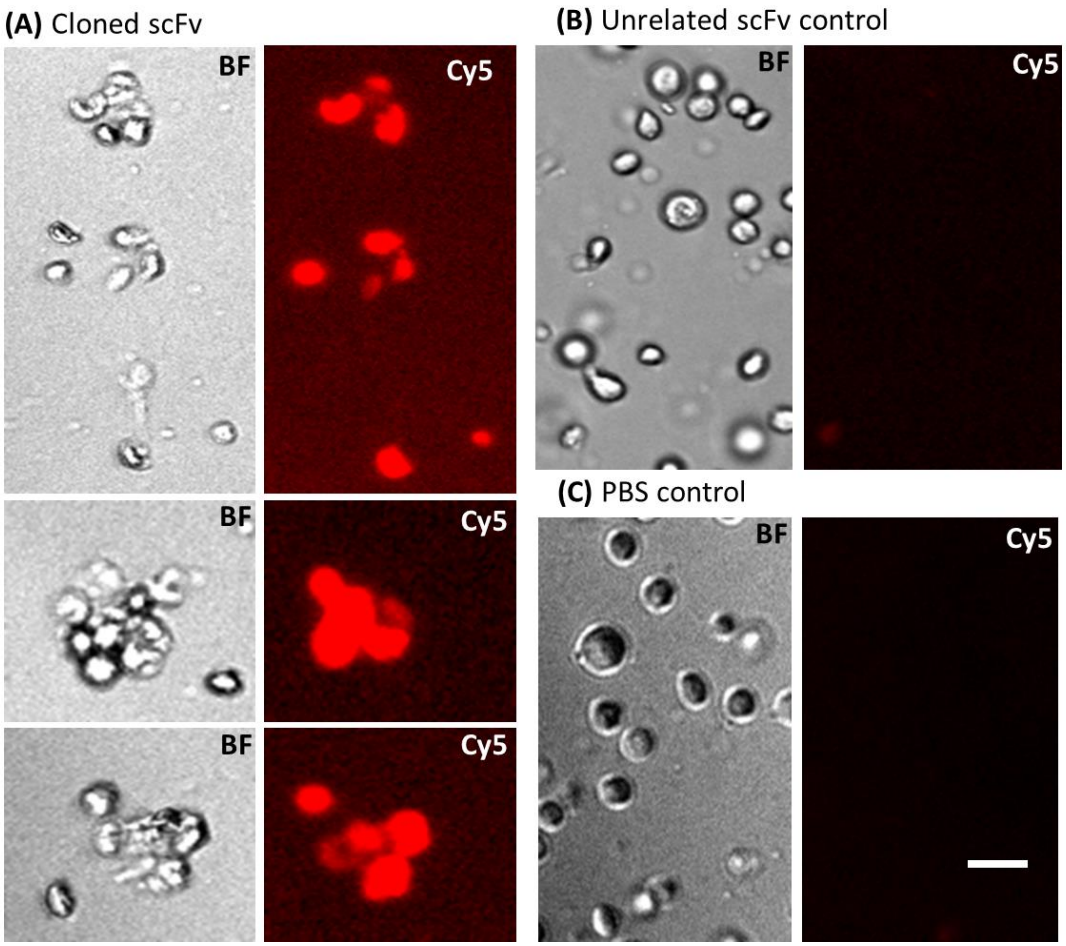

Figure 5.18: Binding of the anti-Jurkat scFv to Jurkat cells. *The variable regions were amplified from a single B cell and the scFv cloned and expressed for testing of maintained binding.* ***(A)*** *Assay performed with the cloned anti-Jurkat scFv* ***(B)*** *Assay performed with an unrelated scFv in place of the specific scFv* ***(C)*** *Assay performed with PBS in place of the specific scFv. Magnification is 10X. BF represents the brightfield view and Cy5 the fluorescent view. Scale bar is 25 µm.*

Based on the images obtained in Figure 5.18, it could be seen that one of the cloned scFvs did maintain binding to the intact Jurkat cells. It was compared with several controls (i.e. PBS in place of cloned scFv and an un-related scFv (anti-CRP) in place of the cloned scFv) to ensure binding was genuine and specific. The remaining five clones did not exhibit any fluorescence upon visualisation. It was also noted that if clumps of cells were present in the control samples, they exhibited a low level of non-specific fluorescence. This should be considered in future screens and could possibly be eliminated by additional washing steps and reduction of clumped cells in the sample prior to visualisation.

5.4.5 Summary for key challenge 5.4

The aim of this section was to determine if DiCAST was compatible with the use of whole cells as an antigen. The feasibility experiments showed that it was possible to bind cells specifically to the assay surface. A screening campaign was then carried out and while recovery rate was low, a specific antibody from a single B cell retrieved was successfully cloned, expressed and shown to exhibit maintained binding, as a scFv fragment, to its original whole cell antigen.

Key Challenge 5.5 Functional Bioassay proof-of-concept

5.5.1 Experimental approach

The ability to detect functional effect in place of or alongside detection of specific binding to a target of interest is highly desirable in screening campaigns. Due to the complexity of functional assays, they are often incorporated late in the process and used only on final drug candidates, not the entire library/cell population. By including functional screening (e.g. induction of apoptosis) for the entire population, in the initial screen on DiCAST, candidates which may otherwise be discarded in earlier screening steps are also captured. This opens up the possibility for identifying unique, and most importantly therapeutically effective candidates, which may have been overlooked in standard screens based on other criteria.

This section is concerned with the establishment and development of a functional assay (detection of apoptosis) and its execution using the microcapillary arrays. The majority of apoptosis assays rely on the detection of cell surface markers (e.g. phosphatidylserine and Annexin V). While it is possible to execute this using the DiCAST system (with modifications to the standard methodology) it is more ideally suited to methods such as fluorescence activated cell sorting (FACS) or fluorescence microscopy. The method employed in this section describes the use of an assay system which allows for the detection of cell death (i.e. necrosis vs apoptosis) from the supernatant of target cells.

5.5.2 Monitoring cell death in the microcapillary array.

During apoptosis, vital intracellular proteins are cleaved by cysteinyl-aspartic acid proteases (caspases) that are expressed as zymogens and are activated by different apoptosis inducers. Once activated, a single caspase activates a cascade of caspases. Circulating full-length and caspase-cleaved cytokeratin 18 (CK18) are considered biomarkers of chemotherapy-induced cell death measured using a combination of the M30 and M65 ELISAs provided by Peviva, shown in Figure 5.19. The M30 ELISA measures caspase-cleaved CK18 produced during apoptosis and the M65 ELISA measures the levels of both caspase-cleaved and intact CK18,

the latter of which is released from cells undergoing necrosis. Previous studies have highlighted their potential as prognostic, predictive, and pharmacological tools in the treatment of cancer (Caulín *et al*., 1997; Hägg *et al*., 2002; Leers *et al*., 1999; Linder, 2011; Schutte *et al*., 2004).

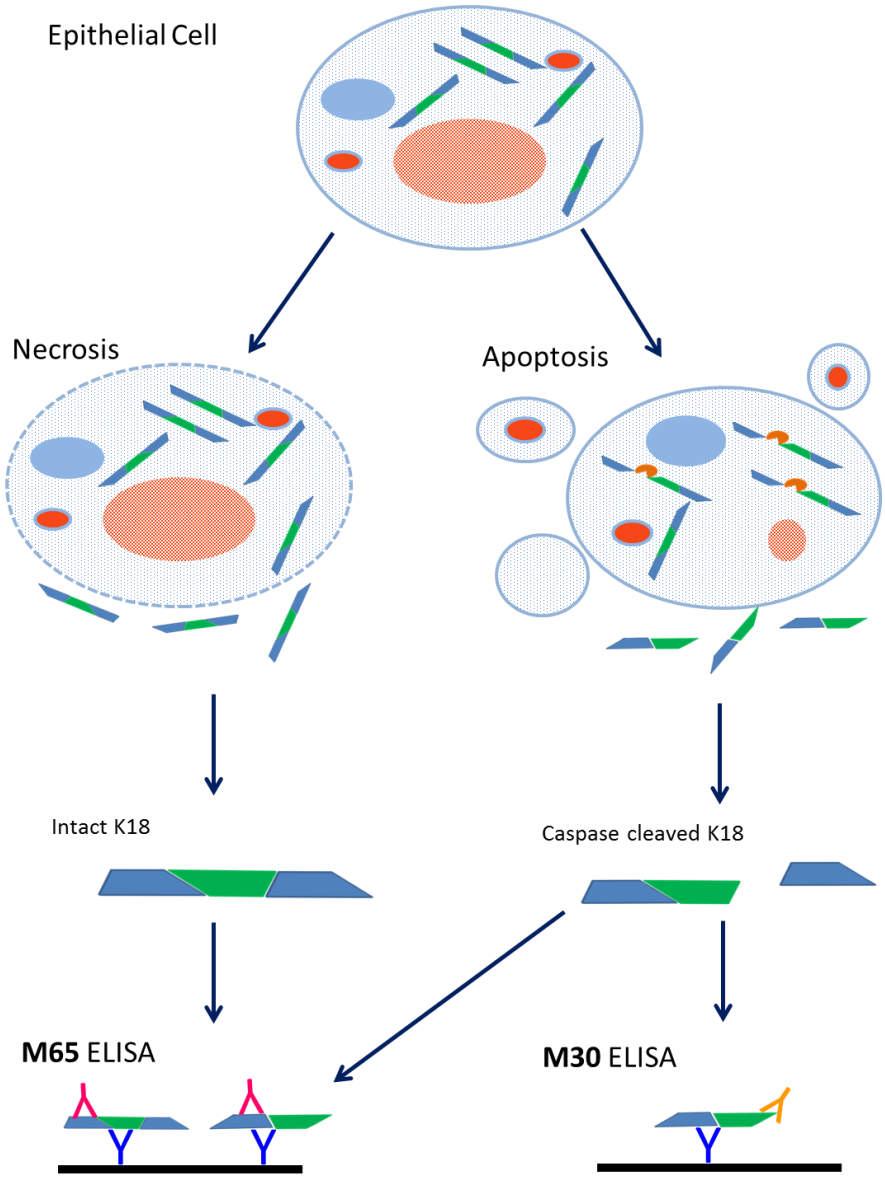

Figure 5.19: Schematic of assay approach for detection of apoptosis in target cells. *Using commercially available monoclonal antibodies from Peviva it is possible to detect necrosis and/or apoptosis of epithelial cells. The M65® ELISA uses two anti-K18 mouse monoclonal antibodies of the IgG type and is primarily intended to be used together with the M30 Apoptosense® assay for detection of apoptotic cells. M30 Apoptosense® is an ELISA assay based on the M30 monoclonal antibody* (Leers *et al*., 1999). *This antibody specifically binds caspase-cleaved keratin 18 (ccK18, caspase-cleaved cytokeratin 18, ccCK18 or K18-Asp396) but does not bind native, uncleaved, K18* (Hägg *et al*., 2002).

To demonstrate the detection of apoptosis using DiCAST a capture immunoassay, to measure total cell death (apoptosis and necrosis) of a Jurkat T cell line induced to undergo cell death with a monoclonal anti-Fas antibody, was performed. This antibody demonstrates cytolytic activity on human cells that express Fas (e.g. Jurkat). Binding of the Fas ligand (Fas-L) induces trimerisation of Fas in the target cell membrane. Activation of Fas causes the recruitment of Fas-associated protein with death domain (FADD) via interactions between the death effector domains (DED) of FADD and Fas. Procaspase 8 binds to Fas-bound FADD which in turn leads to the activation of caspase 8. Activated caspase 8 cleaves (activates) other procaspases, in effect beginning a caspase cascade that ultimately leads to apoptosis.

In this study, Jurkat T cells were added to a 40 micron diameter microcapillary array and sealed with an analysis surface coated with an anti-keratin 18 monoclonal antibody (clone M6, binds intact and cleaved K18). A multi-analyte assay (Figure 5.20) was then employed, using alternately labelled monoclonal antibodies M5 and M30 to observe the occurrence of necrosis and apoptosis, induced by anti-Fas antibody present in the capillaries.

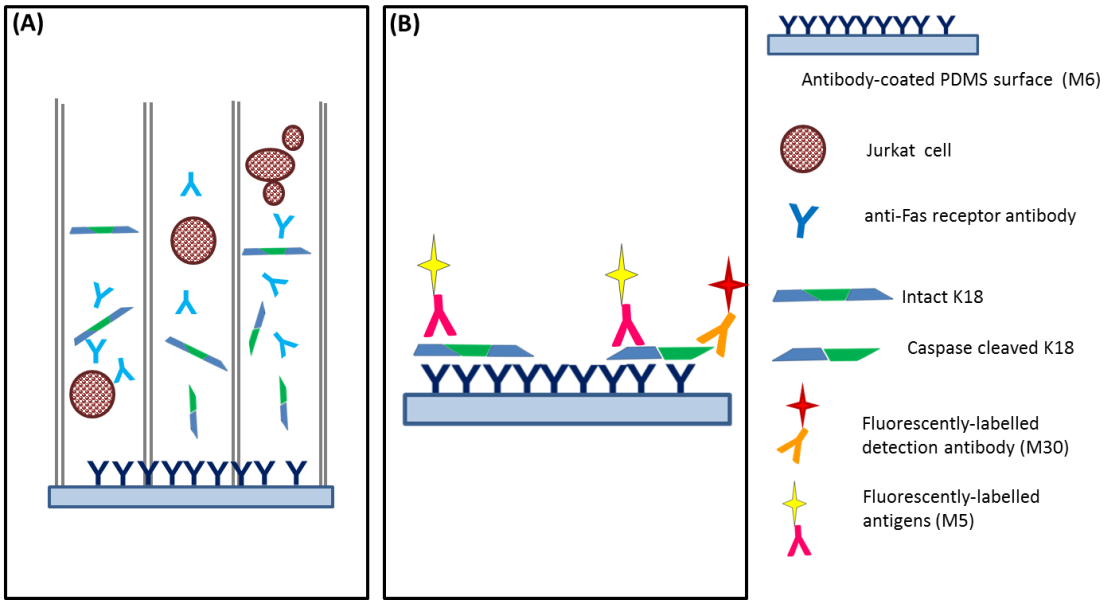

Figure 5.20: Schematic for use of a multi-analyte assay detecting cell death in the microcapillary array. *Utilising the Apoptosense ELISAs from Peviva, in a multi-analyte format, it will be possible to detect cells undergoing necrosis (Cy3-labelled M5) and apoptosis (Cy5-labelled M30) in the wells of the microcapillary array.*

On analysing the fluorescence signals from the assay Jurkat cells undergoing cell death can again be viewed as a 'traffic-light system' with Necrosis (Cy3 signal only), Apoptosis (Cy5 signal only) and necrotic/late stage apoptotic cells (dual Cy3/Cy5 signal) all visualised simultaneously (Figure 5.21).

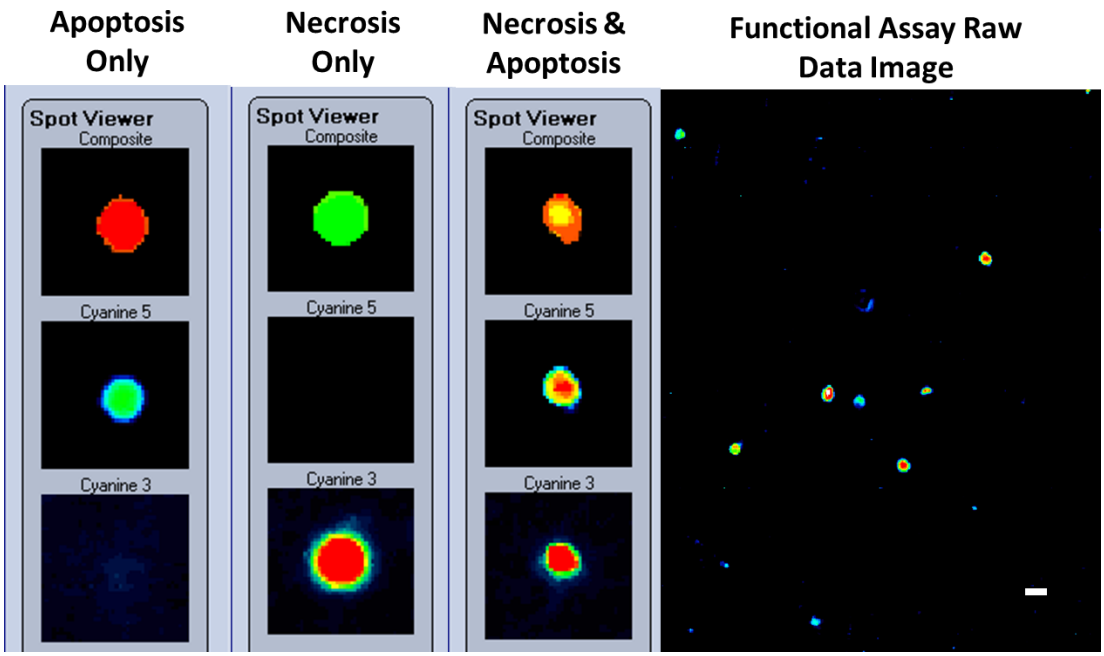

Figure 5.21: Measuring cell death on DiCAST using a combination of the M30 and M65 fluorescence immunoassays. *The M30 antibody specifically measures caspase-cleaved K18 produced during apoptosis and M65 kit antibodies measure the levels of both cleaved and intact K18, the latter of which is released from cells undergoing necrosis. On DiCAST, multiplexed detection of anti-Fas-induced cell death of Jurkat cells was measured simultaneously in the microcapillary array. Scale bar is 100 µm.*

5.5.3 Summary for key challenge 5.5

The aim of this section was to determine if it was possible to carry out a functional bio-assay in the capillaries of the array. A proof-of-concept approach with a commercial anti-Fas monoclonal antibody known to induce apoptosis in Jurkat cells was employed for this purpose. By utilising commercially available ELISA kits and employing them in a multi-analyte format DiCAST assay it was possible to identify capillaries where Jurkat cells were undergoing necrosis, apoptosis or both. The use of such an assay in a screening campaign could offer greater insights into the functional activity of a greater portion of candidate antibodies much earlier in the screening process.

Conclusion and Significance:

In this chapter the ability to analyse mammalian cells, using DiCAST, was assessed and shown to be achievable for both immortalised cell lines and for native B cells.

Firstly the ability to detect antibody secreted from mammalian cells was tested by analysing the supernatant of multiple myeloma cells for secreted hIgE. Using a sandwich ELISA, the supernatant of cells which had been incubated on the array was compared with the supernatant from a flask of the same cells. While levels of hIgE were lower in the supernatant retrieved from the array, there was a detectable level present from the 84,000 capillaries (Figure 5.3). This served to prove that the cells could survive in the reduced volume of the capillaries long enough to produce detectable levels of antibody in the supernatant.

Following on from this a time-course for optimal incubation times of cells was carried out in microtitre plates, as shown in Figure 5.4. While detectable levels of antibody were present after 24 hours, the 48 hour and 72 hour time-points produced similar and much higher levels of antibody. To allow for saturation of the surface and to ensure no slow-growing or low-expression cells are captured, an incubation time of at least 48 hours will be used going forward.

The time-course carried out on plate gave an indication of appropriate incubation time but was not directly transferable, so a single cell assay using the same assay components was carried out on the array. It was shown, by adding a concentration of cells that single cell analysis could be carried out (Figure 5.5) and that signals obtained from each single cell housed in an individual capillary were easily distinguishable above background.

The second challenge of this chapter was to ascertain if it was possible to retrieve the desired cell from the target capillary. While shown to be possible to pass cells through the array (Figure 5.6), under nitrogen flow, with little loss of the overall cell number, retrieval of the contents of a single capillary was not highly successful. The alignment approaches and prototype, discussed in Chapter 3, were again employed but retrieval rates were shown to be very low when addressing a single capillary. This is not surprising given the results obtained using bacteria, where only 10% of the contents of each capillary were retrieved. Whereas bacteria can replicate quickly and a workable culture be obtained within hours, mammalian cells are larger, more fragile and divide at a much slower rate. Thus, the retrieval of mammalian cells proved highly difficult and unreliable. An alternative retrieval prototype, designed specifically for the retrieval of intact mammalian cells, employing the use of a

liquid-liquid removal approach is currently in development and will hopefully greatly improve the yield of cells from the array.

Due to the fact that retrieval of intact and viable cells was shown to be troublesome, a means to retrieve the genetic material of the cell and potentially build the antibody recombinantly was investigated. The RNA was extracted from varying dilutions of cells, the RNA reverse transcribed into cDNA and a specific region amplified from each dilution as a proof-of concept for the method. It was shown to be possible to amplify product from the range of cell dilutions down to the equivalent of approximately 10 cells/mL (Figure 5.8). The potential requirement of this approach would be amplification from one cell and while this would challenge the sensitivity of the approach described above it could possibly be improved by nested-PCR (Tickle *et al*., 2009) or other techniques described in the literature (Kurosawa *et al*., 2012; Kurosawa *et al*., 2011; Yoshioka *et al*., 2011).

Despite requiring an improved method of retrieval, several screening campaigns using native B cells were carried out. Screening of B cells directly without the use of traditional immortalisation strategies is highly desirable as it negates the loss in the original population, caused by the inefficiencies associated with fusion and EBV transformation, and also reduces the time taken for screening (Lightwood *et al*., 2006).

In the first instance, IgG secreted from the splenic B cells of a non-immunised mouse was carried out to ascertain if single B cells could produce a detectable level of secreted IgG to allow detection on DiCAST. Based on the miniaturised format and the high sensitivity of the microarray scanner it was not envisioned that there would be any issue in detecting even small amounts of antibody and this was shown to be the case (Figure 5.9).

Following on from this initial experiment the ability to analyse a population of murine splenic B cells (300,000 at single cell per capillary occupancy), taken directly from an immunised mouse, without prior immortalisation or manipulation was demonstrated (Figure 5.11). This screening campaign involved a multiplex format, which allowed the B cells obtained from a mouse which had been immunised with three different proteins to be screened, for antibodies specific to each of the proteins, simultaneously. This multiplexing capability offers advantages, not only in terms of cost and time-saving, but also in the reduction in animal use with multiple antigens immunised and screened from one animal.

In an effort to add to the functionality of the DiCAST screening offering further, the ability to screen for cell binders using intact cells as the antigen was explored. The desire to select cell binding antibodies is driven by the fact that they can be used as very successful therapeutic and imaging reagents as well as potentially identifying cancer-specific markers

(i.e. present on cancer cells but not on regular cells) (Yoon *et al*., 2012). While cell markers can sometimes be identified and expressed recombinantly they do not remain in their native format, as they would appear on the cell, and lack the presence of potentially important post-translational modifications (e.g. glycosylation) (Siva *et al*., 2008). All too often it does not prove possible to express the protein required and while whole cell panning has proven successful (Hoogenboom *et al*., 1999; Yoon *et al*., 2012) it has limitations in terms of non-specific binding of phage and issues with washing steps which can result in the loss of the cells (Wang *et al*., 2011).

To begin with, the ability of cells to bind specifically to an appropriately coated area of the PDMS surface was investigated. It was demonstrated to be possible (Figure 5.13) and, as a result, a screening campaign against whole cells was undertaken. The spleen cells of a chicken immunised with intact Jurkat cells were analysed using DiCAST. While proving difficult to identify positives with a high level of certainty, due to the irregular shape of the antigen (i.e. fluorescently labelled Jurkat cells), it proved possible to retrieve a number of cells from the array for further analysis. The rate of recovery was low and of those cells retrieved only six were successfully cloned and expressed.

On testing of these cloned recombinant fragments, only one scFv maintained binding to the cell antigen (Figure 5.18). This loss in maintained binding is not unusual, with a number of authors describing between 12 and 50% of cells originally selected producing a recombinant fragments which binds to the original antigen (Jin *et al*., 2009; Lightwood *et al*., 2006). While a worrying observation, if anything it highlights the need to screen more cells in a high-throughput manner to increase the chances of identifying and producing the desired antibody. The identification of a cell binding antibody was a successful result but improvements in the screening approach would absolutely be needed to progress with this approach. An enhanced imaging system (i.e. greater resolution), which would remove uncertainty in the identification of positives on the scanned image, would be a priority for development.

As a final challenge in this chapter, the ability to carry out a functional bio-assay on array was examined. The majority of functional assays require visualisation of the cell, in addition to being complex and expensive. As a result they are only carried out on the small number of final selected antibodies at the end of a screening campaign. With actual functional effect being of such great importance for the success of a therapeutic antibody, the ability to incorporate functional screening at a much earlier stage could prove advantageous in identifying a greater pool of functionally effective candidates.

A bio-assay which detected functional effect in the supernatant of cells (i.e. induction of apoptosis) was identified, the assay components sourced and a proof-of concept experiment for ultra-high throughput functional screening attempted using DiCAST.

By inducing apoptosis of Jurkat cells using a commercially available anti-Fas antibody, of known properties, it was shown to be possible to differentiate necrosis and apoptosis of the Jurkat cells in the capillaries of the array (Figure 5.21). The inclusion of an assay of this type on the entire population of cells, at such an early stage, could offer assurance to those developing potential therapeutics. Typically, using traditional methods, only the final candidate clones are tested for functional activity and there is a potential that investigators may have missed potent candidates because of reducing the pool in earlier steps based on non-ideal criteria (e.g. binding or expression level).

Based on the findings of the experimental work carried out in this chapter, although not fully validated, DiCAST offers a very attractive alternative to the traditional methods employed for the screening of mammalian cells. The time, labour and cost-saving elements are only one consideration. The potential to find a unique antibody by screening all of the cells without bias is perhaps the greatest advantage of employing single cell technologies, such as DiCAST, in the place of the typically used cloning by limiting dilution approach.

Chapter 6

Development of automated and high-throughput screening protocols

Hypothesis

In the recent past the move towards the use of robotics and liquid handling systems, to increase screening throughput, has become more commonplace (Layton *et al*., 2012). The protocols once established and verified can help to reduce workload on operators and often mean that performance of the system, in terms of robustness and reliability, is improved. Typical screening approaches attempt to reduce the number of clones for characterisation (e.g. binding properties, on-rate, off-rate and affinity) to a manageable number. This approach potentially means that important antibodies can be lost early in the selection process. This chapter demonstrates the development of automated and high throughput screening protocols for antibodies

Rationale

The ability to build large highly diverse repertoires of monoclonal antibodies (mAbs) increases the likelihood that an antibody with the desired characteristics will be generated. The challenge is finding the requisite unique antibody, which can often be both costly and time consuming. Antibodies and their derivatives are increasingly important as biotherapeutics, precision diagnostics, and essential tools for biological research. Techniques to screen and characterize large numbers of antibodies and provide confident selection of the best candidates for development are therefore essential. As discussed extensively in preceding subject areas of this thesis, the move towards miniaturisation, single cell interrogation and technologies such as DiCAST are becoming the leaders in the field for successful screening of large populations of monoclonal antibodies, from multiple sources.

While the initial selection using these "microtools" can screen thousands, up to millions in the case of DiCAST, of clones simultaneously the screening campaign can often result in thousands of desirable clones. This is evidenced by the outcome of the C-Reactive protein library screen, described in Chapter 4, where 1631 positive hits were obtained. While the use of a more restrictive antibody selection screen might be useful to reduce the number of finally selected clones, there still exists a requirement for final characterisation and deployment of the selected antibodies.

The aim of this chapter is to develop a set of protocols (automated ELISA and high-throughput SPR) which can be employed to improve on traditional methods of screening and characterisation for a particular set of antibodies (Figure 6.1). In this first portion of this chapter, the design and implementation of a set of protocols to efficiently carry out an automated, plate-based sandwich ELISA on a laboratory liquid handling workstation was

undertaken. The assay, against CRP, was fully optimised and tested with true clinical samples to determine its suitability for purpose.

In the second part of this chapter, a high-throughput screening campaign for a set of antibodies (960) which had been previously selected, against cardiac Troponin I (cTnI), by phage display were interrogated using the Biacore 4000. The antibodies were first ranked based on binding stability and the top ten percent were then brought forward for kinetic characterisation. In addition an epitope mapping experiment was employed to identify binders to highly specific regions of the target antigen. All screening was designed to be both time and cost efficient, as well as delivering the most appropriate antibodies for the specific project.

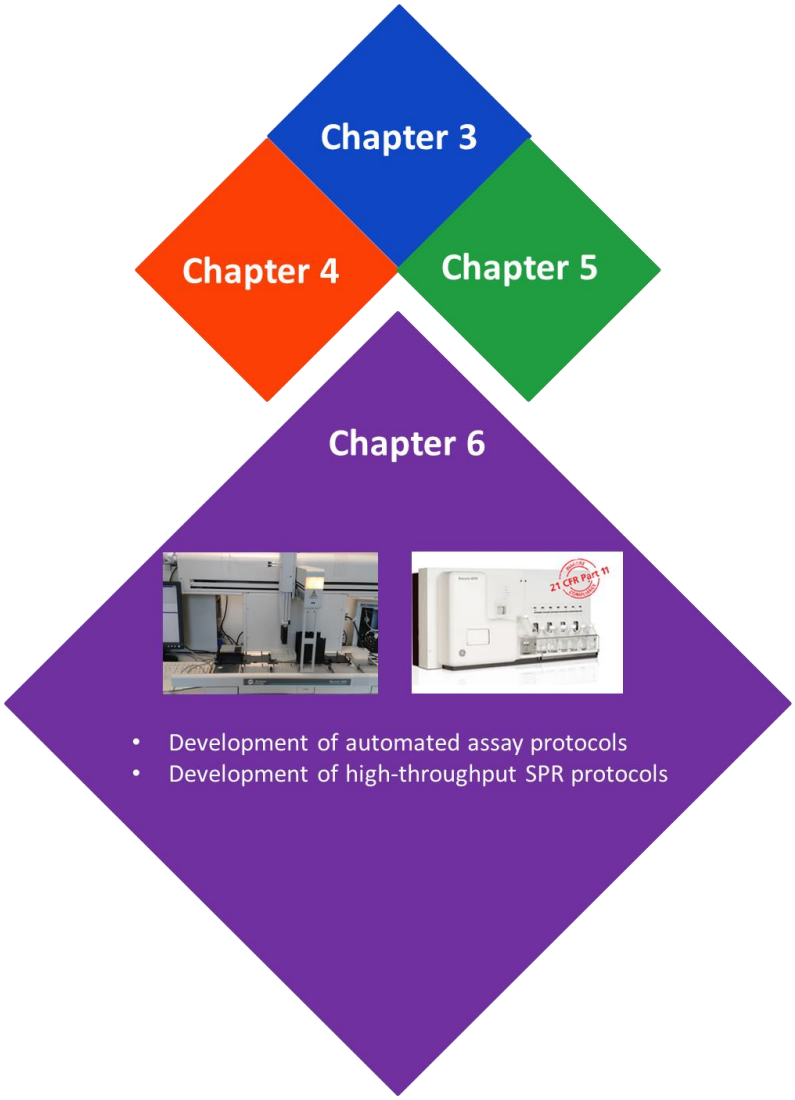

Figure 6.1: Schematic overview of thesis layout. *Chapter 6 explores the establishment of an automated ELISA screening system using the Biomek 2000 and the development of protocols for efficient high-throughput SPR analysis, using the Biacore 4000.*

Key Challenges

6.1 Key Challenge 1-Development of an automated anti-CRP sandwich assay using the Beckman Coulter Biomek 2000 laboratory automation workstation.

6.1.1 Experimental Approach

The use of the Biomek 2000 allows for a more robust assay system and reduces the time spent by the user on completing tasks such as dilution series and washing steps. The workflow for the design of programmes on the Biomek 2000 begins with defining the layout of the work surface, shown in Figure 6.2A. This involves placing all pipetting tools (single-channel, multi-channel or wash heads) and required pipette tips, reservoirs, microtitre plates and tube racks for all operations to be carried out. Once all lab ware has been selected and placed in an appropriate layout, the second step in the process is to define the functions to be carried out from the function palette (Figure 6.2B) such as filling plates with coating, blocking or washing solutions and serially diluting samples. All pipetting steps are carried out as detailed in the pipette transfer tab, as shown in Figure 6.3. This allows for exact conditions to be replicated between methods and days, helping to improve the precision, reproducibility and robustness of assays carried out.

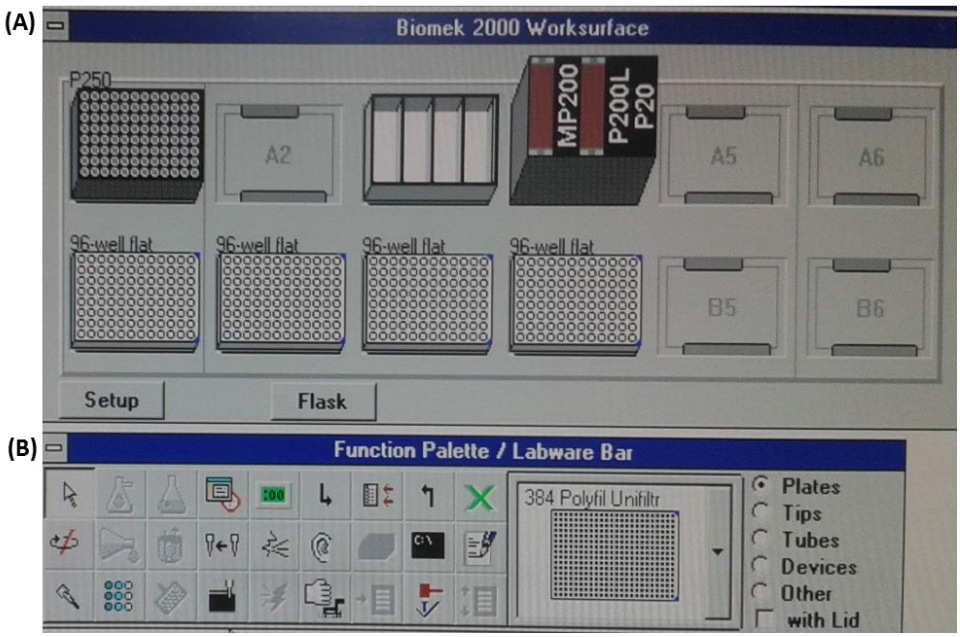

Figure 6.2: Beckman Coulter Biomek 2000 laboratory automation workstation work surface and function definition user interfaces. *The work surface tab (A) allows definition of location for each of the required elements (i.e. pipetting tools, buffer reservoirs, pipette tips, microtitre plates etc.) from the defined list in the lab ware bar. The function palette (B) allows for precise definition of operations (i.e. serially dilute, transfer, fill etc.) within the work surface.*

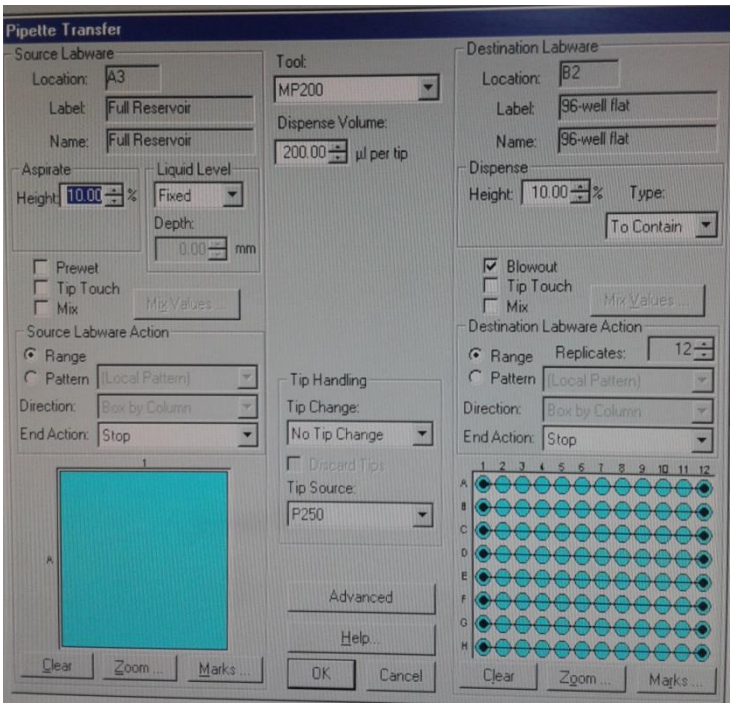

Figure 6.3: ***Beckman Coulter Biomek 2000 laboratory automation workstation pipette transfer function tab.*** *Each criterion of the particular pipetting step is specifically defined within this tab including; source and destination lab ware items, tools used, volumes to be transferred, pattern of transfer, tip change requirements, mixing etc.*

A range of programmes were designed and tested, on the Biomek 2000, to allow for the development and validation of an assay which would detect levels of C-Reactive protein (CRP) in human serum. CRP is used as a sensitive marker for systemic inflammation and elevated levels have been linked to risk of future myocardial infarction (Ridker *et al*., 1998). The sandwich assay for detection was optimised and its accuracy and precision determined. The recombinant anti-CRP scFv A6 selected using DiCAST in Chapter 4, was used in combination with a commercially available polyclonal antibody. Because of the high sensitivity of the recombinant scFv the range of detection for the plate assay was well below typical levels of CRP normally detected in serum, which are between 1 and 5mg/L in healthy individuals and >10mg/L for individuals with inflammation (Food and Drug Administration, 2005). This meant that the sample could be diluted quite significantly for use which might be useful in the case of sample taking (i.e. finger prick in place of actual blood draw). In addition, while the antibody might prove to be too sensitive in a plate assay, the use of such a highly sensitive antibody could be beneficial in non-typical devices such as the mobile phone assay device developed in the BDI. Due to the limited nature of the detection system on the phone a more sensitive antibody could help in the improvement of assay function. The developed assay could also be useful if employed for the detection of high sensitivity (hsCRP) or cardiac CRP (cCRP). Cardiac CRP assays, like hsCRP assays, have

measurement ranges that extend below the measurement range typical of most conventional CRP assays, typically ≤1mg/L (Food and Drug Administration, 2005).

6.1.2 Purfication of anti-CRP scFv A6 using immobilised metal affinity chromatography (IMAC).

Purification of the filtered bacterial lysate was performed by immobilised metal affinity chromatography (IMAC), as described in section 2.2.4 IMAC works by interaction between specific amino acid side chains on the protein of interest and a transition metal ion, in this case Ni^{2+}, immobilized on a matrix (Bornhorst & Falke, 2000). The pCOMB 3XSS vector in which the anti-CRP scFv A6 is expressed contains a 6X histidine fusion tag. Histidine has a strong interaction with Ni^{2+} metal immobilized on the matrix due to the formation of coordination bonds between the immobilized transition metal and the electron donor groups on the histidine imidazole ring (Bornhorst & Falke, 2000). Those proteins which contain consecutive histidine residues are retained within the IMAC column matrix; washing with buffers containing lower levels of free imidazole allows for non-specifically or loosely bound proteins to be removed from the column. In addition, wash buffer A, detailed in section 2.2.4, contains glycerol, sodium chloride and Triton-X100 which serve to reduce hydrophobic interactions between non-specific proteins and the column matrix allowing for fewer contaminants in the final eluted protein. The desired protein can then be eluted from the column by adding a buffer with a higher level of free imidazole. Imidazole is a histidine analogue and its addition competitively elutes the His-tagged protein from the purification column. An aliquot of each of the fractions from the purification process was retained and analysed by SDS-PAGE, as detailed in section 2.2.1.

6.1.3 Characterisation of purified anti-CRP scFv A6 by SDS-PAGE.

The purity of the anti-CRP scFv A6 was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE allows the separation of proteins according to their size. A picture of the SDS-PAGE gels is presented in Figure 6.4 and shows the high level of purity obtained from the IMAC purification. The expected size of the desired protein is approximately 27 kDa. The final eluent was buffer exchanged into PBS, concentrated, aliquoted and stored at -20^{0}C until further use.

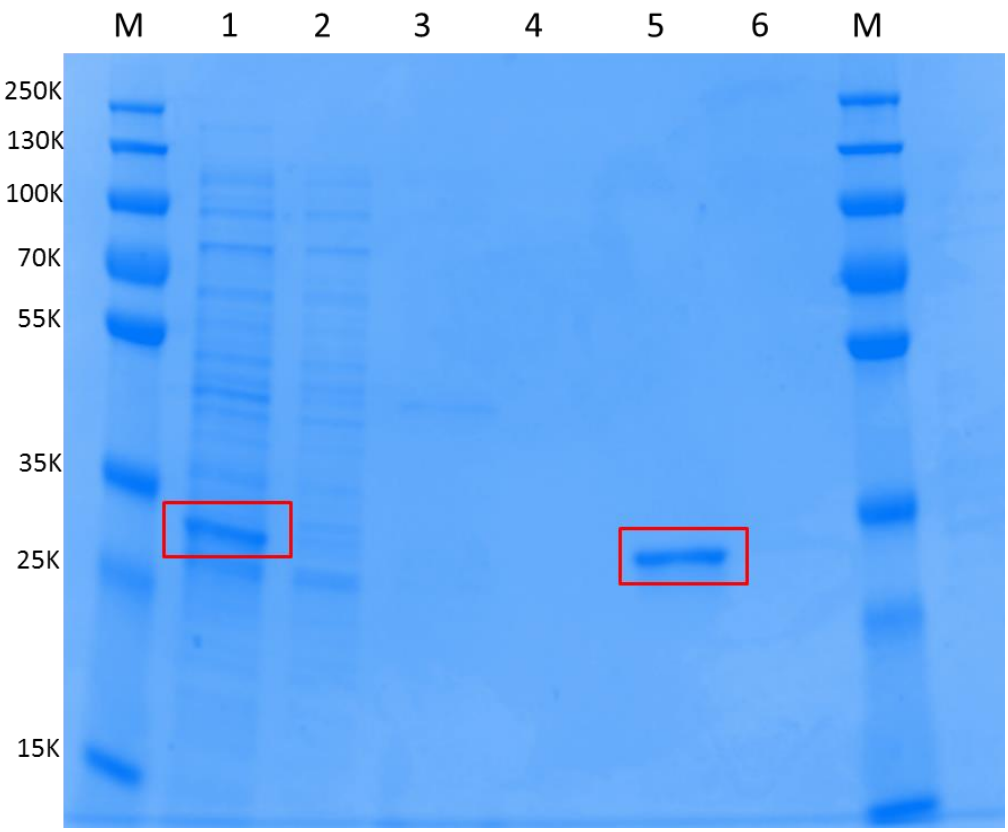

Figure 6.4: SDS-PAGE analysis of IMAC purification for anti-CRP scFv A6. *Lanes designated 'M' contain the protein marker (PageRuler plus pre-stained protein ladder, sizes for each of the bands are indicated to the left of the gel picture). The desired band is highlighted on the image, in both the lysate and elution fractions. Lane 1 contained the crude lysate of scFv A6, generated as described in Section 2.2.2. Lane 2 represents the flow-through from the IMAC column, containing all proteins that did not bind to the IMAC column. Lane 3 contained Wash A, which removes non-specifically bound proteins to the column. Lane 4 contained Wash B which removes any additional non-specific proteins. Lane 5 contained the eluted, IMAC-purified scFv A6 (expected size is approximately 27 kDa).*

6.1.4 Optimisation of anti-CRP sandwich assay.

The process of optimisation is integral to the development, and possibly validation, of any assay. It will allow for a more reliable assay and gives confidence to the user that the assay will perform as expected throughout all operations. To optimise this indirect sandwich ELISA, a series of checkerboard ELISAs were carried out to determine the optimal concentrations of the reagents used.

A checkerboard titration involves the dilution of two reagents against each other to examine the interactions between all resultant combinations (Crowther, 2000). For any checkerboard investigation the maximum number of reagents that can be varied per assay is two (Crowther, 2000). As a result two separate checkerboard ELISAs were carried out to optimise this sandwich assay, as described in sections 2.6.2 and 2.6.3. The first was utilised

to determine the correct coating concentration of the polyclonal anti-CRP antibody as well as the appropriate range of CRP to use and the second assay was used to determine the optimal dilution of primary detection antibody (scFv A6). Throughout the assay development the secondary detection antibody (HRP labelled anti-HA) was used at a 50 ng/mL dilution, as recommended by the manufacturers.

Based on the initial checkerboard, which is shown in Figure 6.5, the optimal coating concentration for the assay was determined to be 4 µg/mL. This was based on the fact that there was little difference between the higher concentration of 20 µg/mL. The titer and the overall absorbance levels were non-optimal below 4 µg/mL.

In terms of the range of CRP which could be reliably detected using the assay, there was a plateau effect evident after approximately 0.6 µg/mL of CRP and there was still a response above background at 0.001 µg/mL. A range of 1-0.0001 µg/mL CRP was determined at optimal for the assay going forward.

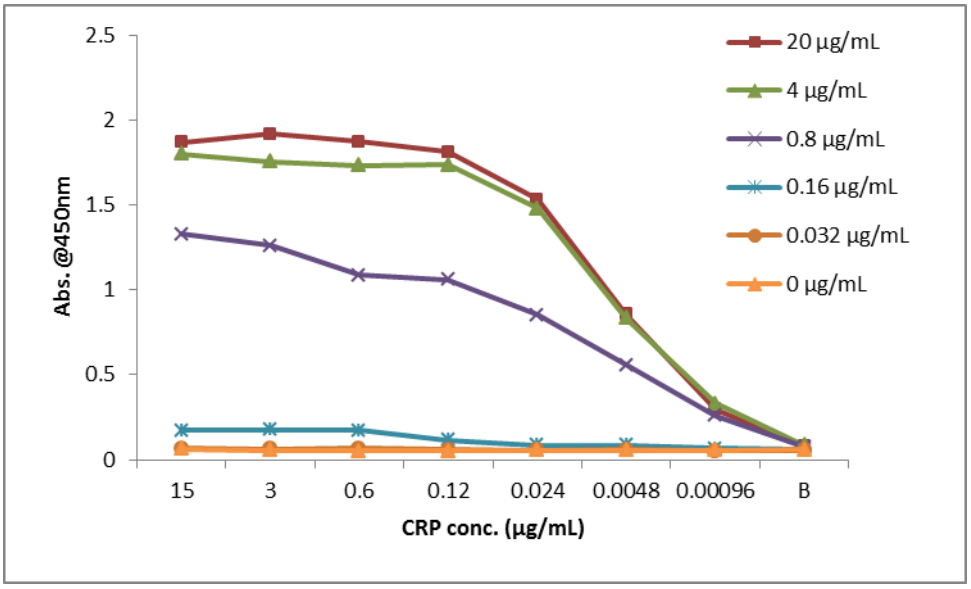

Figure 6.5: Checkerboard ELISA for the determination of appropriate coating concentration of the polyclonal anti-CRP antibody. *The coating antibody was diluted in series (1 in 5) and a sandwich assay carried out using a range of CRP concentrations (15 µg/mL to 0.0001ug/mL; 1 in 5 series). The recombinant scFv A6 was used as the primary detection antibody at a concentration of 1.04 µg/mL and secondary detection was carried out using 50 ng/mL anti-HA tag antibody which was HRP labelled.*

In the second checkerboard ELISA (Figure 6.6) the dilution of primary detection antibody was investigated. Three different dilutions were employed, as described in Section 2.6.3. Based on the responses obtained a concentration of 1.04 µg/mL appears to be optimal by giving sufficient absorbance values while also titering well.

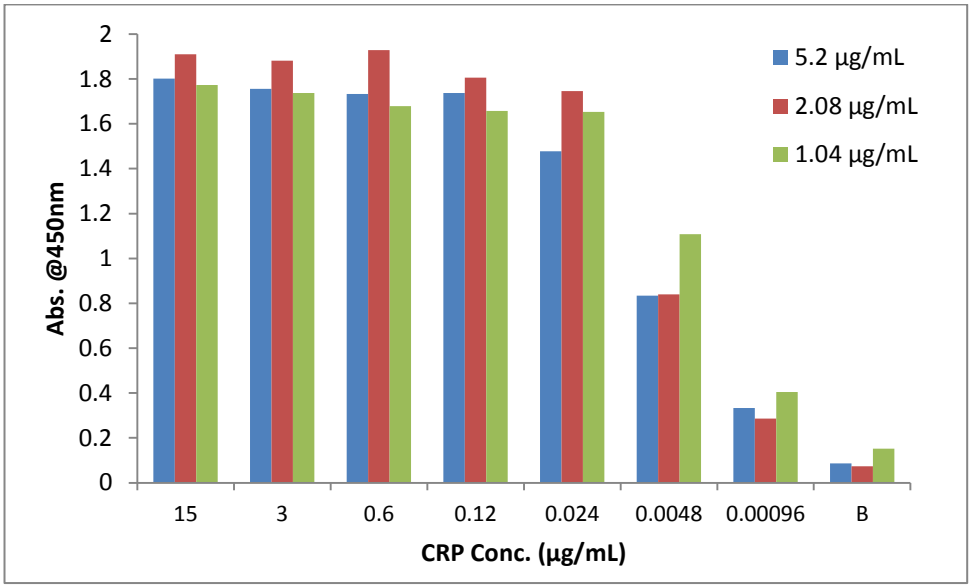

Figure 6.6: Checkerboard ELISA for the determination of appropriate concentration of primary detection antibody (anti-CRP scFv A6). *The coating antibody was used at 4 µg/mL as determined in section 6.1.4. The recombinant scFv A6 was used as the primary detection antibody at a range of dilutions (1in 100 (5.2 µg/mL), 1 in 250 (2.08 µg/mL) and 1 in 500 (1.04 µg/mL)) and secondary detection was carried out using anti-HA tag antibody which was HRP labelled, as per manufacturer's instructions.*

6.1.5 Determination of the performance of the optimised sandwich assay.

In order to measure the accuracy of the immunoassay, intra-day assay variability studies were performed. Three sets of standards were prepared and assayed as per section 2.6.4. The results were plotted and are shown in Figure 6.7. The standard deviations, for each of the points of the standard curves, were expressed as error bars on the graph.

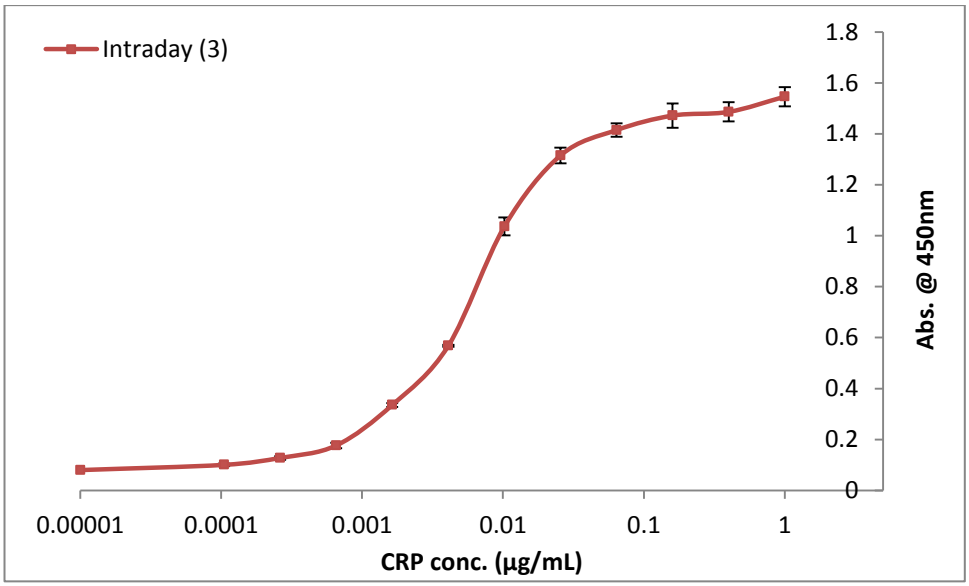

Figure 6.7: Intraday assessment of the standards used in the CRP sandwich assay. *The error bars indicate the deviation in the absorbance over three separately prepared standard curves assayed on the same day. Each standard curve was carried out on a separate plate and was composed of three replicates for each standard. The % CV values ranged from 1.12 to 6.12% which is within the accepted ranges for this type of analysis* (DeSilva *et al*., 2003).

To measure the intermediate precision (precision of repeated measurements, taking into account sources of variation such as runs, days, reagents etc.), inter-day assay variability studies were performed using the optimised assay. The assay was carried out, as described in section 2.6.4, on three separate days using freshly prepared reagents for each day. The results obtained are displayed in Figure 6.8, where once again the standard deviations are shown for each point of the standard curve using error bars. While it is evident that there was a larger degree of variance interday than intraday, it could be expected that this would be the case.

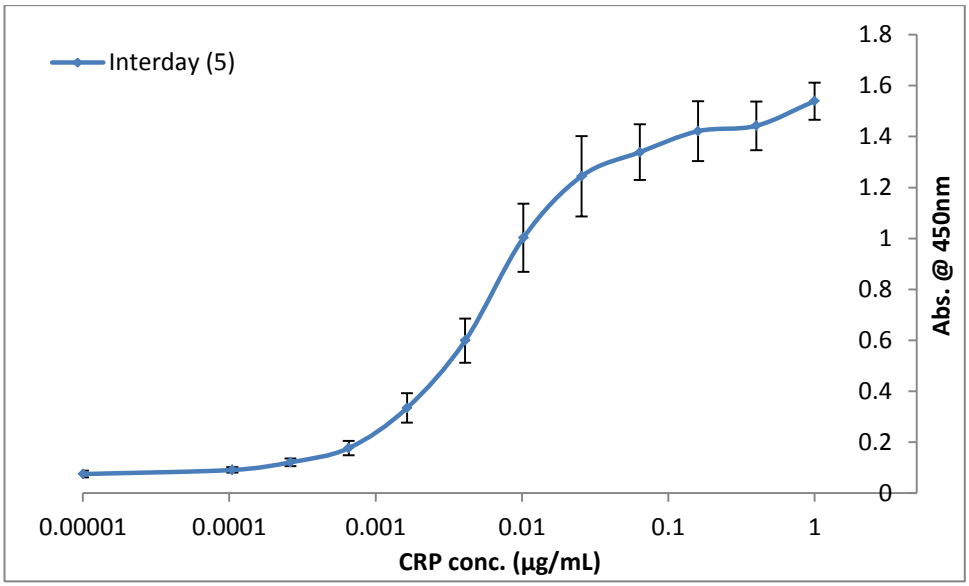

Figure 6.8: Interday assessment of the standards used in the CRP sandwich assay. *The error bars indicate the deviation in the absorbance over three separately prepared standard curves assayed on the three different days. Each standard curve was carried out on a separate day and was composed of three replicates for each standard. The % CV values ranged from 0.92 to 16.83%. While this is still within the accepted guidelines for precision* (DeSilva *et al*., 2003) *some of the values were on the higher end of the scale. This was predominantly evident in the lower end of the curve where any deviation would represent a higher percentage of the mean absorbance for that standard.*

To further evaluate the accuracy of the assay, spiked samples were prepared at a concentration of 0.001 µg/mL, in both buffer (PBS with 1% (w/v) BSA) and serum, and subsequently assayed using the optimised ELISA method. The concentration of 0.001 µg/mL was chosen as a potential LOQ for the developed assay, by observation of the standard curve. The results obtained were plotted using BIAevaluation software and a four parameter logistic equation fitted to the data. The four parameter function provides an accurate representation of the sigmoidal relationship between the measured response and the logarithm of concentration observed for many immunoassays (Findlay *et al*., 2000)

The concentration for each of the spiked samples was then back-calculated based on the standard curve obtained. This was carried out for 10 separate spiked samples on each of three consecutive days. The data obtained from this analysis is summarised in Table 6.1 (intraday) and Table 6.2 (interday). The method proved to be very accurate in both the cases of intra- and inter-day analysis, with calculated concentrations for all samples (buffer and serum) ranging between ~88 and 103% recovery. The precision of the method was also well within acceptable ranges, with % CV for each set of data being below 7%. For analysis of this type a % CV that is ≤20% tends to be considered acceptable (DeSilva *et al*., 2003). It

could be noted that, while within acceptable values, the analysis in serum was slightly less accurate and precise, most likely due to the more complex nature of the matrix.

Table 6.1: Inter- and Intra-day data for back calculations and % recoveries for both buffer and serum spiked samples. *Actual spiked concentration is 0.001 µg/mL CRP. Samples were assayed alongside a standard curve, values for each of the samples were then calculated based on the standard curve (four parameter logistic fit).*

Intraday	Absorbance (450nm)	Calculated conc. (µg/mL)	Recovery (%)
Buffer			
1	0.3289	0.000934	93.37
2	0.3317	0.000947	94.70
3	0.3325	0.000951	95.08
4	0.3454	0.001012	101.22
5	0.3300	0.000939	93.89
6	0.3305	0.000941	94.13
7	0.3247	0.000914	91.37
8	0.3419	0.000996	99.55
9	0.3244	0.000912	91.22
10	0.3311	0.000944	94.41
Summary			
Mean	0.3321	0.000949	94.89
SD	0.0067	3.19E-05	3.19
CV (%)	2.02	3.36	3.36
Serum			
1	0.3232	0.000943	94.28
2	0.3327	0.000989	98.94
3	0.3227	0.000940	94.04
4	0.3306	0.000979	97.90
5	0.3217	0.000936	93.55
6	0.3121	0.000889	88.92
7	0.3162	0.000909	90.89
8	0.3284	0.000968	96.82
9	0.3188	0.000922	92.15
10	0.3085	0.000872	87.21
Summary			
Mean	0.3214	0.000935	93.47
SD	0.0078	3.8E-05	3.80
CV (%)	2.43	4.06	4.06

Table 6.2: Interday data for back calculations and % recoveries for both buffer and serum spiked samples. *Actual spiked concentration is 0.001 µg/mL CRP. Samples were assayed alongside a standard curve. Values for each of the samples were then calculated based on the standard curve (four parameter logistic fit). The interday values are representative of the mean and standard deviation from 10 replicates assayed on three separate days.*

Interday	Calculated conc. (Mean)	Calculated conc. (SD)	Recovery Mean (%)	Recovery SD (%)	CV (%)
Buffer					
Day1	0.000932	4.29827E-05	93.19	4.29	4.61
Day2	0.000958	5.55126E-05	95.76	5.55	5.79
Day3	0.000950	5.67111E-05	94.96	5.67	5.97
Summary	0.000946	1.31862E-05	94.64	1.31	1.39
Serum					
Day1	0.000921	4.64978E-05	92.13	4.64	5.04
Day2	0.001032	5.94022E-05	103.22	5.94	5.75
Day3	0.000965	6.73871E-05	96.50	6.73	6.98
Summary	0.000973	5.58901E-05	97.29	5.58	5.74

6.1.6 Analysis of clinical samples using the optimised CRP assay

In order to assess the performance of the assay for its final purpose, a number of clinical samples of known CRP concentration were analysed with fully optimised assay. The samples were obtained from the Mater Misericordiae University Hospital, Dublin. They were previously analysed using Abbott ARCHITECT c16000 Clinical Chemistry Analyser, in a turbidometric assay format. The samples were then titered and assayed, as described in section 2.6.6. Once again the results obtained were plotted using BIAevaluation software and a four parameter logistic equation fitted to the data, which is displayed in Figure 6.9. The values for each of the dilutions were then back-calculated using the standard curve, as shown in Table 6.3, with the aim of identifying a dilution which allowed for accurate calculation of the CRP levels in the clinical samples. Due to the wide range of samples concentrations (2-21 mg/L or µg/mL) for the clinical samples it was important to identify a single dilution that could be applied to all samples/unknowns that would allow for their accurate and precise quantitation, in an actual blind analysis. On observation of the titer, carried out as detailed in section 2.6.6, for the clinical samples (Figure 6.9) it can be seen that the greatest differentiation between samples of different concentrations can be seen at roughly a 1 in 3125 dilution.

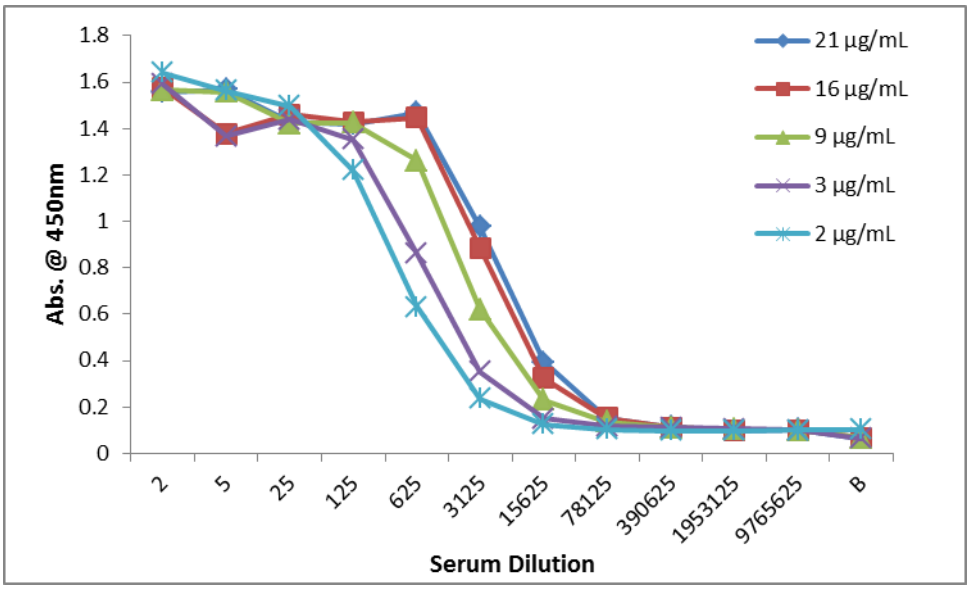

Figure 6.9: Titer of clinical samples in sandwich format*. The serum samples (containing 21, 16, 9, 3 and 2 mg/L or µg/mL, as determined in the hospital clinic) were each diluted in series (1 in 5) to determine a dilution which will allow for all concentrations to be accurately quantified using a standard curve, as previously described. On observation of the curve it can be seen that the greatest differentiation between samples can be seen at a 1 in 3125 dilution.*

The back calculation of sample concentrations from the curve provides a more detailed analysis of the data and identifies the most accurate dilution ranges, highlighted within Table 6.3, for all of the clinical sample concentrations. The higher concentration samples appear to have a wider range of suitable dilutions for accurate quantitation while the lower concentration samples (2 and 3 µg/mL) are only accurate within a narrower range (1 in 625 to 1 in 3,125).

Table 6.3: Determination of correct dilution factor to allow accurate quantification of CRP levels in clinical samples. *Each of the values from the standard curve were used as hypothetical samples and the CRP level calculated based on the standard curve. The accuracy of the calculation was then determined and the ideal dilution range for each sample concentration defined (highlighted in blue in the table).*

Sample	Dilution	Actual Conc. (µg/mL)	Absorbance (450 nm)	Calc. Conc. (µg/mL)	Accuracy (%)
21	2	10.50	1.5565	ND	ND
	5	4.2	1.5680	ND	ND
	25	0.84	1.4267	0.0259	3.0943
	125	0.168	1.4205	0.0250	14.9031
	625	0.0336	1.4697	0.0354	105.5860
	3125	0.0067	0.9783	0.0061	91.3808
	15625	0.00134	0.3933	0.0014	111.4070
	78125	0.0002	0.1507	0.0002	104.5423
	390625	5.38E-05	0.1154	5.00E-05	92.9363

	1953125	1.08E-05	0.0965	ND	ND
	9765625	2.15E-06	0.1022	ND	ND
16	2	8	1.5739	ND	ND
	5	3.20	1.3781	0.0200	0.6253
	25	0.64	1.4612	0.0330	5.1665
	125	0.128	1.4266	0.0259	20.2942
	625	0.0256	1.4473	0.0297	116.3430
	3125	0.00512	0.8857	0.0050	98.0280
	15625	0.001024	0.3239	0.0011	112.2518
	78125	0.000205	0.1520	0.0002	140.7955
	390625	4.1E-05	0.1129	2.61E-05	63.7881
	1953125	8.19E-06	0.0994	ND	ND
	9765625	1.64E-06	0.0998	ND	ND
9	2	4.5	1.5658	ND	ND
	5	1.8	1.5583	0.2534	14.0799
	25	0.36	1.4231	0.0254	7.0636
	125	0.072	1.4262	0.0259	35.9901
	625	0.0144	1.2608	0.0127	88.3996
	3125	0.00288	0.6206	0.0028	97.3701
	15625	0.000576	0.2301	0.0006	119.9788
	78125	0.000115	0.1370	0.0002	174.1953
	390625	2.3E-05	0.1114	8.11E-06	35.1898
	1953125	4.61E-06	0.1038	ND	ND
	9765625	9.22E-07	0.1004	ND	ND
3	2	1.5	1.5906	ND	ND
	5	0.6	1.3665	0.0189	3.1609
	25	0.12	1.4374	0.0278	23.1910
	125	0.024	1.3543	0.0179	74.8939
	625	0.0048	0.8605	0.0047	99.0625
	3125	0.00096	0.3534	0.0012	134.9635
	15625	0.000192	0.1506	0.0002	146.0643
	78125	3.84E-05	0.1164	5.86E-05	152.5355
	390625	7.68E-06	0.1124	2.07E-05	269.2913
	1953125	1.54E-06	0.1052	ND	ND
	9765625	3.07E-07	0.0999	ND	ND
2	2	1	1.6393	ND	ND
	5	0.4	1.5638	0.5972	149.3205
	25	0.08	1.4979	0.0471	58.9053
	125	0.016	1.22	0.0112	70.1658
	625	0.0032	0.6321	0.0028	90.0220
	3125	0.00064	0.236	0.0007	112.5089
	15625	0.000128	0.1246	0.0001	94.1025
	78125	2.56E-05	0.1027	ND	ND
	390625	5.12E-06	0.0985	ND	ND

1953125	1.02E-06	0.0974	ND	ND
9765625	2.05E-07	0.0996	ND	ND

Based on the detailed accuracy analysis, discussed above, the clinical samples were all diluted 1 in 2000 (ten replicates for each sample) and assayed as described in Section 2.6.7.

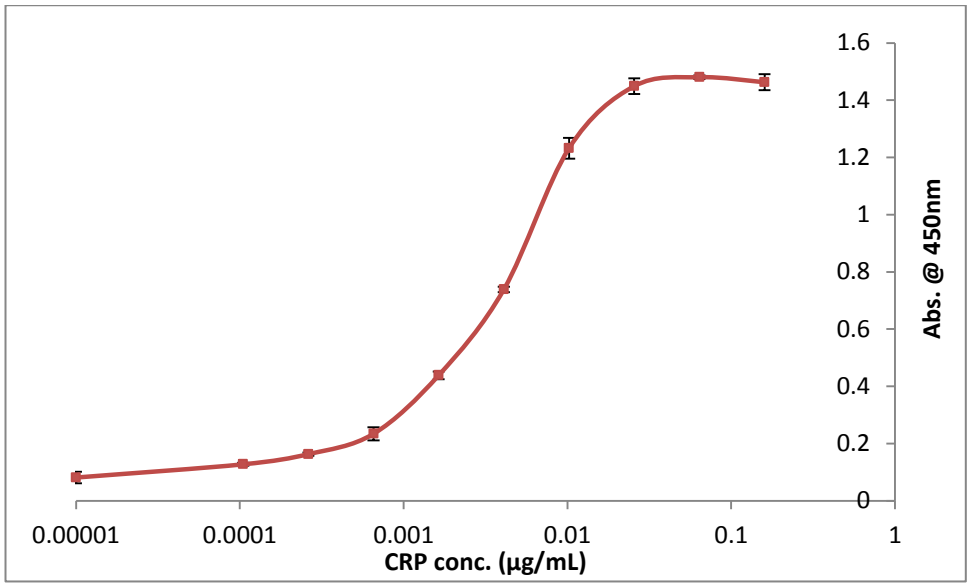

Figure 6.10: Standard curve for determination of clinical serum sample CRP concentration. *The curve was generated as described in section 2.6.4, and the data fitted with 4-parameter equation using BIAevaluation software. Absorbance values from the diluted clinical samples were calculated based on this standard curve. The result of the analysis can be seen in Table 6.3. Error bars denote the standard deviation from the mean of three replicates for each standard.*

A standard curve was generated using the BIAevaluation software (Figure 6.10), as previously discussed, and was used to quantify the level of CRP in the clinical samples. The results of this quantification, for ten replicates of each clinical sample, are detailed in Table 6.4. Overall there was an extremely high level of accuracy (Mean accuracy ranged from 100 to 105%) and precision (% CVs for the ten replicates for each sample ranged from 1.22 to 3.61%) for all of the samples analysed.

Table 6.4: Calculation of CRP levels in clinical patient samples using the optimised sandwich assay. *Samples were diluted 1 in 2000, which was determined as the optimal dilution range from Table 6.3. Each sample was assayed 10 separate times, the concentration calculated based on the standard curve (Figure 6.10) and the sample concentration corrected for dilution factor before calculating the recovery and precision values. All performed very well and had low % CV, indicating a very high level of precision when calculating the amount of CRP in clinical samples.*

Actual Conc. (µg/mL)	Abs (450nm)	Calculated Conc. (µg/mL)	Dilution corrected (x 2000)	% Recovery
21	1.189	0.010	20.55	97.84
	1.198	0.011	21.07	100.34
	1.200	0.011	21.20	100.97
	1.199	0.011	21.13	100.62
	1.201	0.011	21.22	101.05
	1.199	0.011	21.12	100.60
	1.193	0.010	20.78	98.94
	1.189	0.010	20.54	97.81
	1.211	0.011	21.87	104.14
	1.196	0.010	20.92	99.63
Mean	1.197	0.011	21.04	100.19
SD	0.006	0.00019	0.39	1.84
CV (%)				1.84
16	1.092	0.008	15.98	99.89
	1.098	0.008	16.21	101.34
	1.099	0.008	16.24	101.49
	1.089	0.008	15.87	99.21
	1.099	0.008	16.26	101.62
	1.099	0.008	16.25	101.57
	1.099	0.008	16.25	101.55
	1.101	0.008	16.33	102.09
	1.108	0.008	16.60	103.77
	1.099	0.008	16.25	101.58
Mean	1.098	0.008	16.23	101.41
SD	0.005	0.00010	0.19	1.22
CV (%)				1.2
9	0.838	0.005	9.06	100.70
	0.846	0.005	9.22	102.42
	0.840	0.005	9.09	101.04
	0.859	0.005	9.48	105.35
	0.844	0.005	9.17	101.89
	0.837	0.005	9.04	100.46

	0.851	0.005	9.32	103.54
	0.850	0.005	9.30	103.30
	0.842	0.005	9.14	101.59
	0.851	0.005	9.31	103.47
Mean	0.846	0.005	9.21	102.38
SD	0.007	0.00007	0.139	1.540
CV (%)				1.50
3	0.390	0.001	2.96	98.66
	0.396	0.002	3.02	100.61
	0.390	0.001	2.96	98.76
	0.401	0.002	3.07	102.35
	0.399	0.002	3.06	101.91
	0.390	0.001	2.96	98.59
	0.401	0.002	3.07	102.42
	0.401	0.002	3.08	102.56
	0.399	0.002	3.05	101.81
	0.389	0.001	2.95	98.32
Mean	0.396	0.002	3.02	100.60
SD	0.005	0.00010	0.05	1.82
CV (%)				1.81
2	0.299	0.001	2.05	102.54
	0.300	0.001	2.07	103.37
	0.309	0.001	2.15	107.43
	0.313	0.001	2.22	111.21
	0.290	0.001	1.97	98.30
	0.310	0.001	2.16	108.02
	0.299	0.001	2.06	102.84
	0.299	0.001	2.05	102.69
	0.310	0.001	2.16	108.17
	0.301	0.001	2.07	103.67
Mean	0.303	0.001	2.10	104.82
SD	0.007	0.00004	0.08	3.78
CV (%)				3.61

6.1.7 Summary for key challenge 1.

The objective when approaching key challenge 1 was to develop an automated, highly sensitive, accurate and precise assay for the detection of CRP in clinical samples, utilising the liquid handling workstation Biomek 2000.

The accuracy and precision of the method were thoroughly investigated through intra- and interday analysis. Once deemed to be within acceptable limits the assay was applied to clinical samples of known CRP concentration for verification of the assay. An ideal dilution factor was also identified to allow for accurate quantitation of a varied range of CRP concentrations, in true clinical samples. Based on the data described above it can be seen that the objective has been achieved with an optimised and robust assay tested and proven with actual clinical samples.

6.2 Key Challenge 2- Development of a high-throughput SPR screening protocol using the GEHC Biacore 4000.

6.2.1 Experimental Approach

High-quality antibodies are key components in diagnostic and therapeutic applications. Today, secondary screening of recombinant antibodies or fragments (e.g., scFv and Fabs) from different selection platforms is often an analysis bottleneck due to large datasets of low quality. In this section, the Biacore 4000 equipped with Biacore 4000 Antibody Extension Package was used for rapid screening, characterisation, and ranking of antibodies against a marker for cardiovascular disease (troponin I) as detailed in Figure 6.11.

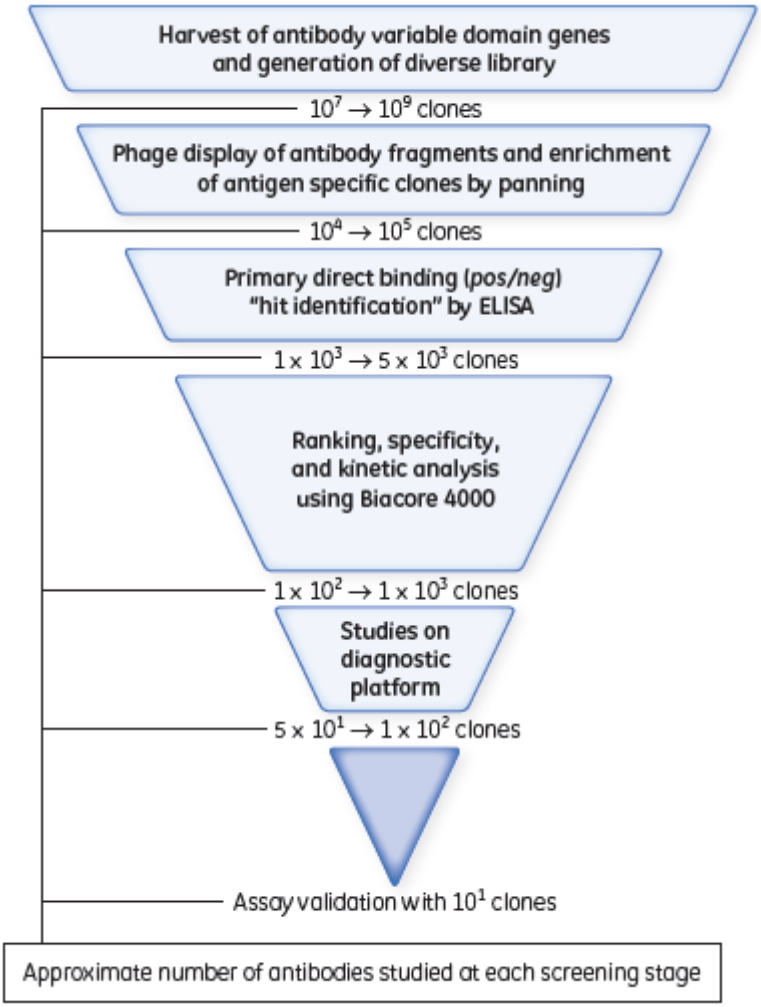

Figure 6.11. Schematic overview of the process for selecting recombinant antibodies for diagnostic use. *Biacore 4000, used for secondary screening, has the throughput required to resolve the analysis bottleneck and provides high quality binding, specificity, and kinetic data.*

The antibodies were initially developed to be incorporated into a point-of-care (POC) diagnostic device for the detection of cTnI in patients with suspected, recent myocardial infarction (MI). To serve as high quality diagnostic reagents for POC applications, ideal antibodies should have a high specificity and a high affinity that is temperature-stable. Additionally, the antigen of interest may require a very stringent screening protocol to ensure that the correct antibodies are selected for further characterisation and promotion to validation in appropriate assays and/or devices.

In this study the antigen to be studied was cardiac Troponin I (cTnI) which exists *in vivo* as part of a complex. The Troponin complex is a heteromeric protein playing an important role in the regulation of skeletal and cardiac muscle contraction. It consists of three different

subunits – troponin T (TnT), troponin I (TnI) and troponin C (TnC). Each subunit is responsible for a part of troponin complex function (Gomes *et al*., 2002).

Human troponin I is presented in cardiac muscle tissue by a single isoform with molecular weight 23,876 Da and it consists of 209 amino acid residues. The cTnI molecule contains two serine residues in the 22 and 23 positions. Both amino acid residues can be phosphorylated *in vivo* by protein kinase A, so four forms of protein – one dephospho, two monophospho and one bisphospho – can coexist in the cell. Phosphorylation of cTnI changes the conformation of the protein and modifies its interaction with other troponins as well as the interaction with anti-TnI antibodies (Zhang *et al*., 2011).

Because of the complexity of the antigen this section will not only develop a high throughput screening protocol for the identification of specific, high-affinity recombinant fragments but also identify the epitopes bound by each of the antibodies in a high throughput epitope mapping experiment.

6.2.2 Screening of scFv fragments based on stability

The antibodies screened throughout this section were selected from a large immune antibody library of 10^9 clones, described in section 2.2.5, using phage display. The library was panned against cardiac Troponin I (cTnI) and the plates screened contained monoclonal stocks from output plates obtained after four rounds of panning. All plates were obtained from the Applied Biochemistry Group (ABG) in DCU.

The antibody panel screened, analysed using the set-up depicted in Figure 6.12., consisted of ten 96-well plates of crude bacterial extracts, expressed from the stocks as described in section 2.6.8, containing avian-derived scFv antibody fragments.

The polyclonal anti-HA antibody was covalently immobilized to spot 1, 2, 4, and 5 in all four flow cells on Series S Sensor Chip CM5 with amine coupling to a level of approximately 7000 RU. Each of the crude cell lysates were centrifuged, diluted 1:4 in HBS-EP+ buffer, and captured onto spots 1 and 5 of each flow cell. A solution of troponin I (50 nM) was injected over all spots. During each cycle, 8 individual scFv samples were analysed simultaneously.

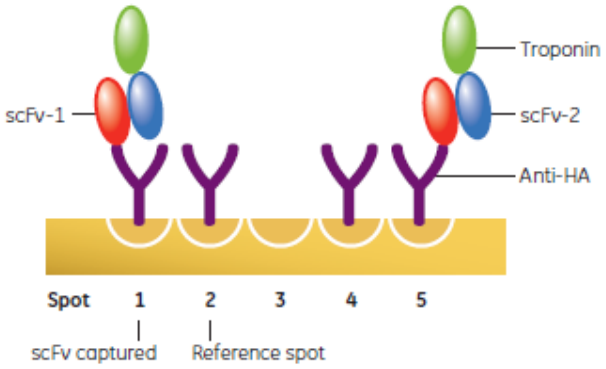

Figure 6.12: Biacore 4000 assay setup for screening of scFv fragments based on stability. *The anti-HA capture antibody is immobilised by amine coupling to the CM-dextran surface of the sensor chip. The scFv fragments are then passed over the surface (Spot 1/5), followed by the antigen cTnI at 50nM (All spots). This set-up allows for a high level of referencing (both anti-HA and CM-Dextran surface controls for non-specific antigen binding are inherently included) and removal of any responses as a result of non-specific binding. The surface is then regenerated using 20mM NaOH, removing the scFv and cTnI complexes entirely and making the surface available for the next cycle.*

The antibody capture responses ranged from 0 to 890 RU depending on the antibody expression level and antigen responses varied between 0 and 210.

The sensor surface was regenerated using 20 mM NaOH and the surface was used for 120 cycles without any loss of binding activity. Ranking of binding stability was evaluated based on report points, placed early (stability_early) and late (stability_early) during antigen dissociation. A selection of sample sensorgrams (120, representing all scFvs passed over 1 of the 8 available analysis spots) with the report points indicated as coloured bars are shown in Figure 6.13.

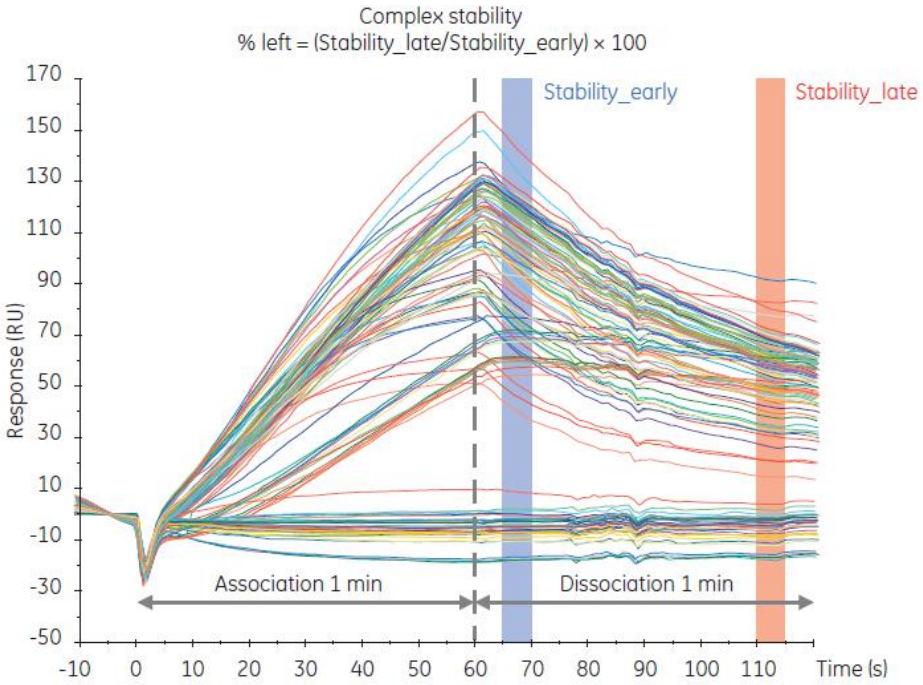

Figure 6.13: Ranking of anti-cTnI scFv fragments based on complex stability. *Sensorgrams shown represent those samples (120) passed over one particular analysis spot during each of the analysis cycles. Complex stability is determined by expressing the response units at the stability_late (Red) time as a percentage of the response units at the stability_early point (Blue).*

Typically those antibodies which exhibit a higher complex stability are those which are the most desirable. The scatterplot, shown in Figure 6.14, displays report points stability_early plotted against stability_late and allows the ranking of scFvs based on binding stability. The figure shows two main groups of scFvs, non-binders close to the origin and binders higher along the diagonal.

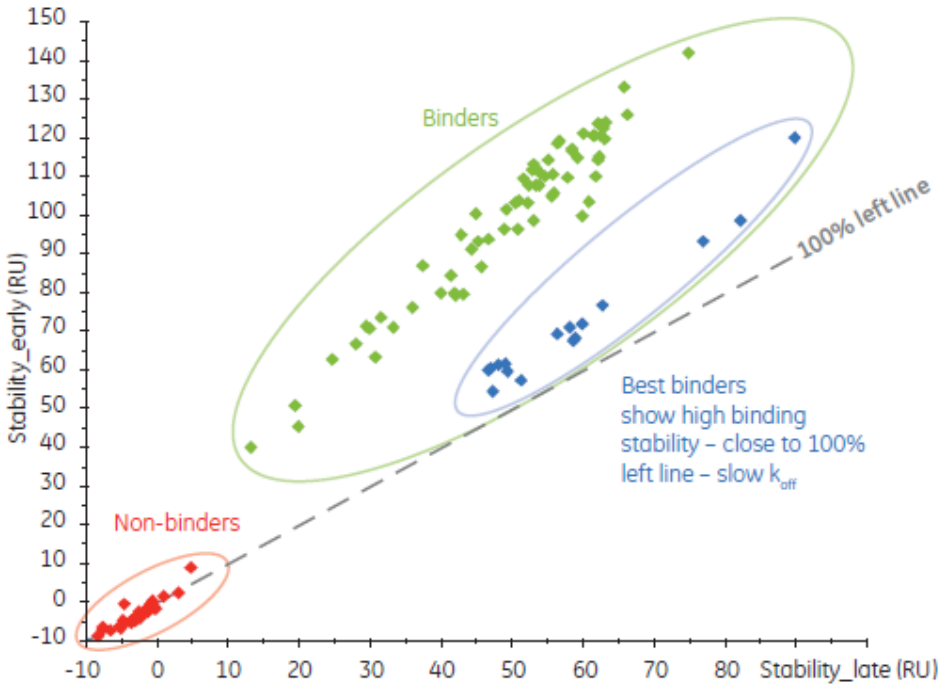

Figure 6.14: Scatterplot for identification of stable binders from a population of clones screened. *The plot is obtained by graphing Stability_early vs Stability_late. The dotted grey line denotes a 100% left (i.e. no dissociation once injection of antigen ceases) which would represent a very strong binding event. Those clones (blue squares) which are close to the 100% left line exhibit high binding stability and have a very slow 'off-rate'. Those clones denoted as green squares exhibit binding to the antigen but are less stable.*

The best binders, with a high binding stability and slow dissociation, are shown as blue squares. In total, 960 samples were analysed and ranked with respect to binding stability in 27 h. Response levels early and late in antigen dissociation are plotted on the y-axis to show the quality of the interaction of captured scFv with antigen. Those clones which exhibit poor binding are distinguished from the best clones (close to the dashed lines) which have the greatest binding stability (slow off-rates). Importantly, Biacore analysis of antibodies and their fragments allows both the expression levels of the clones and quality of the antibodies to be measured simultaneously.

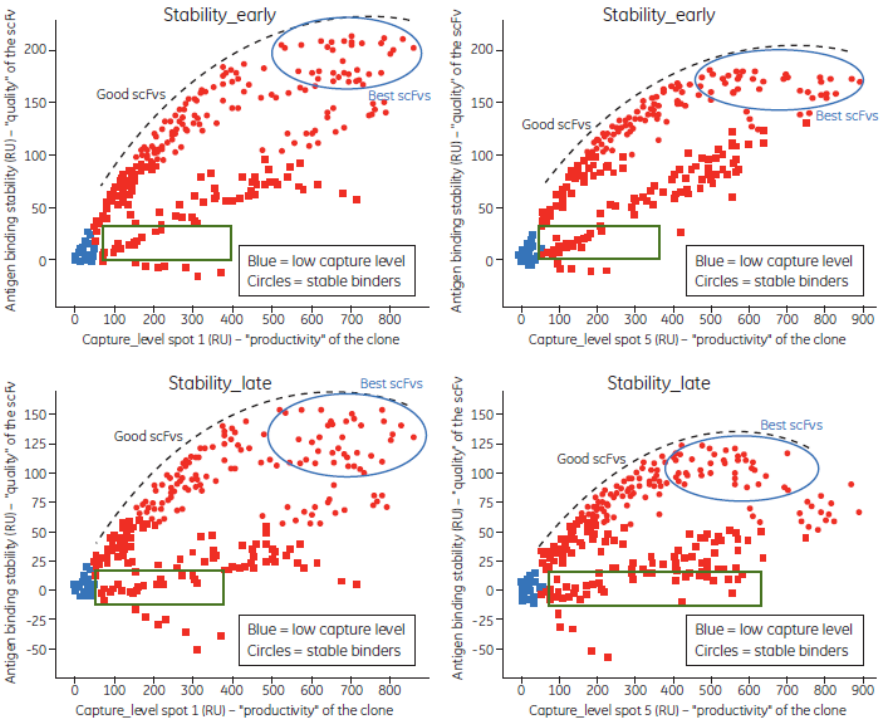

Figure 6.15: Ranking of scFvs based on antibody expression and antigen binding. *Antigen binding stability, stability_early (top) and stability_late (bottom), is plotted against capture level in spot 1 (left) and spot 5 (right). Wells without scFv binding (blue squares), with significant capture (red squares), and scFvs showing a stable antigen response (red circles) are displayed. The green box indicates scFvs that are captured, but do not exhibit antigen binding. The best scFv clones show high capture level and high binding stability (circled in blue).*

Alternative methods to analyse each antibody is shown in Figure 6.15, which gives an example of how report point data from the scFv screen can provide an excellent overview of the performance of different clones. It correlates binding levels to the initial capture level (related to level of expression) of the scFv. In certain cases a scFv with high production rates might be desirable and may alter those clones selected for further characterisation. These are important criteria when selecting high quality antibodies and antibody fragments for further development. In this study the method of ranking by complex stability was employed and used to select the scFvs for 2-over-2 kinetic characterisation, which is discussed further in Section 6.2.3.

6.2.3 Affinity ranking of scFv fragments by employment of 2-over-2 kinetics on Biacore 4000.

The top 10% binders (96) from the screening experiment, discussed in Section 6.2.2, were analysed using 2-over-2 kinetics. The Biacore 4000 Antibody Extension Package enables the setup of 2-over-2 kinetics using an additional spot-addressing functionality.

The assay setup, shown in Figure 6.16, involved the immobilisation of anti-HA antibody at two different levels (~4000 RU on spots 1 and 5, and ~2000 RU on spots 2 and 4) across each flow cell. The scFvs in cell lysate were diluted 1:4 and captured on spots 1+2 and 5+4. The antigen (cTnI) diluted to 100nM, 25nM and 0nM in HBS-EP+ was injected over the captured surface for 1 minute with dissociation monitored for 10 minutes. Data from the reference spot during each cycle were subtracted and each antigen response was then double referenced by subtracting the buffer response (zero antigen concentration) for each antibody tested, using the dedicated Biacore 4000 evaluation software. The analysis was performed at both 25°C and 37°C.

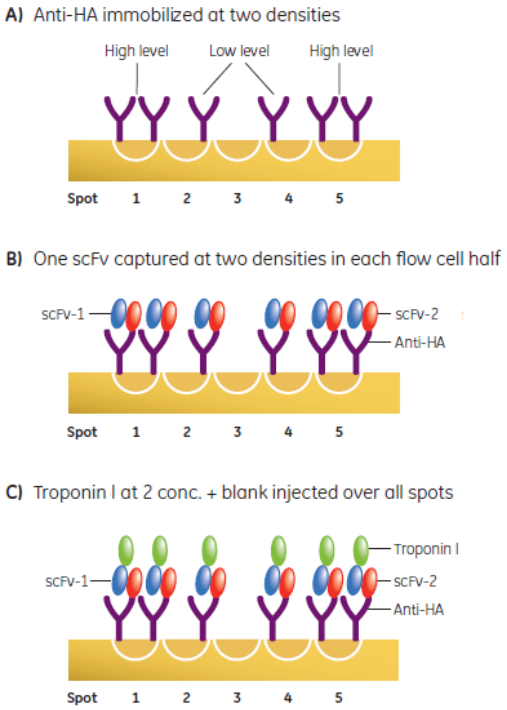

Fig 6.16: ***Biacore 4000 assay setup for "2 over 2" kinetic screening of scFv fragments.*** *Two densities of capturing molecule are immobilized on the spots in each flow cell half (higher density on spots 1 and 5, lower density on spots 2 and 4). This enables capture of two levels of antibody in each flow cell half. The 2-over-2 kinetics analysis requires 3 antigen cycles, 2 antigen concentrations plus a blank per antibody. The data are then combined and evaluated together for generation of the kinetic constants.*

Obtaining kinetics data early in the development process enables better-informed selection, since kinetic properties have consequences for both therapeutic and diagnostic agents. The 2-over-2 kinetics approach is particularly suitable for providing kinetic information already during screening, as the data can be obtained in half the time using significantly fewer reagents and smaller sample volumes. Evaluation was performed with the kinetic evaluation tool in the Biacore 4000 Evaluation Software, by combined kinetic fitting of the sensogram data from spots 1-2 and 4-5, respectively. The evaluation combines the different surface

densities with the different analyte concentrations in one fit. To investigate temperature stability of the binding, all samples have been analysed at 25°C (Fig 6.17A) and 37°C (Fig 6.17B).

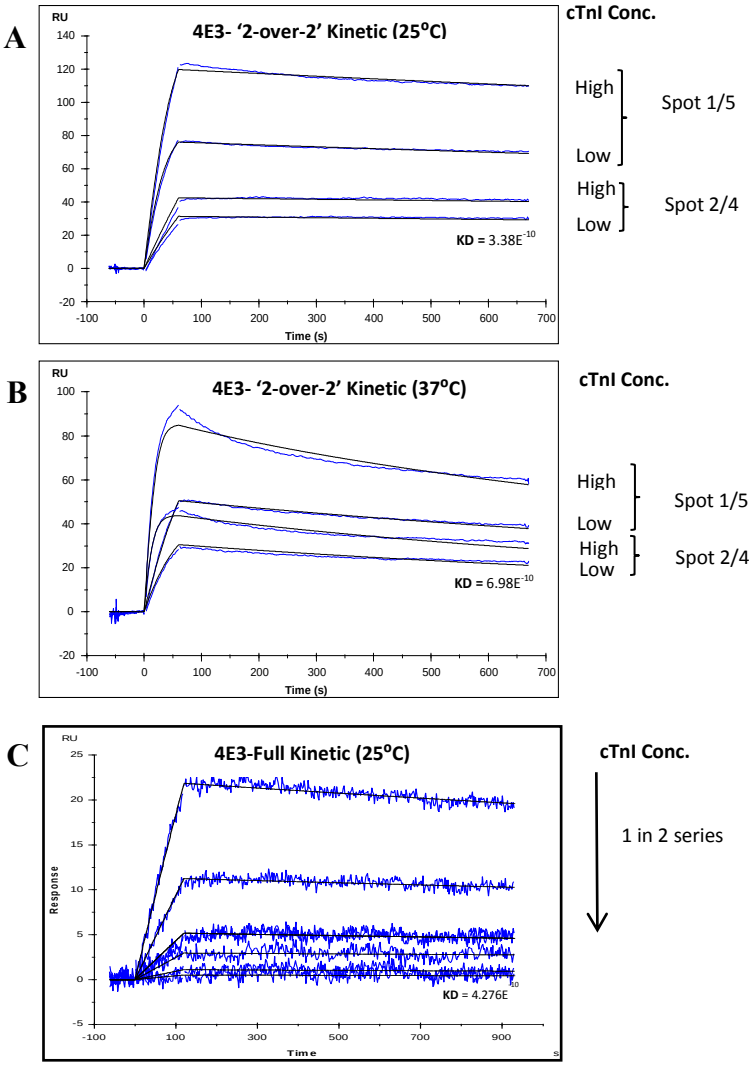

Figure 6.17: Kinetic fits for a selected anti-cTnI clone (4E3). *The overlay plots compare the performance of the same clone using a 2-over-2 kinetics run at two different temperatures to investigate stability of scFv/Troponin binding at 25°C (A) and 37°C (B) During a '2 over 2' kinetics experiment, two levels of antibody are captured in each flow cell half and two concentrations of antigen are injected over the captured antibody. The data are then combined for the kinetic evaluation. A global fit of a 1:1 interaction model to the response data is shown; the black lines represent the global fit to the response data (blue lines). The performance of the 2-over-2 approach to a traditional 'full' kinetic (C) shows that the method provides an appropriate alternative to full kinetics screening and is a beneficial method for increasing throughput in screening campaigns.*

The data was also compared in a kinetic distribution plot (Figure 6.17C) which shows the association rate versus the dissociation rate, over the range of temperatures analysed. Temperature-stable, high-affinity scFvs were identified in Spotfire using on/off-rate maps (Fig 6.18). Due to licencing issues all Spotfire analysis was carried out in GEHC and was kindly shared with DCU.

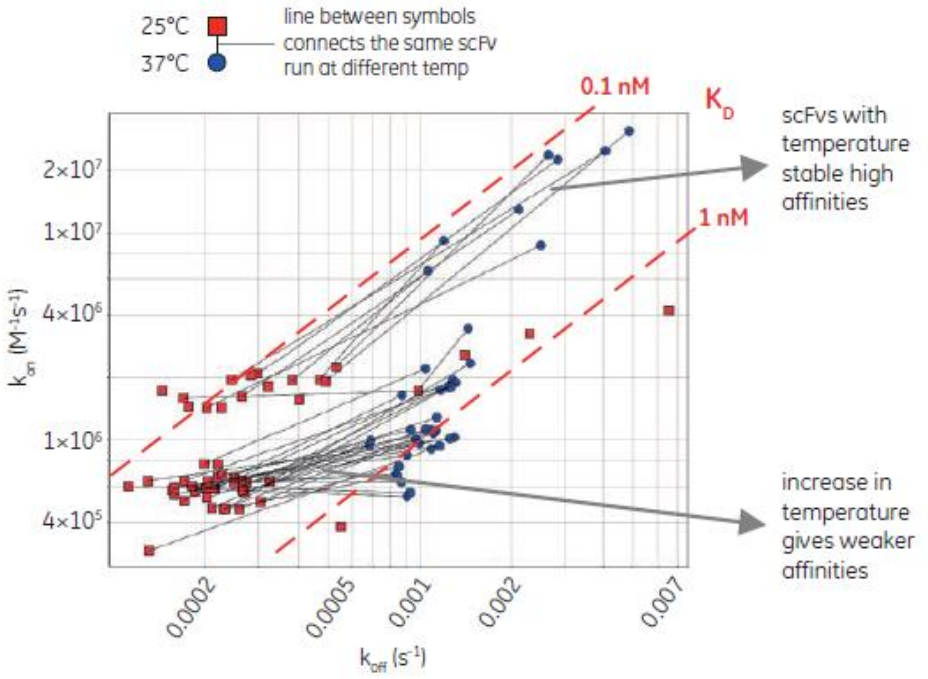

Figure 6.18: On/off-rate map for scFv antibodies specific to troponin I. *The group with temperature-stable high affinities is most likely to be robust, and preferable for use in diagnostic analyses run at varying temperatures.*

All of the antibodies displayed in Figure 6.18 have high affinities in the sub-nanomolar range. However, two groups of antibodies displaying different kinetic behaviour were identified. One group compensated for an increase in off-rate at increased temperature by a parallel increase in on-rate, thereby displaying a temperature-stable affinity, while the other group showed weaker affinity at higher temperature. Because the analyses are typically performed between room temperature and 37°C on this diagnostic platform, suitable reagents must behave similarly at both temperatures. Temperature analysis is considered important as an antibody which is sensitive to temperature fluctuations may cause variation in the final assay, dependent on the environment in which the analysis is carried out. Those antibodies which exhibit stable binding affinity over a range of temperatures, and as such are not susceptible to fluctuations in ambient temperature, are the most desirable (Leonard *et al*., 2011).

In less than 11 h, close to 100 samples were characterized with regards to on/off-rates and KD. Using the 2-over-2 kinetics approach, the best candidates, which showed temperature stable affinity, were quickly identified and could be advanced rapidly to the next stage of characterisation.

6.2.4 Epitope Mapping of cTnI scFvs

Cardiac Troponin I (cTnI) is considered a gold standard marker for damage and death of cardiac muscle. A great number of diagnostic tests have been developed and deployed but there did exist, at least initially, a large discrepancy in the results obtained for the same sample when analysed across multiple test methods. This was often attributed to the heterogeneity of cTnI forms in human blood and the difference in the epitope specificity of the antibodies utilized in different assays. This means that results obtained using different assays can differ greatly (Apple & Collinson, 2012).

Often the concentration of cTnI measured with different assays could be found to vary by orders of magnitude (10-1000 fold), for the same sample (Tate *et al*., 2010). Through efforts between national and international organizations, scientists, clinical practitioners and industrial assay manufacturers, the discrepancies observed are currently far less (Tate *et al.*, 2010).

During the standardisation process of the cTnI assays there were two important factors identified and addressed. These included the introduction of the international cTnI standard (SRM 2921) developed by the National Institute of Standards and Technology (NIST) and also a gradual "standardisation" of the epitopes detected in commercial assays. Most assays now utilise antibodies which are specific to three regions of the cTnI molecule: 23–43, 41–56 and 83–93 (Apple & Collinson, 2012). These regions are illustrated in Figure 6.19, which was kindly provided by BDI researchers following its generation using a protein modelling program, Deepview.

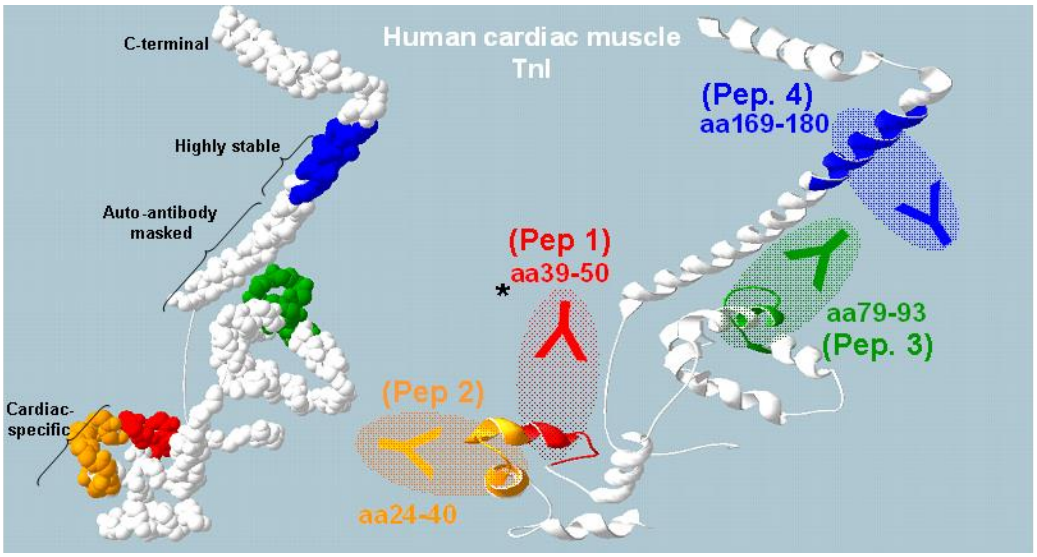

Figure 6.19: Human cardiac Troponin I (cTnI) ribbon structure. *The structure was modelled, using the amino acid sequence, in the DeepView molecular graphics program. The regions from where the three peptides used for cloning were selected are highlighted and the amino acid numbers indicated.*

6.2.4.1 Cloning of cTnI peptides

The cloning strategy, described in Section 2.6.12, involved the cloning of a cTnI peptide-GFP gene fusion into pET28b vector using *Bam*HI and *Hind*III. The peptides listed in Table 6.5 were incorporated into the 5' primer to yield the following construct 6xHis-T7 tag-Peptide-GFP-6xHis-STOP. The same back primer was used for all peptides with the forward primer (encoding peptide) changing for each peptide.

Table 6.5: Details of cloned cTnI peptides. *The amino acid sequence for each of the cloned peptides is included as well as the location of the peptide within the native antigen. The specific antibody listed is the clone name of the commercially available Hytest antibody.*

Peptide Name	Amino acid sequence	Location	Specific antibody (Hytest)
Peptide 1	KISASRKLQLKT	39-50	19C7
Peptide 2	NYRAYATEPHAKKKSKI	24-40	228
Peptide 3	QPLELTGLGFAELQ	80-94	560

Each of the peptides was confirmed to be present, by pick-PCR (section 2.2.12 and Figure 6.20) and expressing correctly by small scale expression (2mL culture) of the clone and observation of the green colour obtained from expression of the GFP, prior to large-scale expression and purification.

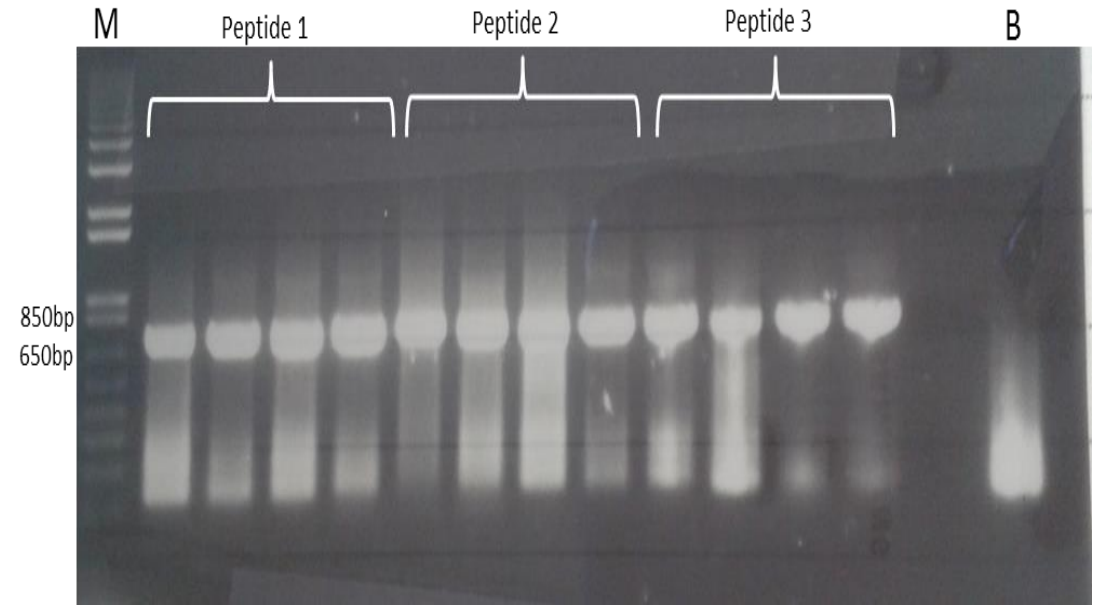

Figure 6.20: Colony pick-PCR for confirmation of insert present in transformation colonies following cloning. *This involved carrying out PCR on individual colonies, from the transformation plates, to confirm that that particular clone contained the gene of interest. Three colonies were investigated for each of the cloned peptides and all colonies contained the appropriately sized insert (~750bp). Marker used is TrackIt™ 1 Kb plus DNA Ladder (Invitrogen). B represents a PCR reaction which contained no colony material (i.e. template DNA).*

6.2.4.2 Expression and purification of cTnI peptides

Each of the three cTnI peptides was expressed in large-scale culture, as described in section 2.2.3. The level of purity obtained for each was assessed by SDS-PAGE analysis, as described in section 2.2.1, and one of the images obtained (Peptide 3-GFP) is shown as an example in Figure 6.21. Purified proteins were buffer-exchanged and concentrated using a Vivaspin column with a 10KDa MWCO. The protein concentration of each sample was then quantified at 280nm, using a Nanodrop instrument and they were aliquoted and stored at -20^{0}C for future use.

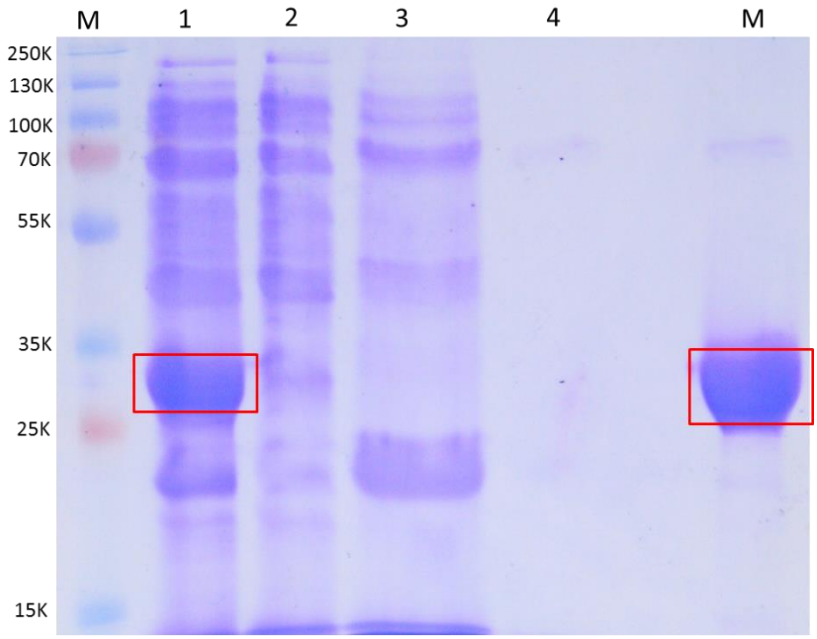

Figure 6.21: SDS-PAGE analysis of the IMAC purification of cTnI peptide 3-GFP. *Lanes designated 'M' contain the protein marker (PageRuler plus pre-stained protein ladder, sizes for each of the bands are indicated to the left of the gel picture). The desired band is highlighted on the image. Lane 1 contained the crude lysate of cTnI peptide 3- GFP, generated as described in Section 2.2.3. Lane 2 represents the flow-through from the IMAC column, containing all proteins that did not bind to the IMAC column. Lane 3 contained Wash A, which removes non-specifically bound proteins to the column. Lane 4 contained Wash B which removes any additional non-specific proteins. Lane 5 contained the eluted, IMAC purified cTnI peptide 3- GFP which had been buffer exchanged and concentrated (expected size is approximately 28 kDa).*

6.2.4.3 Screening of anti-cTnI scFv fragments against cloned and purified cTnI peptides.

Each of the purified cTnI-derived peptides was immobilised, through amine coupling, to a single spot in each of the four flow cells of a series S sensor chip. In order to test the performance of the immobilised surface, the commercial Hytest antibodies specific to each of the peptides were passed over the surface. The resulting binding profiles can be seen in Figure 6.22. In case of the Hytest anti-peptide 1 antibody there was a very large level of binding to immobilised peptide 1 (1382 RU), a certain amount of binding to peptide 2 (190RU) and no binding to peptide 3. This cross reactivity could easily be expected as the region of cTnI that the antibody targets is adjacent to peptide 1. In terms of the both the anti-peptide 2 and anti-peptide 3 commercial antibodies, their binding performance was as would be expected, showing no cross-reactivity with any of the non-targeted peptides.

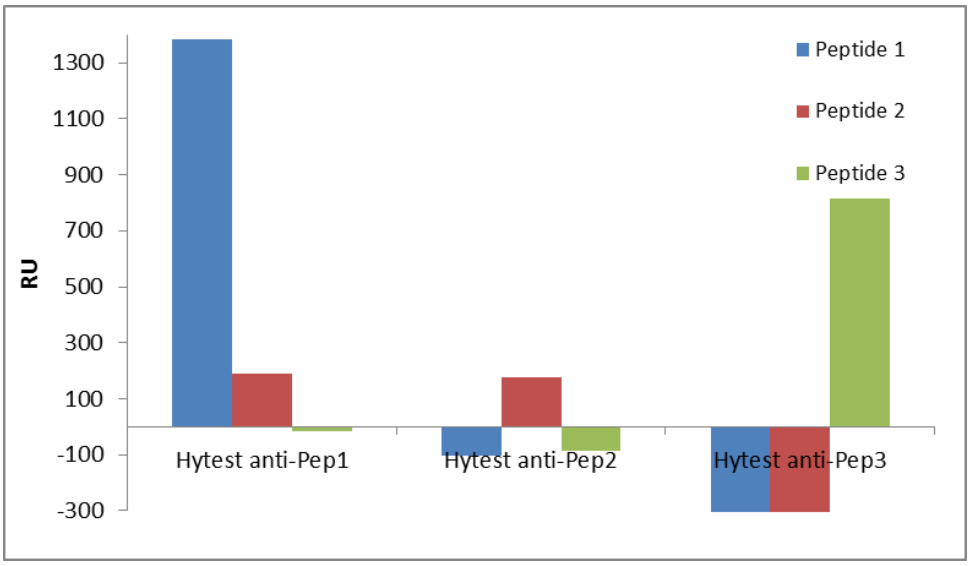

Figure 6.22: Verification of peptide binding to commercial antibodies on Biacore 4000. *The graph depicts the binding response (RU) for each of the commercial antibodies against the three cTnI peptides. This analysis was carried out prior to epitope mapping experiments on anti-cTnI in-house generated scFvs. Each of the peptides was immobilised to a CM5 sensor chip and the Hytest monoclonal antibodies passed over the surface. The peptide 1-specific antibody was 4T21-19C7, the peptide 2-specific antibody was 4T21-228 and the peptide 3-specific antibody was 4T21-560.*

The 960 anti-cTnI scFv fragments, from the high throughput stability screen, were tested against the immobilised peptides. Crude lysate for each fragment was passed over the entire flow cell, allowing four antibodies to be analysed in parallel, as shown in the raw sensogram in Figure 6.23. Binding levels for each of the peptides against each antibody were obtained following evaluation with the Biacore 4000 evaluation software.

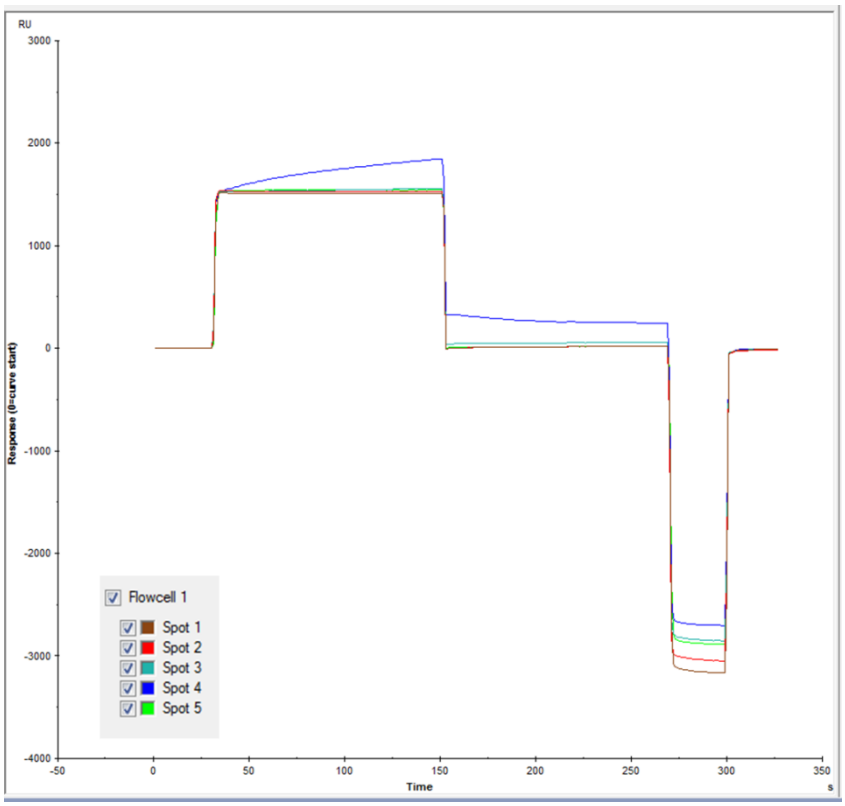

Figure 6.23: Raw sensogram taken from the Biacore 4000 epitope screen. *This view represents one scFv passing over one flow cell (FC1) with each spot/peptide represented by a different colour sensogram as detailed in the key at the bottom left. This particular clone solely binds to peptide 3.*

The binding to native antigen, obtained from the initial 10 plate screen data described in Section 6.2.2, was combined with the data obtained from the peptide screen to provide an epitope binding profile for each of the 960 antibodies screened. The resulting breakdown of binders can be seen in Figure 6.24.

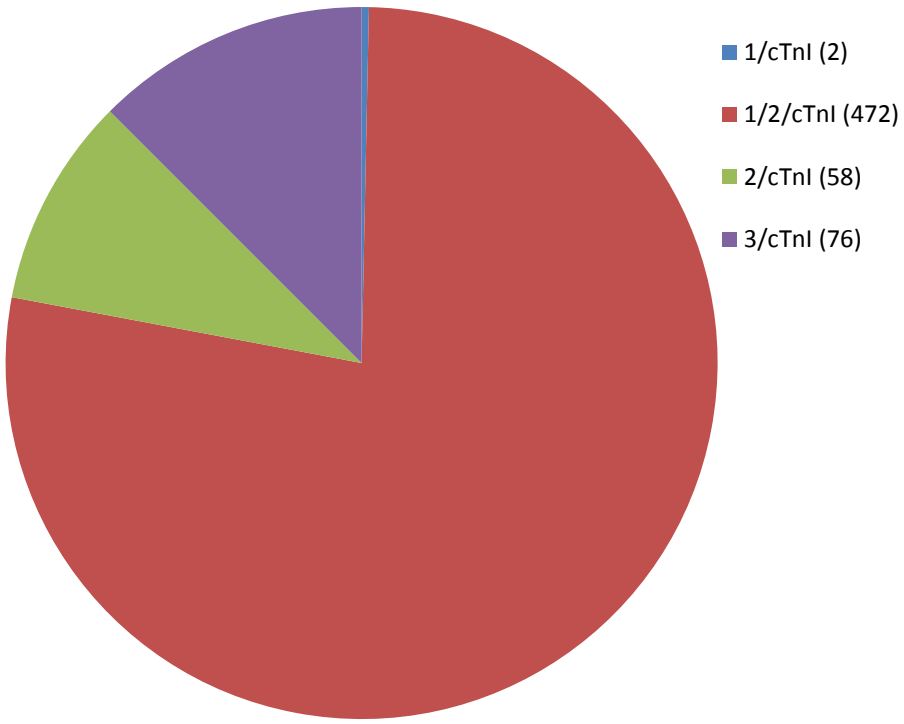

Figure 6.24: Epitope mapping of 960 anti-cTnI scFvs against three cTnI-derived peptides and the native antigen using the Biacore 4000. *Each of the antibody fragments was screened against 3 antigen derived peptides as well as the native antigen. All data was combined and the trend of binding profiles plotted. The predominant group appears to comprise those fragments which bind peptide 1 and 2 as well as the native antigen (472).*

Of the 608 fragments which bound the native antigen, the peptide specificity of each of the fragments could be defined. The data provide a detailed breakdown of the antibodies present within the library. In terms of value, this screen can be very useful in identifying the correct antibodies for a potential assay while quickly discounting a large number of unsuitable fragments (e.g. cross reactive with peptide 1 and peptide 2). What is perhaps most interesting to note is that when this library was screened using traditional methods (phage display followed by monoclonal ELISA analysis) the library was thought to be hugely biased towards peptide 3 specific fragments as those in the final selected clones were all directed toward that region. By looking at this screen one can see that peptide 3 binders account for only 76 of the total 960. What is interesting when seeing the full analysis is that peptide 3 binders show very high levels of capture on the surface (related to antibody expression), explaining why they may be have selected as the "best" binders in ELISA. In addition only peptide 3 binders have a level of binding which exceeds 250 RU (up to a maximum 2000RU); the majority of other binders have a response close to 100RU for their respective epitopes. This point helps to reiterate the need to carry out high-throughput screens, of this type, on larger numbers of antibodies early in the screening and selection process to ensure rare and/or valuable antibodies are not overlooked.

6.2.5 Summary for key challenge 6.2

The high capacity of Biacore 4000 allowed sampling of a large diversity pool from phage displayed combinatorial antibody libraries. In just over 27 hours, 960 samples were screened and ranked based on binding stability. The top 10% from this analysis were then taken forward for 2-over-2 kinetic analysis which provided on-rates, off-rates, and affinity for 96 scFvs in approximately 11 hours. Crude sample preparations were analysed without the need for purification, saving both time and resources. The capture-based approach minimizes assay development time and allows the convenient analysis of multiple analytes or antibody batches in a single analysis. The results show that kinetic data enables better-informed selection of antibody fragments. Off-rate-based screening for initial selection and rapid on- and off-rate mapping for characterisation, over a range of temperatures, are the keys to success in antibody development. In a separate epitope mapping experiment of 960 scFv fragments, carried out in just over 14 hours, a valuable insight into the specific binding properties of each of the individual fragments was provided. All this taken together demonstrates that the Biacore 4000 can provide the throughput and data quality needed to resolve the analysis bottleneck typically observed in the screening workflow of this type.

Conclusion and Significance:

In this chapter two key research challenges were addressed, both concerned with demonstrating the benefit of utilising improved methods (i.e. Automated or high-throughput) in the selection and characterisation of recombinant antibody fragments.

The first challenge involved the use of Beckman Coulter Biomek 2000 laboratory automation workstation for completion of a standard ELISA. Typical ELISA analysis can be both labour intensive and prone to human error or variation. By automating the process it was hoped that these factors could be reduced or even eliminated. The entire assay development was carried out by implementing specifically written programmes on the Biomek 2000. Each programme was designed to utilise the dedicated workspace and labware in the most simple and efficient way. All programmes were also tested several times with buffer prior to use with actual reagents to ensure that all operations performed as expected over multiple iterations. The workstation was used to perform all filling (coating, blocking), dilution (serial dilutions for standard curves) and washing steps, significantly reducing the required input from the end user and allowing for a high level of robustness overall from the assay.

The accuracy and precision of the method were thoroughly investigated through intra- and interday analysis and found to be suited to the final application for the assay (i.e. determination of CRP levels in serum). The precision of the standard curve generated was tested and performed within recommended guidelines in terms of intraday (1.12 to 6.12% CV) and interday (6.92-16.83% CV) precision, Figures 6.7 and 6.8, respectively. The accuracy of the method was also investigated by testing the ability to correctly quantify the level of CRP in spiked samples using the standard curve generated. In terms of accuracy the method performed particularly well, in both buffer and serum, with values shown in Table 6.1 and Table 6.2. The accuracy of calculation for intraday analysis in buffer were between 4.61% and 5.97%, with interday values at 1.29 % based on three different analysis days. In serum, while slightly less accurate than in buffer, the assay also performed at a high level in both intraday (5.04-6.98%) and interday (5.74%) analysis.

Once both the programmes and assay had been completely optimised and verified, as described above, the assay was employed for use with clinical samples. These samples were obtained from the Mater Misericordiae University Hospital in Dublin. Prior to their arrival in DCU they were analysed as per standard clinic practices, and the levels of CRP determined, using Abbott ARCHITECT c16000 Clinical Chemistry Analyser in a turbidometric assay format.

Due to the high-sensitivity of the CRP sandwich assay developed in this chapter and the wide range of CRP levels (2-21 mg/L) in the samples, it was necessary to carry out preliminary investigations on the level of dilution required to allow for accurate quantitation of the levels of CRP in future "unknown" clinical samples. By carrying out this analysis it was possible to identify a dilution factor (between 1 in 625 and 1 in 3125) which would allow for use of the assay for all CRP levels (Table 6.3). The samples were then diluted to a value within this range (1 in 2000), tested and the CRP levels quantified. The assay performed particularly well in this set of experiments and the quantitation of CRP across the range was highly accurate (100-105 %) and precise (1.2-3.78 % CV), as shown in Table 6.4.

Based on the findings of this study, the use of the anti-CRP recombinant scFv A6 combined with the Beckman Coulter Biomek 2000 laboratory automation workstation helped to deliver a highly precise, accurate and robust assay for the determination of CRP levels in serum samples of patients.

The second challenge addressed in this chapter was to establish a high-throughput screening protocol, for recombinant antibody fragments, utilising the Biacore 4000. A set of antibodies, against cardiac troponin I, were analysed throughout the study. The ultimate goal of the analysis was to develop a method for high throughput screening that could be used to streamline the antibody discovery process irrespective of target analyte. With this in mind the screening campaign was designed to provide the greatest amount of information, for each individual antibody, in the most efficient manner.

In terms of this particular analysis, desirable qualities of antibodies would include a high level of binding stability (fast on-rate and slow off-rate), high affinity and binding to specific areas within the native antigen. The analysis involved the ranking of 960 scFv fragments based on their binding stability (Figure 6.13), the top ten percent of those ranked (96) were then affinity ranked utilising a "2-over-2" kinetic screen which proved much faster than a traditional kinetic screen while providing a similar output in terms of kinetic constant values for each of the clones (Figure 6.17). Due to the high-throughput nature of the "2-over-2" approach it was carried out over several analysis temperatures (Figure 6.18), further narrowing the number of clones which behaved in a desirable fashion (i.e. affinity and on/off-rates remained stable despite a shift in analysis temperature). All antibodies demonstrated picomolar affinity for the native antigen at 25^0C and ranged from high nanomolar to picomolar affinity when analysed at 37^0C.

In addition to ranking and definition of the affinity of the antibodies, an epitope mapping experiment was carried out to identify exactly where on the antigen the scFv fragment bound. This involved the cloning and expression of three different cTnI-derived peptides,

described in section 2.6.12, and their subsequent immobilisation on a Biacore sensor chip. The scFv lysate were then passed over all the peptides and their specificity profiles determined (Figure 6.24). It was interesting to note that, when compared with the typical screening approach usually undertaken in the laboratory (i.e. predominantly ELISA characterisation with Biacore kinetic analysis for a small number of clones), the clones identified were more diverse in their binding properties.

In addition to the advantage of a greater level of knowledge obtained for each individual clone, all of the screening programmes mentioned were carried out in relatively short time frames. There is a minimal time commitment from the user once programmes are designed and reagent and sample plates prepared and loaded to the instrument, as the analysis does not require any additional input until evaluation. In all, the entire screening campaign, taking into account the need to immobilise three different chips to allow for the analysis, could theoretically be completed within a week. This offers a very attractive alternative to the traditional ELISA-based approaches which can often take considerable time and effort to complete effectively. These and further anti-cTnI antibodies developed at the BDI were screened, using this very tailored approach, by the dedicated cardiac biomarker team and the best antibodies licensed to industry. This further development and characterisation was reported in Conroy *et al*. (Conroy *et al*., 2012).

Overall, the two key challenges of this chapter have been rigorously investigated. The establishment of suitable screening protocols, automated (Biomek 2000) or high-throughput (Biacore 4000), for each set of equipment was completed. The use of these protocols and procedures could easily be implemented for all users to allow for a higher degree of certainty of finding and accurately characterising the optimal antibody for each individual application or project. Also noteworthy is the higher level of repeatability afforded by the introduction of robotics/liquid handling allowing for more reliable results and improved efficiencies within traditional screening approaches.

Chapter 7

Overall Conclusion

Monoclonal antibodies are now the largest class of biological drugs with at least 36 monoclonal antibody drugs approved by the FDA, and are currently responsible for almost 40% of the market (Aggarwal, 2014) . With the antibody discovery field expanding year on year, in the area of biological therapeutics, and hundreds of potential new antibody drugs in clinical trials it is expected that antibodies will drive in excess of 50% of total market growth within the pharmaceutical industry over the next 3-4 years.

The numerous ways by which to generate monoclonal antibodies have been discussed in the earlier chapters of this thesis and it can be said that there are a plethora of options available to the community, each with advantages and disadvantages associated. The two gold standards have been phage display and hybridoma technology coupled with cloning by limiting dilution. They have demonstrated through their uptake across the general community that they are amenable to use in all research situations, whether based in commercial, industrial or academic laboratories.

In comparing DiCAST with the traditional methods, the benefits of utilising a single cell screening tool of its type are clear as discussed further below.

The disadvantages of phage display as an antibody selection tool, such as the potential high level of non-specific phage and the presence of truncated clones, have been discussed in Chapter 4 and provide evidence of the need to establish new approaches for the screening of antibody libraries which have been generated recombinantly and are expressed in bacteria. DiCAST offers this option, by allowing the spatial addressing of millions of individual bacterial cells each secreting a monoclonal antibody. A direct comparison of the two approaches is shown in Table 7.1.

In terms of recombinant screening, the ability to address cells on a single cell basis is highly novel. The large size and diversity of the libraries generated means that only ultra-high throughput methods, such as DiCAST, FACS, next generation sequencing or micro-droplet platforms, can screen the number of cells required to properly probe any given library.

Table 7.1 Comparison of phage display and DiCAST for recombinant screening.

Recombinant Screening		
Antibody Source	Bacterial antibody fragment library	
No. of starting cells	1×10^8 - 1×10^{10}	
Method	**Phage display method**	**DiCAST method**
Selection process	2 to 4 rounds of enrichment by biopanning	0-1 round of enrichment by biopanning
Time (days)	3 – 8	0 – 2
No. of wells per plate	96-384 wells	950,000 - 10 million capillaries
No. of single cells per plate	96-384	~300,000 – 3 million
Primary screening assay type	Binding	Binding, cross reactivity and/or functional in one screen
No. of surfaces for analysis	1	2 or more
Min. time for mAb detection	1 day	<6 hours
Antigen required per plate	24 – 48 µg	1 – 4 µg
Test volume per well	75 – 200 µL	80 pL – 1.2 nL

As discovered in Chapter 4 (Section 4.1.2) it is entirely possible to find the same as well as superior antibodies from a particular library, in a much improved time frame, when comparing DiCAST to phage display. An additional advantage is the fact that antibodies which are selected are suited to soluble expression, which is important when moving on to characterisation and potentially large-scale expression. Furthermore, all antibodies can be addressed without a bias towards those which are more suited to expression on the surface of a bacteriophage.

Practical considerations such as the amount of antigen required for screening and the opportunity to increase the numbers of assays carried out simultaneously by harnessing the unique multiplexing features available also make DiCAST a favourable alternative to the traditional approach of phage display.

In terms of screening of mammalian cells, which include hybridoma cells, immortalised cells or native B cells, the traditional approach that is often taken is cloning by limiting dilution which has been described in Chapter 1. While progress in areas of laboratory automation and liquid handling robotics have allowed for the laborious and time-consuming nature of this method to be addressed, there are still questions pertaining to the overall effectiveness of plating cells at densities which tend to favour those that grow quickly without any preference for those that are secreting a desirable antibody.

When comparing the DiCAST approach for screening of mammalian cells with the typical cloning by limiting dilution method (Table 7.2) it is not difficult to identify the distinct advantage that microtools, such as DiCAST, present. The first distinct advantage is the time taken for screening. Because of the need to grow cultures for between 2 and 3 weeks per round of cloning and the fact that several rounds of cloning are typically required, DiCAST

provides a highly attractive substitute to the traditional approach. When this is coupled with the added improvements in areas of throughput and multiplexing as well as reduction in antigen required and overall footprint in terms of laboratory space (i.e. A microscope slide sized microcapillary array is equivalent to 10,000 x 96 well plates) the use of DiCAST for mammalian cell screening can be seen as an much more efficient method.

Table 7.2 Comparison of cloning by limiting dilution and DiCAST for mammalian cell screening.

Mammalian screening		
Antibody Source	Heterogeneous mix of hybridomas, native B cells or immortalised cells	
No. of starting cells	1×10^5 - 1×10^8	
Method	**Traditional method**	**DiCAST method**
Selection process	Culture of thousands of cells per well followed by 1-3 rounds of cloning by limiting dilution	Direct immunoanalysis of single cells in microcapillaries
Time (days)	14-56	1-3
No. of wells per plate	96-384 wells	950,000 - 2.5 million capillaries
No. of single cells per plate	96-384	~300,000 – 900,000
Primary screening assay type	Binding	Binding, cross reactivity and/or functional in one screen
No. of surfaces for analysis	1*	2 or more
Min. time for mAb detection	3 – 7 days	<6 hours
Antigen required per plate	24 – 48 µg	1 – 4 µg
Test volume per well	75 – 200 µL	300 pL – 1.2 nL

*Supernatant from each well could technically be analysed multiple times in separate ELISA-based assay but is limited by the amount of supernatant available from each well and the labour intensive nature of the screening.

Despite its limitations, the use of cloning by limiting dilution and other microtitre plate-based screens continue to be utilised, alone or in combination with other techniques, to screen antibody-secreting cells for antibody discovery. Indeed several companies which are leaders in the field (e.g. AdiMab, AiMM Therapeutics, Anaptys Bio, Biotem, Humab Biomed, Immunome, Novimmune, Fabrus, Theraclone, Trellis Bioscience and UCB) utilise some form of microtitre plate based method as part of their screening programme. It should be mentioned that each of these companies hold their own unique selling points and have amended and improved the typical process in one way or another.

For instance UCB Pharma, carry out an initial ELISA-based screen on a particular B cell population, plated at a high density of cells per well, to identify cells which contain a B cell clone of interest. Once identified the well is screened using what is termed the fluorescent foci method, which involves placing the cells on a glass slide with a labelled antigen (e.g. an antigen immobilised on a bead or a surface bound receptor on a cell) and allowing them to incubate together for approximately 2 hours (Lightwood *et al*., 2013). As the B cells begin to

secrete IgG, those producing antibody specific to the labelled antigen will develop a halo of fluorescence. These individual cells are then removed, and the genes captured by RT-PCR, using a micromanipulator (Clargo *et al*., 2014). UCB Pharma also employs a high level of automation within their screening facility to enable efficient initial screening (Presentation given by Daniel Lightwood at HAH conference in 2014).

Another interesting approach is that used by Trellis Biosciences, who employ their CellSpot™ assay, which utilises beads with a number of different biological components attached in a highly multiplexed manner. Cells are placed on a membrane and overlaid with a semi-solid medium effectively immobilising them in the well. The antibody secreted from each cell is captured on an antibody-coated slide beneath the membrane and is probed with multiple labelled beads. Cells of interest are allowed to reach a colony of approximately 10-50 cells and are retrieved for processing by micropipette (Harriman *et al*., 2009).

While these traditional approaches have stood the test of time and remain in use today, there has been a shift in the focus of the community towards increasing the throughput, sensitivity and information garnered for each antibody-secreting cell screened. As single cell analysis has become more commonplace, novel ways in which to screen and interrogate each cell within a population have emerged and are now becoming the cornerstone technologies and platforms of leading companies in the antibody discovery space, either replacing or complementary to traditional approaches (Table 7.3). A number of these single cell, high throughput techniques are discussed in greater detail below.

FACS has developed from its conception in 1969 (Hulett *et al.*, 1969) into a very well established and supported technology for the high-throughput analysis of heterogeneous cell populations from various sources (e.g. blood, tissue, antibody-secreting cells). It is widely used alone or in combination with complementary methods by a number of antibody discovery companies (e.g. AdiMab, AiMM Therapeutics, Anaptys Bio, Biotem and ImmunoQure among others) and with sorting times as fast 10,000 cells per second it is not difficult to see why it is so popular.

The major advantage of using FACS is the ability to sort a very large number of cells on a single cell basis in a relatively short space of time. The technology is also very amenable to multiplexing, with typical analysis capturing up to 20 different criteria (e.g. cell size/shape, nucleic acid content, membrane integrity, cells undergoing apoptosis etc.) in a highly sensitive and efficient manner. The limitations of the technology as a means to screen antibody libraries tends to focus on the sensitivity of the method, with loss of potentially interesting clones often attributed to high levels of noise (Harriman *et al*., 2009). In addition the ability to detect secreted products is somewhat limited, as reliable detection is generally

carried out using intracellular or cell surface based targets. A previous disadvantage of FACS arose in the retrieval of cells, as cells are typically collected as bulk populations and would require subsequent characterisation of the sorted populations. Machines which allow single cell retrieval are now available and have begun to negate this issue.

The relatively recent emergence of novel microtools to circumvent the limitations of traditional screening approaches has led to the discovery of many very interesting antibodies and also has allowed a much more in-depth analysis of the immune system (Ogunniyi *et al*., 2009; Varadarajan *et al*., 2012).

All microtools, irrespective of their classification (i.e. microwell, microdroplet or microfluidic chamber) have inherent advantages such as lower consumption of reagents, shorter analysis time (attributed to faster accumulation of detectable target in the reduced volumes), compatibility with multiple cell types, amenability to multiplexing and the all-important ability to screen cells on an individual basis. The different classifications each present their own advantages and disadvantages when compared to each other in Table 7.3, and as discussed below.

Microtools utilising microwells, described in Chapter 1, involves the seeding of individual cells into a chip containing microengraved wells and analysing their response either directly in the well or by capturing secreted proteins on a slide sealed to the chip. Once identified cells of interest are retrieved using a micromanipulator and are either expanded in culture or the genetic information captured by RT-PCR. Some approaches, such as those carried out by the company Single Cell Technologies (http://www.single-cell-technology.com/) lyse the target cell in the microwell and carry out RT-PCR *in situ*. The variable regions in each well are then sequenced and the antibody sequence related back to the response obtained in the assay.

A number of successful applications of this approach are described in the literature. For instance, the ISAAC (Immunospot Array Assay on a Chip) method described by Jin *et al*. describes the use of microwells for the discovery of biological therapeutics and is the cornerstone technology of the antibody discovery company Valneva (Jin *et al*., 2009). Valneva markets a vaccine against Japanese encephalitis which was approved in the U.S., Europe, Canada, Hong Kong, Singapore, and Israel under the trade name IXIARO® and in Australia and New Zealand, where it is marketed as JESPECT® (http://www.valneva.com/).

Table 7.3 Comparison of microtools currently available

Name	Microtool Category	Throughput	Assay format	No. of assay surfaces	Assay Type	Cell recovery	Publication/Website
DiCAST	Microcapillaries (80 pL - 2 nL)	1-10 million capillaries per chip	Surface Homogenous	≥2 *	Binding Functional	Automated (every 3 seconds); Single cell	Fitzgerald *et al*. (2015). Analytical Chemistry; 87, pp 997-1003
HiFiBio	Droplets (50 pL)	>1000 droplets per second	Homogenous	N/A	Binding	Collected as sorted by FADS; Bulk or single cell	Mazutis *et al*. (2013) Nature Protocols; 8 (5), pp 870-891
Sphere Fluidics	Droplets (pL to nL)	Up to 1 billion droplets per screen	Homogenous	N/A	Binding Functional	Collected as sorted by FADS; Bulk or single cell	http://www.spherefluidics.eu/index.php
Enumeral Biomedical Inc.	Microwells (100 pL- 1 nL)	25,000-234,000 per chip	Surface	≥1 *	Binding	Micromanipulator (>1 minute); Single cell	Love *et al*. (2006) Nature Biotechnology 24 (6); pp703-7
Single cell technologies	Microwells (100 pL- 1 nL)	30,000 cells per chip	Surface	≥1 *	Binding Functional	RNA extraction carried out on chip; single cell (VH and VL paired)	http://www.single-cell-technology.com/
Valneva	Microwells (100 pL- 1 nL)	62500 wells per chip	Surface	1	Binding Functional	Micromanipulator (>1 minute); Single cell	Jin *et al*. (2009) Nature Medicine; 15 (9), pp1088-1092
Abcellera	Microfluidic chambers (4.1 nL)	8092 chambers per chip	Homogenous	N/A	Binding Functional	Automated micromanipulator (>1 minute); Single cell	Lecault *et al*. (2011). Nature Methods; 8 (7), pp 581-586

Notes: All technologies are utilised for single cell screening. All technologies are amenable to multiplexing assays. All technologies can be used to screen native B cells, immortalised cell lines and hybridomas with DiCAST and the droplet technologies also capable of screening bacterial cells (mostly due to throughput required). (* Surfaces can be removed and replaced with additional prepared surfaces to allow for added assay throughput and functionality).

The numerous papers from Christopher Love's laboratory describe the utilisation of microwell arrays for the interrogation of not only antibody-secreting cells (Love *et al*., 2006; Ogunniyi *et al*., 2009; Story *et al*., 2008) but also the dissection of the immune response by profiling the T cell population on a single cell basis (Varadarajan *et al*., 2012). Their work formed the basis for the company Enumeral, who are currently generating novel antibody immunotherapies, or immunomodulators, targeting checkpoint proteins (e.g. PD-1, OX40 and Lag3) (http://www.enumeral.com/).

In comparison with DiCAST the microwell approach has several disadvantages, of which the main element is throughput, as chips must be fabricated and the number of wells is currently limited to approximately 90,000 or fewer individual microwells. The ISAAC method is limited to only one analysis surface, as the halo of fluorescence is detected on the chip, which again negatively effects throughput and information that is obtained from each screen.

Microdroplets are another microtool approach which has been applied to the discovery of antibodies in recent years. It involves the isolation of individual cells by capturing them in two-phase droplets which range in volume from picolitre to nanolitre (Mazutis *et al*., 2013). They can be used for single cell analysis in a range of applications from antibody and cytokine secretion to gene expression or enzymatic activity (Love *et al*., 2013). Droplets are sorted by FADS (Fluorescence activated droplet sorting) which works on the same principle as FACS but overcomes the limitation of detection of secreted product as the secreted product is also contained within the droplet. Interestingly, passive droplet fusion, electro-coalescence, and pico-injection have all been utilised to allow addition of reagents at a certain time point which adds to the diversity of assays which can be carried out in droplets (Mazutis *et al*., 2013). Desirable features of this screening approach include the highly rapid analysis (i.e. >1000 droplets per second) and also the diversity of analyses that can be carried out in the droplets, including the ability to lyse the cell in the droplet and carry out RT-PCR *in situ* (Hatch *et al*., 2011).

In comparing the approach with other microtools there are a number of disadvantages when adopting the use of microdroplets for screening of antibody-secreting cells. The approach is only compatible with homogenous assays which can be carried out in solution without the need for washing steps. Additionally the generation and manipulation of the droplets requires very precise conditions and cells are typically collected in bulk as they are sorted, likely requiring additional rounds of screening to isolate the desired monoclonal antibody.

Nevertheless, El Debs *et al*. describe the use of micro-droplets to successfully screen approximately 300,000 individual hybridoma cells against ACE-1 which is associated with

blood pressure regulation and the development of vascular pathology and remodelling (El Debs *et al*., 2012).

There are also a number of companies which describe the use of microdroplets in biological discovery. HiFi Bio offer a range of screening options that include antibody discovery (Droplet mAb) as well as other interesting application of microdroplets such as whole genome chromatin DNA mapping from a single cell (Droplet ChIP-seq) and single cell, high fidelity dose response screening (http://www.hifibio.com/).

Another company, Sphere fluidics, offer a service model which can either carry out the discovery project for a customer or facilitate the design of a specific analysis biochip. They also supply a number of off-the-shelf products including specialised chemical reagents and polymer picodroplet biochips to facilitate single micro/picodroplet screening (http://www.spherefluidics.eu/index.php).

The use of microchambers constitutes the third microtool category that is currently available for single cell screening. It involves the capture and of single cells in microchambers, linked by a microfluidic system, and the performance of bead-based assays to detect specific antibodies produced. This approach, while limited to utilising homogenous assays, overcomes the associated lack of sensitivity seen in microdroplet or *in situ* microwell assays by allowing washing steps to be incorporated. Additionally, medium exchange can be carried out, if required, facilitated by the unique microfluidic valve system.

Some disadvantages associated with this approach when compared to other methods include the requirement for manufacture of highly specialised chips. To scale-up such a complex device would be difficult, if possible at all, and the chip size is currently limited to somewhere in the range of 10^3 chambers per chip (Lecault *et al*., 2011). Other limitations include the difficulty of single cell seeding as the chambers are linked and also that manual visualisation of the chip is required to detect a specific response. This factor slows down the overall processing time and ultimately reduces throughput.

Microchambers have been extensively employed for diverse uses such as antibody discovery and affinity ranking (Singhal *et al*., 2010), stem cell proliferation (Lecault *et al*., 2011), cell signalling (Ricicova *et al*., 2013), RT-qPCR of single cells (White *et al*., 2011) and miRNA analysis of single cells (Petriv *et al*., 2010). These studies, among others, have led to the establishment of the company Abcellera which offers an antibody discovery model through collaboration with pharmaceutical companies using their microchamber platforms (http://abcellera.com/).

Our newly developed Direct Clone Analysis and Selection Technology (DiCAST) represents a new class of microtool and delivers fully customisable deep screening of cell libraries. By isolating, culturing and interrogating up to 10 million antibody-secreting cells in a nanoscale self-contained environment per experiment, the screening avoids any bias typically introduced in traditional methods, which analyse cells in bulk, and is applicable to both prokaryotic and eukaryotic library systems.

DiCAST represents the first described incidence of screening single antibody-secreting cells using microcapillaries (PCT Patent Application No: PCT/EP2011/062015) and is entirely novel in that respect. DiCAST offers an enhanced option when compared with the other microtools currently described, within the industry as a whole, as the throughput is much greater than many of them (i.e. they are typically limited to numbers close to 10^5 single cells) and the analytical output has the potential to also exceed those of close competitors.

The DiCAST technology has several advantages over its direct competitors including the fact that microcapillary arrays are an off-the-shelf product available commercially, meaning that capillary size and density can be varied, making it highly scalable to increase throughput. The straw-like nature of the arrays also offers up unique advantages in two respects. The first is the ease of retrieval, with contents easily removed by application of force on an opening and capture of the ejected contents on the opposite side. The second is that dual openings lend themselves to very efficient multiplexing with the associated methods (i.e. multi-analyte, multi-Lift and especially double-sided analysis) which have been described in Chapter 3.

Apparent disadvantages such as the requirement for high precision alignment, a custom prototype for accurate retrieval of the contents of a single target capillary, the need for specialised software for interpretation of data and the ability to carry out complex assays on a hydrophobic surface have all been overcome in the process of developing the technology. This demonstrates that they were not insurmountable and that the unique advantages afforded by the use of microcapillaries for antibody discovery far outweigh any potential limitations.

In screening such a high number of antibody-secreting cells the final number of selected, interesting antibodies can also be high. Through clever design of the screening assay on DiCAST, by building in several assays through the multiplexing options available, it is possible to select only those antibodies which meet a very specific list of criteria. It is hoped by screening in this way that the number of antibodies that will be taken forward following this initial selection round will be kept to a manageable level. Even so during the course of

this work a number of high throughput and automated approaches for antibody characterisation to fully complement the DiCAST screening process have been developed.

The first approach was the development and testing of protocols for the implementation of high-throughput Biacore screening. Biacore has become the household name for SPR analysis and their range of instruments cater to users of varied requirements (i.e. low throughput research instruments amenable to troubleshooting and fine-tuning as well as high throughput instruments with standardised reagents kits allowing ease of use and a high level of reproducibility). The techniques discussed in Chapter 6 allow for an efficient and informative analysis of a large number of clones (960) in a relatively short period of time, utilising the high throughput Biacore 4000 instrument. The use of crude lysates for both binding and kinetic analysis, using a capture format, allows for the antibodies to be screened without the need for any complicated or laborious purification procedures, further reducing the time to select and characterise antibodies of interest.

The second involved performing assay development using a Beckman Coulter Biomek 2000 laboratory automation workstation. Optimisation of programmes for automated platforms such as this gives the opportunity to further streamline the entire process by removing the need for users to carry out ELISA-based methods manually. The correct use of automation, implementing fully optimised protocols, can also allow for a greater level of precision and reduces the variability often encountered between different users and laboratories. Its description within this thesis aimed to demonstrate that automation, in this case of an assay for C-reactive protein in clinical samples, helped to provide a robust and reliable assay for potential clinical use.

Table 7.4 Comparison the antibody discovery technologies from companies generating monoclonal antibodies for therapeutic use.

Name	Disease Area	Approach	Additional Offerings
Abcellera	As requested (service model)	Microfluidic Chamber single cell screening	
Adimab	As requested (service model)	Yeast Display (Surface/FACS or Secretion/ELISA modes available)	Ultra HT FACS (10, 000 cells per second)
AiMM Therapeutics	Cancer Infectious diseases	FACS/ELISA	Proprietary immortalisation method AIMProve (Improve affinity/stability) AIMLink (Develop bispecifics or ADCs)
Anaptys bio	Cancer Inflammation	FACS/ELISA	SHX-XEL (In Vitro somatic hypermutation platform)
Arsanis Biosciences	Infectious Diseases Toxins (*S.aureus* toxin, *E.coli*, *Shigella*)	Hybridoma/Yeast Display	
Enumeral Biomedical Inc.	Checkpoint proteins (PD-1, OX40 and Lag3)	Microwells single cell screening	
Fabrus/Sevion	Cancer Autoimmune diseases Chronic pain Diabetes	FACS/ELISA	FAST (Fabrus arrayed screening technology) arrays the members of their in-house library (Fab with longer CDR3 regions) in the wells of microtitre plates for screening.
Genmab/Medarex	Cancer Autoimmune diseases CNS diseases Infectious diseases	Not stated	Duobody® platform (bispecifics); Unibody® platform (silencing effect); Hexabody® platform (increased potency)
HiFiBio	As requested (service model)	Droplet single cell screening	Single cell whole genome chromatin DNA mapping, Single cell, high resolution dose response screening
Humab Biomed	Cancer Infectious disease inflammatory disease	CellClone (EBV immortalisation with HT Microwell screening)	
Immunome Inc.	Cancer	Hybridoma (proprietary cell fusion method)	
Intrexon (mAbLogix)	Not specified (Service provided)	LEAP® (Laser enabled analysis and processing)	
Kenta Biotech	Hospital acquired infection	Hybridoma (MabIgX platform with unique specific heteromyeloma fusion	

		cell line)	
Mabvax therapeutics	Cancer	Not stated	
Morphosys	Cancer Inflammatory diseases Autoimmune diseases	Phage Display (HuCal high content library)	Yscreen (Robotic liquid handling and screening); Slonomics (Genetic engineering platform)
NovImmune	Cancer Inflammatory diseases Autoimmune diseases	Phage Display/ELISA screening	Fully human bispecific IgG generation using 'Kappa-Lambda' body
Patrys	Cancer	Not stated	Optimised expression and purification of IgM in Per.C6 cells
Single cell technologies	As requested (service model)	Microwells single cell screening, cells lysed and RT-PCR carried out in situ	Affinity Ranking available
Sphere Fluidics	As required (technology model)	Droplet single cell screening	Alternative analysis available includes bacterial cell screening (antibiotic resistance etc.)
Theraclone Biosciences	Cancer Infectious diseases	Single cell ELISA screening	
Trellis Bioscience	Cancer Infectious diseases	Proprietary CellSpot platform (multiplexed probes on latex beads)	Single cell screening in plates
UCB	CNS and immunology disorders	ELISA screening	SLAM (Selected Lymphocyte antibody method) and Fluorescent foci method. High level of automation available
Vaccinex	Cancer Multiple sclerosis	Recombinant cDNA libraries in Vaccinia virus (panning/cell sorting screened)	
Xbiotech	Infectious Disease	Microwells single cell screening	Competency in vaccine development (EB66 cell line for production, IC31 approved adjuvant)

Note: Where fields remain blank information is not readily available.

Future Outlook:

In terms of future work there are several areas of development and validation that are required for DiCAST to reach its full potential. The first of these is in the identification, optimisation and implementation of a number of other assay offerings, such as affinity ranking on array.

The second is concerned with the modification and improvement of the retrieval device to increase the rate of successful retrieval for mammalian cells. Due to their fragile nature a new approach using liquid-liquid retrieval where a small jet of buffer or medium is used in place of the high pressure nitrogen gas is envisioned and will hopefully become a realised entity in the coming year. In connection with this, a microscope-based imaging system is being considered which will help to increase the throughput of the system in two ways, the first being the ability to use larger arrays, where the sample size will not be limited to a microscope slide size (as is currently the case with the microarray scanner). The second way being that the enhanced resolution of this type of imaging system will mean that the capillary size and the distance between capillaries can both be reduced.

The final element that will be enhanced in the DiCAST process is the software packages for ranking, sorting and correlation of the data. Employing this type of data mining generates a very powerful tool to select unique subsets of antibodies from potentially tens of thousands of hits.

Once these new amendments have been incorporated the ultimate aim is to begin to utilise the technology to screen human B cells, ideally isolated from patients who are recovering from diseases such as cancer or have been recently exposed to infectious agents. This will help researchers to decipher the way in which the immune system fights off these attacks and will hopefully lead to the discovery of much needed future medicines.

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