

Towards single cell protein analysis of cardiac progenitor cells

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Declaration of Authorship

I, Gemma Milman, declare that this thesis contains my own work unless otherwise stated, all information sources are referenced accordingly.

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Abstract

Studying single cell protein expression is becoming increasingly important; however it is limited by the availability of highly sensitive technology. In adult cardiac progenitor cells (CPCs), expression of all the key cardiac transcription factors can be found, and both *in vivo* and *in vitro* studies have shown they can form cells of the cardiac lineage. However, effective mobilisation of these cells *in situ* for heart regeneration remains elusive. Close examination of CPCs for the important interactions and modifications may reveal the control mechanisms holding them in their arrested state, and therefore could lead to more effective cell therapies for heart failure.

Total internal reflection microscopy is a super-resolution technique, allowing the visualisation of individual protein molecules through fluorescent labelling. It uses an immunoassay to pull down the protein of interest, detecting the protein with a secondary fluorescently labelled antibody. Whilst the assay sensitivity relies heavily on the antibody kinetics, it avoids the need to genetically modify proteins with fluorescence. Thus the assay can be adapted to look at any protein, in any cell, and can be multiplexed to examine many proteins simultaneously.

In this thesis, an assay for the detection of the cardiac transcription factor, Gata-4, is developed. Gata-4 is pivotal for cardiac development; it is a part of an extensive gene regulatory network, interacting with many other key transcription factors to confer precise heart morphogenesis. It is expressed in cardiac progenitor cells, and the aim here was to quantify protein expression from single CPCs.

Another facet examined here was the potential use of alternative protein capture agents. DNA oligonucleotides, encoding the consensus binding sequence for Gata-4 was investigated for its suitability in an assay for single cell protein expression. Whilst this yielded promising results it was constrained by the lack of a suitable negative control.

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Friends are like feathers, with them you can fly, without them you are just a sole feather floating in disgrace

Jalal ad-Din Muhammad Rumi

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Table of Contents

Declaration of Authorship	2
Copyright Declaration.....	3
Abstract	4
Acknowledgements	5
Chapter 1	
1. Introduction	19
1.1 Single cell proteomics overview.....	19
1.1.1 Advances in proteomic technologies.....	20
1.1.2 Single molecule counting with Total Internal Reflection Microscopy.....	25
1.2 Cardiac progenitor cells	26
1.2.1 Cell population heterogeneity	29
1.2.2 What is the key to unlocking the cardiogenic potential of cardiac progenitor cells? ...	29
1.2.3 Stochasticity in stem cell differentiation.....	31
1.2.4 Deterministic mechanisms for differentiation of cardiomyocytes from cardiac progenitor cells.....	33
1.3 Thesis overview: Application of single cell proteomics to cardiac progenitor cells	39
Chapter 2	
2. Methods and materials.....	41
2.1 Cells.....	41
2.2 Cell culture	41
2.3 Cloning: creating a flag tag Gata4 expression vector	43
2.4 Protein expression systems	43
2.5 Non-denaturing protein isolation	44
2.6 Preparation of cellular fractions	44

2.7	Western blotting	45
2.8	Immunofluorescent staining of cultured cells	48
2.9	PCR of cell lines	48
2.10	Design of double stranded DNA probes for capture of Gata4 protein.....	49
2.11	Printing of capture agents.....	50
2.12	Photolithography	50
2.13	PDMS microfluidic device fabrication.....	50
2.14	TIRFM experiment setup	51
2.15	Optical setup	51
2.16	Optical trapping of single cells	52
2.17	Laser lysis.....	52
Chapter 3		
3.	Single molecule counting algorithm.....	53
3.1	Background correction of single molecule datasets.....	53
3.2	Setting the threshold for counting single molecules	56
3.3		56
3.4	Counting single molecules.....	57
Chapter 4		
4.	Validation of Gata-4 antibodies.....	59
4.1	Methods.....	60
4.1.1	Western blot.....	60
4.1.2	Immunofluorescent cell staining.....	60
4.2	Results.....	61
4.2.1	Western blotting to validate antibodies against target protein	61
4.2.2	Immunofluorescence of murine cells to detect native Gata-4 protein	64
1.1.1	Immunofluorescence to test cross-reactivity between Gata factors.....	67
4.3	Discussion.....	68

4.3.1	Antibody validation.....	68
4.3.2	Antibody cross-reactivity	68
4.3.3	Gata expression in cardiac progenitor cells.....	68
4.3.4	Immunofluorescent cell staining demonstrates native protein detection.....	69
4.4	Summary.....	69
Chapter 5		
5.	Optimisation of antibody immobilisation and labelling	70
5.1	Overview.....	70
5.1.1	Determining the concentration of the detection antibody	70
5.1.2	Fluorescent conjugation of detection antibodies.....	70
5.1.3	Antibody immobilisation strategies	73
5.1.4	Surface passivation.....	75
5.2	Methods.....	75
5.2.1	Antibody conjugation.....	75
5.2.2	Surface passivation with PEG.....	76
5.2.3	Experimental set up.....	76
5.3	Results.....	77
5.3.1	Concentration of detection antibody	77
5.3.2	Comparison of direct and indirect antibody labelling methods	78
5.3.3	Antibody immobilisation with Protein G	80
5.3.4	Results of PEG surface passivation	81
5.4	Discussion.....	82
5.4.1	Detection antibody must saturate the free antigen	82
5.4.2	Comparing fluorescent antibody labelling by Fab fragments and conjugation by the primary amines.....	83
5.4.3	Immobilising antibody with protein G.....	84
5.4.4	Surface passivation with PEG.....	84

5.5	Summary.....	85
Chapter 6		
6.	Determining assay sensitivity	86
6.1	Overview.....	86
6.1.1	Antibody affinity and avidity	86
6.1.2	Sensitivity and limit of detection.....	88
6.1.3	Estimating protein copy number per cell.....	89
6.2	Methods.....	89
6.2.1	Gata4 calibration curve in large volume chambers.....	90
6.2.2	Gata4 calibration in microfluidic chips	90
6.2.3	Calibration with cardiac progenitor cell lysate	90
6.3	Results.....	91
6.3.1	The calibration curve retains the ambient analyte regime	91
6.3.2	Antibody affinity, activity and saturation	91
6.3.3	Fraction of Gata4 bound in the microfluidic chips	92
6.3.4	Determining the sensitivity of Gata4 sandwich assay in nanolitre volumes.....	93
6.3.5	Application of the assay to cell lysate: Gata4 copy number and single cell sensitivity .	94
6.4	Discussion.....	96
6.4.1	Assay kinetics sufficient for single cell sensitivity.....	96
6.4.2	Counting Gata4 copy number.....	96
6.4.3	Sensitivity and limit of detection.....	97
6.5	Limitations.....	99
6.5.1	True concentration of pull down antibody unknown.....	99
6.5.2	Antibody affinity is estimated.....	99
6.5.3	Limited data input.....	100
6.5.4	Cardiac progenitor clones are small cells	100
6.5.5	Gata-4 protein purity	100

6.6	Summary.....	101
Chapter 7		
7.	Analysis of Gata-4 expression in cells with TIRF microscopy	102
7.1	Overview.....	102
7.1.1	Single cell proteomics in microfluidic devices	102
7.2	Methods.....	103
7.2.1	Microfluidics for cell handling and on chip lysis	103
7.2.2	Cell handling	104
7.2.3	On-chip cell lysis methods.....	104
7.2.4	Large volume cell experiments	105
7.3	Results.....	105
7.3.1	Single cell experiments with GFP-tagged Gata-4 expressing cells	105
7.3.2	Single cell analysis of cardiac progenitor clone, SP ₃	106
7.3.3	Limit of detection in large volume chambers	110
7.3.4	Limit of detection in the microfluidic antibody chip.....	112
7.4	Discussion and limitations.....	113
7.4.1	Variable expression of Gata-4 in GFP tagged Gata-4 transfected cells	113
7.4.2	Challenges with single cell proteomics with cardiac progenitor clones	114
7.4.3	Directly lysing cells in large volumes indicates single cell sensitivity	114
7.4.4	Improving the lysis methods on the MAC chip	116
7.4.5	Reducing the cell chamber volume	117
7.5	Summary.....	118
Chapter 8		
8.	DNA Capture of Gata-4 protein as an alternative to antibodies	119
8.1	Overview of aptamers as protein capture agents.....	119
8.1.1	Alternatives to antibodies	120
8.2	Methods.....	123

8.2.1	Design of the Gata-4 Capture agents.....	123
8.2.2	Surface modification for printing	124
8.3	Results.....	125
8.3.1	Optimisation of DNA concentration	125
8.3.2	DNA probe development	126
8.3.3	Gata-4 protein pull down with printed oligonucleotides	126
8.4	Discussion.....	127
8.4.1	Optimisation of printing density	127
8.4.2	DNA oligonucleotides for Gata-4 pull down.....	128
8.4.3	Does DNA capture provide single cell sensitivity	128
8.4.4	Transcription factors inherently bind nonspecific DNA	129
8.4.5	Further development of negative controls is required	130
8.5	Summary.....	131
Chapter 9		
9.	Discussion and conclusion	132
9.1	Antibody validation and optimisation.....	132
9.1.1	Antibody immobilisation directly affects their activity	133
9.1.2	Antibody conjugation.....	134
9.2	Assay kinetics and calibration	135
9.2.1	Assay kinetics sufficient for single cell sensitivity.....	135
9.2.2	Sensitivity and limit of detection.....	135
9.2.3	Sensitivity	137
9.2.4	Variable expression of Gata-4 in GFP tagged Gata-4 transfected cells	138
9.2.5	Single cell sensitivity indicated by on-chip cell lysis in microlitre volumes	138
9.2.6	Challenges with single cell proteomics with cardiac progenitor clones	139
9.3	Future work and improvements	139
9.3.1	Improving the lysis methods on the MAC chip	139

9.3.2	Reducing the cell chamber volume	141
9.3.3	Development of alternative capture agents	141
9.3.4	Transcription factors inherently bind non-specific DNA.....	142
9.4	Limitations.....	142
9.4.1	True concentration of pull down antibody unknown.....	142
9.4.2	Antibody affinity is estimated.....	143
9.4.3	Limited input data.....	144
9.4.4	Estimating cellular protein concentration for calibration.....	144
9.5	Applications of TIRFM single molecule counting to cardiac progenitor cells.....	144
9.6	Conclusion.....	146

Table of Figures

Figure 1.1 Schematic showing the capabilities of the most sensitive proteomic technologies.....	21
Figure 1.2. Illustration of different antibody immobilisation and detection techniques.....	22
Figure 1.3. Illustration of total internal reflection microscopy.....	26
Figure 1.4. Heatmap showing the heterogeneous expression of the key cardiac transcription factors in cardiac progenitor cells.....	30
Figure 1.5. Waddington epigenetic landscape.....	31
Figure 1.6. Illustration of the concept of attractor states.....	32
Figure 1.7. A schematic overview of the gene regulatory network governing cardiac development.....	36
Figure 1.8. Schematic illustrating some of the important protein-protein interactions which regulate the activity of the key cardiac transcription factor Gata-4.....	38
Figure 2.1. Optical setup for TIRF microscopy, the optical trapping and the laser lysis.....	52
Figure 3.1 shows the effect of different filter sizes on single molecule count.....	55
Figure 3.2. Outline of the background correction process.....	56
Figure 3.3. A comparison of different filter sizes to identify single molecules.....	58
Figure 4.1. Schematic of Gata-4 protein highlighting the regions.....	59
Figure 4.2. Immunoblot of monoclonal anti-Gata-4 antibodies validated against either HeLa Gata-4 transfected lysate (+) and untransfected lysate (-).....	62
Figure 4.3. Reverse transcription PCR of the untransfected HeLa cell line (HeLa) for Gata-4 expression.....	62
Figure 4.4. Western blot testing the two Gata-4 antibodies on cardiac progenitor cells.....	63
Figure 4.5 Reverse transcription PCR showing Gata-4 expression in the murine cardiac progenitor cells, SP3 and SP6.....	64
Figure 4.6. Immunofluorescent staining of clonally derived cardiac progenitor clones.....	66
Figure 4.7. Immunofluorescent staining of HeLa cells transfected with Gata-4, -5 or -6.....	67
Figure 5.1. Illustration of the three different labelling mechanisms to fluorescently conjugate antibodies.	72
Figure 5.2. Illustrates functionalising the glass surface with GPTS.....	75
Figure 5.3. Optimisation of the detection antibody concentration.....	77
Figure 5.4. Comparing total single molecule count with two separate antibody labelling strategies, Fab indirect labelling and amine direct labelling.....	78
Figure 5.5. Comparison of the signal to noise ratio of the two labelling strategies.....	79
Figure 5.6. Comparison of Gata-4 binding versus detection antibody alone to pull down antibody immobilised by protein G.	80

Figure 5.7. Total single molecule count from cardiac progenitor lysate incubated with printed antibody on PEG passivated surface.....	81
Figure 5.8. Comparison of signal-to-background ratio of PEG passivated surface and that of BSA.....	82
Figure 6.1. Illustrates antibody multivalency, depicting the two identical binding sites found on the IgG antibody subtype.....	87
Figure 6.2. Layout of microfluidic chip used for lysate experiments.....	90
Figure 6.3. Comparison of the total input Gata4 protein with the number of molecules counted.....	91
Figure 6.4. A calibration curve with Gata4 purified protein in large volume chambers (10 ul).....	92
Figure 6.5. Reducing the volume can increase the capture by pushing the system out of the ambient analyte regime.....	93
Figure 6.6. To determine the assay sensitivity, total input of Gata4 protein molecules is compared to the output single molecule count.....	94
Figure 6.7. Calibration curve was conducted with SP3 cell lysate.....	95
Figure 7.1. Diagram of the microfluidic design used for single cell experiments.....	104
Figure 7.2. Titration of cells transiently transfected with GFP tagged Gata-4 expression vector.....	106
Figure 7.3. Analysis of Gata-4 protein expression after laser lysis of cardiac progenitor clones.....	107
Figure 7.4. Chemical lysis of cardiac progenitors in microfluidic chips.....	107
Figure 7.5. Tile scan images of single cell chambers 2 hours after laser lysis.....	109
Figure 7.6 Direct cell lysis of cardiac progenitor cells in microlitre volume chambers.....	110
Figure 7.7 Lysis of control cardiac progenitor cells, which do not express Gata-4 protein, in microlitre chambers.....	111
Figure 7.8. Titration of cardiac progenitor cells in microfluidic chambers and chemically lysed.....	112
Figure 7.9. Illustration of high local concentration of Gata-4 protein in region of printed antibody.....	113
Figure 8.1. Generation of RNA aptamers by the technique of Systematic Evolution of Ligands by EXponential enrichment (SELEX).....	121
Figure 8.2. Immobilisation of DNA onto GPTS functionalised glass surface.....	124
Figure 8.3. Titration of printed Gata-4 DNA oligo concentration.....	125
Figure 8.4. Compares Gata-4 binding to three different oligonucleotides, the Gata-4 ANF enhancer (ANF), Gata-4 consensus sequence (Gata-4) and the mutated ANF sequence (Mutant).....	126
Figure 8.5. Testing the DNA capture sensitivity, incubating with lysate at either 7.5 or 75 ng/nl; this equates to 1 cell and 10 cells per chamber respectively.....	127
Figure 8.6. Illustration of transcription factors searching for DNA binding sequence.....	129

Appendix, Figure A. Polyacrylamide gel electrophoresis of Gata-4 purified protein under denaturing conditions.....	168
Appendix, Figure B. Binding of Gata-4 antibody to printed purified protein, Gata-4 and P10.....	169
Appendix, Figure C. A cell experiment, where chambers are loaded with 3, 5, 10, 15 or 0 cells.....	170
Appendix, Figure D. As a priori to conducting cell experiments with lysis solution, the assay was tested by comparing lysate in BSA only with Lysate in 1x lysis solution.....	171
Appendix, Figure E. DNA-protein binding assay with cell lysate that endogenously expressed Gata4.....	172
Appendix, Figure F. Open chip comparison of different DNA oligonucleotides to capture Gata4 protein.....	173
Appendix, Figure G. Comparison of binding to two different DNA oligos, the Gata4 binding sequence (Gata4) and the retinoic acid receptor response sequence (RAR).....	174
Appendix, Figure H. Here Gata binding was compared between a Gata4 oligo and that of the oestrogen receptor (ER).....	175

List of Tables

Table 2.1. List of all cells used in this study.	41
Table 2.2. Recipe for clonal growth media (CGM).....	42
Table 2.3. Culture media for all cells except cardiac progenitor cells.	42
Table 2.4. Details of collagen coating solution.....	42
Table 2.5. Primer sequences to amplify Gata4 insert.....	43
Table 2.6. Reagents and conditions for digesting DNA vector and Gata4 insert.....	43
Table 2.7. Non-denaturing lysis buffer recipe	44
Table 2.8. List of all antibodies used in both Western blot (WB) and immunofluorescent cell staining (IF).	46
Table 2.9. List of ingredients for 4x Laemmli sample buffer.....	47
Table 2.10. Recipe for running buffer used to run protein samples on acrylamide gels.....	47
Table 2.11. Recipe for transfer buffer.....	47
Table 2.12. Recipe for acrylamide gels.....	48
Table 2.13. Recipe for 4% stacking gel.....	48
Table 2.14. Conditions for RT PCR.....	49
Table 2.15. Recipe DNA PAGE.	49
Table 4.1. Details of the Gata-4 antibodies tested in this study.....	61
Table 6.1 Estimates the number of Gata4 proteins per cell based on the lysate calibration curve.....	96
Table 8.1. List of DNA sequences used as protein capture agents.....	123
Appendix, Table A. Summary of single cell techniques and their limits of detection.....	176

Abbreviations

TIRFM	Total internal reflection microscopy
TIRF	Total internal reflection
FBS	Foetal bovine serum
DMEM	Dulbecco's modified eagle media
CGM	Clonal growth media
PBS	Phosphate buffered saline
EMSA	Electromobility shift assay
PAGE	Polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
GFP	Green fluorescent protein
SBR	Signal to background ratio
SP3/16	Cardiac side population cells, clone 3/16
K _d	Dissociation constant
Hep G2	Hepatocarcinoma derived cell line
MCF7	Mamary cancer derived cell line
HEK	Human embryonic kidney cell line
HeLa	Human cervix epitheloid carcinoma cell line
CPC	Cardiac progenitor cell
LOD	Limit of detection

MAC chip	Microfluidic antibody capture chip
Da/ kDa	Dalton/ kilo Daltons
MS	Mass spectrometry
BMP	Bone morphogenetic proteins
TGF- β	Transforming growth factor- β
HATs	Histone acetyl transfereases
TFs	Transcription factors
FOG	Friend of Gata (TF)
SRF	Serum response factor (TF)
HAND	Heart- and neural crest derivatives-expressed protein (TF)
MEF-2	Myocyte enhancing factor - 2 (TF)
NFAT	Nuclear factor of activated T-cells
GSK3 β	Glycogen kinase synthase 3 beta
HDACs	Histone deacetylases
siRNA	Small interfering RNA
miRNA	micro RNA
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
SBR	Signal to background ratio
scFv	Single chain variable domain fragment
Nd :Yag	Neodymium-doped yttrium aluminium garnet (laser crystal)

Chapter 1

1. Introduction

1.1 Single cell proteomics overview

Proteomic analysis of cell populations has elucidated a huge amount of information. However, fluctuations in protein expression are known to occur across cell populations. This heterogeneity can be instructive, but is masked by bulk analysis. In the fields of cancer and stem cell research, this heterogeneity is very important. In the case of cancer, it can lead to drug resistance, and in stem cell niches it is the source of self-renewal as well as multipotency (Easwaran et al. 2014; Enver et al. 2009). Thus, being able to analyse protein expression of individual cells within a population will be instructive. In the case of stem cells, elucidating the mechanisms of control would permit manipulation of the differentiation pathways, which is essential for producing specified cells of high purity for therapies.

Protein analysis at the single cell level is fundamentally difficult. Unlike mRNA it cannot be amplified, in addition, the copy number can vary by orders of magnitude, from a few hundred to tens of thousands. Whilst the housekeeping genes are very abundant, consisting of 25% of the total cellular protein, other important proteins such as transcription factors and signalling proteins tend to have low expression levels and have temporal activity (van Hoof et al. 2012). Thus, extremely sensitive techniques are required to analyse proteins from a single cell.

Protein expression does not correspond to mRNA expression (Taniguchi et al. 2010a; Schwanhäusser et al. 2011; Lu et al. 2009), and whilst single cell mRNA studies are gaining ground, it overlooks the plethora of regulatory mechanisms that control protein activity. These include post translational modifications (protein acetylation, phosphorylation, sumoylation and ubiquitination), cellular localisation, protein-protein interactions, inhibition by micro RNA and epigenetic regulation. A good example of regulation separated from simple transcription-translation is that of Gata-4. Gata-4 activity is regulated by phosphorylation by the mitogen activated kinases. However, phosphorylation by GSK3 β marks the protein for nuclear export and subsequently is degraded. This demonstrates how it is not simply about protein expression levels. Examining protein modifications and interactions can be insightful to the pathways at play, particularly in stem cell differentiation.

Our group is investigating endogenous cardiac progenitor cells as source of new cardiac tissue. These cells have been demonstrated by many groups to generate cells of the cardiac lineage. They exist in

small numbers in the heart, expressing cardiac transcription factors but not structural genes. Understanding the cues which keep these cells in an arrested state will be important in harnessing these cells as a therapy for cardiac repair.

Traditional approaches to protein analysis, such as mass spectrometry and gel electrophoresis, are not amenable to single cell protein analysis. These techniques require large samples, encompassing whole populations of cells, and are not very quantitative. Whilst progress has been made with cell mass cytometry and epifluorescent microscopy there is still an unmet need in single cell proteomics.

Our group has developed a single cell proteomic platform using an immunoassay coupled to total internal reflection microscopy (TIRFM), a highly sensitive technique that can detect single molecules. Together with microfluidic cell handling this platform is capable of single cell protein detection. Currently the system works with p53 protein detection (Salehi-reyhani et al. 2011; Burgin et al. 2014) and the group is working towards detecting other proteins as well as protein modifications and protein-protein interactions. This thesis aims to develop the assay for detection of protein in cardiac progenitor cells, which exist in small numbers in the heart, focusing on the transcription factor Gata-4.

1.1.1 Advances in proteomic technologies

There is a whole range of proteomic technologies that have been used to study proteins on the population level. Whilst some of these have been adapted to single cell studies, the progress has been slow and many advances still need to be made. The technologies developed with high sensitivity and capability for single cell analysis are illustrated in Figure 1.1. With the rise of microfluidics, the handling of small volumes has become easier. However, at the single cell level, sample loss through lysis, separation or detection stages, will severely impact results (Wang & Bodovitz 2010a). This obstacle is clearly seen by the lack of studies that have achieved single cell proteomics (Figure 1.1). Thus, the integration of sample manipulation is paramount to the success of single cell proteomics.

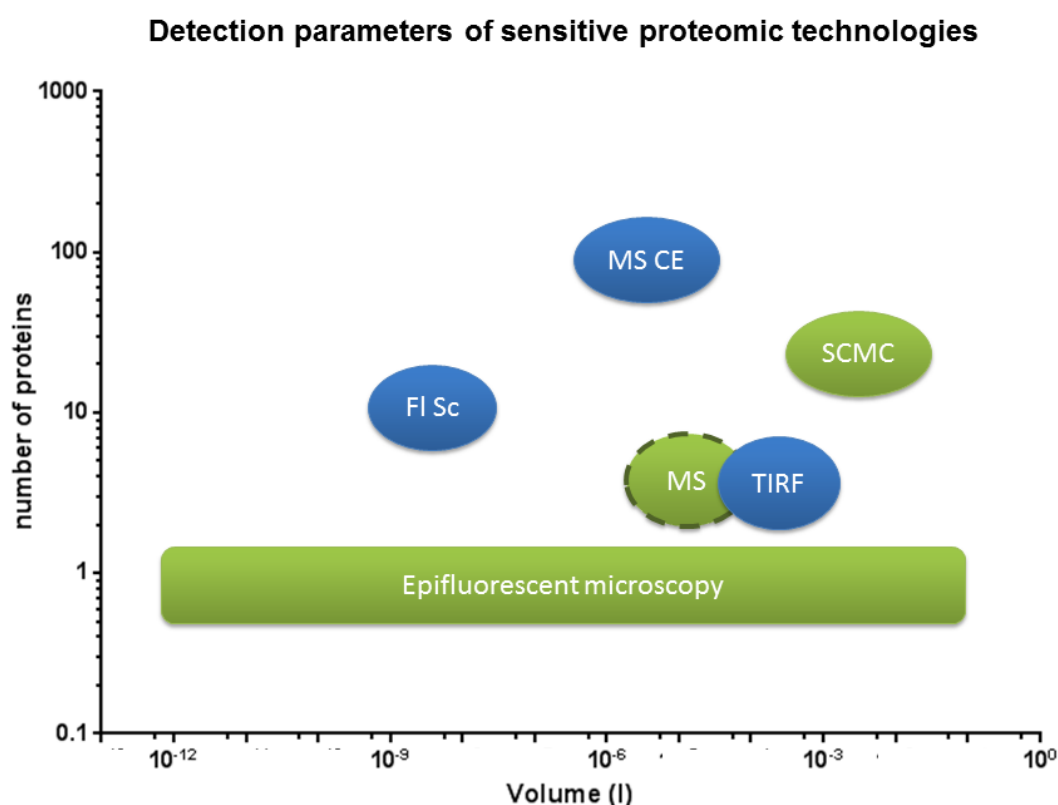


Figure 1.1 Schematic showing the capabilities of the most sensitive proteomic technologies. The plot illustrates the number of different proteins that can be detected in a single sample (y axis) and the operating volume (x axis), with the latter often impacting the overall sensitivity. Technologies highlighted in green have achieved detection from individual cells. A dashed border indicates only peptide or small molecule detection and not protein detection. Other technologies are working toward improving sensitivity, with some achieving attomolar and zeptomolar sensitivity but this work is mainly conducted with, small populations of cells, cell lysate or secreted proteins (blue). The acronyms for the technologies are as follows: Fluorescent scanning (FI Sc), Mass spectrometry with capillary electrophoresis (MS CE), mass spectrometry (MS), total internal reflection microscopy (TIRF), single cell mass cytometry (SCMC). References for the studies using these technologies are listed in the text, with further details listed in the appendix (table A).

Antibodies have been instrumental in protein studies, whether via the traditional immunoassay or by flow cytometry. Some of the bottle necks to using antibodies include their affinity, labelling and immobilisation strategies and will be discussed further in Chapter 6.

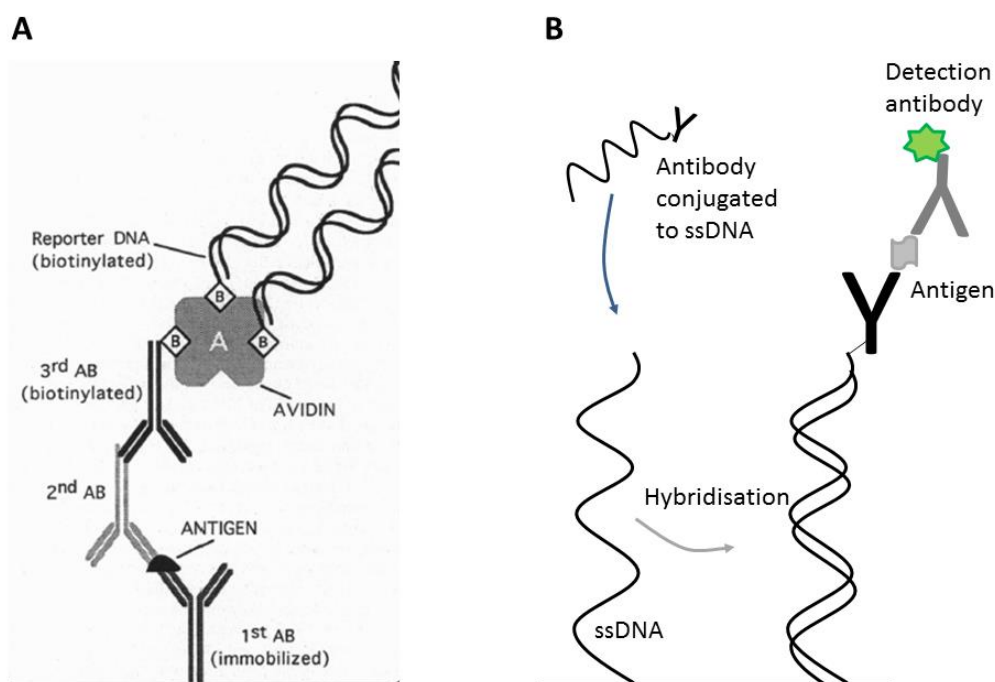


Figure 1.2. Illustration of different antibody immobilisation and detection techniques. A) Schematic of immune PCR chemistry. This technique is very similar to the traditional ELISA except with DNA PCR amplification to develop the signal. Here a tertiary antibody conjugated to DNA via biotin-streptavidin is used to detect the antibody-antigen binding. The presence of the DNA molecules permits PCR and the resulting products therefore correlate to the presence of antigen. *Taken from Sanna et al. 1995.* B) illustrates of the technique termed DNA barcoding used by Shin et al. 2011, Shi et al. 2012, Ma et al. 2011 for single cell analysis. Here single stranded DNA (ssDNA) is immobilised on glass and hybridised to its complement which is conjugated to an antibody. Antigen detection is conducted with fluorescently conjugated antibodies. It allows for multiplexing of antibodies as well as functional antibody immobilisation.

Developments in immunoassay detection chemistry led to increased sensitivity in the early 90's. These included processes such as immunoPCR, rolling circle amplification and immuno-detection amplified by T7 RNA (Sano et al. 1992; Zhou et al. 1993; Mweene et al. 1996; Sanna et al. 1995; Zhang et al. 2001). These chemistries harnessed the power of amplification by PCR and linked it directly to antibody-antigen detection, permitting a lower limit of detection of 580 antigen molecules (Sano et al. 1992). At the time, all these developments demonstrated remarkable increases in sensitivity in comparison to the standard ELISA but were still limited by detection technologies.

Mass spectrometry is the most powerful proteomic technology, possessing the advantage of whole proteome analysis which can detect interactions as well as modifications and with no labelling requirements. Despite this, it is difficult to analyse small volumes and thus not well suited to single cells studies. This technology is currently limited to the analysis of peptides and small molecules

from single cells (Lapainis et al. 2009; Rubakhin et al. 2000; Jo et al. 2007; Wang & Bodovitz 2010b). Steadily, advances are being made into single cell protein detection. To date, the highest resolution was obtained from single pancreatic islets, containing up to 4000 cells (Waanders et al. 2009a). By coupling mass spectrometry with separation by liquid chromatography, Waanders et al. (2009) were able to resolve 6,873 proteins. However, the ability to detect low abundant proteins is still very limited (Galler et al. 2014).

Whilst a diverse range of modifications has been trialed with mass spectrometry (see appendix table A) with the aim of achieving single cell sensitivity the limitations remain the same. These include the high sample loss during manipulation, the ability to detect low abundant proteins without labelling and the huge amounts of data processing which is computatively intensive, taking days in some instances.

Fluorescent activated cell sorting (FACS), is one of the most developed and utilised form of single cell proteomics. This powerful technology couples high throughput, sorting at 10,000 cells per second (Galler et al. 2014; Bendall et al. 2012), with single cell resolution. Whilst not hugely quantitative, it can sort cells based on fluorescence into high and low protein expression, and has the ability to simultaneously deal with multiple colours, permitting detection of up to 15 different proteins within a single cell (Galler et al. 2014; Wu & Singh 2012a; Bandura et al. 2009). Conversely, cells either require fixing and staining with antibodies or genetic modification to fluorescently tag the protein of interest. Both instances can pervert the cells natural homeostasis.

One big drawback is that the technique requires manual manipulation of large cell populations, and is therefore not suitable for rare cell populations. Microfluidic handling helps to circumvent this and there have been many developments in this area (Galler et al. 2014; Wu & Singh 2012b). Despite these drawbacks, some nice studies tallying mRNA and protein expression used FACS, highlighting the stochasticity and disparity between mRNA and protein (Newman et al. 2006)

Single cell mass cytometry is quickly closing the gap between whole proteome analysis and single cell studies. This technique combines the single cell handling by flow cytometry and protein analysis by mass spectrometry. Essentially, cells are labelled with up to 30 different antibodies conjugated to metal element tags via metal chelating polymers (Lou et al. 2007a). These tags are then detected by induced coupled plasma time-of-flight mass spectrometry (ICP MS; Wu & Singh 2012; Bandura et al. 2009). The availability of different metal-polymer tags overcomes the limited number of fluorophores and their confounding overlap in emission wavelengths (Wu & Singh 2012b; Bandura et al. 2009). In addition, the sensitivity of mass spectrometry in this setup is capable of distinguishing between protein expression levels that differ by orders of magnitude and is working toward absolute quantification (Lou et al. 2007b; Bandura et al. 2009).

Although being a high throughput technology, processing 1000 cells per second (Bendall et al. 2012), it still requires large sample quantities (Bandura et al. 2009), and is thus not suitable for rare cells. In theory, this technique can detect as low as 100 ions; however, this has yet to be achieved in practise (Bendall et al. 2012). Advances in antibody conjugation will shortly circumvent this: by adding conjugation with more and heavier metals, thereby decreasing the limit of detection (Bendall et al. 2012).

Single cell studies are readily conducted by epifluorescence. Using time lapse fluorescence, Cohen et al. (2008) demonstrated differential expression and localisation of up to 1000 proteins from live individual cancer cells. Although the method demonstrated high throughput capacity, it required the use of a fluorescently tagged protein library. As a consequence, the method cannot be conducted on clinical samples without genetic modification. Further still, this characterised differential expression rather than absolute quantification. In addition, the use of reporter library limits analysis to a few proteins per cell.

With simple tweaks such as coupling with flow (Huang et al. 2007a; Todd et al. 2007) or simply using intensity measurement (Taniguchi et al. 2010b), epifluorescence could be used to quantify protein in single cells or at low concentrations in blood plasma. It was through this method that the first direct comparisons between mRNA and protein counts in a single cell were made, showing that at any given time the two do not correlate (Taniguchi et al. 2010b). The main limitation of epifluorescence is that it illuminates everything in the field of view. Thus without constraining counting to genetic fluorescent tags or having the benefit of a step wise increase in protein concentration (Cai et al. 2006), this technique demands a great deal of sample processing. Requiring complex microfluidics to capture, wash, lyse and separate the proteins before imaging.

Whilst it might be very complex to harness epifluorescence for single cell studies, it is feasible. The Heath group (California Institute of Technology, USA), uses a fluorescent scanner, as is commonly used for DNA microarrays. They conducted on-chip single cell lysis and were able to detect 10 different proteins from one cell (Shin et al. 2010; Shi et al. 2011). Furthermore, they use DNA barcoding as their antibody immobilisation technique, which permits multiplexing and facilitates functional immobilised antibody (Figure 1.2). Although this technique only compared relative expression levels and not absolute quantification, it shows the steady progression in the field.

Surface plasmon resonance has been used for many years in determining antibody-antigen reactions but until very recently it has not had single molecule resolution (Zijlstra et al. 2012; Mayer et al. 2010). As yet, this has not been applied to single cell studies but with its label-free advantage, it presents exciting possibilities to the proteomics field.

Total internal reflection fluorescent microscopy (TIRFM) has the benefit over Epifluorescence in that it excludes bulk fluorescent illumination by only illuminating materials at the glass-liquid interface. It can thus help circumventing the complexities of sample separation and washing in small volumes. Further still, it is perfectly adapted to single molecules studies: with its super resolution it can image single fluorescent molecules. In live cell studies, it is a powerful tool for studying cell surface proteins and can be applied to both protein tracking and interactions (Leake et al. 2006; Ohara-Imaizumi et al. 2004; Sako et al. 2000; Douglass & Vale 2005). Unfortunately, its shallow depth of field means it is not well suited to intracellular studies. Consequently, intracellular proteins must be released and brought close to the glass-liquid interface in order to be detectable by TIRFM. This means that the cells must either secrete the proteins, such as free protein found in blood samples (Lee et al. 2009), or be lysed (Salehi-reyhani et al. 2011).

Another useful application of TIRFM is in the study of protein-protein interactions. Jain et al. (2011) demonstrated this in a study using cell lysate, and were able to detect interactions from a minimum of 10 cells. Although this study does not show single cell sensitivity, with the current advances in technology we believe this is an attainable goal.

Single cell proteomics is an ever evolving field that is technically very demanding. There is as yet no gold standard in the field for single cell protein analysis. Given the different modalities of proteins, there is unlikely to be one solution, but rather a collection of techniques that complement each other. In this thesis, TIRF is used to explore the possibility of conducting protein analysis in cardiac progenitor cells. We work towards achieving single sensitivity by reducing the reaction volumes by using microfluidic technology coupled with high affinity antibodies

1.1.2 Single molecule counting with Total Internal Reflection Microscopy

Our group has developed a single molecule counting algorithm, with which we can quantify protein molecules bound to the surface and visualised using TIRFM. Total internal reflection microscopy works by directing a laser at a glass-liquid interface at an angle such that the laser beam is totally reflected. At the angle of reflection a small evanescent wave is emitted with a maximum height between 100-300 nm. This shallow depth of excitation means that any fluorescent molecules in the bulk volume are excluded and only molecules near the surface boundary are being imaged, as illustrated in Figure 1.3. A pull down antibody was used to capture the protein of interest, and a second fluorescently conjugated antibody was used to label the protein.

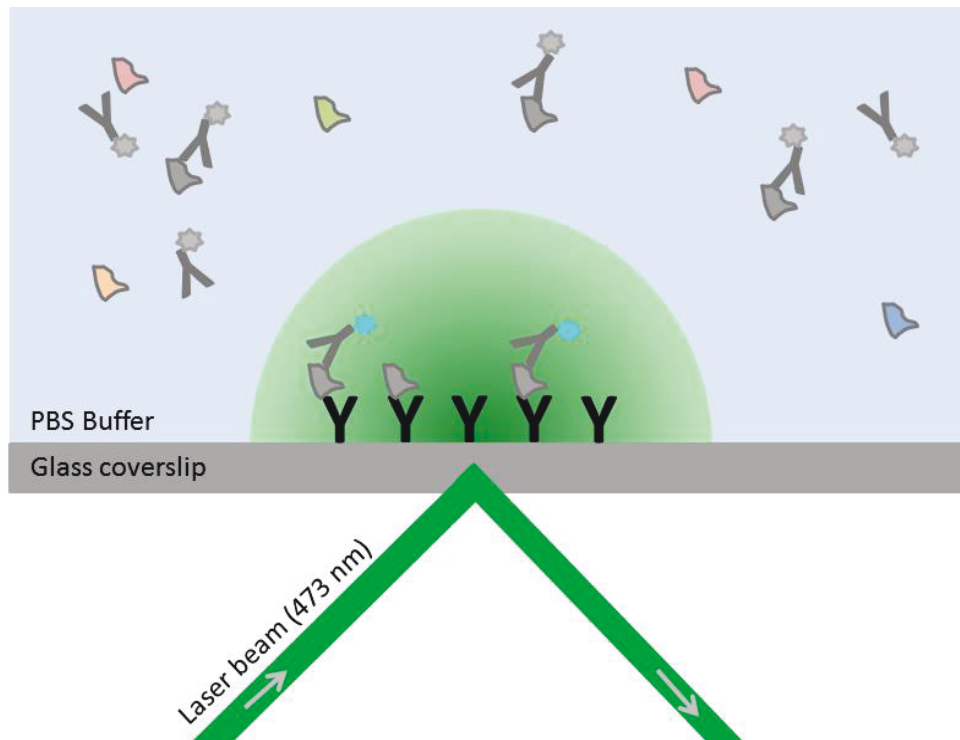


Figure 1.3. Illustration of total internal reflection microscopy. The laser beam (line in green) is directed at a glass interface at such an angle that the beam is totally reflected. At the point of reflection an evanescent wave of light is emitted, that decays exponentially (semi-circle of green). This excites any fluorescent molecules within this 100-300 nm region. The fluorescent molecules contained within the bulk volume remain dark. Antibody is printed onto the glass surface (in black) and used to pull down the protein of interest so that it is within the evanescent wave. A second fluorescently conjugated antibody (grey) is used to detect the bound proteins, with the fluorescent molecules only becoming excited within the evanescent wave.

1.2 Cardiac progenitor cells

The adult mammalian heart possesses scant ability to regenerate itself. It can respond to work overload via muscular hypertrophy and it heals tissue damage (e.g. myocardial infarction) by scar tissue formation. This is often an inadequate replacement for the lost beating tissue, with the increased strain eventually leads to heart failure. Although it is reported that the human heart has a 1% annual turnover (Bergmann et al. 2009), replenishing the cardiac tissue with new cardiomyocytes does not offset injury under normal (unassisted) conditions.

To counter this inadequacy to regenerate, a range of stem cells have entered clinical trials for cardiac repair, with limited efficacy. The first to be trialed were skeletal myoblasts. Although they were seen to improve function in *in vivo* experiments and in clinical trials, there were serious doubts to their safety. Whilst being autologous, these cells were unable to make electrical connections with cardiomyocytes and were reported to cause arrhythmias in transplanted patients (Dimmeler et al.

2005; Menasché 2004; Ptaszek et al. 2012). Haematopoietic stem cells were the next cells to be trialled in infarct patients. Various haematopoietic cell types have been tested, with bone marrow mononuclear cells being the most common. Whilst a mild improvement was seen in cardiac function in some studies, a recent meta-analysis strongly indicates that this has no effect on improving cardiac function or reducing further cardiac events (Jong et al. 2014). The reported improvements are more likely due to paracrine effects; increasing angiogenesis and supporting existing cells and not formation of new cardiomyocytes (Zimmer et al. 2012; Mirotsoy et al. 2011; Ptaszek et al. 2012). Results from Jong's meta-analysis suggest that mesenchymal stem cells are more likely to be beneficial and that these would have better scope as off the shelf treatment. Being an immune privileged cell implies that cells from younger, healthy donors could be used, thereby offering a potentially more efficacious treatment, than using the patient's own cells, which are likely less responsive (Jong et al. 2014).

Most recently there are two trials using cardiac progenitor cells, harnessing stem cells endogenous to the heart for cardiac repair. Cardiac progenitor cells (CPCs) are a diverse population of cells, which are identified by a selection of different markers including c-kit⁺, Sca-1⁺, Isl-1⁺, PDGFR α as well as the side population phenotype (Beltrami et al. 2003; Oh et al. 2003; Moretti et al. 2006; Pfister et al. 2005; Oyama et al. 2007; Chong et al. 2013). As a whole, these populations of cells are defined by their ability to differentiate into cells of the cardiac lineage, such as cardiomyocytes, endothelial and smooth muscle cells.

As yet, the role that these CPCs play in the adult heart is unclear. Evidence through lineage tracing suggests that they act in cardiac homeostasis, with Sca-1⁺ cells being shown to replenish the pool of cardiomyocytes, though at a low frequency, in the adult murine heart (Uchida et al. 2013). Both the Sca-1⁺ CD31⁻ and ckit⁺/lin⁻ pools of CPCs have been shown to increase in the early stages after infarction and home to the insulted area, suggesting they play a role in the response to pathology (Wang et al. 2006; Ellison et al. 2013). In particular, stimulation by prostaglandin E₁ in myocardial infarction revealed that Sca-1⁺ could contribute to the formation of new cardiomyocytes in the border zone (Hsueh et al. 2014).

Further to this, a Sca-1⁺ knockout study showed that these cells play a role in mediating the response to stress, with KO mice displaying age related cardiac dilation and dysfunction induced by chronic overload (Rosenblatt-Velin et al. 2011).

Whilst both ckit⁺ and Sca-1⁺ cells appear to transdifferentiate *in vivo*, different studies have reported varying degrees of efficacy in response to myocardial damage (Wang et al. 2006; Ellison et al. 2013). Some report that administration of c-kit⁺/lin⁻ cells have a marked capacity for regeneration and improvement of cardiac function (Beltrami et al. 2003; Ellison et al. 2013). However, Molkentin et al.

(2014) recently reported a very minimal contribution of the ckit⁺ cells to the formation of new cardiomyocytes. In addition, a study by Bailey et al. (2012) showed that the ability of CPCs to repair the heart diminished upon loss of Sca-1⁺.

Supporting the use of Sca-1⁺/CD31⁻ cells in cell therapy, Wang et al. (2006) showed that they attenuated the decline in cardiac function and observed modest regeneration of cardiomyocytes. However, moderation of the adverse remodelling processes is proposed to be mediated mostly by paracrine effects as seen by an increase in angiogenesis (Wang et al. 2006).

Work in our group has focused on cardiac progenitors defined by Sca-1⁺ expression and the side population phenotype. In our lab, these cells have been shown to be clonogenic and express a variety of cardiac transcription factors that are important during heart development; these include Gata4, Mef2c, TBX5 and NKX2.5 (Figure 1.4). However, these cells do not express the cardiac structural genes. Work by others have shown that these cells differentiate *in vitro* to form cardiomyocytes, endothelial cells and smooth muscle cells (Oh et al. 2003; Wang et al. 2006; Matsuura et al. 2004; Pfister et al. 2005).

The expression of Sca-1⁺ also exists outside the heart in other stem cell niches and in some somatic cells, most notably in the haematopoietic stem cell population (Bradfute et al. 2005; Ito et al. 2003). Many studies have tried to elucidate the role of Sca-1⁺ in these populations and indicate that it plays a role in stem cell lineage determination and self-renewal, although the latter is disputed by Bradfute et al. (Ito et al. 2003; Bradfute et al. 2005).

More recent studies on the function of Sca-1⁺ in the heart indicate that this population of cells helps to maintain the pool of cardiac progenitors and acts as a regulator of cardiomyocytes renewal in the heart (Rosenblatt-Velin et al. 2011). Sca-1⁺ cells tend to reside in stroma along with mesenchymal cells, endothelial cells and pericytes, though staining indicates some reside in close contact with cardiomyocytes, akin to that seen of satellite cells and skeletal muscle (Uchida et al. 2013).

As yet, a human homologue to Sca-1⁺ has not been identified and initial attempts to isolate human cardiac progenitors used the ckit⁺ marker (Bearzi et al. 2007). However, cross reactivity with the Sca-1⁺ antibody indicates an orthologue likely exists (Goumans et al. 2007). In fact the murine Sca-1⁺ antibody was used to purify a population of human adult cardiac progenitors (Goumans et al. 2007; den Haan et al. 2012). These cells were shown to differentiate *in vitro*, expressing cardiac structural genes, forming functional gap junctions that enable electrical coupling and responded to L-channel calcium inhibitors and β adrenergic stimulation (Goumans et al. 2007). These differentiated cardiomyocytes appear more mature than those obtained from foetal ventricular cardiomyocytes (Goumans et al. 2007). This strongly supports the development of these cells as a clinical application,

or at least provides a good model for pharmacological, physiological or developmental studies (Goumans et al. 2007).

Cardiac progenitor cells are rare in the adult heart, representing only 0.03% of the heart's cell population (Oh et al. 2003). Even though their response to insult is inadequate, their role in tissue homeostasis and their capacity to ameliorate the pathological response in *in vivo* studies, demonstrates their great potential in the clinic. In the current CPC clinical trials, the cells are harnessed by growing them outside the patient after isolating the cells from a heart biopsy, before injecting the cells back into the heart. As to the efficacy of this, only time will tell.

1.2.1 Cell population heterogeneity

The importance of transcription factor expression in these cells is that it indicates that they are committed to the cardiac lineage, although they appear to be in arrested state. As mentioned earlier, *in vivo* and *in vitro* experiments show that these cells differentiate into cells of the cardiac lineage, but the exact mechanisms controlling this are unknown.

1.2.2 What is the key to unlocking the cardiogenic potential of cardiac progenitor cells?

As yet there is not a definitive method for differentiation of murine CPCs to cardiomyocytes. Whilst there exists a variety of different approaches, attaining high purity cardiomyocytes is a challenging task. Common reagents employed for differentiation include dexamethasone, 5-azacytadine, oxytocin, co-culture with neonatal cardiomyocytes or the combination of different growth factors (Yang et al. 2011; Chong et al. 2013; Oh et al. 2003; Goumans et al. 2007; Beltrami et al. 2003; Miyamoto et al. 2010; Matsuura et al. 2004). Part of the problem with these approaches is their efficiency since the reagents 5-azacytadine and oxytocin both convert CPCs to cardiomyocytes at a low rate (Oyama et al. 2007; Matsuura et al. 2004). The maturity of the cells is limited, particularly with 5-azacytadine, where sarcomeres remain unorganised, and do not form striations (Beltrami et al. 2003; Matsuura et al. 2004). Further still, these cells do not seem restricted to the cardiac lineage with Sca-1⁺ cells expressing osteogenic and adipocyte markers (Matsuura et al. 2004). Whilst oxytocin seems to produce a more thorough result with spontaneously beating cultures, the efficiency of this is only 1% (Matsuura et al. 2004).

Culture with neonatal cardiomyocytes is more efficient, with Miyamoto et al. (2010) reporting complete conversion of ckit⁺ cells to cardiomyocytes with good structural organisation. However, the risk of cross contamination makes this an unlikely candidate for clinical applications.

Differentiation using co-culture is most likely due to the growth factors expressed by the exogenous cardiomyocytes. A combination of different factors has been used to develop differentiation protocols. In the mouse, Rosenblatt-Velin et al. (2005) showed that FGF-2 is an important factor. However, in different species different factors are in operation. For instance, Goumans et al. employed TGF- β with 5-azacytidine to achieve 94.7% cardiomyocytes from human CPCs. In comparison, 5-azacytidine alone produced less than 15% cardiomyocytes (Goumans et al. 2007).

Despite the quest for efficient differentiation procedures, the correct ratio of cell types for therapy is not defined. Considering the benefit of paracrine effects that the clinical trials have shown, it is likely a combination of cell types are needed, augmenting the vascularisation with endothelial cells as well as increasing contractile tissue with new cardiomyocytes.

Further to this, harnessing potential of adult CPCs is much more difficult than that of neonates (Rosenblatt-velin et al. 2005). Expression of the key cardiac transcription factors alone is not sufficient to induce differentiation (Olson & Schneider 2003), and thus it is important to understand the control mechanisms in place.

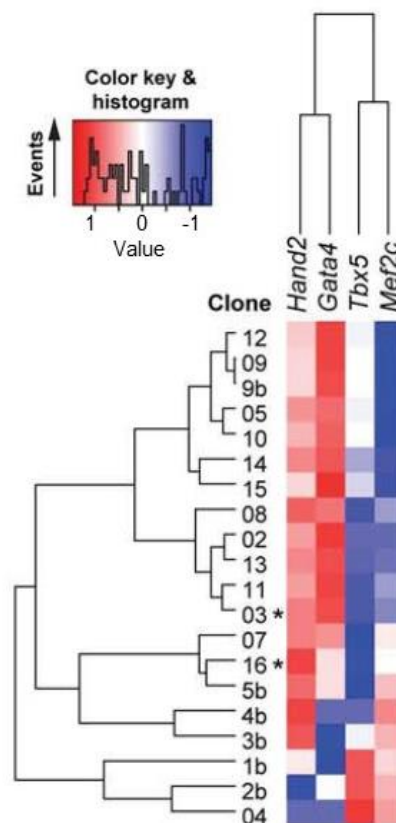


Figure 1.4. Heatmap showing the heterogeneous expression of the key cardiac transcription factors in cardiac progenitor cells. Twenty different clones were compared by microarray analysis and showed different transcription factor expression patterns, and z-scores were calculated from δ CT values. The asterix (*) indicates the clones which were used in this thesis. *Figure obtained from Nosedá et al. 2015.*

The Sca-1⁺ CPC clones isolated in our lab express a mosaic of the cardiac transcription factors (Figure 1.4). It is speculated that having an incomplete set of the key cardiac transcription factors might be holding them in the arrested state. Filling the key gaps might push the cells towards the cardiomyocytes cell fate. Of course, there is more at play during differentiation than just the expression of cardiac transcription factors alone.

Much has been elucidated on the differentiation of stem cells to cardiomyocytes, demonstrating an importance of transcription factor interactions in addition to chromatin modifications.

1.2.3 Stochasticity in stem cell differentiation

Waddington was the first to describe an epigenetic landscape for stem cell differentiation, proposing that pluripotent cells start at the top of the hill. As they roll down, they deviate along different differentiation pathways becoming ever more restricted by their lineage choice, as shown in Figure 1.5 with the valleys representing the ever increasing lineage restriction.

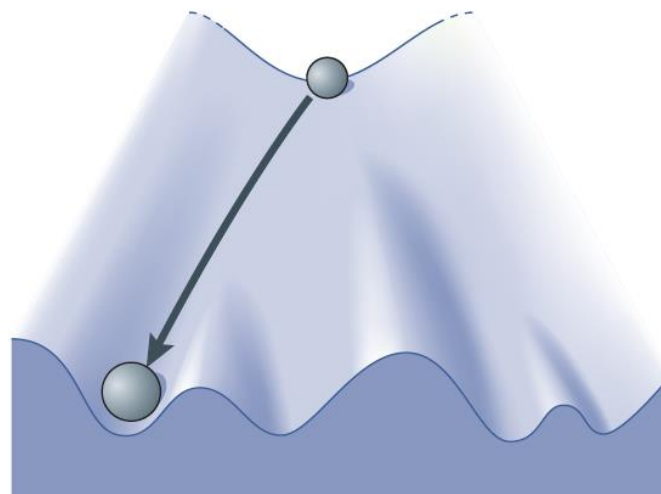


Figure 1.5. Waddington epigenetic landscape, illustrating how a pluripotent stem cell starts at the top of the valley, and as it progresses downhill, it may take various paths (or valleys) which increasingly restrict the cell fate along a certain lineage. *Taken from Ladewig et al. 2013*

There have been some developments in this story, particularly with regard to the transdifferentiation and pluripotent reprogramming of somatic cells. Originally, reversing cell commitment was considered impossible, but the breakthrough in induced pluripotent stem cells showed that the somatic cell state is reversible with the right stimuli (Takahashi et al. 2007; Okita et al. 2007). In accordance with this, the landscape theory has progressed, indicating that this transdifferentiation does not have to occur along the same forward pathway, nor does the commitment pathway follow the steepest gradient (Wang et al. 2011). A good example of the latter is the differentiation of HL60

promyelocytic progenitors to neutrophils where differentiation can be triggered by two different chemicals, DMSO or all-*trans* retinoic acid. Whilst achieving the same outcome, the two chemicals activate different pathways (Macarthur et al. 2009)

Another more recent development in the theory is that of attractor states. These are not necessarily the fully differentiated cell, but can be any stable phenotype. For example, whilst the undifferentiated stem cells are considered rather unstable and transient, the subsequent progenitor cells attain a more stable state and may rest in an “attractor” state before further lineage specification (Wang et al. 2011; Enver et al. 2009). This is most notably seen in studies of haematopoietic progenitors where the lineage decision between erythroid and myeloid cell fates is governed by the expression of the transcription factors PU.1 and Gata1. These transcription factors can skew the commitment decision either way, but their simultaneous low expression produces a stable state, through which only the expression above a threshold will kick further lineage specification (Chickarmane et al. 2009; Huang et al. 2007b). This concept is illustrated in Figure 1.6

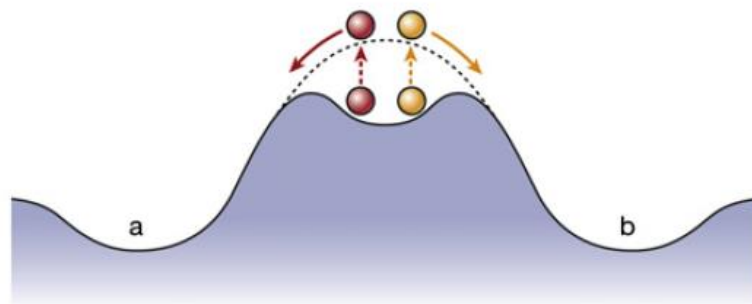


Figure 1.6. Illustrates the concept of attractor states, which are wells in the epigenetic landscape in which cells can fall into along the differentiation pathway. These attractor states are fairly stable, and may represent a progenitor cell. Cells can still form a variety of cell types (a and b valleys), within a certain lineage, but require expression above the threshold of the determinant transcription factors. *Taken from Enver et al. 2009*

This is an interesting concept with regard to CPCs, which seem stable yet are able to differentiate into the three cell types of the cardiac lineage in addition to reports of osteogenic and adipocyte marker expression. Whilst CPCs are more complicated than the bistable switch seen in haematopoietic expression of PU.1 and Gata1, the expression levels of key transcription factors may play a part.

Deterministic versus stochastic gene expression: which is most effective in determining stem cell differentiation? The likely answer is both. Differentiation pathways are governed by a network of transcription factors. In addition, noise is inherent to all organisms, and arises from many different

sources from chromatin conformation to the number and availability of transcription factories, as well as the number and location of transcription factor binding sites (Raj & van Oudenaarden 2008).

In unicellular organisms this phenomenon is beneficial, by having a subset of the population that can immediately respond to changes in the environment (Enver et al. 2009; Newman et al. 2006). This is not only cost efficient, meaning that only some cells are in a sensing mode at the same time, but also ensures survival of the population (Raj & van Oudenaarden 2008).

Although noise may be minimised there are some examples where it has been shown to play a key role in development. For instance, it was demonstrated in the development of the murine olfactory sensing system where a diverse range of sensory receptors are required but which are manifested by simple stochasticity of gene expression (Vassar et al. 1993)

How much do stem cells try to minimise noise? Whilst expression of genes determines the lineage decision, the levels of expression appear to be inherently stochastic. This was shown by Chang et al. (2008) who demonstrated that Sca-1⁺ haematopoietic stem cells could regenerate a heterogeneous population of Sca-1⁺ expression from a single clone. Sca-1⁺ low and high expressing have different lineage preferences; however, the population attractor state is that where there is a distribution of protein expression, from low to high.

It would be interesting to study different levels of protein expression within the cardiac progenitor cell population. The CPC cells are a population selected on the basis of Sca-1⁺, CD31⁻ expression, with the side population phenotype. Despite this refined selection procedure, there is still a great amount of variability in the transcription factor expression and within that, most likely a heterogeneous expression of each protein.

1.2.4 Deterministic mechanisms for differentiation of cardiomyocytes from cardiac progenitor cells

The heart is the first organ to develop in the body, the mechanisms behind which are evolutionarily conserved across species (Olson 2006). Heart formation starts with the specification of cardiac mesoderm, from the lateral plate mesoderm and defined by the expression of Mesp1 (Saga et al. 1996; Saga et al. 1999; Saga et al. 2000). These cells migrate to form the cardiac crescent, and subsequently form the linear heart tube through lateral folding of the embryo. This tube then undergoes looping and septation to form the four chambered heart. To begin with, the tube consists of two layers of cells, the internal endocardial cells and the exterior myocardial cells which form the myocardium. Migration of the neural crest forms the final layer of epicardium, through which arises the coronary vasculature and cardiac fibroblasts, and migration of second heart field forms the right ventricle and outflow tracts.

Cardiomyocytes form only 1/3 of the total population of cells in the heart. The remaining 65-70% of cells are non-myocytes and consist of fibroblasts, endothelial cells, pericytes, smooth muscle cells and macrophages (Nag 1980). Unmistakably, heart formation is an intricate process and governing this is a complex network of transcription factors and chromatin remodelling factors. The core cardiac transcription factors include Nkx2.5, Gata-4, 5 and 6; the TBX family, Hand 1 and 2, Isl-1, and the MEF family. These are supported by the addition of serum response factor (SRF) and myocardin. This complex gene regulator network works through transcription factor interactions and in regulating each other's expression (Figure 1.7). In concert, these control expression of cardiac structural proteins, gap junctions, calcium signalling proteins, as well as cell proliferation, hypertrophy, chamber septation and patterning.

Nkx2.5 is one of the first transcription factors to mark the onset of cardiomyocyte differentiation (Komuro & Izumo 1993). It is an evolutionary conserved gene; the first orthologue was identified in *Drosophila* and called tinman, as without this factor the heart did not develop (Bodmer 1993). Whilst Nkx2.5 is essential in *Drosophila*, in mice the null phenotype leads to abnormal heart morphogenesis, with lethality by E9.5 (Lyons et al. 1995). This suggests a more redundant role for transcription factors in the mammalian heart (Olson 2006; Olson & Schneider 2003)

Gata-4 is another important regulator of cardiac development. Whilst it does not appear to determine cardiomyocyte lineage specification, it plays an important role in migration and formation of the linear heart tube (Molkentin et al. 1997; Kuo et al. 1997). Its significance is highlighted by Gata-4 knockout studies which showed embryonic lethality between E8.5-10.5, with mice lacking heart tube formation and irregular distribution of procardiomyocytes (Molkentin et al. 1997; Kuo et al. 1997). However, tetraploid complementation clearly demonstrated that Gata-4^{-/-} cells are able to differentiate into cardiomyocytes (Kuo et al. 1997). From the onset of its expression at E7.0 (Heikinheimo et al. 1994), Gata-4 is active during all stages of heart development, continues to maintain the adult heart after birth (Charron & Nemer 1999) and is pivotal to hypertrophic response in adult heart failure (Oka et al. 2006; Suzuki 2011; Morimoto et al. 2001; Molkentin et al. 1998; Liang, De Windt, et al. 2001; Liu et al. 2008). It is a potent transactivator, promoting expression of many cardiac structural genes including ANF, BNP, cTnI, α -MHC, β -myosin heavy chain and platelet-derived growth factor receptor β (Charron & Nemer 1999; Oka et al. 2007).

Whilst the transcription factors Nkx2.5 and Gata-4 are globally expressed in the heart, and are potent transactivators of the cardiac muscle program, they are not sufficient to induce cardiomyogenesis on their own (Charron & Nemer 1999). However, interactions between transcription factors have been shown to potentiate cardiac gene expression. For instance, an early study by Durocher et al. (1997) showed that both Nkx2.5 and Gata-4 can interact and synergise to promote ANF and BNP expression. In more recent studies, reprogramming fibroblasts to cardiomyocytes showed that Gata-4

was indispensable, supporting Gata-4 as a key player in the formation of cardiomyocytes (Ieda et al. 2010; Nam et al. 2013; Song et al. 2012).

Gata4 is known to interact with a number of different transcription factors, which include SRF, Mef2, Tbx5, NFAT and FOG-2 (Dai et al. 2002; Garg et al. 2003; Lu et al. 1999). Some of these transcription factors have restricted expression within the developing heart, being deployed in certain chambers but not others. For instance HAND2 is restricted to the right ventricle whereas HAND1 is expressed in the left (Mcfadden et al. 2000; Dai et al. 2002). Gata-4 is known to directly regulate HAND2 expression (Mcfadden et al. 2000), interacting with the protein to synergistically promote cardiac gene expression (Dai et al. 2002). Another example of chamber restricted expression is that of Tbx5. Whilst it is expressed throughout the cardiac crescent, its expression becomes graded upon tube formation and is subsequently further restricted to the left ventricle and atria upon looping and septation (Bruneau et al. 1999).

Interactions not only mediate protein expression but, in a top down approach, also confer responsiveness to key signalling pathways. Specifically, the interaction of Gata-4 with NFAT permits responsiveness to calcium signalling, a pivotal player in pathological cardiac hypertrophy (Molkentin et al. 1998; Molkentin 2004; Oka et al. 2005; Morimoto et al. 2001; Kakita et al. 2001). Calcium signalling responds to growth stimuli, and feeds into the gene transcription by the dephosphorylation of NFAT by calcium/calmodulin dependent protein phosphatase, calcineurin (Oka et al. 2005). This permits translocation to the nucleus where it can interact with other transcription factors, such as Gata-4, and promote gene expression (Molkentin et al. 1998; Taigen et al. 2000)

In addition to the synergistic interactions, Gata-4 can also be negatively regulated by the interaction with the zinc finger protein FOG-2 (Lu et al. 1999). FOG-2 is important for heart morphogenesis and vascular development but can only bind DNA through its interaction with Gata-4 (Crispino et al. 2001). Whilst *in vitro* studies show that it predominantly inhibits Gata-4 transcriptional activity (Lu et al. 1999; Svensson et al. 1999), this is cell-type and gene dependent and may activate transcription in some instances.

Gata-4 itself is expressed in a variety of other tissues including the visceral endoderm, as well as the definitive mesendodermal derived tissues such as the developing liver, lungs, gonads and gut (Molkentin 2000). It is the unique combinatorial expression of these transcription factors that confers tissue specificity, as seen in the heart (Dai et al. 2002).

Schlesinger et al. (2011) demonstrated this high degree of combinatorial regulation in a comprehensive transcriptome study of four of the cardiac transcription factors; Gata-4, Nkx2.5, SRF and Mef2a. They analysed the DNA binding sites using ChIP-chip and used siRNA to examine the

functional consequence of transcription factor knockdown. From this they identified that these four transcription factors simultaneously control 91 genes, 3 of them control 121 genes, and 143 genes by just Gata4 and Nkx2.5 (Schlesinger et al. 2011). Inhibition with siRNA only produced a mild disruption in the expression of the target genes; thereby demonstrating the redundancy that can occur during development. This study also showed the importance of acetylation, with histone 3 acetylation markedly increasing Gata-4 and SRF target gene expression.

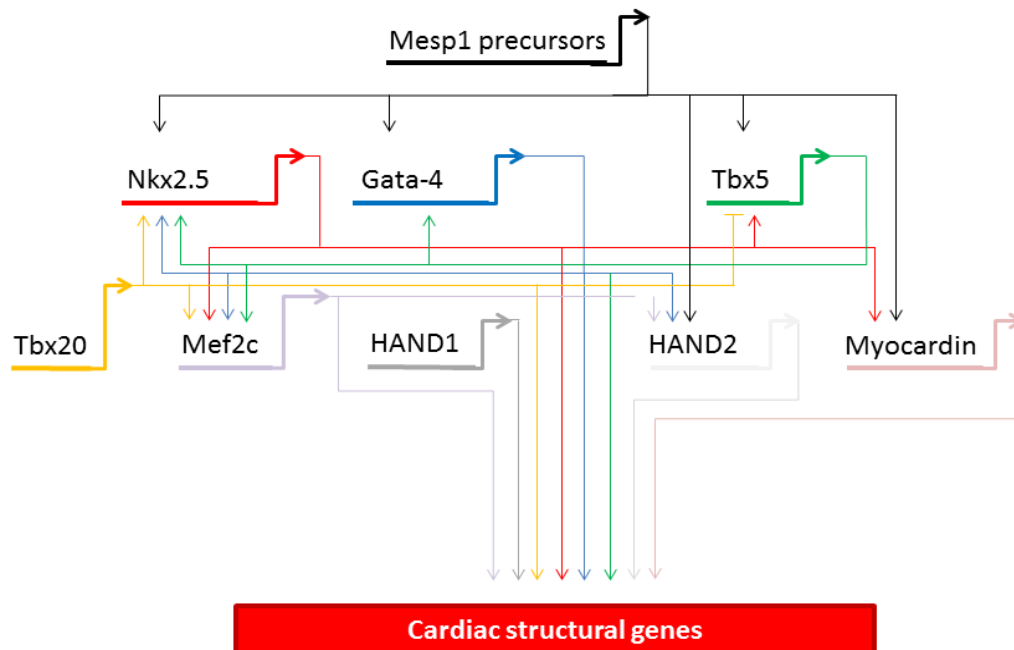


Figure 1.7. A schematic overview of the gene regulatory network governing cardiac development. This diagram is restricted to the interplay that occurs in the first heart field, which forms the left ventricle and atria. Whilst many of the genes regulate each other's gene expression, they also interact to promote expression of the structural genes. In this way the transcription factors act in a feed forward loop. For example, whilst Gata-4 promotes expression of Nkx2.5, Mef2c and HAND2 it also physically interacts with these proteins to promote heart development. Arrows designate gene activation and bars inhibition. Adapted from Herrmann et al. 2012

Undeniably, there are many other considerations besides transcription factor interactions. These include protein modifications such as phosphorylation, acetylation and sumoylation; as well as the chromatin conformation which is controlled by remodelling agents and more recently on the scene is the interplay of microRNAs.

Phosphorylation of Gata-4 plays a large role in regulating its activity. Notably, there are two distinct mitogen activated kinase cascades that activate Gata-4; the p38 and the MEK1-ERK12 signalling pathways. These have been shown to relay the response of hypertrophic stimuli, such as

phenylephrine and endothelin-1, increasing the Gata-4 transcriptional activity of B-type natriuretic peptide and A-type natriuretic peptide (Kerkelä et al. 2002; Liang, Wiese, et al. 2001). Conversely, phosphorylation by GSK3 β negatively regulates Gata-4 activity by targeting the transcription factor for nuclear export (Morisco et al. 2001)

miRNA have been described as fine-tuners of gene expression, down regulating low expression proteins and honing protein expression in specific cell types (Liu & Olson 2010). A single type of miRNA can target many different transcripts, most often working by inhibiting mRNA translation (Liu & Olson 2010). miRNA1 and 133 have been shown to be important in cardiac development and maintenance, with dicer knockout causing embryonic lethality (Liu & Olson 2010). This emphasises the importance of monitoring protein levels directly, and not only mRNA levels, as it is protein production that is most commonly disrupted by miRNAs.

Another important player in controlling gene expression is that of chromatin modifications. In the closed state, chromatin exists in tightly coiled formation around histones. Opening of the chromatin by relaxing these coiled structures is known to enhance gene expression in that region by permitting access of the transcription factors to the gene promoter binding sites. There are a large number of regulators of chromatin conformation. These include the histone acetylases and histone deacetylases, methylation, and ATPase based factors such as SWI/SNF-like complex.

In the liver, Gata-4 is known as the 'pioneer' factor being able to access promoter regions in compacted chromatin (Cirillo et al. 2002). However, this does not necessarily occur in heart development as there is mounting evidence for the role of chromatin remodelling agents. In particular Baf60c, a cardiac specific subunit of the SWI/SNF-like complex, was shown to facilitate attachment of Gata-4 to promoter sequences, although this was in non-cardiac mesoderm (Takeuchi & Bruneau 2009).

Transduction of hESC with factors to direct cardiomyocyte formation showed that the minimum required was Tbx5 and Gata4, but the four factors, Tbx5, Gata4, Nkx2.5 and Baf60c were the most efficient (Dixon et al. 2011). The need for the chromatin remodelling agents is corroborated by earlier work on Baf60c, which showed its global expression in the heart of the developing embryo (Lickert et al. 2004). Silencing of this subunit resulted in lethality and severe cardiac malformations, reportedly due to the underdevelopment of the first and second heart field progenitors (Lickert et al. 2004). *In vitro* experiments showed that Baf60c fostered the interaction between cardiac transcription factors Nkx2.5, Tbx5 and Gata-4 with Brg1, the BAF complex ATPase (Lickert et al. 2004; Puri & Mercola 2012).

Other instances indicating chromatin modification are that of the interaction between HAND2 and Gata-4, which simultaneously recruited p300, a protein with histone acetyltransferase activity

(HATs; Dai et al., 2002). p300 also directly acetylates Gata-4 enhancing its DNA binding and transcriptional activity and subsequently demonstrated its importance in regulating cardiogenesis (Kawamura et al. 2005).

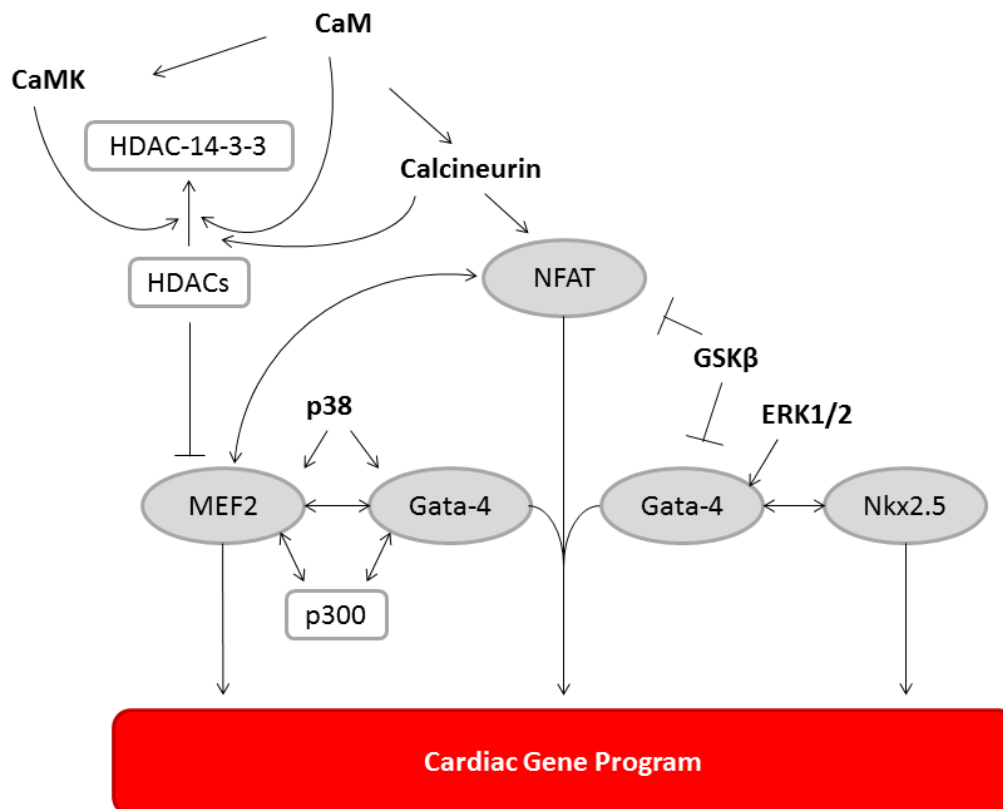


Figure 1.8. Schematic illustrating some of the important protein-protein interactions which regulate the activity of the key cardiac transcription factor Gata-4. Calcium signalling responds to growth stimuli and feeds into the gene network in multiple ways. It acts via calmodulin (CaM) which activates calcineurin to dephosphorylate the transcription factor NFAT. This permits its translocation to the nucleus where it can interact with Gata-4. Calcium signalling also controls HDAC activity, where phosphorylation mediates its nuclear export allowing MEF2c to interact with other transcription factors. The mitogen activated kinases, p38 and ERK1/2, are also involved in promoting cardiac gene expression by phosphorylating Gata-4. However, Gata-4 activity is countered by phosphorylation with GSK3 β which promotes its nuclear export and subsequent degradation. These interactions and protein modifications demonstrate the complexity of protein regulation. *Adapted from (Akazawa & Komuro 2003)*

Opposing the action of HATs are the histone deacetylases (HDACs), which act to deacetylate the amino tails of histones. This restores their positive charge and promotes the compact conformation of the chromatin (Haberland et al. 2009; Grunstein 1997). HDACs are highly implicated in mediating pathological hypertrophy in response to heart failure. Studies have shown that the class II HDACs repress the transcription factor Mef2c through direct binding, thereby inhibiting its transcriptional activity and interaction with other transcription factors such as Gata-4 (Zhang et al. 2002; Olson et

al. 2006; Chan et al. 2003). In cardiogenesis specifically, inhibition of HDAC 4 with trichostatin in P19 embryonic carcinoma stem cells (Karamboulas et al. 2006) up regulated expression of the transcription factors Nkx2.5, Gata-4 and Mef2c as well as the structural protein cardiac α -actin. Thus, this demonstrates the significance of HDACs in cardiogenesis.

The gene regulatory network controlling cardiac development is complex (Figure 1.7). The examples here focus on the pivotal role of the transcription factor Gata-4; highlighting the vast regulatory networks in which it is involved and which is far more complicated than gene expression alone (Figure 1.8). Gata-4 transcriptional activity is controlled by interactions with other transcription factors and regulatory proteins, some of which confer post translation modifications. These modifications include acetylation, phosphorylation and sumoylation and act not only to control its activity but the cellular location and susceptibility to degradation, all of which impact the transcriptional activity. Another facet of transcription factor control is access to the enhancer regions, which is controlled by chromatin conformation and modulated by proteins families such as HATs, HDACs and SWI/SWIF like complex. Thus, it is clear that protein analysis is necessary, and that studying protein interactions and modifications will be instructive as to the pathways controlling differentiation of cardiomyocytes.

1.3 Thesis overview: Application of single cell proteomics to cardiac progenitor cells

There is compelling biomedical need to regenerate cardiac tissue. Clinical trials with bone marrow stem cells had limited efficacy in improving cardiac output in patients who had suffered a myocardial infarction. Current trials are using endogenous cardiac progenitor cells, expanding the cells *ex vivo* from a biopsy before re-administration. Although they have been shown to form cardiomyocytes in *in vivo* studies, these cells are poorly characterised. The great challenge is to activate the cardiac progenitor cells *in situ* for cardiac repair (Vieira & Riley 2013; Bollini et al. 2011).

Already, it is known that forced expression with the key cardiac transcription factors can direct fibroblasts to the cardiac cell fate. Elucidating the key steps that control differentiation would be preferable to the use of retroviral transformation (Vieira & Riley 2013). The CPCs express the key cardiac transcription factors whilst maintaining a stable progenitor phenotype. Rigorous examination the status quo of these cells is needed in order to understand the cues holding these cells in their arrested state. By understanding the activity status of transcription factors or their protein interactions will facilitate unlocking their cardiogenic potential.

Given that cardiac progenitor cells are so few in number in the heart, studying the protein expression of the fresh cells is almost impossible without clonal expansion. Further still, clonal expansion of these cells has shown that there is a heterogeneous expression of the core cardiac transcription

factors. Therefore being able to study these cells at the single cell level would be greatly beneficial, and is currently not met by other technologies.

The aim of this thesis was to develop an assay that could detect protein from a single cardiac progenitor cell using total internal reflection microscopy, with a view to looking at protein interactions in the future.

Gata-4 has a prominent role in cardiac development, being a key component of the gene regulatory network and is known to be expressed in the cardiac progenitor cells. There are many important other players in cardiac differentiation; however, their inclusion in such an assay as this is highly dependent on the availability of specific, high affinity antibodies. An initial search of the antibodies commercially available for the key cardiac transcription factors revealed that there was limited availability of monoclonal antibodies for the factors of interest. Since it is known that polyclonal antibodies have lower affinity for their target to monoclonals, it was decided to setup the assay using the cardiac transcription factor Gata-4, which had a large repertoire of monoclonal antibodies.

The assay is based on the traditional antibody sandwich system, in which proteins are pulled down with printed capture agents and detection performed through fluorescently conjugated secondary antibody. Two approaches were used for the development of this assay; the first was using antibodies as is typical, and the second approach focused on developing a DNA based protein-capture agent.

The chapters in this thesis describe the different stages of assay development, and detail further background information for each section. Chapter 2 lists all the materials and methods, whilst chapter 3 explains the single molecule counting algorithm developed by the group and used to process the TIRFM data.

Chapter 4 details the initial stages of assay development, with validating suitable Gata-4 antibodies, using the standard techniques of Western blotting and immunohistochemistry. For optimising such a sensitive technique, several parameters need to be considered and are discussed in Chapter 5. These include surface chemistry, immobilisation strategies, and types of conjugations as well as antibody concentration. This is followed by the calibration of the assay in Chapter 6 and discusses the issues of affinity and other parameters which affect the assay's limit of detection. Chapter 7 shows the development of single cell experiments with cardiac progenitor clones, then details all the permutations and finally discusses possible improvements in addition to the limitations. Chapter 8 focuses on the possible development of alternative protein-capture agents, and details the development of the DNA capture agents for Gata-4. Finally, Chapter 9 rounds up all the discussion points and summarises the main findings and limitations, improvements and future work.

Chapter 2

2. Methods and materials

2.1 Cells

All cells used throughout this study are listed in the table below (Table 2.1).

Name	Source	Tissue origins	Purpose
HEK 293 FT	Invitrogen (R700-07)	Human embryonic kidney cell line, immortalised. Contains the SV40 viral antigen	Transfection for protein expression
Hep G2	HPA (85011430)	Human hepatocyte carcinoma	Protein isolation
MCF7	Willison, Imperial College London	Human breast adenocarcinoma	Protein isolation
HeLa	Tate group, Imperial College, London	Human cervix epitheloid carcinoma	Transfection for protein expression
Scal⁺ clonal cardiac progenitors cells	Schneider et al. Imperial College London	Adult mouse heart, derived from a single cell	Protein isolation, TIRFM single cell experiments

Table 2.1. List of all cells used in this study.

2.2 Cell culture

All cells were grown at 37 °C, in 5% CO₂, in DMEM containing 10% FBS (Table 2.3), except cardiac progenitor clones which were cultured in CGM (see Table 2.2 for details) on collagen I coated flasks. Flasks were coated in the tissue culture hood using collagen I solution (Table 2.4), adding 1 ml per 10 cm² dish and leaving for 30 minutes before aspirating. Coated flasks were left in the hood overnight to dry. Flasks were washed once in PBS (PAA, UK # H15-002) prior to use. All cells were passaged at about 80% confluency with 0.25% trypsin-EDTA (Life technologies, UK, # 25200-056).

Clonal Growth Medium (CGM)	Manufacturer (Catalogue Number)
65 % DMEM / Ham's F-12	Life technologies, UK (#I1320074)
35 % Iscove's Modified Dulbecco's Medium	Life technologies, UK (#I2440046)
3.5 % Bovine growth serum	Merck Millipore, UK (# SH30541)
100U/ml Antibiotics-Antimycotics	Life Technologies, UK (# I5240096)
2 mM L-Glutamine	Life Technologies, UK (# 25030024)
0.1 mM β -Mercaptoethanol	Sigma aldrich, UK (# M7522)
1.3 % B27 media supplement	Life technologies, UK (# I7504044)
6.5 ng/ml Epidermal growth factor	Peptotech, UK (# AF-100-15)
13 ng/ml fibroblast growth factor-basic	Peptotech, UK (# I00-18B)
0.0005 U/ml thrombin	Roche Applied Biosystems, UK (#I0602400001)
0.345 ng/ml human cardiotrophin-1	Cell Sciences, UK (# CRC700B)

Table 2.2. Recipe for clonal growth media (CGM) used to culture cardiac progenitor cells, SP₃ and SP₁₆.

DMEM culture medium	Manufacturer (Catalogue Number)
Dulbecco's modified eagle medium	Sigma Aldrich, UK (# D6429)
10% Foetal bovine serum	PAA, UK (# A15-151)

Table 2.3. Culture media for all cells except cardiac progenitor cells.

Collagen-coating solution	Manufacturer (Catalogue Number)
50 μ g/ml Type I rat tail collagen	BD Biosciences, UK (# 354236)
547 μ l Glacial acetic acid	Thermo Fisher, UK (# A/0400/PB17)
500 ml Distilled water	Life Technologies, UK (# I5230162)

Table 2.4. Details of collagen coating solution used to coat culture flasks for growing cardiac progenitor cells.

2.3 Cloning: creating a flag tag Gata4 expression vector

An N terminus Flag tagged Gata4 expression vector, was created using expression vectors pCDNA3.1 containing the Flag sequence (from Dr. D Mann's group) and pCMV6-XL4 containing the human Gata-4 expression sequence (Origene, USA # SC124037). The Gata-4 insert was created by PCR, with a TEV cleavage site using the primers detailed in Table 2.5. The primer sequences encoded 5' restriction sites XhoI and XbaI on the forward and reverse strands, respectively. The pCDNA3.1 vector encoding the flag tag and the PCR product were cut using the restriction enzymes, XhoI and XbaI, for which the conditions are listed in Table 2.6

Primer	Sequence
Forward	CTCGAG TTGAAAACCTGTATTTTCAGGGAGGA GGAATGTATCAGAGCTTGGCCATG
Reverse	TCTAG ATTACGCAGTGATTATGTCCCC

Table 2.5. Primer sequences to amplify Gata4 insert. In bold are the restriction 5' sites encoded, XhoI for the forward sequence and XbaI in the reverse sequence. The TEV cleavage site encoded in the forward primer is italicised.

Digestion
1xNEB buffer 4
0.1mg/ml BSA
2 units XhoI
2 units XbaI
2ug of DNA
Incubate at 37°C for 1.5 hours
Heat inactivate at 65°C for 20 minutes

Table 2.6. Reagents and conditions for digesting DNA vector and Gata4 insert.

2.4 Protein expression systems

Protein was expressed in HEK 293T cells transfected with the plasmids using Fugene 6 transfection reagent (Promega), at a ratio of 3:1 reagent to DNA. Media was changed prior to transfection and cells grown for up to 48 hours before harvesting (see protein isolation protocol in section 2.5).

The FLAG tag on the Gata-4 protein allows isolation using a FLAG specific antibody, and the TEV cleavage site allows for protein elution. To purify the protein, magnetic protein G coated beads were cross-linked with an anti-Flag antibody (F1804, Sigma Aldrich) according to the manufacturer's instructions. Cell lysates were then incubated with the beads for 10 minutes at room temperature, followed by washing in PBS. Upon transferal to a new tube the beads were treated with Pro-TEV plus

(#V6101, Promega) to cleave the protein. The enzyme is then removed from the protein solution via its HQ tag, which binds to nickel when passed through a HisLink affinity column (#V8823, Promega). The resulting purified protein was aliquoted and stored at -80°C.

2.5 Non-denaturing protein isolation

To retain protein function, cell lysates were made with non-denaturing conditions. Whole cell lysates were made by washing cells three times in cold PBS, before incubating with non-denaturing lysis buffer contain 1% triton X-100, for 10 minutes with shaking (see below for buffer details). Cells were then collected by scraping and sonicated to release nuclear proteins. Sonication was done at 4°C, with 5 repetitions of 5-10 seconds of sonication with a pause of 30 seconds in between to avoid overheating. The cell lysates were then spun at 14,000 rpm for 10 minutes at 4°C to pellet cell debris. The supernatant was collected and stored at -20°C or -80°C for long term storage.

All protein samples were stored with 10% glycerol to preserve protein function upon freezing. Protease and phosphatase inhibitors (#78442, Pierce) were added to all buffers and contained aprotinin, sodium fluoride, sodium orthovanadate, β -glycerophosphate, bestatin, E64 and leupeptin.

Non-denaturing lysis buffer
50mM Tris HCl pH7.4-8.0
150mM NaCl
10% glycerol
1% Triton X-100

Table 2.7. Non-denaturing lysis buffer recipe

2.6 Preparation of cellular fractions

Cells were placed on ice and washed three times with cold PBS, scraped and pelleted at 3000 rpm for 5 minutes in a swing bucket centrifuge. Cells were then resuspended in 6 volumes of Harvest buffer (10 mM HEPES pH7.9, 50 mM NaCl, 0.5 M Sucrose, 0.1 mM EDTA, 0.50% Triton x 100). After incubation for 5 minutes on ice, the nuclei was pelleted at 1000 rpm in a swing bucket centrifuge for 10 minutes. The cytosolic supernatant was carefully collected and subsequently cleared of cellular debris by centrifugation at 14,000 rpm for 15 minutes. The nuclei pellet was washed and resuspended in buffer A (10 mM HEPES pH7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) and re-pelleted at 1000 rpm for 5 minutes. The supernatant was discarded and 4 volumes of buffer C (10 mM HEPES pH7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA) added, and then vortexed at 4°C for 15 minutes to release nuclear protein. The nuclear lysate was then repelleted at 14,000 rpm for 10 minutes at 4°C and the nuclear extract collected. Protease and phosphatase inhibitors (#78442,

Pierce) were added to all buffers prior to use and contained aprotinin, sodium fluoride, sodium orthovanadate, β -glycerophosphate, bestatin, E64 and leupeptin.

2.7 Western blotting

Prior to loading, protein samples were denatured by adding 4x sample buffer containing SDS and heated to 94°C for 10 minutes. Samples were loaded on to polyacrylamide gels at between 20-30 μ g per lane and run on 10% gels with a 4% stacking gel, in running buffer containing SDS, at 75-90V for up to 2 hours in mini vertical gel tanks (Hoefer, USA # SE250). Proteins were then transferred from the gels to nitrocellulose membranes in tris-glycine transfer buffer containing methanol at 90v, 4°C for 1.5 hours in Mighty Small Transfer Tank (Hoefer, USA # TE22). Nitrocellulose membranes were blocked in 5% skimmed milk powder (Sigma Aldrich, UK # 70166) in PBS-tween 20 (PBST). Membranes were then probed with primary antibody over night at 4°C on a rotator. Membranes were washed three times in PBST, for 5 minutes each wash. Membranes were then incubated, whilst shaken, with the secondary antibody conjugated to horse radish peroxidase, followed by washing three times in PBST. Signal was developed using ECL western blotting detection reagents (GE Healthcare Life Sciences, UK # RPN2109) and visualised either by x-ray film (Amersham Hyper Film ECL, GE Healthcare Life Sciences, UK # 28-9068-35) or with the G:BOX Chemi XX6 imaging system (Syngene, UK).

Target	Host Species	Type	Application used in (ug/ml)	Company (Product code)
Anti-Gata4 antibodies	Mouse	Monoclonal (IgG2b)	WB (1), IF(10)	R&D systems, UK (# MAB2606)
	Mouse	Monoclonal (IgG1)	WB (0.5), IF(0.5)	BD biosciences, UK (# 560327)
	Rabbit	Monoclonal (IgG)	WB (1/250)	Abnova, Taiwan (# ab134057)
	Mouse	Monoclonal (IgG2b)	WB (1/250)	Abnova, Taiwan (# ab86371)
	Mouse	Monoclonal (IgG3)	WB (0.4)	Sigma Aldrich, UK (# SAB3300090)
	Mouse	Monoclonal (IgG2a)	WB (0.5)	Santa Cruz Biotech., USA (# sc25310X)
Gata - 5	Goat	Polyclonal	IF	Santa Cruz Biotech, (# sc-7280)
Gata - 6	Rabbit	Polyclonal	WB	Thermo Fisher, UK (#PA1-104X)
HSP90	Rabbit	Polyclonal	WB (1 in 1000)	Cell signalling tech., USA (# 4874)
Beta-Actin	Mouse	Monoclonal (IgG1)	WB (1 in 2000)	Sigma Aldrich, UK (# A54441)
Conjugated secondary antibodies				
Anti-mouse	Horse	HRP, polyclonal	WB (1 in 1000)	Cell signalling tech., USA (# 7076)
Anti-rabbit	Goat	HRP, polyclonal	WB (1 in 1000)	GE Healthcare, UK (# RPN4301)
Anti-goat	Donkey	HREP, Polyclonal	WB (0.4)	SantaCruz Biotech., USA (# sc-2020)
Anti-Mouse	Goat	FITC, polyclonal	IF (1 in 1000)	SantaCruz Biotech., USA (# sc-2010)
Isotype controls				
IgG1	Mouse	Monoclonal	IF(0.5)	R&D systems, UK (# MAB002)
IgG2B	Mouse	Monoclonal	IF(10)	R&D systems, UK (# MAB004)
IgG	Rabbit	Polyclonal	IF	R&D systems, UK (#AB-105-C)
IgG	Goat	Polyclonal	IF	R&D systems, UK (#AB-108-C)

Table 2.8. List of all antibodies used in both Western blot (WB) and immunofluorescent cell staining (IF).

4x sample buffer	Company (product number)
0.5 M Tris (pH 6.8)	Sigma Aldrich, UK (# T1503)
36% Glycerol	Sigma Aldrich, UK (# G5516)
18% ml 20% SDS	Sigma Aldrich, UK (# L3771)
0.04% Bromophenol Blue	Fisher Scientific, UK (# I0040040)
4% Beta-Mercaptoethanol	Sigma Aldrich, UK (# M3148)

Table 2.9. List of ingredients for 4x Laemmli sample buffer, used to for running protein samples on acylamide gels.

1x running buffer	Company (product number)
25mM Tris Base	Sigma Aldrich, UK (# T1503)
192mM Glycine	Sigma Aldrich, UK (# G8898)
0.1% SDS	Sigma Aldrich, UK (# L3771)

Table 2.10. Recipe for running buffer used to run protein samples on acrylamide gels

1x Transfer buffer	Company (product number)
25mM Tris Base	Sigma Aldrich, UK (# T1503)
192mM Glycine	Sigma Aldrich, UK (# G8898)
20% Methanol	VWR Int. Ltd., UK (L13255)

Table 2.11. Recipe for transfer buffer used to transfer proteins from the acrylamide gel to a nitrocellulose membrane using electrophoresis.

10% resolving gel	Company (product number)
10% Acrylamide/Bis (19:1)	AppliChem, UK (# UN3426)
1.5 M Tris (pH 8.8)	Sigma Aldrich, UK (# T1503)
10% SDS	Sigma Aldrich, UK (# L3771)
10% APS	Sigma Aldrich, UK (# A3678)
0.1% TEMED	Sigma Aldrich, UK (# T9281)

Table 2.12. List of ingredients for making the acrylamide gels. This is made first and then the 4% stacking gel poured on top.

4% stacking gel	Company (product number)
4% Acrylamide/Bis (19:1)	AppliChem, UK (# UN3426)
0.5 M Tris (pH 6.8)	Sigma Aldrich, UK (# T1503)
10% SDS	Sigma Aldrich, UK (# L3771)
10% APS	Sigma Aldrich, UK (# A3678)
0.1% TEMED	Sigma Aldrich, UK (# T9281)

Table 2.13. Recipe for 4% stacking gel which formed the top 2 cm of the polyacrylamide gels.

2.8 Immunofluorescent staining of cultured cells

Glass coverslips were placed in 24-well plates and coated in collagen I as with coating cell culture plates (see section 2.2). The night before fixation, cells were plated between 50-100,000 cells per well. The following day cells were washed three times in PBS and fixed with 4% formaldehyde, 20 minutes on ice followed by three PBS washes. Cells were permeabilised with a 1:1 mixture of methanol and acetone at -20°C for 10 minutes. Cells were blocked in PBS containing 4% FBS for 1 hour at room temperature. Cells were then incubated overnight with primary antibodies in blocking solution, shaking overnight at 4°C. Cells were then washed three times in PBS and then incubated in fluorescently conjugated secondary antibodies solution whilst shaking for 1 hour at room temperature (for antibody concentrations see Table 2.8).

After the final three PBS wash steps, coverslips were mounted on microscope slides using Fluoroshield™ mounting medium containing DAPI (Sigma Aldrich, UK # F6057) and visualised using Zeiss Observer Z1 epifluorescence microscope.

2.9 PCR of cell lines

Reverse transcription PCR was run in order to check gene expression. Separate primers were used for mRNA detection in mouse and human cell lines. The murine primers were GapDH (Integrated DNA Technologies) for the control and Gata-4 (# RDP-345, R&D Systems) producing a band at 479

bp. The annealing temperature for the GapDH (452bp) and Gata-4 primers were 64 and 54.4°C respectively. For the human cell lines a Gata-4 primer with an amplicon of 68bp was used (#4331182, life technologies) with an annealing temperature of 60°C. For low the low weight amplicons (less than 100 bp), products were run on 10% acrylamide TBE gels with an Ultralow Range DNA Ladder, spanning 10-300bp (GeneRuler). otherwise products were run on 1% agarose gel containing gelred using a 1 kb DNA ladder (Gene ruler) and on a 4% TBE gel.

RNA was extracted using the Qiagen shredder kit according to the manufacturer's instructions. Human Universal and Murine Universal Reference RNA was used as the positive controls and Human Colon and Murine Kidney RNA was used as the negative controls (eBioscience, UK). PCR was performed using the Qiagen One-Step PCR kit and the conditions optimised for the primers.

Stage	Temp	Time	Repeat 30 times
Reverse transcription	50	30 mins	
Initiation	95	15 min	
Melting	95	30 sec	
Annealing	54.4-64	30 sec	
Extension	72	1 min	
Final Extension	72	10 min	

Table 2.14. Conditions for RT PCR, annealing temperatures different for Gata-4 and GAPDH primers respectively.

10% TBE gel	Company (product number
1xTBE	Fisher Scientific, UK (# FQ-B52)
10% acrylamide (19:1)	AppliChem, UK (# UN3426)

Table 2.15. Recipe for gel used for DNA PAGE.

2.10 Design of double stranded DNA probes for capture of Gata4 protein

Double stranded DNA oligonucleotides were designed for Gata-4 protein capture. The DNA binding regions were chosen based on consensus sequences found for Gata-4 in the Transfac® data base (Biobase GmbH). The ANF Gata4 binding site and the ANF mutant negative control were obtained from (Charron et al. 1999). DNA was bought as single strands and then hybridised by heating to 94°C and cooling over several hours. Details of the sequences are listed in Chapter 8.

2.11 Printing of capture agents

Capture agents were printed with the OmniGrid Micro, microarray (Digilab, UK) using micro spotting pins (ArrayIt, USA # 946MP2). All capture agents were printed in 3x SSC, 1.5M Betane (Diehl et al. 2001) on clean glass coverslips, thickness no. 1.5 (VWR, UK #631-0147), unless otherwise stated. The SSC-betane buffer was the chosen as it improved spot homogeneity both in DNA and, in our experience, antibody printing (Diehl et al. 2001). Amine modified DNA capture agents were printed on GPTS modified glass coverslips. All capture agents were printed at 50-60% humidity.

GPTS modified glass coverslips were prepared by cleaning in methanol, followed by 0.5 M sodium hydroxide and then plasma cleaned. Glass coverslips were then incubated with GPTS solutions, containing 2.5% GPTS, 5% acetic acid and ethanol with pH2-3. Slides were incubated for 1 hour at room temperature before washing in distilled water and dried with nitrogen gas. Unmodified glass coverslips were cleaned in three changes of methanol with sonication of at least 30 minutes.

2.12 Photolithography

In order to micropattern PDMS with micrometer scale features a template is made on a silicon wafer using photolithography. This is formed by placing a photomask onto light sensitive photoresist and the design is transferred by exposure to UV light. In order to coat the silicon wafer with photo resist, the wafer is spun with SU-8 50 (MicroChem), ramping the speed to 500rpm at an acceleration of 100rpm/second and held for 5 to 10 seconds, the spin coater is then ramped to 2000 rpm at an acceleration of 300 rpm and held there for 30 seconds. This gives a coating with a thickness of 50 μm . The photoresist is then soft baked to remove the solvents and densify the film by pre-baking at 65°C for 6 minutes followed by soft baking at 95°C for 20 minutes. The photoresist is then exposed, with the photomask on top, to UV (350-400 nm) to a total of 400 mJ/cm². The wafer is then baked once more to cross link the exposed regions, heating at 65°C for 1 minute followed by a post exposure bake at 95°C for 5 minutes. The SU-8 mask is then developed in SU-8 developer (MicroChem) for 6 minutes, followed by washing in isopropyl alcohol and finally dried in nitrogen gas.

2.13 PDMS microfluidic device fabrication

PDMS devices were made by casting PDMS at a 1:10 ratio with curing agent onto the silicon wafer masks. The PDMS was placed in a desiccator to de-gas and then allowed to set at room temperature, overnight on a flat surface. The devices were then cut out and ports drilled using 300 μm drill bits. The PDMS was then cleaned in ethanol with sonication followed by drying with nitrogen gas. Devices were then aligned to printed coverslips using a jig (made in house). The designs of the microfluidics are in the relevant results chapters.

2.14 TIRFM experiment setup

Microfluidic device were connected to an electronic pump set (Labsmith, USA) that can administer microfluidic volumes and was controlled by the accompanying computer software (Labsmith, USA). The devices were connected to samples with peek tubing, inner diameter 160 μm , outer diameter 360 μm . Devices required desiccation with fluid before connecting to pumps.

2.15 Optical setup

An inverted microscope (Nikon Ti-E, Nikko, Japan) with a 60x oil-immersion objective (apo, 1.49 NA, Nikon) was used for single molecule microscopy. The specifications of this microscope include widefield white light and epi-fluorescent imaging provided by halogen and mercury lamps. The turret housed the following objectives: achromat 10x 0.25 NA dry, plan fluor 20x 0.5 NA dry, plan fluor 40x 0.9 NA dry, as well as the 60x objective mentioned above. The motorised stage could translate in x,y directions and the objectives translated in the z axis. This enabled the perfect focusing system (PFS) which automatically adjusted the focal plane during imaging, thereby eliminating focal drift. The microscope has two filter carousels; the upper contained the standard Nikon FITC, DAPI, and TRITC dichroic filters plus the TIRF illumination dichroic (Chroma, USA). The lower carousel contained a filter set for the pulsed laser source used for laser lysis, a single-edge dichroic (LPD01-532R-25; Laser 2000, UK) and long pass filter (LP03-532RS-25; Laser 2000, UK) with wavelength at 532 nm or 1064 nm, respectively. The single edge filter prevents reflected or scattered light from reaching the detector.

The microscope is connected to a motorised TIRF unit (MTU) to convert the solid-state continuous wave laser beam with the wavelength of 473 nm (MBL-473-200; Laser 2000, UK) into TIRF. Two other lasers were attached through the back port; a pulsed Nd-YAG at 1064 nm for laser lysis and a Ytterbium fibre laser at 1070 nm for optical trapping of cells. Figure 2.1 illustrates the optical setup.

For fluorescent detection an IXON DU-897E electron-multiplying charge-coupled device (EM-CCD; Andor Technologies, Ireland) is used. Intrinsic noise primarily results from dark current and read noise which is minimised by thermoelectric cooling. Single molecule detection is achieved due to the background discrimination coupled with the high sensitivity of the EM-CCD.

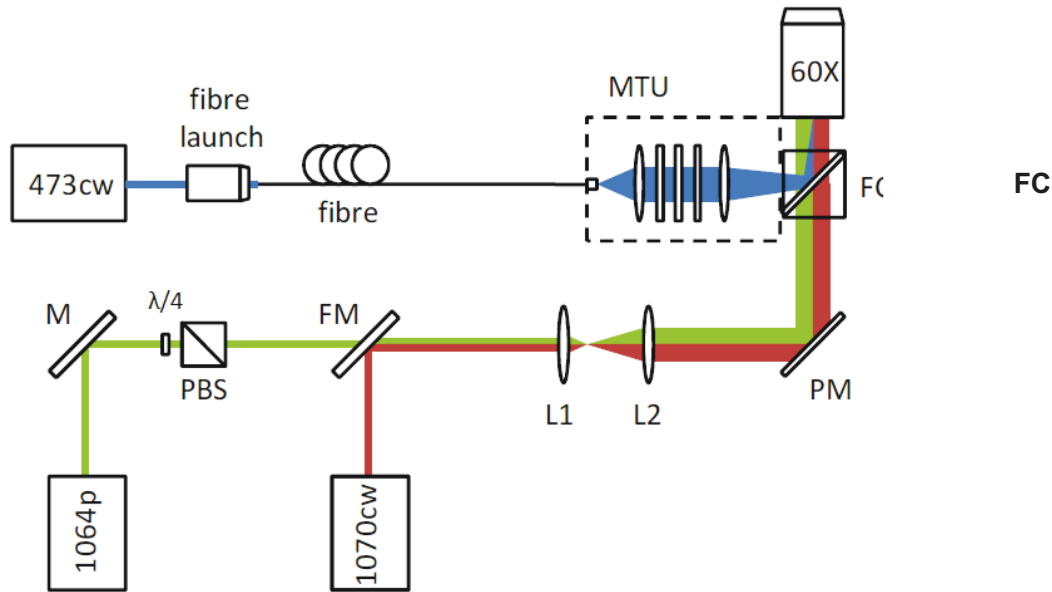


Figure 2.1. Optical setup for TIRF microscopy, the optical trapping and the laser lysis. The lasers; 473nm continuous waver laser, Nd:YAG pulsed laser at 1064 nm and Ytterbium fibre laser at 1070 nm are coupled into the back port of the microscope. EM-CCD = electron multiplying charge-coupled device camera, PM= periscope mirrors, M = mirror, $\lambda/2$ = half-wave plate, PBS = polarising beam splitter, FM=flip mirror L₁ & L₂ = lenses in beam expander , MTU = Nikon motorised TIRF unit, FC = filter cube. *Taken from Salehi-reyhani et al. 2011.*

2.16 Optical trapping of single cells

Here a single beam of wavelength 1070 nm, power set at approximately 50 mW, from a Ytterbium fibre laser, is highly focused using the 60x 1.49 NA, oil-immersion objective to provide attractive and repulsive forces. Where the focused beam is most narrow, there is a very strong electric field gradient. Dielectric particles (like cells) are attracted along the gradient to the strongest point. The x,y translation of the motorised stage allows manipulation and movement of the cells through the microfluidic device.

2.17 Laser lysis

A high energy solid-state Nd:YAG pulsed laser (Surelite SL I-10; Continuum, USA) was used for cell lysis. This laser can deliver a single pulse, of 6 ns in duration, at a wavelength of 532/1064 nm. The pulse fire is operated by a manual joystick, delivering a pulse of maximum 1.43 ± 0.13 J. A 60x NA=1.49 oil-immersion objective is used, focusing pulses to 10 μ m above the cells.

Chapter 3

3. Single molecule counting algorithm

The counting algorithm used in this work was developed by other members of the research group (Burgin et al 2014). The Matlab program I specifically used for my data sets was developed by Dr. Aidan Brown. The algorithm consists of three parts; i) background correction of the laser illumination profile, ii) setting a threshold, iii) counting of single molecules based on pixel size and intensity.

3.1 Background correction of single molecule datasets

For image detection we used a sensitive digital detector and an electron multiplying current coupled device (EMCCD, Andor 897). This technology is capable of detecting single photons and designed to detect weak signals above intrinsic noise. The intrinsic camera noise includes photon shot noise, dark current, read noise and the analogue to digital conversion, and clocked induced charge. Much of the intrinsic noise is overcome by the electron multiplying gain function which is able to multiply weak signals such that the intrinsic noise is negligible. The cooling feature in the device ensures that dark noise is kept to a minimum. The EMCCD corrects for gain fluctuations, so that the peak of the distribution is the same for each frame.

The next step in background correction is to flatten the field of view. The laser beam illuminates the field of view with uneven intensity due to fibre alignment (see Figure 3.1.B). Photon excitation from a fluorophore scales with laser intensity; therefore, in a single image fluorescent molecules in the centre of the field of view will have a greater intensity than those in the periphery. It is therefore important to eliminate this uneven intensity, otherwise weaker signals will not have the same weighting as stronger signals.

The background correction is applied by taking a set of control images, in this case frames containing fluorophore only, and taking the average across the set. A broad gaussian kernel filter is applied with a radius of 60 pixels. This gives the averaged distribution of laser intensity across the images. All frames are divided by this background image, resulting in an even intensity distribution across the image. Since the intensity of each frame can vary across the data set, for example between time

points or different frames, the background correction applied is scaled so that the intensity of the background image matches the intensity of each individual frame. Background intensity of each frame was measured in regions where there was no printed antibody and few real single molecules. The algorithm used these intensities to scale the correction image to match of the image being corrected.

A second filter, a clustering mask, was applied to the data set to discriminate true signal from background noise. This is a disc filter with a radius of 1.5 pixels, it identifies local maxima in both the x and y direction. Fluorescence of a single molecule (fluorophore conjugated antibody) will display over more than one pixel, displaying intensity over a group of connected pixels. Typically, background noise does not cluster and occurs on individual pixels that are not connected. This filter dilutes background noise and blurs real single molecules across a broader range of pixels.

Figure 3.2 shows the effect of different filter sizes on single molecule count. By applying a filter of radius 0.5, no discrimination is made between background noise and true signal, and thus produces a high false positive count. By applying a larger filter, akin to the size of true single fluorescent molecule, reduces the signal by 3 fold. There is negligible difference between a filter size of radius 1 or 1.5. Hence, discriminating single molecules based on size is a useful tool decrease the false positive count.

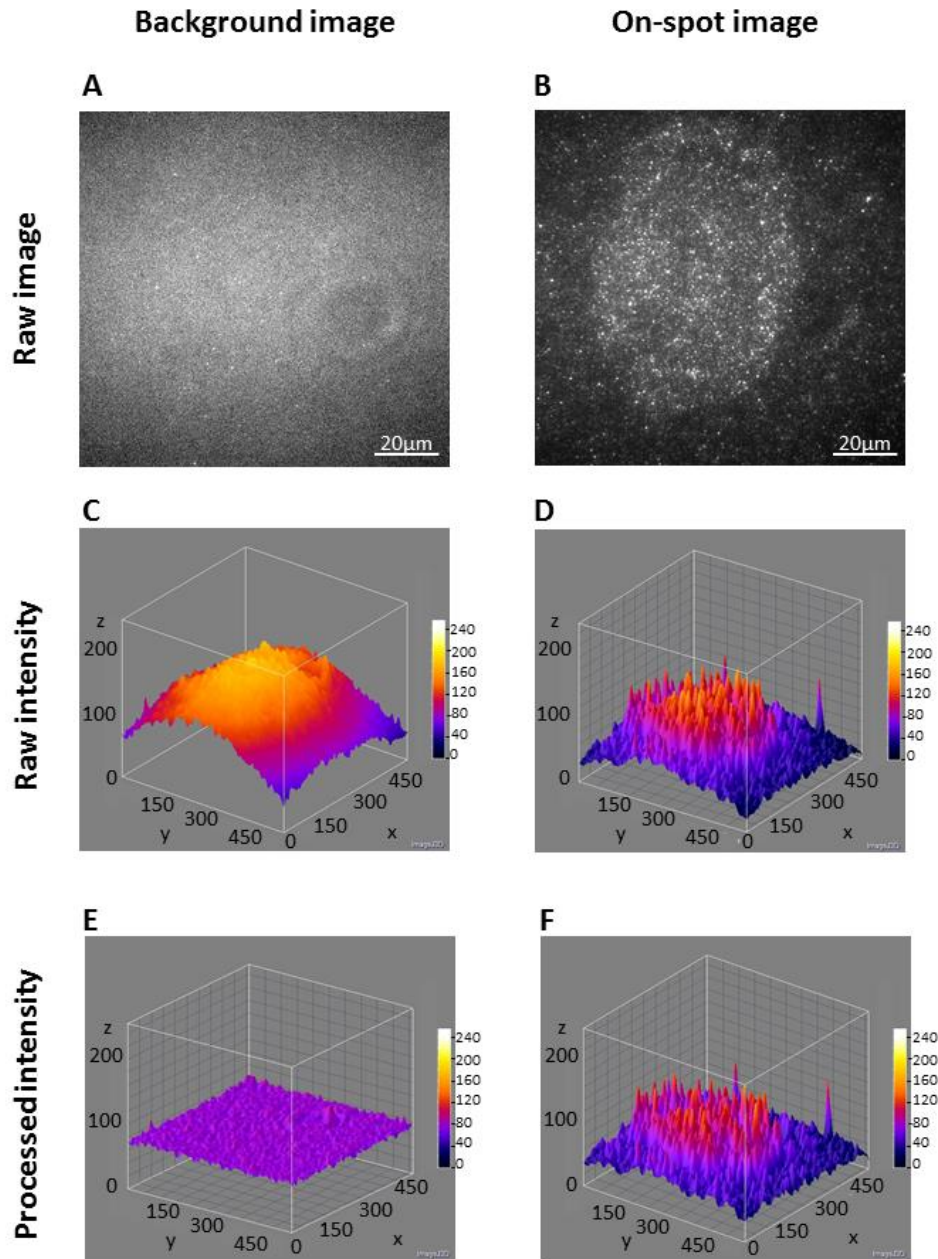


Figure 3.1. Outline of the background correction process. Starting with the raw images taken of background on glass with only fluorophore present (A) and of protein binding to printed antibody (B). C and D show the corresponding pixel intensities of the raw data (Z axis), where the x,y axes represent position across the field of view. C, essentially shows the Gaussian profile of the laser beam, resulting in uneven illumination across the field of view. D shows the intensity across the printed antibody spot after incubation with Gata4. It can be seen that pixels in the centre of the spot are more intense than at the periphery. E and F are the intensities after dividing through by the blurred background image. The Gaussian profile of the laser beam has become planar (E) and the pixel intensity across the antibody spot is normalised (F).

Comparing single molecule count with different filter sizes

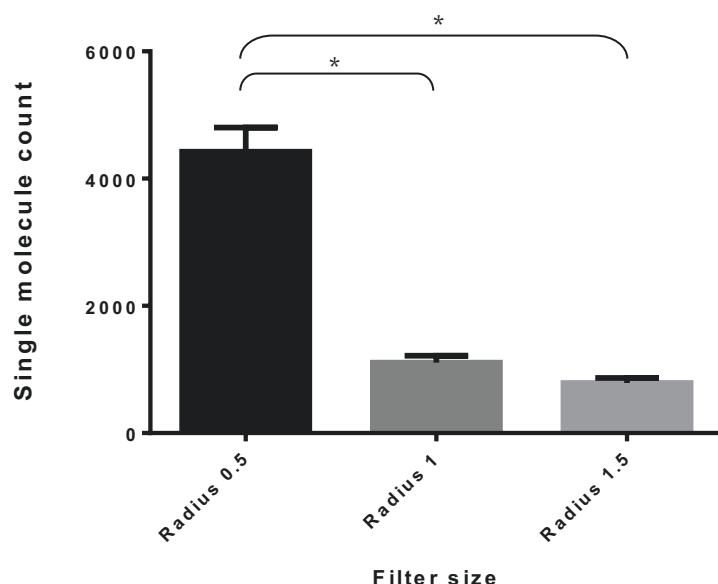


Figure 3.2. A comparison of different filter sizes to identify single molecules. Filters are applied to sample data; printed DNA incubated with Gata4 protein and secondary ant-Gata4 detection antibody. Filter radius 0.5 identifies single pixels and therefore all intense pixels will be counted, producing a high single molecule count. There is a significant difference between filter with radius 0.5 and that of radius 1 and 1.5. There is no observable difference between filter with radius 1 or 1.5. Filter of 1.5 was applied to all further data sets.

3.2 Setting the threshold for counting single molecules

Setting the threshold above background requires measuring the intensities on a background region in each frame, a region of plain glass, where there are few single fluorescent molecules. There are two methods to determining the threshold; the first takes a histogram of the total intensities in each background region. The second method, which this algorithm used, finds the local maxima in each background regions. This essentially sums the single molecules found in this region. In both cases, the mean plus three times the standard deviation is taken to be the threshold ensuring a confidence interval of 99.7%; thereby reducing the false positive signal to 0.3%. Anything above this threshold is considered real single molecules and everything below is taken to be just background.

Counting single molecules

The algorithm calculates the single molecule count based on the density of fluorescence in each frame. If the density of single molecules is low such that they are spatially separated then single molecules can be counted using a clustering mask which is able to pick out single molecules against background. However, this method is limited to approximately 700 molecules (figure 3.3), after which the molecule count deviates from the true molecule count and plateaus at 2000 molecules. This particular method is limited by the density of single molecules increases, as spatial resolution is lost as molecules begin to overlap. Instead, another method is used where the cumulative intensity of the single molecules is scaled to determine the number of molecules.

To demonstrate the accuracy of the algorithm, a data set was simulated (Ovesný, P. et al. 2014) varying the number of input molecules from approximately 50 – 30,000. For each image generated, the true number of molecules is known which allows for the comparison of the two molecule counting methods: 1) using the clustering mask to identify single molecules, 2) scaling the image intensity. This data shows that the scaling method is highly accurate at calculating the number of molecules present across the entire range. In contrast, the single molecule identification method is only accurate up to approximately 700 molecules, plateauing at 2000 molecules. As mentioned above, this is due to the lack of spatial resolution when molecules begin to overlap. The, the scaling method was used in this thesis to calculate the molecule count for all subsequent data sets.

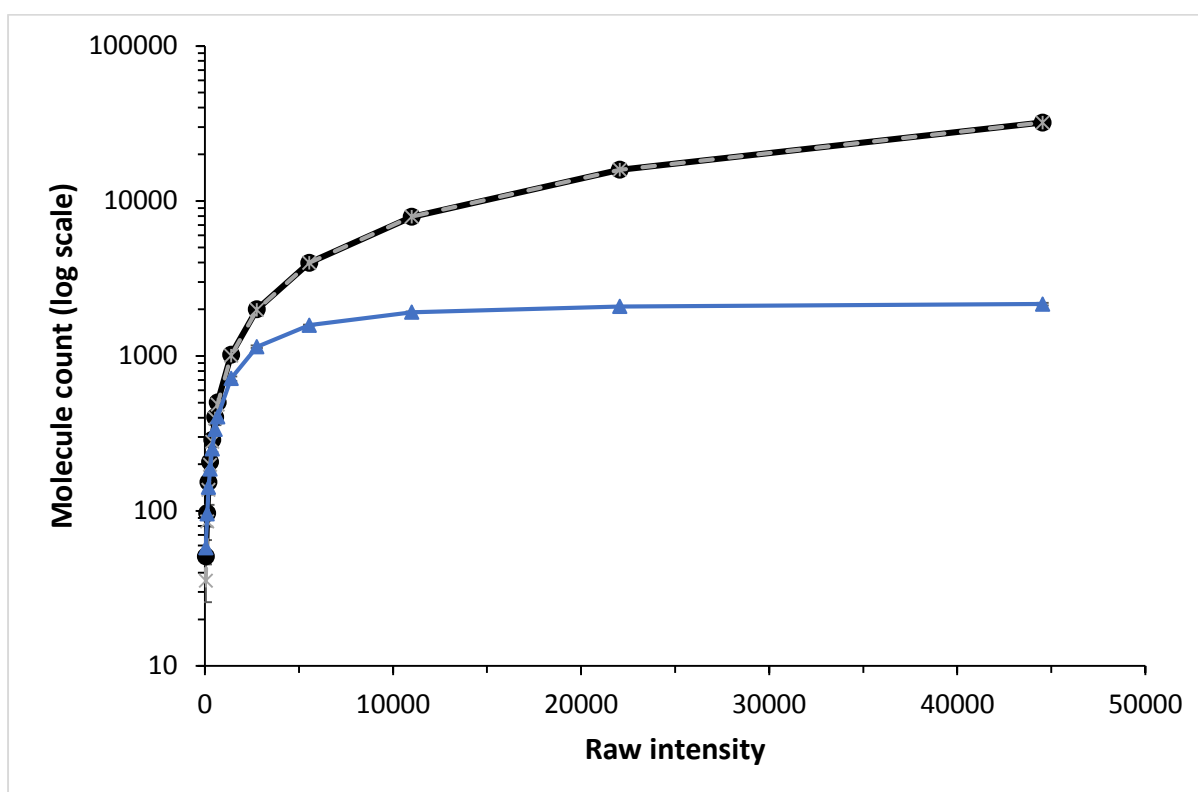


Figure 3.3 uses simulated data to show the accuracy of the single molecule counting algorithm. The raw intensity of each simulated image was plotted versus the molecule count, with the true number of input molecules shown in black. The intensity scaling method (grey) and single molecule identification method (blue) are compared against the input reference for accuracy. The scaling method is highly accurate over the entire range of intensities and molecule counts, fitting very closely with that of the input molecules. The single molecule identification method is accurate up to approximately 700 molecules after which it deviates from the input curve.

Chapter 4

4. Validation of Gata-4 antibodies

Using TIRFM for detecting single protein molecules requires antibodies with both high specificity and affinity to its target protein. By using two antibodies to the target protein, in a conventional sandwich assay format, the specificity of the assay is increased. In this assay antibodies directed at different epitopes of Gata-4 were used. Information on the exact epitope target is often restricted, and often only specified as a large region of the target protein. It is important that the two antibodies used in the sandwich assay recognise different epitopes so that the two antibodies can bind the same antibody simultaneously. Although this may be less important if the protein forms dimers.

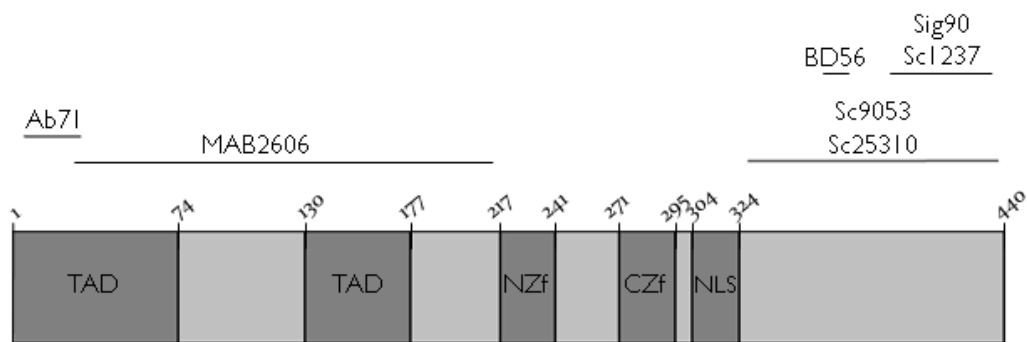


Figure 4.1. Schematic of Gata-4 protein highlighting the regions containing the transactivation domains (TAD), N-terminal Zinc finger (NZf), C-terminal zinc finger (CZf) and the nuclear localisation signal (NLS). The epitopes recognised by the antibodies tested in this study are denoted by the black line (-), with its respective antibody above. Not all antibody binding regions are depicted as some of this information is restricted. *Diagram adapted from Molkentin 2000.*

Typically, antibodies recognise a region of 15 amino acids; however these may not necessarily be continuous amino acids but rather those brought closer together by protein folding (Frank 2002). The Gata-4 transcription factor has many active domains and Figure 4.1 illustrates its two transactivation domains, the two zinc fingers that permit DNA binding and a nuclear localisation signal (Ip et al. 1997). The antibodies validated in this chapter were raised against short peptide sequences and are depicted in Figure 4.1, of which specific details are in Table 4.1.

The Western blot is a well established protocol for protein detection, developed in the late 70's by three independent groups simultaneously (Burnette 1981; Towbin et al. 1979; Renart et al. 1979). It is a denaturing technique whereby proteins are sorted by size. Whilst the field of DNA and RNA analysis has progressed vastly since the invention of the Southern and Northern blots, the Western blot still remains the gold standard for analysis protein expression. Here, the Western blot is used to assess the specificity of the anti-Gata-4 antibodies for the Gata-4 protein.

4.1 Methods

4.1.1 Western blot

Methods of making cell lysate and western blotting are listed in Chapter 2, section 2.5-2.6. Cell lysates were loaded equally, with each lane containing 20 µg of protein and purified protein at 100 ng. The concentration of each antibody used is listed in the Table 4.1 below. Primary antibodies were incubated with the immunoblots for 1 hour at room temperature before washing and incubating with the corresponding secondary detection antibody.

4.1.2 Immunofluorescent cell staining

Cells were fixed using 4% formaldehyde and permeabilised using methanol. Cells were stained either with the anti-Gata-4 antibody MAB2606 or BD along with their respective isotype control antibodies, IgG2b and IgG1, at the same concentrations. All antibody concentrations are listed in Chapter 2, Table 4.1.

Shorthand name	Target epitope (amino acid position)	Host Species	Type	Company / Product code
MAB2606	Met27-Phe211	Mouse	Monoclonal / IgG2b	R&D / MAB2606
BD	354-367	Mouse	Monoclonal / IgG1	BD biosciences / 560327
Ab57	5-30	Rabbit	Monoclonal / IgG	Abnova / ab134057/ EPR4768
Ab71	27-211	Mouse	Monoclonal / IgG2b	Abnova / ab86371
Sig90	296-442	Mouse	Monoclonal / IgG3	Sigma Aldrich / SAB3300090
Sc-25310	328-439	Mouse	Monoclonal / IgG2a	Santa Cruz / sc25310X

Table 4.1. Details of the Gata-4 antibodies tested in this study. Information on the immunogen sequence was used to identify which would be suitable for use in an antibody sandwich assay. The shorthand names are used in all other figures and text.

4.2 Results

4.2.1 Western blotting to validate antibodies against target protein

Antibodies were validated against a positive and negative control lysate. For the positive control, either HeLa cells transfected with a Gata-4 expression vector (+) was used and compared to the untransfected counterpart (-). Expected molecular weight of Gata-4 is 45 kDa and whole blots are shown here to demonstrate the specificity of the antibody. In total six monoclonal antibodies were tested.

Three antibodies of the six tested appeared to be specific for the Gata-4 protein. These were MAB2606, Sig90 and BD (Figure 4.2), all of which produced a band near the expected height of 45 kDa and gave a clean blot with the negative control. The three remaining antibodies did not appear

specific to Gata-4 protein as they either produced bands that were too low in molecular weight (sig90), to high (ab71) or produced bands in both lanes (ab57). This suggests that the antibodies ab71, sig90 and ab57 are not suitable.

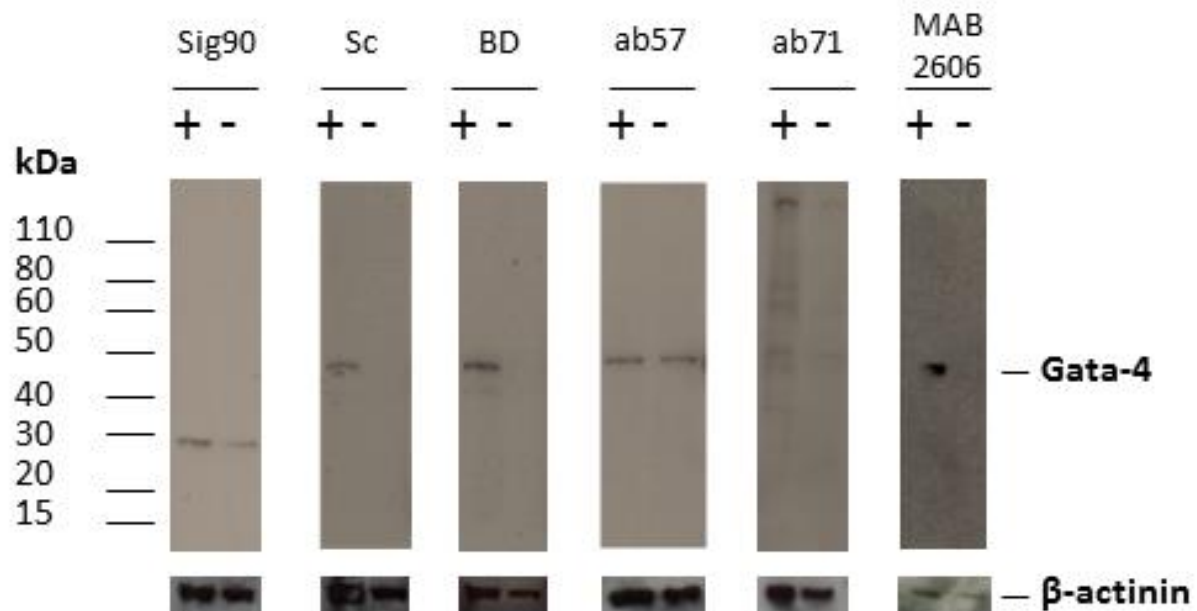


Figure 4.2. Immunoblot of monoclonal anti-Gata-4 antibodies validated against either HeLa Gata-4 transfected lysate (+) and untransfected lysate (-). Three antibodies gave positive signal at the expected height for Gata-4 protein (45 kDa), these were Sc, BD and MAB2606. The remaining monoclonal antibodies either produced bands at the wrong height for Gata-4 (Sig90, ab71) or produced bands in both the positive and negative control lanes (ab57). All blots were tested for the loading control, β -actinin, which produced an expected band at 42 kDa for each lysate, and which was very slightly lower than the band expected for Gata-4 protein.

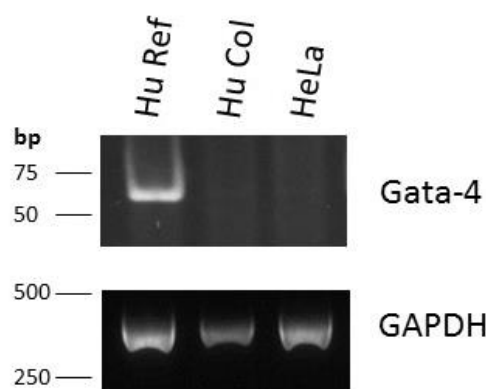


Figure 4.3. Reverse transcription PCR of the untransfected HeLa cell line (HeLa) for Gata-4 expression, testing using primers that produce a product of 68bp and therefor run on acrylamide gel in order to resolve bands. This was compared to Human Reference (Hu Ref) RNA as positive control and Human Colon (Hu Col) as a negative control for Gata-4 mRNA expression. GapDH was used as a loading control with an expected height of 452 bp.

All blots were stained with the loading control Beta-actinin to ensure that both the positive and negative control lysates were loaded equally. The untransfected HeLa cell line demonstrated no

Gata-4 RNA expression by reverse transcription PCR (Figure 4.3), and was compared to a Universal Human reference sample containing all human tissues and which was positive for Gata-4. Human Colon RNA, identified using the Array Express database (EMBL-EBI; Kolesnikov et al. 2015), was used as the negative control.

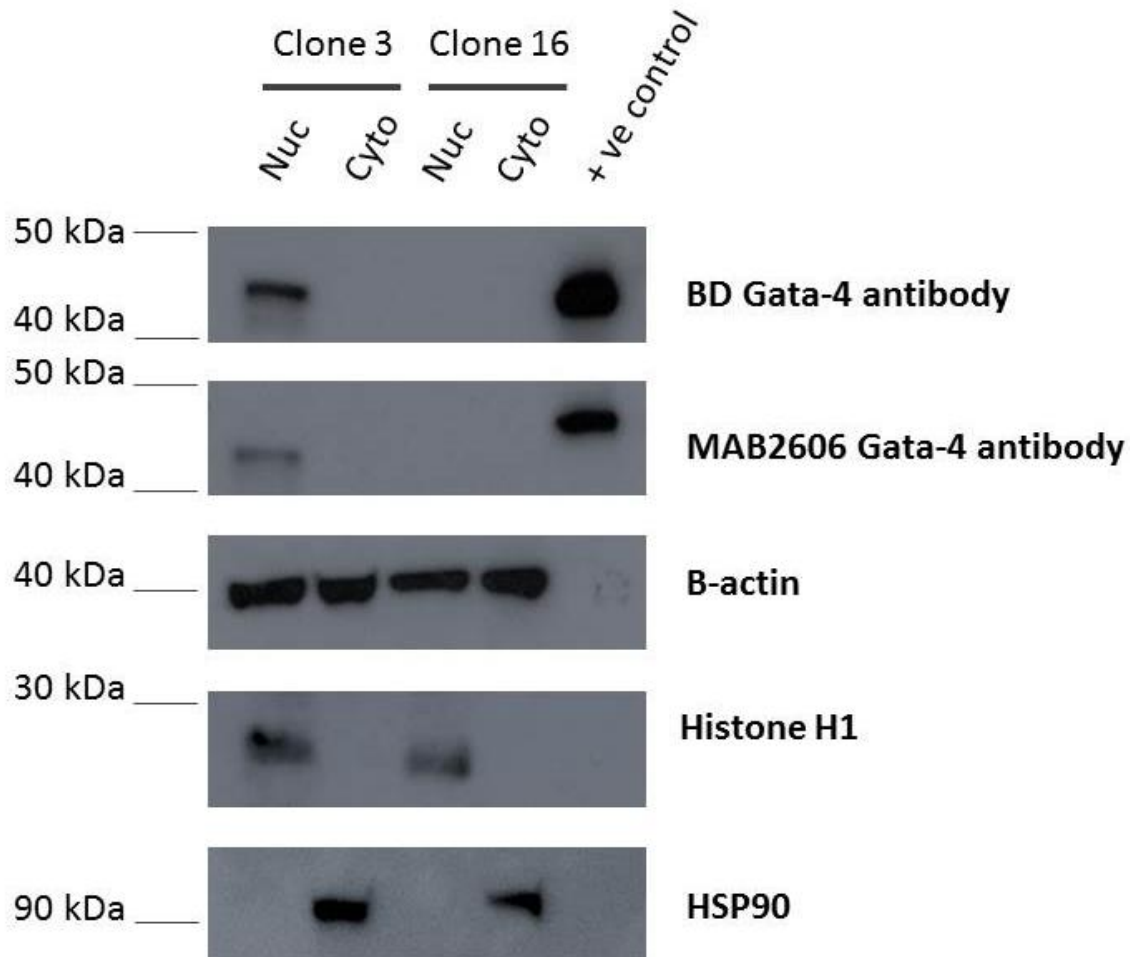


Figure 4.4. Western blot testing the two Gata-4 antibodies on cardiac progenitor cells. The two clones tested were clone 3 and 16, and nuclear (Nuc) and cytosolic (Cyto) fractions were tested for Gata-4 protein expression. As expected the Gata-4 was found to be localised to the nucleus and was only detected in Clone 3 and not Clone 16. Gata-4 purified protein was included as positive control (+ve). B-actin expression was used as the loading control, demonstrating even loading in all lysates and no expression in the purified protein.

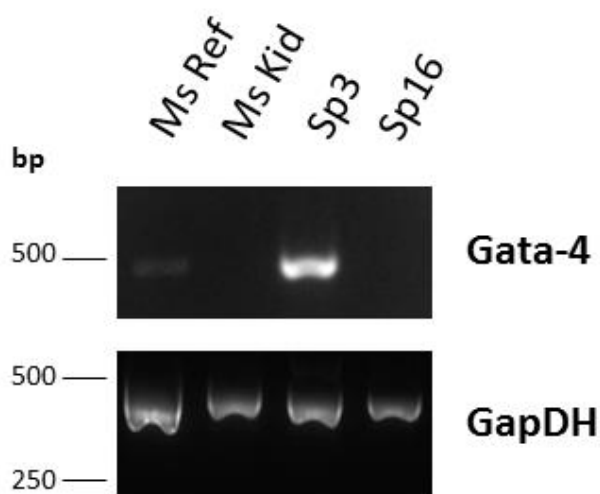


Figure 4.5 Reverse transcription PCR showing Gata-4 expression in the murine cardiac progenitor cells, SP3 and SP16 as compared to a positive and negative control, Mouse Universal Reference (Ms Ref) and Mouse Kidney (Ms Kid) RNA. Both the Mouse Universal Reference and the cardiac progenitor clone SP3 proved positive for Gata-4 with a band near the expected height of 479bp. Mouse kidney and cardiac progenitor clone SP16 showed no detectable Gata-4 expression by routine RT-PCR in accordance with the disparate levels of Gata-4 mRNA by qPCR. (Nosed et

al. 2015). GapDH was used as the loading control

Two of the cardiac progenitor clones, Clone 3 and Clone 16 were tested for Gata4 expression (Figure 4.4). Cell lysates were separated into their cellular and nuclear fractions and tested against the two Gata-4 antibodies, BD and MAB2606. Gata-4 purified protein was used as a positive control. Both the BD and MAB2606 antibodies detected protein expression in the nuclear fraction of Clone 3, but not in Clone 16. The band detected is of the same height as the Gata-4 protein for the BD antibody. The band produced by the MAB2606 antibody is slightly lower than the purified protein. This could be an artefact of the gel, with slight uneven electrophoresis which often happens due to over-heating. Beta-actin was used as the loading control with an expected height of 42 kDa, Histone 1 protein (H1, approximately 30kDa) and HSP90 (90 kDa) used to show clear nuclear and cytosolic fractionation, respectively. The Gata-4 expression correlates with reverse transcription PCR data which shows Gata-4 mRNA expression in Clone 3 but not Clone 16 (Figure 4.6).

4.2.2 Immunofluorescence of murine cells to detect native Gata-4 protein

One way of assessing whether antibodies can detect native, non-denatured protein is to run a non-denaturing polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting, like in Western blotting. This was used in the early stages of antibody validation but was not successful due to technical issues.

Running basic proteins on native PAGE is challenging. Gata-4 is a relatively basic protein, with a pI of approximately 9.4 (predicted from online bioinformatics resource portal, ExPASy, Swiss Institute for Bioinformatics). This means when run in a buffer of pH 8.8 it is likely to have neutral charge and therefore not migrate. Several runs showed that the protein stacked at the top and didn't run into the gel. This was either due to the basic nature of the protein or that it formed large protein complexes or aggregates too large to migrate into the gel.

Thus, immunofluorescence was used to assess the native binding capacity of the antibodies. Indirect immunofluorescence was performed on the clonally derived cardiac progenitor cells from mouse (Schneider, Imperial College London, UK) which are known to express Gata-4 (see Figure 1.4 for clone details). The cell lines HEK and Hep G2 were not tested as they were not intended to be used for cell experiments in the TIRFM assay. Figure 4.6 shows the staining of the clonal cardiac progenitor cells with the anti-Gata-4 antibodies and the nuclear stain, DAPI. Cells show a distinct nuclear localisation of the Gata-4 protein, with the DAPI signal (blue) and the Gata-4 signal (green) overlapping in the merged image. Staining with the isotype controls gave minimal signal, affirming that the Gata-4 antibodies are detecting the target protein.

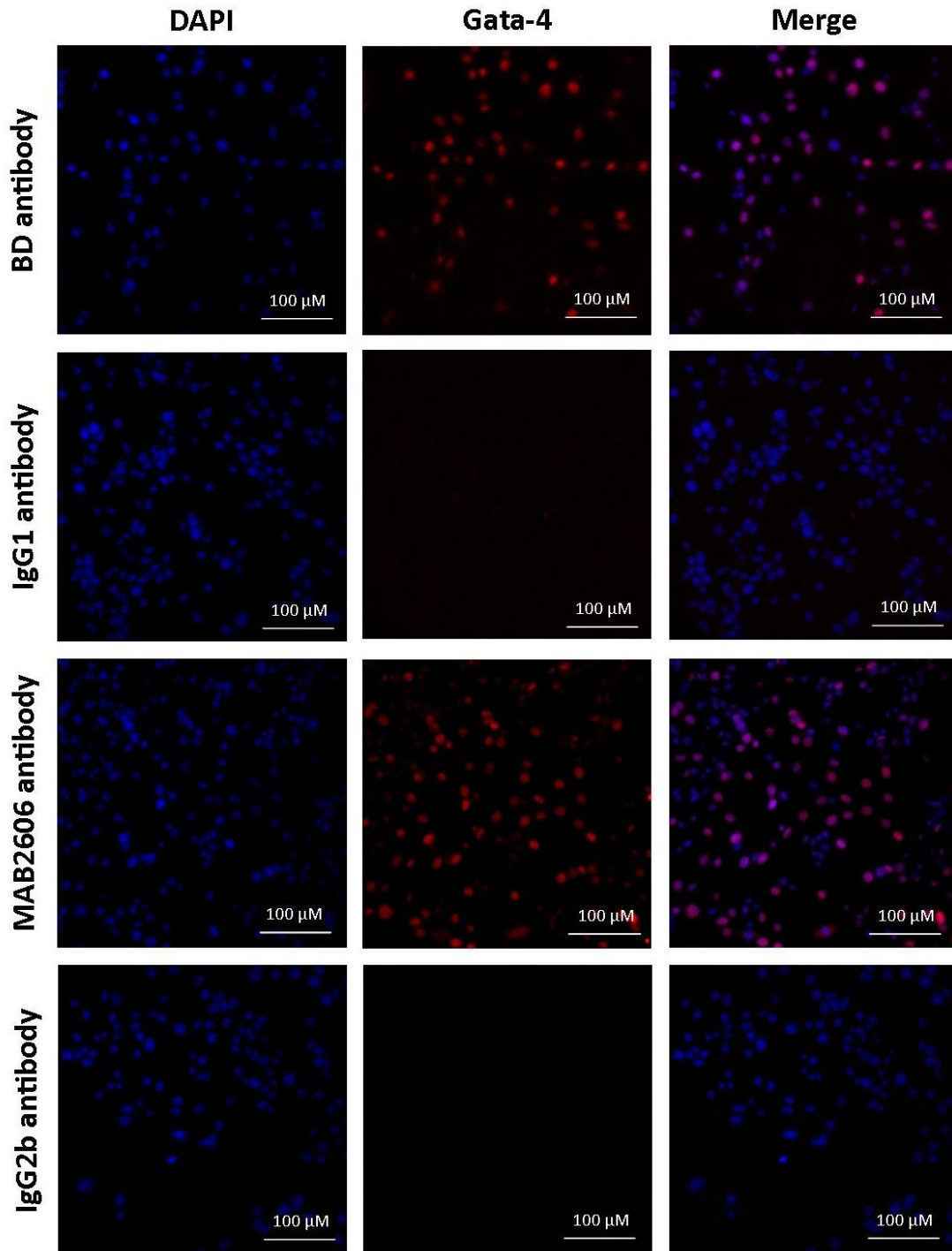


Figure 4.6. Immunofluorescent staining of clonally derived cardiac progenitor clones. Clones known to express Gata-4 protein were probed using two different anti-Gata-4 antibodies, both showing nuclear staining (red) which co-localised with DAPI staining (blue). The Anti-Gata-4 MAB2606 and BD antibodies were compared with their respective isotype controls, IgG2B and IgG1.

4.2.3 Immunofluorescence to test cross-reactivity between Gata factors

To assess if the antibodies detected other Gata proteins, HeLa cells were transiently transfected with either Gata-4, Gata-5 or Gata-6 and counterstained with the two Gata-4 antibodies plus the respective control antibody, Gata-5 or Gata-6 (Figure 4.7). The results show that the Gata-4 antibodies detect protein in the cells transfected with Gata-4 but not the cells transfected with Gata-5 or Gata-6. In addition the IgG control antibodies were negative for all transfected cells.

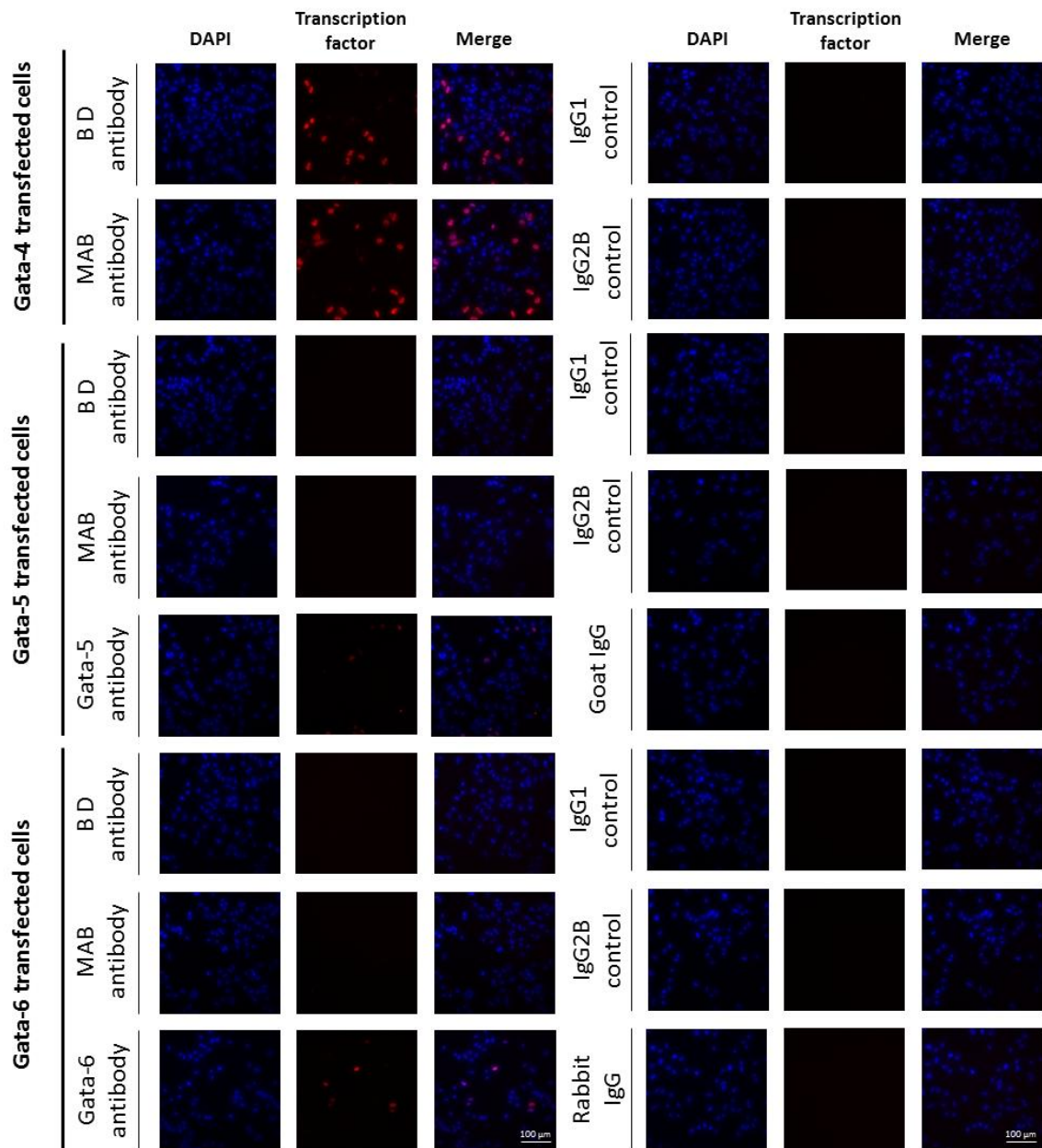


Figure 4.7 Immunofluorescent staining of HeLa cells transiently transfected with either Gata-4, 5 or 6. Cells were then counterstained with the Gata-4 antibodies and the respective control antibodies, Gata-5 or Gata-6 antibody (single experiment, tested in duplicate).

4.3 Discussion

4.3.1 Antibody validation

Of the six antibodies tested only a half proved to be specific for Gata-4 from Western blot analysis. Of the three, BD and MAB2606 were chosen to be tested on chip. Whilst this may seem like a hasty decision, initial Western experiments indicated that the Sc antibody was not specific for Gata-4, it was only after retesting the antibodies with HeLa lysate (initial experiments were with HEK 293T) that Sc was seen to be a possible candidate antibody. Unfortunately, due to time constraints this antibody was not robustly tested on-chip.

4.3.2 Antibody cross-reactivity

There are 6 members of the Gata family, of which Gata-4, 5 and 6 bear the most similarity. Gata-4 shares 45% and 33% identity with Gata-5 and Gata-6, respectively, with the highest degree of similarity occurring in the zinc fingers (basic local alignment search tool; NCBI). These three Gata members often have overlapping expression, particularly in the heart (Charron & Nemer 1999; Charron et al. 1999; Zhao et al. 2008) and Gata-4 and 6 are both expressed in cardiac progenitor cells studied in our group (Chapter 1, Figure 1.4, Nosedá et al. 2015).

Considering the possibility of cross-reactivity of the Gata-4 antibodies with other Gata factors, antibody specificity was tested. The results here indicate that the antibodies are specific to their target, staining positive in the cells expressing Gata-4 but not in cells expressing Gata-5 or 6. An important limitation is that the counterstaining with Gata-5 and Gata-6 was relatively low; however, it was relatively higher than negative control and the Gata-4 antibody staining of Gata-5 and 6 transfected cells. In addition to this, specificity was also suggested by staining in the cardiac progenitor cells (discussed below) and the initial antibody validation with untransfected and transfected HeLa cells (Figure 4.2)

4.3.3 Gata expression in cardiac progenitor cells

Microarray expression data from our group (Chapter 1, Figure 1.4, Nosedá et al. 2015) shows a myriad of transcription factor expression in the cardiac progenitor cells. Based on this data, a clone positive and negative for Gata-4 were chosen to test out this assay, these were Clone 3 and clone 16 respectively. Gata-4 expression was verified using Western Blot analysis (Figure 4.4) with the two chosen Gata-4 antibodies and by PCR (Figure 4.5). This confirmed that Clone 3 expresses Gata-4 in the nucleus whilst Clone 16 was negative for Gata-4 expression.

4.3.4 Immunofluorescent cell staining demonstrates native protein detection

Assessment of antibody binding to native proteins was initially trialled by native PAGE. However, the protein remained in the stacking wells and did not migrate into the gel very far. This could be due to formation of large complexes which under denaturing conditions will dissociate. Alternatively it could be due to the basic nature of the protein, which running in a buffer of pH 8.8 will neutralise the protein's charge. Various other conditions were trialed but did not work, including testing with more alkaline buffer or an acidic buffer and reversing the charge.

Immunofluorescence can be used to detect target proteins within cells. In this instance, it does not indicate the specificity of the antibody but does demonstrate if the antibody can recognise the native form of the protein. Although, cell fixation protocol can modify the proteins, it is good representation of native proteins. The antibodies BD and MAB2606 both demonstrated native detection, staining Gata-4 in the nucleus as would be expected for the transcription factor. This is important with regards to single cell proteomics as antibodies will encounter the native protein form, and not the denatured form.

4.4 Summary

- In total 8 antibodies were tested via Western blotting to assess their specificity to the Gata-4 transcription factor
- Two monoclonal antibodies were identified as specific for Gata-4 protein detection; MAB2606 and BD
- These were tested on the cardiac progenitor clones and showed Gata-4 expression in Clone 3 but not Clone 16

Chapter 5

5. Optimisation of antibody immobilisation and labelling

5.1 Overview

The key to the success of any immunoassay is the antibody immobilisation and labelling strategies (Kusnezow et al., 2003; Wild, 2001). Inefficient immobilisation or over labelling can directly impinge the assays sensitivity and therefore will affect this assays ability to detect proteins from a single cell. The factors considered and optimised in this chapter are: antibody fluorescent labelling, immobilisation of the pull down antibody, the concentration of the detection antibody and surface passivation. Although, the assay is ultimately limited by the availability of antibodies with good affinity, these additional parameters can increase sensitivity by reducing background noise and increasing antibody functionality.

5.1.1 Determining the concentration of the detection antibody

Optimising the concentration of detection antibody is a balance between maximising antigen-antibody interaction and minimising non-specific antibody binding. The concentration must be high enough to saturate all antigen binding epitopes. This is because under-labelling the antigen can lead to undercounting. The caveat here is that non-specific binding is not saturable and increases with increasing antibody concentration, thereby diminishing the signal to noise ratio (Wild, 2001). Therefore, the ratio of signal to background noise will have a maximum, above which more antibody will only decrease the signal-to-background ratio (Wild, 2001).

5.1.2 Fluorescent conjugation of detection antibodies

Antibodies can be labelled directly or indirectly. The indirect method requires a second antibody directed against the species, or isotype, of the first antibody (e.g. mouse, rabbit, goat, IgG₁, IgG₂, IgG₂B). This method is quite often used in immunohistochemistry (as seen in Chapter 4, Figure 4.6) as it is less costly than directly labelling the antigen-specific antibody with fluorophore. It also allows amplification of the signal, since the many anti-species antibodies can bind the Fc region or heavy and light chains of their target antibody.

However, using the indirect labelling method in this system would require using three antibodies simultaneously. This introduces an extra element to non-specific binding, increasing the likelihood of cross-reactivity. It also relies heavily on the affinity of the tertiary antibody for the secondary, as a low affinity antibody would result in unbinding and would not be suitable for this assay. An alternative to using full length antibodies is to use Fab fragments directly conjugated to fluorophores. The fragments used in this chapter were isotype specific and were compared to detection antibodies that were covalently labelled.

5.1.2.a Choice of fluorescence: organic fluorophores versus quantum dots

Quantum dots are nanocrystals with photophysical properties that make them very suitable for multiplexing and long imaging acquisitions. In brief, they have a broad excitation band but are highly tuneable, offering a range of different wavelength emissions distinct from the excitation wavelengths (Giepmans, Adams, Ellisman, & Tsien, 2006; Medintz, Uyeda, Goldman, & Mattoussi, 2005). In addition they are resistant to photobleaching and biological degradation, making them very suitable to biological applications. On the other hand, the quantum dots are very large at 20-30 nm in diameter (Giepmans et al., 2006; Medintz et al., 2005), which swamps the smaller size of antibodies which have an average height only of 14.5 nm and width of 8.5 nm (Lee et al., 2009). The large size of quantum dots would slow the diffusion rate of the antibody, thereby limiting the assay kinetics. In contrast, fluorescent proteins are often only a few hundred Daltons and are thus much smaller than antibodies which are typically 150 kDa.

The structure of quantum dots renders them insoluble, and they require additional coatings to resolve this and enable conjugation to proteins (Medintz et al., 2005). The conjugation strategies involve either covalent linking to amine or thiol groups of proteins or by adsorption (Medintz et al., 2005). There is little control exerted over how the antibodies are orientated and how many are attached per quantum dot (Medintz et al., 2005). This, along with the diffusion limitation and the liability of cross linking, makes this technology unsuitable for this application at present.

5.1.2.b Covalent conjugation of fluorescent molecules to antibodies

The three main strategies for direct labelling with organic fluorophores: via primary amines, the carbohydrate groups or the thiol groups (Figure 5.1). These all involve covalently bonding a fluorescent molecule to the antibody. The primary amines are most commonly used; although this method offers little control over where in the antibody it is labelled and by how much (Haugland,

1995). This means that primary amines near or in the binding region can be targeted and thereby affect the antigen binding capacity.

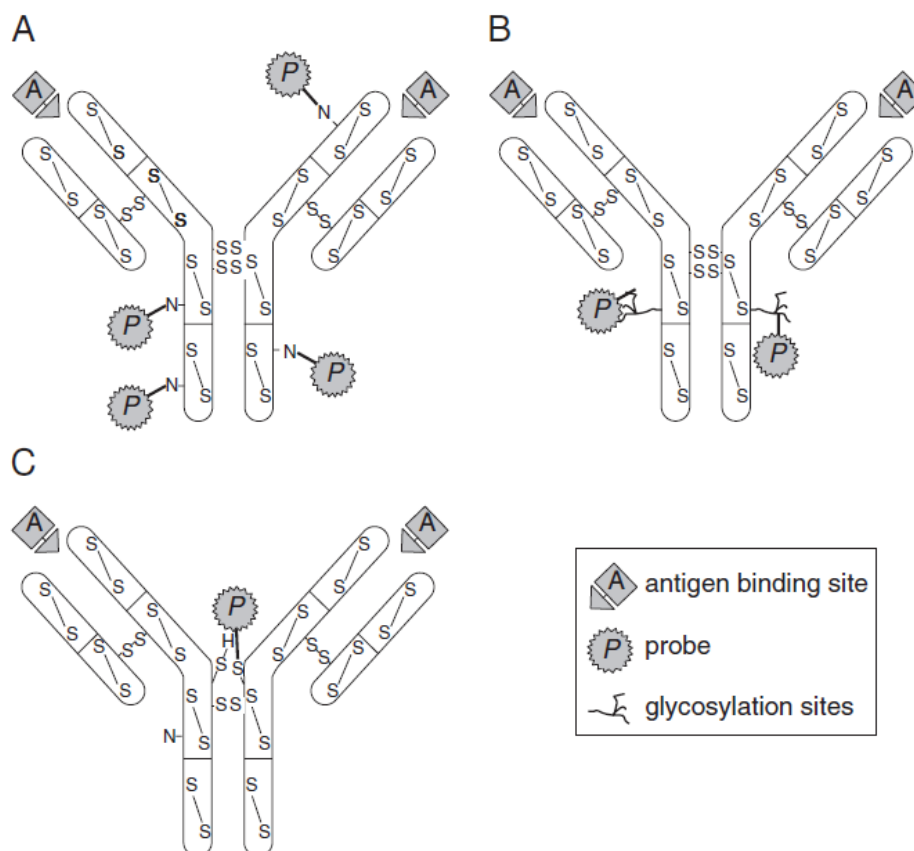


Figure 5.1. Illustration of the three different labelling mechanisms to fluorescently conjugate antibodies. These are covalent bonding via the primary amines in the antibody (A), labelling via the carbohydrate chains located in the Fc regions (B), or labelling via the thiol group which is released by breaking one of the disulphide bonds in the hinge region. *Diagram adapted from Haugland 2001.*

Other labelling targets can be the disulphide bonds that help form the tertiary structure. These link the heavy and light chain, and also form hinge region which connects the two antibody halves. The sulphide bridge in the hinge region is quite accessible and can almost be selectively hydrolysed to expose a reactive thiol (Haugland, 1995, 2001). This can then be used for covalent bonding of a fluorescent molecule, often by using maleimide reactive groups. This offers control over where and how much the antibody is labelled, effectively resulting in a 1:1 ratio of fluorescence to antibody. The down side is that the tertiary structure can be effected and therefore impact binding efficacy.

The final approach is to use the carbohydrate groups which are only found in the antibody Fc region (Haugland, 1995, 2001). This means that antibodies are labelled away from the binding region. However, the process of oxidising the carbohydrates to reveal reactive aldehydes is quite lengthy and requires high starting concentrations (above 1mg/ml) of antibody and often results in large

antibody loss (Haugland, 2001; Kusnezow et al., 2003). Thus, this strategy is unsuitable here because the commercial antibodies used are supplied at less than 1mg/ml and in very small quantities.

Sometimes antibodies are linked to fluorophores by biotin-streptavidin binding. These molecules have very strong affinity for each other; therefore avoid the issue of unbinding. Fluorophores are covalently linked to streptavidin and the antibody to biotin. The biotin is covalently linked to the antibody in the same manner as discussed above, either with primary amines, thiols or to the carbohydrate chain of the antibody. Thus it presents the same problem as with directly labelling but provides an extra processing step, which will result in more antibody loss.

5.1.2.c Artefacts of labelling

Another caveat to antibody labelling is the stabilising buffers the antibodies are provided in. If these contain primary amines such as BSA or glycerol, both common stabilising agents, then labelling will be hindered as it labels these agents. This can result in high non-specific binding as well as inefficient antibody labelling, both affecting the assay sensitivity. These stabilising agents can be removed using a dialysis kit, but this often results in a loss of antibody.

Labelling with amines often requires a greater than 10-20 times molar excess of fluorophores to antibody, resulting in each antibody having multiple fluorophores per antibody, with an optimum of between 3-8 fluorophores per mole of antibody (Haugland, 1995). There are many commercial kits available which are optimised for labelling small quantities of antibody (<20µg). Thus, this method seemed most accessible in the context of this assay.

In this chapter, indirect labelling using Fab fragments was compared with the direct conjugation using covalent bonding to the primary amines.

5.1.3 Antibody immobilisation strategies

Many strategies have been developed for immobilising antibodies on a glass surface (Angenendt, Sobek, Lehrach, & Cahill, 2003; R. C. Bailey, Kwong, Radu, Witte, & Heath, 2007; Boozer, Ladd, Chen, & Jiang, 2006; Cho et al., 2007; Kusnezow & Hoheisel, 2003; Kusnezow, Syagailo, Goychuk, Hoheisel, & Wild, 2006; Seurnyck-Servoss, White, Baird, Rodland, & Zangar, 2007; Vijayendran & Leckband, 2001). However, retaining the functionality of antibodies immobilised on a surface remains the biggest challenge in protein array technology. The questions remain as to whether to orientate the antibody or not, and whether to lift them off the surface and if so by how much (Kusnezow et al., 2003).

5.1.3.a Orientated versus non-orientated antibodies

It is thought that by orientating the antibodies the binding regions remain accessible and thereby increase the functionality of the immobilised protein. The biggest drawback here is the functionalization of the antibody which it often results in huge antibody loss. These orientation techniques include using immobilisation via biotin-streptavidin (Cho et al., 2007), through carbohydrate modification (Cretich, Damin, Pirri, & Chiari, 2006; Kusnezow & Hoheisel, 2003; Kusnezow et al., 2003; Seurnyck-Servoss et al., 2007) or with Protein G (Bae, Oh, Lee, Lee, & Choi, 2005; MacBeath, 2002). There is an added benefit in that both Biotin-streptavidin and Protein G both lift the antibody off the glass and may increase functionality by decreasing steric hindrance.

Non-orientation methods include immobilisation through adsorption, covalent binding using the primary amines (Angenendt et al., 2003) or 3D hydrogels (Olle et al., 2005; Seurnyck-Servoss et al., 2007). Despite possibly having a more suitable environment for antibodies, the latter of these is not suitable for the TIRF application due to thickness of the structure and the reportedly high backgrounds (Kusnezow et al., 2003; Seurnyck-Servoss et al., 2007). Adsorption has the advantage of maximising the amount of antibody possible to print down, though not all the antibody will be functional. Immobilisation via the amines allows extension of the antibodies away from the surface by surface modifications. For instance reacting an epoxide, such as GPTS, with the silicon glass surface and then allowing antibodies to covalently bind to the terminal reactive group extends the antibody away from the surface (Figure 5.2; Kusnezow et al. 2003). This helps to reduce steric hindrance but does not orientate the antibody as any primary amine can react with the epoxide.

A thorough comparison of surface chemistries was conducted by Seurnyck-Servoss, S. L. et al. (2007). Focusing on the lower limit of detection, this work showed that there was little difference in sensitivity of the ELISA assay between covalent and non-covalent adsorption (Seurnyck-Servoss et al., 2007). This was corroborated by another study by Kusnezow et al. (2003) on the surface chemistry of protein microarrays. They showed that whilst antibodies immobilised by their amine groups have an overall higher signal intensity, simple adsorption onto poly-L-lysine slides had lower background and comparable lower limits of detection. In addition, they reported femtomolar sensitivity with covalent immobilisation with either the primary amine with GPTS, or by the thiol groups with MPTS attached to a carbon chain linker (Kusnezow et al., 2003). A caveat of immobilisation via adsorption is that the antibody can exchange with the incubated solution, being only held in place by hydrogen bonding, hydrophobic and ionic interactions (Seurnyck-Servoss et al., 2007).

Bailey et al. (2007) developed a strategy circumventing the direct immobilisation of antibodies to the surface. Instead they conjugated the antibodies to a DNA nucleotide sequence and printed the complementary sequence. By flowing through the antibody complex, the antibody then hybridises

to the printed DNA sequence. This technique was tried in our lab but was found that the preparation resulted in huge loss of antibody, and thus was not very practical for our setup.

Assay sensitivity depends on the amount of pull down antibody effectively available. Therefore, antibody immobilisation is a careful balance between density and functionality. In this thesis immobilisation with protein G and adsorption to the glass surface was tested.

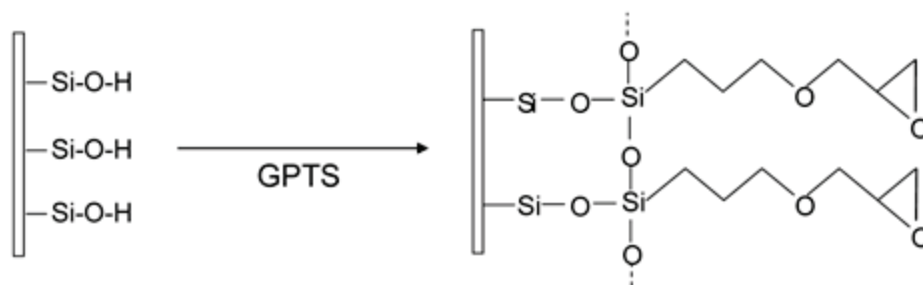


Figure 5.2. Illustrates functionalising the glass surface with GPTS. The silicon glass surface contains numerous hydroxyl groups, cleaning the surface with reducing agents liberates the hydrogen ion, leaving the reactive oxygen to form a covalent bond the GPTS. This epoxide contains a highly reactive oxygen which will covalently bond with amines, aiding the immobilisation of proteins. *Diagram taken from Kim et al. 2007.*

5.1.4 Surface passivation

One way to help increase the lower limit of detection is to decrease the background noise. The standard method of surface passivation is to block the surface with a non-specific protein. In ELISA assays, this is often BSA and was therefore used in this assay. BSA coats the glass surface by adsorption, sticking to the glass by a combination of van der Waals, ionic and hydrophobic interactions (Boenisch et al., 2001). Other methods of blocking are used in other immunostaining techniques; semi-skimmed milk proteins and FBS are commonly used in Western blot and immunofluorescent staining. Coating the surface with polyethylene glycol (PEG) is another method commonly used to reduce background binding. PEG is an amphiphilic molecule which gives it solubility in water whilst retaining its hydrophobic properties. It is this latter property blocks non-specific protein adsorption after attaching it to the glass surface.

5.2 Methods

5.2.1 Antibody conjugation

Direct and indirect antibody labelling methods were compared. Antibodies were labelled either using covalent bonding to the primary amines with alexafluor-488 or indirectly with fluorescently conjugated Fab fragments (Apex and Zenon antibody labelling kits, Invitrogen) according to the

manufacturer's instructions. Both kits permit labelling of miniscule quantities of antibody, and are therefore particularly suitable for this assay where antibodies are supplied in small amounts.

5.2.2 Surface passivation with PEG

Glass coverslips were cleaned with potassium hydroxide for 30 minutes with sonication. After rinsing with water, slides were irradiated in a plasma oven for 1 minute. Plasma treated slides were then incubated in 2.5% aminosilane solution, containing ethanol and 5% acetic acid, slides then washed and incubated with PEG solution in a dark, humidity chamber for up to 2 hours. Slides were subsequently washed and dried with nitrogen air. Antibodies were printed onto the PEG coated slides and chips were assembled with PDMS microfluidic channels as described in section 6.2.2 .

5.2.3 Experimental set up

5.2.3.a Comparison of fluorescent labelling methods

Fab fragment and primary amine fluorescent labelling were compared by lysing cells directly on chip, in 10 μ l perfusion chambers. This was to simulate the cell experiment set-up that the assay is intended for. Chambers were loaded with 222 or 111 cells/ μ l. This was to equate to the theoretical single cell concentration of 0.22 cells per nl (or 1 cell per 4.5 nl chamber). Cells were detached using trypsin EDTA 0.05% and kept on ice in L15 medium with 10%FBS. L15 medium is designed for cells kept out of the incubator for long periods of time. Cells were spun down and diluted to appropriate cell number in L15 medium containing 10% FBS, 4% BSA, protease inhibitors and secondary antibody diluted to the appropriate concentration. Both antibodies were diluted to the optimised concentration of 250 ng/ml. Binding of fluorescently labelled antibodies was compared to printed Gata-4 antibody and the respective IgG isotype control.

5.2.3.b Antibody immobilisation with Protein G

To assess antibody immobilisation Protein G was printed as a series of 10-fold dilutions between 0.5 and 500 μ g/ml. The slide was then incubated with anti-Gata4 pull down antibody at 0.2 mg/ml. Excess antibody was washed away and the sample was incubated with either detection antibody alone or with detection antibody plus Hep G2 lysate. Detection antibody was diluted to 250 ng/ml, as optimised in section 5.1.1, and the HepG2 lysate to 0.75 mg/ml. Signal was analysed after 30 minutes.

5.2.3.c Comparison of surface passivation

To compare surface passivation methods, two independent experiments were performed in the microfluidic chip either treated with PEG or with BSA. These 5 channel chips were then incubated with varying dilutions of cardiac progenitor cell lysate, ranging from 7.5 pg/nl to 112.5 pg/nl. This range of concentrations theoretically spans that of single cell sensitivity.

5.3 Results

5.3.1 Concentration of detection antibody

Here, the concentration of detection antibody to use in the assay was optimised. It is important as too high a concentration interferes with the TIRF signal; from preliminary experiments this upper limit was determined to be 2.5 µg/ml, at which the field of view is distorted, giving spurious image acquisitions (data not shown).

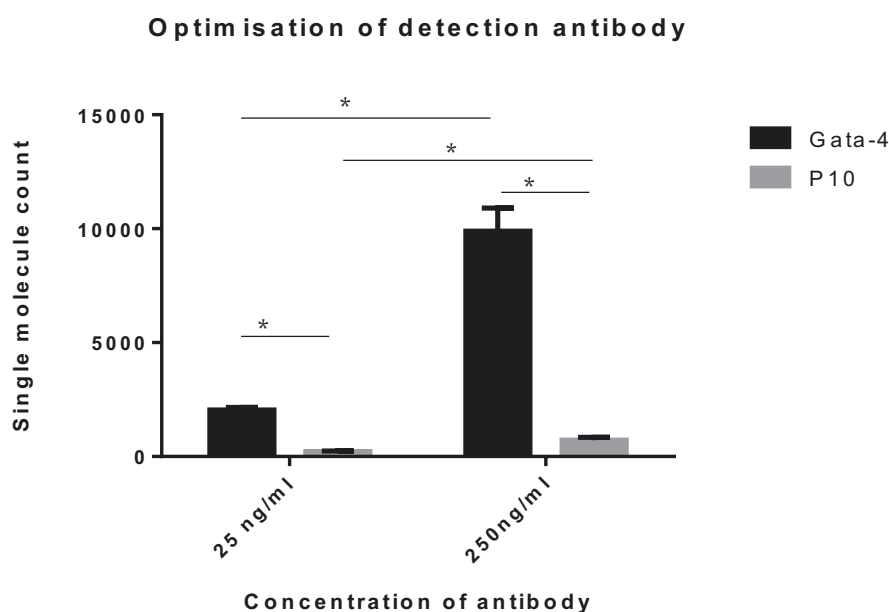


Figure 5.3. Optimisation of the detection antibody concentration. Two independent experiments with antibodies diluted to 25 ng/ml (left) or 250 ng/ml (right) and incubated with printed protein, Gata-4 and p10. There was almost a 10 fold difference in signal between the two concentrations and the signal-to-background ratios were 9.2 and 13.6 for 25 ng/ml and 250 ng/ml respectively. Binding to the Gata-4 protein was significantly higher than to P10 protein with both concentrations of detection antibody.

In these experiments, two different purified proteins were printed onto glass: Gata4 protein and p10 protein, a negative control. They were incubated with fluorescently conjugated anti-Gata-4 at either

25 or 250 ng/ml. These concentrations were determined based on the number of molecules per nl, which correspond to 1×10^5 and 1×10^6 molecules/nl, respectively.

As expected, binding to the Gata-4 protein was significantly higher than to the negative control protein, p10, at both concentrations of antibody. There was a significant difference in binding between the two concentrations, with 10 fold higher binding to Gata-4 protein at 250 ng/ml of detection antibody than at 25 ng/ml (Figure 5.3). This coincided with a slight increase in non-specific binding to P10 protein at 250 ng/ml of antibody. However, the signal to background ratio also increased from 9.2 to 13.6 for antibody concentrations 25 and 250 ng/ml, respectively.

5.3.2 Comparison of direct and indirect antibody labelling methods

Two labelling methods were compared, Fab fragments and primary amine conjugation. The counter antibody was then printed along with the respective isotype controls. Cell solutions were added and lysed directly on chip.

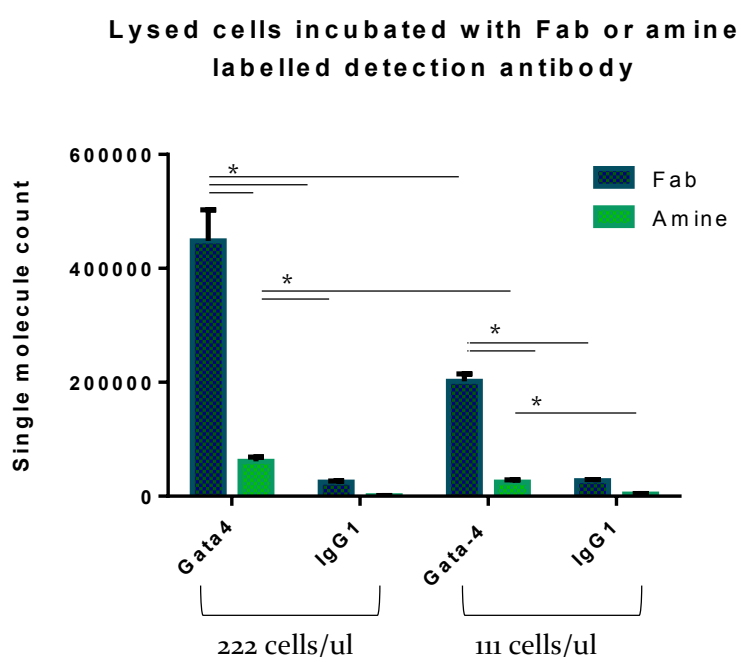
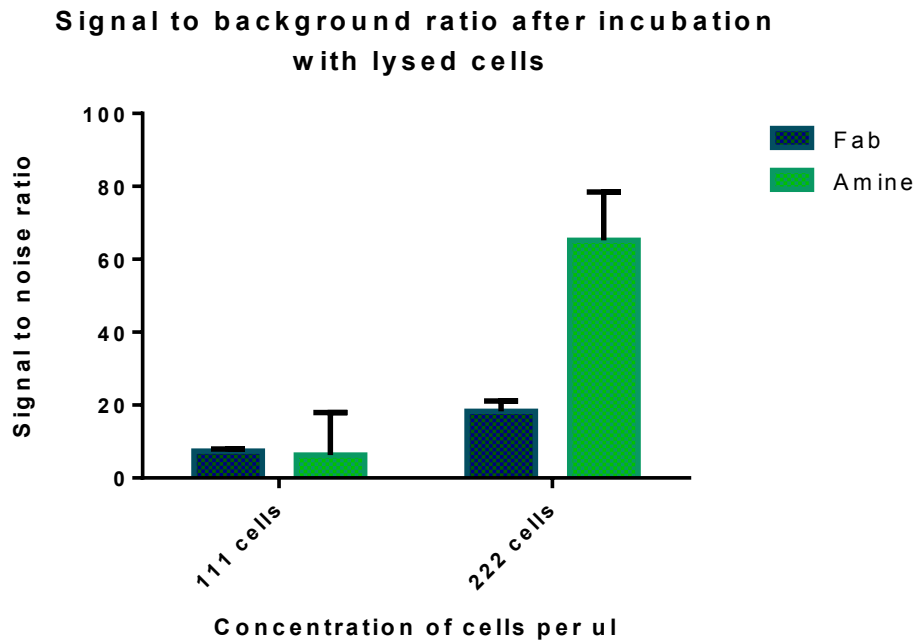


Figure 5.4. Comparing total single molecule count with two separate antibody labelling strategies, Fab indirect labelling and amine direct labelling. Cells were lysed directly on chip and consisted of either 111 cells per μ l (A) or 222 cells per μ l (B). These dilutions are equivalent to having 1 or 0.5 cells in a chamber of a microfluidic chip. Overall a significantly higher signal is achieved with Zenon labelled detection antibodies than Apex, at both cell concentrations. It is worth noting that the overall non-specific binding to IgG control is higher for the Zenon than Apex labelled antibodies, and remains constant despite the different cell concentrations. Error bars are SEM and represent 5 antibody spots of both Gata4 and IgG, replicated 3 times (n=15).

A



B

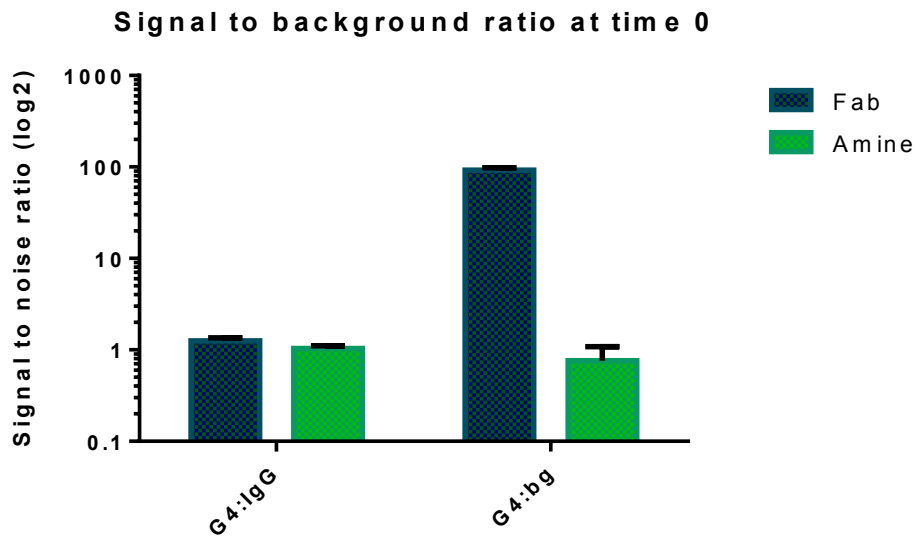


Figure 5.5. Comparison of the signal to noise ratio of the two labelling strategies. SBR for both methods are similar at 111 cells/ μ l but amine labelling has a significantly higher SBR of 65 compared to 18 of Fab labelling at the higher cell concentration (A). The SBR analysed at time 0 (B), before addition of cells shows high initial binding of Fab labelling. SBR was compared for ratio of antibody binding to Gata4 and control antibody (G4:IgG), and Gata4 and background binding to plain glass (G4:bg). Both amine and Fab labelling had similar SBR when comparing non-specific binding to antibodies. However, the Fab labelled antibodies had an extremely high SBR when comparing antibody to non-specific binding on plain glass. Error bars are SEM and represent 5 antibody spots of both Gata4 and IgG, replicated 3 times ($n=15$).

Fluorescent labelling with Fab fragments produced signal far superior to labelling via the primary amines (figure 5.4). However, the non-specific binding to IgG was higher with the Fab labelled antibodies. Non-specific binding did not scale with cell concentration, with each labelling technique having the same amount at both cell dilutions. The amine labelled antibodies had lower signal to both the Gata-4 antibody and negative IgG control, with a significant difference between the two.

Signal to background ratio was also evaluated by comparing the signal at time 0 (before cell lysis) of Gata-4 antibody to that of background on glass. Figure 5.2B, compares this with the SBR attained from comparing Gata-4 antibody to IgG isotype control. The amine labelled antibodies gave similar, low SBR in both situations. However, the SBR for Fab labelling is remarkably different when comparing Gata-4 signal to that of background on glass or to the IgG control. This is a demonstration of antibody-antibody cross reactivity, with SBR of Gata-4 to IgG antibody being very low at 1.25, and very high SBR of 92 for Gata-4 to background on plain glass.

5.3.3 Antibody immobilisation with Protein G

The method of immobilising antibody onto a glass surface is important for antibody function. Here, immobilisation with Protein G was tried as it has high affinity for antibodies, being commonly used in affinity purification of antibodies. It has the added benefit of not requiring any antibody modification and so reduces any antibody loss through antibody functionalisation.

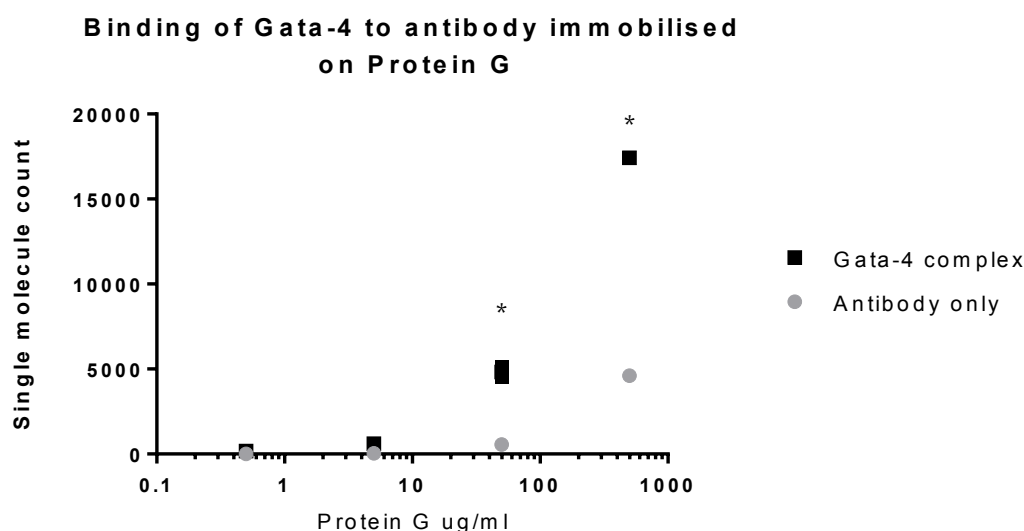


Figure 5.6. Comparison of Gata-4 binding versus detection antibody alone to pull down antibody immobilised by protein G. There is a significant difference between binding of complex and that of detection antibody alone at 50 and 500 µg/ml of protein G. There is no significant difference at 0.5 and 5 µg/ml of protein G. Signal on protein G complexes of 500 and 50 µg/ml is significantly higher than at 5 and 0.5 µg/ml of protein G.

G. The problem lies in the amount of unwanted binding of detection antibody to protein G which may be enough to mask the signal of Gata4 at the single cell level. Starred points (*) indicates $P < 0.01$.

Preliminary experiments suggested that protein G bound the free detection antibody despite being saturated with the detection antibody. Here, I set out to test whether decreasing the protein G concentration reduced the binding of secondary antibody (Figure 5.6).

Negligible binding of Gata-4 is observed at 0.5 and 5 $\mu\text{g/ml}$ of Protein G, with no significant difference between the Gata-4-antibody complex and when the pull down antibody is incubated with detection antibody only (Figure 5.6). At higher concentrations of Protein G, 50 and 500 $\mu\text{g/ml}$, strong signal is observed. Whilst there is a significant difference between the Gata-4 binding and that of antibody only, the cross reactivity remains quite high (Figure 5.6). At the highest concentration of Protein G the detection antibody binding makes up over 25% of the signal, at 50 $\mu\text{g/ml}$ of protein G antibody background binding of detection antibody formed almost 12% of the total signal.

5.3.4 Results of PEG surface passivation

Surface passivation with PEG was assessed for Gata-4 capture with varying concentrations of cardiac progenitor cell lysate (Figure 5.7). In general, the PEG surface had higher signal to background ratio than with BSA alone (see Chapter 6, Figure 6.7) but with huge error (Figure 5.8). Despite the high SBR with PEG, the Gata4 single molecule count is much lower than for BSA, although the background counts are also very low (Figure 5.7). Furthermore, with PEG the signal decreases after 75pg/nl.

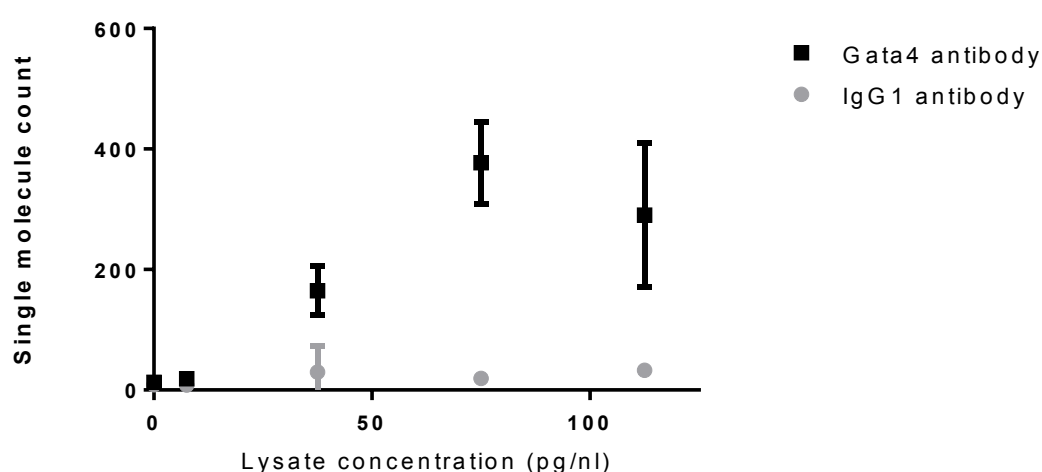


Figure 5.7. Total single molecule count from cardiac progenitor lysate incubated with printed antibody on PEG passivated surface. Clear distinction between binding to Gata-4 antibody and IgG1 control at concentrations above 37.5 pg/nl. At 7.5 pg/nl, that equivalent to a single cell, there is no observable

difference. Furthermore, the total counts are far lower than that normally observed on BSA passivated glass (see figure 5.8).

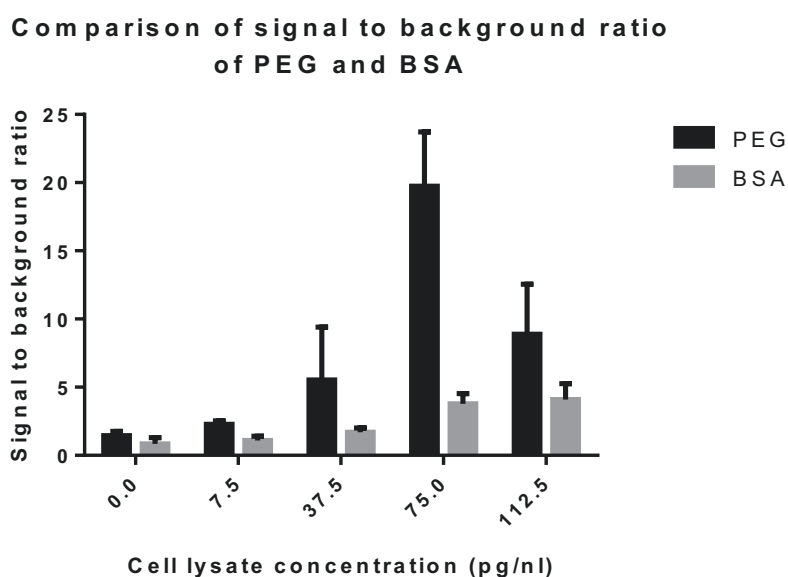


Figure 5.8. Comparison of signal-to-background ratio of PEG passivated surface and that of BSA. Printed antibody was incubated with cardiac progenitor cell lysate at varying concentrations and the SBR was produced from the signal from the Gata4 antibody with that of the IgG control. PEG surfaces produce a higher SBR at both the lower and higher concentrations of lysate. However, there was huge error at higher concentrations than with the BSA passivated surface.

5.4 Discussion

5.4.1 Detection antibody must saturate the free antigen

It is necessary to determine the optimum concentration of detection antibody, taking into account the background binding of the antibody. If the concentration of detection antibody is too low, then there is the risk of undercounting the true value of protein molecules. Conversely, high antibody concentration leads to increased background; therefore, a compromised needs to be reached between the two.

Two concentrations of detection antibody were compared against printed Gata-4 and a non-related protein, P10 protein. A higher single molecule count was achieved at 250 ng/ml of detection antibody. The proteins, Gata-4 and P10, were printed at 0.15 mg/ml. Assuming that the print volume is 200 pl, then approximately 2×10^8 Gata-4 molecules were deposited. Clearly, the detection antibody has not saturated this, detecting only 25% of what was theoretically deposited. This could be for a number of reasons: for instance the print volume could be smaller, the protein could be denatured due to drying and surface immobilisation. Additionally, the epitopes could be masked and

unavailable for binding. Higher concentrations were not trialled here; however, as mentioned earlier, at 2.5 ng/ml the image acquisition is disrupted.

Whilst the increased antibody concentration also increased the non-specific binding to P10, there was also a slight increase in signal-to-background ratio. This indicates 250 ng/ml is more suitable than the lower concentration of 25 ng/ml. It is important that the free Gata-4 antigen in the sandwich assay is saturated; therefore the higher concentration was used in all subsequent experiments.

5.4.2 Comparing fluorescent antibody labelling by Fab fragments and conjugation by the primary amines

The Fab fragments are specifically targeted to the antibody isotype. In this manner, the barriers presented by the antibody stabilising agents such as BSA and glycerol can be overcome. Traditionally, one would have to remove the stabilising agents by dialysis; however this strategy avoids this, circumventing antibody loss. In addition, one can label small quantities of antibody, μg instead of the mg that is usually needed.

Initially, the Fab labelled antibodies presented very promising results with extremely high molecule counts. The downside of this kit is the high degree of non-specific binding to the antibodies, which reduces the signal to background ratio at higher cell concentrations. Further still, the SBR at time 0 is extremely high, indicating that there is a high degree of antibody-antibody cross reactivity. In contrast, the SBR at time 0 for amine labelled antibodies is very low, both when compared to the isotype control and background on plain glass, showing that there is little antibody cross-reactivity.

During the labelling process with the Fab fragment, unbound Fab fragments are not removed but quenched by addition of IgG isotype antibody. This might be the cause of the high non-specific signal: either free Fab fragments binding to the printed antibody or the Fab-bound-IgG binding to the printed antibody. It should be noted that the printed antibody is of a different isotype to that of the detection; therefore, the free Fab fragments should not bind.

The cell concentration of 222 cells per μl is equivalent to the concentration of a single cell in a microfluidic chip. The fact that the amine labelled antibodies have a higher SBR at this concentration suggests it might be better to use in the long run.

5.4.3 Immobilising antibody with protein G

The aim of immobilising antibody via protein G was to increase the antibody functionality. This system also requires the sensitivity to detect low levels of Gata4 protein above background.

The concentration of detection antibody is set at 250 ng/ml. Any background signal from this will therefore always be constant. In contrast, the binding of Gata4 protein is proportional to its concentration. Hence, at low concentrations as would be seen at the single cell level, the Gata4 signal would be swamped by the background binding of the detection antibody to protein G.

Other immobilisation strategies would not necessarily improve the sensitivity of the assay. Although, there have been many thorough reviews of surface immobilisation (Angenendt et al., 2003; Kusnezow et al., 2003; Seurynck-Servoss et al., 2007; Vijayendran & Leckband, 2001), it appears that whilst these strategies increase the maximal signal and produce high signal to noise ratios they also increase the background noise, without much improvement in the lower limit of detection. In addition, these surface chemistries are not necessarily suitable for all antibodies. Surface chemistry would have to be optimised for each antibody, something to be avoided if you wish to multiplex.

5.4.4 Surface passivation with PEG

Surface passivation with PEG initially seemed very promising with incredibly low background and high signal to noise ratio. However, the total signal intensity is very low in comparison to BSA passivation alone (see chapter for graphs).

PEG coating seemed to inhibit high density antibody immobilisation. Though this may not be surprising, it is reported the PEG is only resistant to non-specific binding of up to 100 nM (Jain et al., 2012). Other immobilisation strategies can be employed alongside PEG such as biotin-streptavidin. Alternatively, a species specific antibody conjugated to biotin can be used to immobilise pull down antibody (Jain et al., 2012). There are a few caveats to this: firstly this assay requires a dense spot of antibodies which might not be achieved with diffuse biotin availability in PEG. Secondly, this introduces an extra binding partner which complicates the stability of the protein-antibody complex. In fact preliminary experiments using this assay in the converse, with a tertiary detection antibody, indicated this was not an effective method of detection. In this method a high level of blinking was observed, indicating a fast off rate.

Whilst the PEG surface does have a high signal to background ratio, the error in the single molecule counts is quite large in comparison to the signal. This might indicate that the PEG surface is not stable for antibody printing and cannot give reproducible single molecule count, particularly at the higher lysate concentration.

A technical caveat that should be mentioned is the leakiness of PEG microfluidic devices. PEG being a non-stick surface means that often microfluidic devices will fail. In order to circumvent this high device failure rate and in favour of increasing overall signal output, I chose to use glass surfaces passivated with BSA. Whilst these surfaces have higher backgrounds, this scales with the lysate concentration and is adequately low at low lysate concentrations. In addition, at the theoretical single cell level of 7.5 pg/nl, there was no difference between PEG and BSA passivated surfaces as neither distinguished signal between the Gata-4 antibody and the IgG isotype control. At the opposite end of the scale, the PEG surface seems to saturate at higher concentrations of lysate (Figure 5.7), suggesting that inadequate amounts of antibody is immobilised on the surface. In addition, the single cell experiments had unrivalled low background counts, as will be discussed in chapter 7.

5.5 Summary

- Bountiful options available for both antibody immobilisation and fluorescent conjugation
- Here we chose non-specific adsorption as the method of immobilisation, producing high density spots
- For fluorescently labelling antibodies, amine conjugation was the protocol of choice though giving a lower overall signal, it produced good signal-to-background ratio, with lower non-specific binding
- BSA incubations was chosen for surface passivation, permitting high antibody immobilisation

Chapter 6

6. Determining assay sensitivity

6.1 Overview

The premise of this chapter is to assess the parameters which define the suitability of the TIRF assay for single cell protein analysis. These parameters included antibody affinity, assay sensitivity and limit of detection and were assessed by constructing Gata4 purified protein calibration curves. Finally, applying the assay to cell lysate gives an indication of the assay sensitivity, but also protein copy numbers to be expected from individual cells.

6.1.1 Antibody affinity and avidity

Affinity represents the strength of interaction between two binding partners (Wild 2001). In the case of antibody interaction, affinity is determined by the measurement of the on and off-rates of antigen from its binding partner. The affinity of an antibody for its antigen is the main factor which defines the sensitivity of the assay, although other factors can be improved to increase overall binding.

Avidity is a very similar concept to affinity, but refers to the strength of the reaction between multivalent antigens and multivalent antibodies. In this thesis the antibody subtype IgG was used which contains two identical binding domains (Figure 6.1A). Despite this multivalency, it is standard practise to measure affinity assuming only one binding domain (Goodrich & Kugel 2007).

A further complication to measuring affinity in this assay is the use of two antibodies: one for antigen pull down and another for detection. Measuring affinity in a system with trimolecular binding is difficult, and was simplified by applying the detection antibody in saturating concentrations (Figure 6.1B). Consequently, the complex formed by the antigen and detection antibody is treated as a single entity (Goodrich & Kugel 2007).

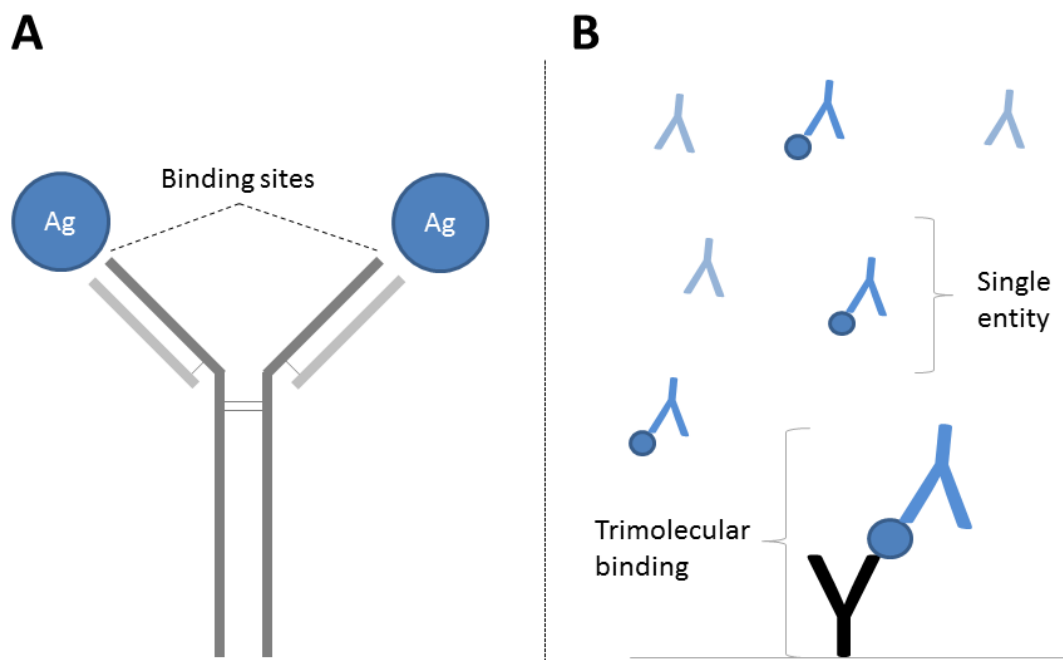


Figure 6.1. Illustrates antibody multivalency, depicting the two identical binding sites found on the IgG antibody subtype (A). The assay is a trimolecular system, containing two antibodies and an antigen; the detection antibody (Υ) is applied at a concentration in which the antigen will be saturated, allowing the antigen-detection antibody complex to be treated as single entity (B).

There are several techniques available to measure antibody affinity, including chromatography, isothermal titration calorimetry and surface plasmon resonance. Here I used the existing TIRF technique in place of the alternative label-free methods.

The experimental protocol is similar in all techniques. Essentially, this is to measure the binding of the antigen to the antibody at equilibrium, varying the concentration of antigen whilst keeping the antibody concentration constant. Ideally, the antigen concentration should be varied across four orders of magnitude and should span across the dissociation constant, K_d .

Another consideration is the concentration of pull down antibody which must be lower than the K_d . This ensures that the assay is in the ambient analyte regime, which is when the antigen bound has a negligible effect on the concentration of total free antigen. This simplifies measuring the K_d by avoiding the need to measure the concentration of unbound antigen as it can be treated as the original, total concentration. Thus, only the bound antigen needs to be measured. This only occurs when the concentration of printed antibody is lower than that of the K_d (Goodrich & Kugel 2007). The dissociation constant is the ratio of reactants to the product at equilibrium:

Equation 1
$$K_d = \frac{A \cdot B}{AB}$$

Where A is free antigen, B is antibody and AB is the portion of antigen bound to antibody. The K_d can be determined by plotting the fraction bound versus the input antigen concentration, where half maximal binding is the K_d .

The time to equilibrium is determined in part by the off rate. If the antibody has a slow off rate, then equilibrium will take longer to establish. Time to equilibrium is not only defined by the kinetics, but in large volumes it is also limited by mass transport. This is where the rate of antigen diffusion directly impacts the antibody-antigen binding kinetics (Kusnezow, Syagailo, Rüffer, et al. 2006; Nygren & Stenberg 1989). Depletion of antigen at the antigen-antibody interface means that the reaction is limited by the diffusion of new antigen into the vicinity, and thus depends on the diffusion coefficient of the antigen molecule which in turn is determined by the protein size and the fluid viscosity. In large volumes the limitation of mass transport necessitates long incubation times, sometimes up to days (Kusnezow, Syagailo, Rüffer, et al. 2006). It is imperative that the measurements are taken at equilibrium, this time can be determined with preliminary experiments or by calculating the diffusion time of the molecules in the proposed volume.

The benefit of using microfluidics means that you can minimise assay volumes. In our setup, we use 4.5 nl volumes for the cell experiments and 10 nl volumes for the calibration experiments. The low volumes means that the reaction is no longer mass transport limited as the diffusion times are reduced, and equilibrium is reached rapidly (Burgin et al. 2014). Simulations of time to equilibrium made by our group indicate that this occurs within 10-20 minutes in the microfluidic chambers (Salehi-reyhani 2011).

6.1.2 Sensitivity and limit of detection

In general terms, the sensitivity of an assay is the ability to detect small concentrations of target antigen. This can be broken down into a few different components: the errors in measurement when no analyte is present, the minimum signal that can be detected and the responsiveness of the assay to increases in antigen.

In reality, the assay sensitivity is less dependent on the antibody kinetics and more dependent on the level and precision of non-specific binding in the system as well as the error in signal measurement. For example, if you were to apply high concentrations of antibody such that all antigen binding sites were occupied, sensitivity would be improved. However it would be limited by the increased non-specific binding which increases concomitantly with increasing antibody

concentration (Wild 2001). Thus, the equilibrium constant is not the limiting factor whilst there is significant measurement errors and non-specific binding. (Wild 2001).

In this study, sensitivity is defined as the slope of the calibration curve, essentially how responsive the detection is to the protein put in, or minimum change detectable. The limit of detection (LOD) is the minimum amount detectable above background. This minimum signal must be 3 standard deviations above the background signal, giving a 99.7% confidence that the observed signal is real. The LOD hence defines the minimum signal detectable from single cells.

Whereas affinity is measured in the ambient analyte regime; the sensitivity and LOD is measured in the mass sensing regime. To improve protein capture efficiency we use nanolitre volumes; this pushes the binding from ambient analyte to the mass sensing regime. This occurs because the concentration of printed antibody exceeds the K_d in these small volumes. Therefore, it is essential to measure the assay sensitivity in the same regime, giving a true reflection of what is occurring in the MAC chip experiments.

6.1.3 Estimating protein copy number per cell

To estimate the approximate number of Gata4 proteins per cell, and assess the assay sensitivity to cellular protein, a calibration assay with SP3 cardiac progenitor cell lysate was performed. The difficult nature of single cell protein analysis has meant protein copy number per cell is relatively unknown. By conducting a calibration with lysate, the cells were assessed to see if they expressed enough protein to be detected in the single cell assay; a priori to doing experiments on single cells.

The protein concentration in a cell is dependent on cell type and cell volume and is hard to quantify absolutely. The volume of mammalian cells can vary between 1-100 pL (Williams et al. 2012) and the cellular protein concentration can range between 50-200 pg/pL (Finka & Goloubinoff 2013). The cardiac progenitor clones are small cells, with an estimated volume of 0.2-3.3 pL. We took a conservative estimate of 75 pg per cells as an average quantity of protein in each cell. Therefore to replicate one cell per chamber, lysate was diluted to 7.5 pg/nL, giving 75 pg in a 10nL chamber.

6.2 Methods

General information on how lysates and protein were made is detailed in the Methods chapter. Below is a description of experimental setup for the assay calibrations discussed in this chapter.

6.2.1 Gata4 calibration curve in large volume chambers

The calibration curve was conducted in a large volume of 10 μ l and incubated with a serial dilution of purified Gata4 protein. Chambers were sealed and incubated at 4°C overnight before imaging with TIRF, as a long incubation period is required to establish equilibrium.

6.2.2 Gata4 calibration in microfluidic chips

Using the microfluidic chip (Figure 6.2), five different concentrations of Gata4 protein were incubated with the printed antibody, in solution containing 250 ng/ml detection antibody. Samples were incubated overnight at 4°C before imaging.

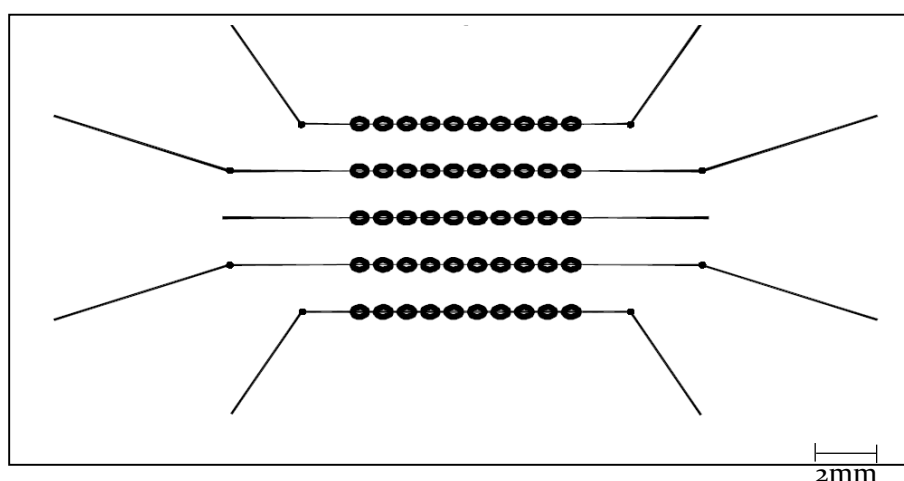


Figure 6.2. Layout of microfluidic chip used for lysate experiments. It contains 5 independent lines, each with 10 chambers of 10 nl each. Each chamber can contain 1 printed antibody spot of approximately 100 μ M in diameter.

6.2.3 Calibration with cardiac progenitor cell lysate

To assess the sensitivity of the assay in small volumes microfluidic chips were used. These were designed to allow lysate to flow through 10 nl chambers. Chips contained 5 individual lines, allowing 5 test subjects each with 10 chambers (see Figure 6.2). Each chamber could contain 1 printed antibody spot. Cardiac progenitor cell lysate was incubated in each line at different concentrations. The effective cell concentration was between 1 and 15 cells per chamber. The microfluidic design was based on work by members of our group (Salehi-reyhani 2011), this thesis used a revised design which was modified by Dr. Natalia Goehring (SCP group, Imperial College London, UK).

6.3 Results

6.3.1 The calibration curve retains the ambient analyte regime

The Gata4 protein calibration was conducted in the ambient analyte regime. This was illustrated by plotting the fraction of Gata-4 bound against the total input Gata-4 (Figure 6.3). The fraction bound at each concentration averaged at 4×10^{-7} , a miniscule portion of the input protein and well beneath Ekins' maximum 1% cut-off limit (Ekins 1998; Saviranta et al. 2004; Wild 2001).

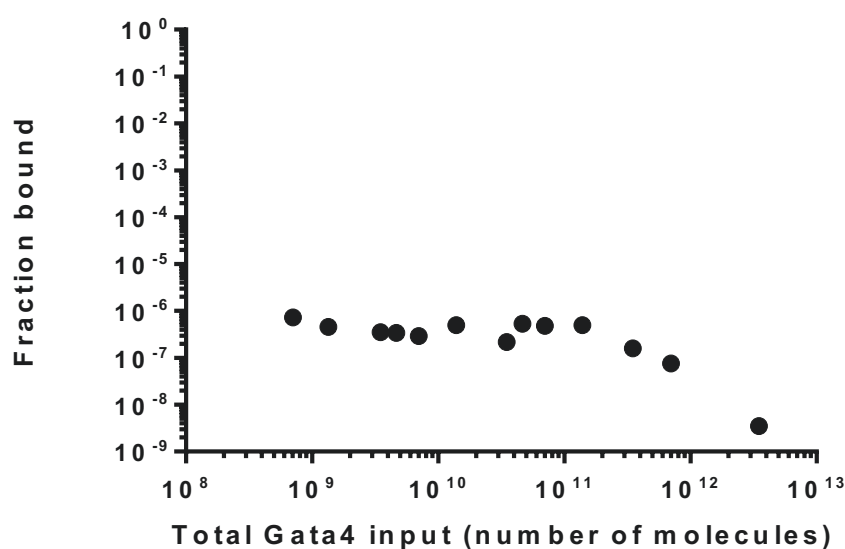


Figure 6.3. Comparison of the total input Gata4 protein with the number of molecules counted. The ratio of input to output is similar at all Gata4 concentrations. The fraction bound 7 orders of magnitude lower than the input protein indicating that the bound fraction would have a negligible effect on the concentration of free analyte. The tail off observed is similar to that seen in Figure 6.4 and is due to the prozone effect.

6.3.2 Antibody affinity, activity and saturation

The Gata4 calibration curve (Figure 6.4) follows sigmoidal binding curve as expected for a semi-log plot. A curve was fitted using software (Prism), the half maximal binding of Gata4 is taken as the affinity and is given as $6 \text{ nM} \pm 2.2 \text{ nM}$. There is a noticeable dip in binding after the plateau, this is known as the prozone phenomenon or hook effect. It is seen commonly in ELISAs and can be misleading, with high concentrations of antigen producing low readouts.

The prozone effect occurs due to high antigen concentrations saturating the binding sites of both liquid and solid phase antibodies. This means that the printed and detection antibody cannot bind the same antigen at the same time, and thus the sandwich assay is inhibited (Wild 2001; Bodor et al. 1989; Haller et al. 1992).

The saturation point of the printed antibodies is about 70,000 molecules (Figure 6.4). Given that the approximate printing volume is 200 pl and the antibody concentration is 0.25 mg/ml, there are approximately 2×10^8 antibody molecules in a printed spot. Therefore, the fraction of these that are active is less than 0.03%. The saturation point in the nanolitre volumes is much increased, attaining 290,000 molecules, giving antibody activity of 0.14% (Figure 6.5).

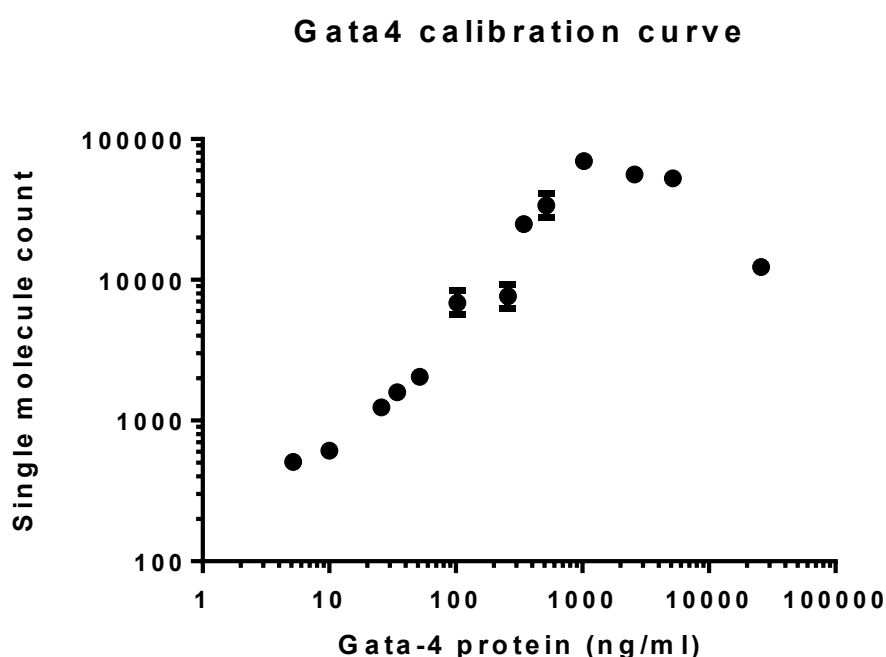


Figure 6.4. A calibration curve with Gata4 purified protein in large volume chambers (10 ul). The lowest concentration tested was 5.2 ng/ml and saturation occurs at approximately 1000 ng/ml. After this, binding to the antibody is inhibited.

6.3.3 Fraction of Gata4 bound in the microfluidic chips

As mentioned earlier, reducing the reaction volume pushes the system out of the ambient analyte regime and into the mass sensing regime. With the 10 nl volume microfluidic chips, the antibodies can capture up to 9% of the total Gata4 protein present (Figure 6.5). This reduces to 0.1% as the

concentration of Gata4 antigen increase, reducing even further once the assay enters the prozone region.

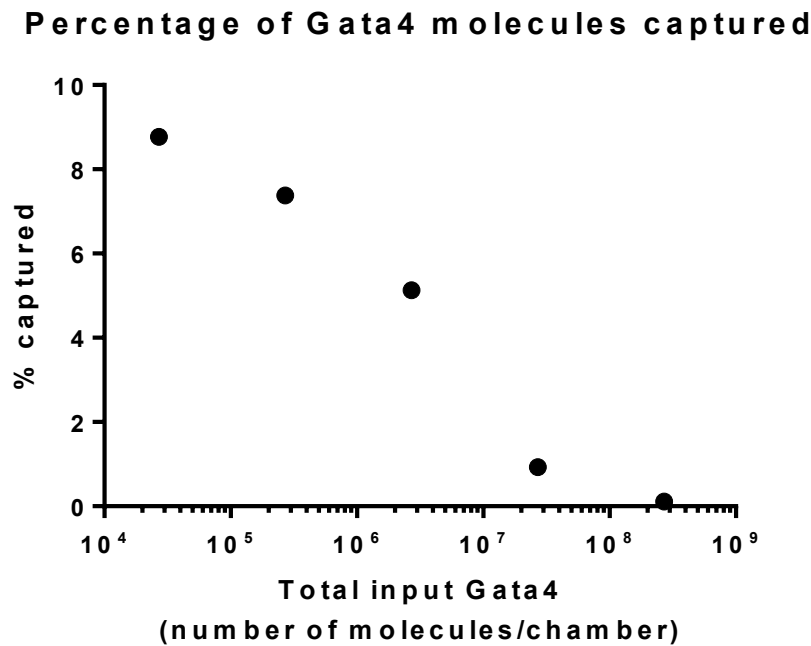


Figure 6.5. Reducing the volume can increase the capture by pushing the system out of the ambient analyte regime. In the 10 nl volume chambers, the volume is still not small enough to push the system towards 100% capture. Instead the antibody sandwich system captures between 0.1 and 9%, depending on the antigen concentration.

6.3.4 Determining the sensitivity of Gata4 sandwich assay in nanolitre volumes

The sensitivity of the assay can be determined by the region of the calibration curve that the assay falls into. For this, a calibration was conducted in nanolitre volumes in the microfluidic chip; the resulting calibration curve can be seen in Figure 6.6. The assay was tested across 5 orders of magnitude with respect to the number of Gata4 protein molecules, and produced a sigmoidal binding curve in a semi-log plot, as expected. The most responsive region, the linear region, is between 20,000 – 250,000 single molecule counts, corresponding to 2.7×10^5 - 10^8 input molecules.

The protein count at the single cell level is approximately between 3-4000 for Gata4, these increases to ~60,000 up to about 15 cells (Table 6.1). This means that the cell experiments fall into the sub-linear region of the calibration curve (Figure 6.6.).

The limit of detection is determined in the presence of antibody only, essentially measuring the cross reactivity. The limit is defined as the signal above background for which you can be 99.7% sure is true signal (3 standard deviations). In the microfluidic set-up, the LOD is 1810 single molecules, and is calculated with incubation with antibody only (i.e. no cells; Figure 6.7).

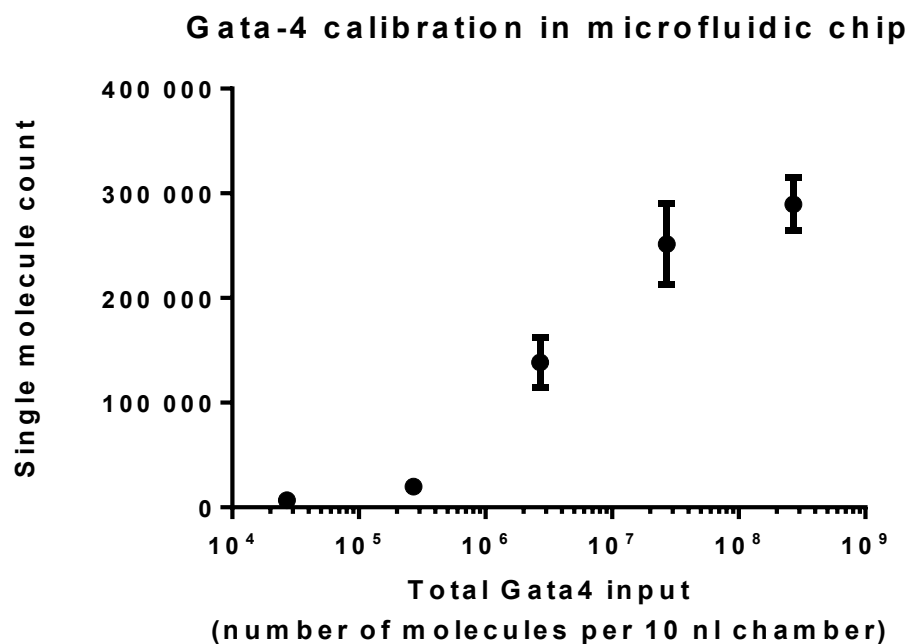


Figure 6.6. To determine the assay sensitivity, total input of Gata4 protein molecules is compared to the output single molecule count. This gives a sigmoidal curve with the linear region between 20,000- 251,000 molecule counts which corresponds to an input of 2×10^5 - 1×10^7 molecules per chamber. According to Figure 6.7 and Table 6.1, the number of molecules from a single cell would fall into the sub-linear region.

6.3.5 Application of the assay to cell lysate: Gata4 copy number and single cell sensitivity

Five lines of the MAC chip were incubated with different concentrations of lysate, including no lysate containing just antibody. Lysate was tested between 1-15 cells per chamber; this was calculated based on the average protein concentration per cell. This produced a linear response to the in-put lysate concentration and the number of molecules counted (Figure 6.7).

Printed IgG1 was used as the negative control and which plateaus at about 14-15000 single molecule counts. This is significantly below binding to the Gata4 antibody at lysate concentrations between 37-112.5 pg/nl. However, there is no significant difference between the control and Gata4 antibody at the lowest lysate concentration of 7.5pg/nl.

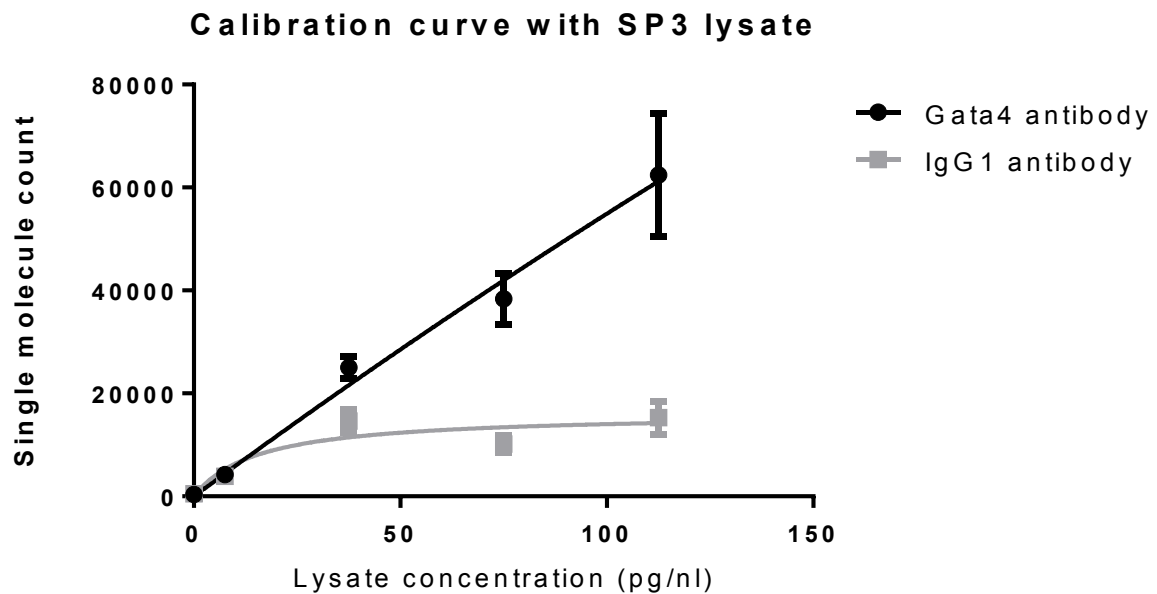


Figure 6.7. Calibration curve was conducted with SP3 cell lysate and diluted between 0 – 112.5 pg/nl. These dilutions equated to 0, 1, 5, 10 and 15 cells per chamber. Cell concentration is estimated at 75 pg/nl. The assay is not single cell sensitive here. The limit of detection lies between 7.5-37.5 pg/nl (1 and 5 cells per chamber).

Total single molecule count	Test conc. (Pg/nl)	Molecule per cell (75pg)
3867	7.5	3867
25054	37.5	5010
38338	75	3834
62461	112.5	4164

Table 6.1 Estimates the number of Gata4 proteins per cell based on the lysate calibration curve. One cell is considered to contain 75 pg of protein. This data indicates there is roughly 3500-5000 Gata4 molecules per cell.

Gata-4 copy number per cell was calculated based on the assumption that a cell contains 75pg of protein (Table 6.1). From the lysate calibration curve (Figure 6.7), Gata4 is measured in the narrow range between 3800-5000 protein molecules per cell. It is interesting to note that at 7.5 pg/nl of lysate, the lowest concentration tested, the Gata4 count per cell is similar to that calculated from other concentrations. Even despite this data point not being significantly different to the IgG control.

6.4 Discussion

6.4.1 Assay kinetics sufficient for single cell sensitivity

Avidity of the assay was measured as 6 nM $\pm$ 2.2 nM. We required antibodies with nanomolar affinity to gain the sensitivity to detect protein from single cells. Whilst we didn't measure the affinity for each antibody, the avidity represents the strength of interaction across the three interacting molecules, and suggests that the assay has the potential sensitivity for single cell protein analysis.

6.4.2 Counting Gata4 copy number

Operating in the mass sensing regime has the benefit of capturing more protein, particularly at lower protein concentrations. Ideally, we would like to bind all the protein expressed in cells and count the absolute protein expression. However, in the current format this assay only captures a maximum

of 9% of the expressed protein (Figure 6.5), decreasing as the protein concentration increases. This is because the concentration of antibody relative to antigen is constant, and whilst there is a proportional increase in binding with increased antigen concentration the number of antibody binding sites are limited.

Improvement in mass sensing is not greatly increased by better affinity antibodies as with high affinity antibodies most the sites are already occupied (Wild 2001). Therefore, one has to increase the number of active printed molecules. Based on the analysis of input and output Gata-4 protein (Figure 6.6), only 0.14% of the printed antibody is active. Whilst this might seem low, this is plausible given the steric hindrance, denaturation and orientation affects that can occur with immobilisation on to a solid surface (Saviranta et al. 2004; Wild 2001). There are many strategies devised to improve this (reviewed in Chapter 5, section o); however, improvements in activity is generally quite limited with full length antibodies although increased activity has been shown with Fab fragments (Saviranta et al. 2004).

An estimate of Gata4 copy number per cell was attained by testing different concentrations of SP3 lysate. This gave copy number per cell between the range of 3500-5000. Since at lower Gata-4 protein concentrations, the total capture is approximately 9% of the input, the actual copy number per cell should be in the region of 38-56,000 per cell.

This is a crude estimate of the copy number per cell, and was measured to give an approximation of what to expect from single cell analysis. This estimate also indicates whether the assay is likely to be sensitive enough to detect protein from single cells. Whilst the lysate calibration curve (Figure 6.7) suggests the assay could possibly be at its limit with respect to single cell analysis, reductions in background might improve the limit of detection and thereby improve sensitivity.

6.4.3 Sensitivity and limit of detection

One important constraint to the overall sensitivity of the assay is the limit of detection. Here the LOD was taken as the signal above the antibody-antibody cross-reactivity, for which it can be 99.7% confident that the single molecule count is due to Gata4 binding.

The LOD in this assay is approximately 1810 molecules; this means that the assay should be capable of detecting Gata4 from single cells. A calibration from cell lysate indicates that there are between 3500-5000 detectable Gata4 protein molecules per cell, which is approximately 2 times the LOD.

The LOD was not assessed in the presence of negative control lysate. The latter control would have been particularly instructive given that the observed binding to IgG isotype control in the presence

of lysate was much higher than the LOD measured in the absence of lysate (Figure 6.7). Therefore, this additional background binding observed might be a feature of adding lysate.

This may indicate that the limit of detection is higher than 1800 molecules counts. However, data from cells lysed directly on chip show that background binding in the presence of Gata4 negative lysate is much lower (Chapter 7, Figure 7.7). This implies that pre-made lysate is inherently sticky, which could be due to various reasons including; inefficient clearing, degradation or even buffer choice which can effect protein charge and thus protein-protein interactions.

Whilst the antibody-antibody cross-reactivity cannot be directly reduced, other factors can be reviewed to improve LOD. The effect of background binding in the presence of sticky lysate significantly increases the LOD. Whilst this might not be an issue with real cell experiments, it might be worth reviewing different blocking reagents, or surface passivation chemistries, such as PEG (Chapter 5, section o). Another useful tool would be to analyse the LOD in the presence of a control purified protein, this would give a good indication as whether the increased background is an artefact of the lysate. In addition, preliminary experiments support this as the Gata-4 antibody negligibly binds to a printed control protein, P10 (appendix, figure B), indicating the antibody is specific to its target, Gata4.

Ideally the sensitivity would lie in the linear, most responsive, region. However, due to the low concentrations being detected the sensitivity lies in the sub linear region. Essentially this impacts the responsiveness of detection to the concentration of input protein. From the lysate calibration curve (Figure 6.7), the assay appears responsive at low concentrations of cell lysate, with single molecule count linearly increasing with lysate concentration. This implies that the assay is sensitive enough to distinguish protein concentration with small numbers of cells and that the overall sensitivity depends on the LOD as previously discussed.

Increased responsiveness could be achieved by increasing the quantity of capture antibody and its activity. Antibody activity could be improved by different surface immobilisation strategies, e.g. orientation by specific coupling in the Fc region. However, this has other drawbacks including loss of antibody during the preparation steps. Hence, there is perhaps increased activity but overall the antibody concentration is reduced.

Another solution could be to simply print a higher concentration of pull down antibody. This could be increased until its maximum packing density; reports from the literature suggest that effective printed concentration is antibody dependent, demonstrating maximum signal anywhere between 0.5-1 mg/ml (Kusnezow, Syagailo, Goychuk, et al. 2006). A caveat to this is that excess antibody will

just be washed away or, in the case of the closed chambers, will just be freely floating in solution binding antigen. A final solution could be to reduce the chamber volume, but this is discussed later (Chapter 7, section 7.4.5).

6.5 Limitations

6.5.1 True concentration of pull down antibody unknown

Printing with a micro-contact printer enables antibodies to be printed at high density, maximising the number of capture sites and is therefore clearly beneficial to this assay. However, the exact printing volume cannot be controlled and is therefore an unknown variable. Further to this, antibody immobilisation can impinge on the antibody function, reducing their activity (section 6). The combination of these two factors means that we do not know exactly how much pull down antibody is immobilised.

According to the manufacturer the delivery volume is estimated to be around 200 pL (Arrayit Corporation, USA). If this is true then the effective concentration of printed antibody in a 10 μ L volume will be approximately 56 pM. Given that immobilised antibody usually has very low activity, maximum 1%, the effective antibody concentration is likely to be much lower than this. The average monoclonal antibody is estimated to have nM affinity for its target protein. Therefore, in this setup the effective pull down antibody concentration will be at least 100-fold less than the K_d , and should be suitable for conducting the assay in the ambient analyte regime (Goodrich & Kugel 2007).

This also impacts the concentration in the microfluidic chips, with maximum concentration of 56 nM. This concentration could in fact be much lower due to limited antibody activity. This impacts the mass sensing regime, which requires high printed antibody concentration in order to increase the percentage of antigen captured.

6.5.2 Antibody affinity is estimated

Although the affinity of the two antibodies was not measured individually, the affinity obtained is a good representation of the assay strength as a whole. It is possible to attain affinities of the individual antibodies, and label free techniques that are commonly used include chromatography, isothermal titration calorimetry, and surface plasma resonance (Hearty et al. 2012). The limitation of the first two techniques is that they require large amounts of protein and antibodies (mg amounts) and were not suitable here.

Surface plasmon resonance is a powerful, label free technique which can measure effectively antibody- antigen affinity in a bimolecular system. It uses small volumes and flow to assess the binding at equilibrium whilst avoiding the limitations of mass transport (Hearty et al. 2012). In addition, measurements in real time mean that it can even give kinetic data (Hearty et al. 2012). It wasn't pursued in this study due lack of access to a Biacore facility and cost. In effect, the TIRF system can perform a similar experiment with the only limitation of fluorescently labelling for detection purposes.

The TIRF set up could be arranged so that it measures the affinity in a bimolecular system, either by printing the antigen and detecting with a fluorescently labelled antibody or alternatively by immobilising the antibody and detecting with a fluorescently labelled antigen. This would require fluorescently tagging the antigen which could adversely affect antigen-antibody binding. Additionally, printing Gata4 directly onto a glass surface may give spurious affinity readings as the binding epitopes are often obscured.

6.5.3 Limited data input

A big draw back with assessing the sensitivity and LOD is the number of data points. Because the microfluidic chips only contained 5 independent lines, only 5 different concentrations could be tested at any one time. It would be ideal to have more data points to populate the standard curve.

6.5.4 Cardiac progenitor clones are small cells

The Gata4 copy number provides only an estimate of the true copy number you would expect in a single cell. The cardiac progenitor cells are quite small, having a radius of between 4-9 μ M. Therefore the volume approximates to between 0.2-3.3 pL. Reported values of protein content per cell volume is approximately 0.2g/ml (Albe et al. 1990), giving a range of 40-660 pg per SP3 cell. It is hard to estimate the true single cell protein concentration, but in the absence of concrete Gata4 copy number, the calibration does provide a good indicator.

6.5.5 Gata-4 protein purity

The purity and quality of the protein sample used will affect the affinity read out. This was assessed using silver staining (see appendix, figure A), which showed that the sample was fairly pure but may have slight contamination. The amount of contamination was not determined. In addition lower bands suggest slight protein degradation. Both these factors will have an impact on the affinity reading.

6.6 Summary

- The Gata-4 detection assay has nanomolar affinity and should therefore be capable of detecting protein from single cells
- Calibration with cardiac progenitor cell lysate estimates the detectable Gata-4 copy number to be between 3-5000 per cell
- The limit of detection is below the estimated Gata-4 copy number per cell
- Single cell sensitivity is not obtained in the calibration with cell lysate; however, cell lysate is inherently sticky and increases the background binding
- Assay sensitivity could be increased by reducing background noise

Chapter 7

7. Analysis of Gata-4 expression in cells with TIRF microscopy

7.1 Overview

Single cell proteomics is inherently difficult for many reasons and to date only a few research groups have succeeded in achieving this (Shi et al. 2012; Bendall et al. 2011). The prominent limitations to single cell proteomics are; the lack of amplification that is readily available for RNA and sensitive enough technology that can detect few proteins. Whilst TIRFM is a highly sensitive technique, it in turn is restricted by the availability of good antibodies. These need to be both specific and have high affinity and is further compounded by the challenges encountered with antibody immobilisation. Despite over two decades of development, miniaturised proteomics still remains one of the greatest challenges in current molecular biology

Moving toward single cell proteomics, this chapter builds on the lysate and protein calibrations covered in the previous chapters, progressing to experiments with live cells. A few parameters make this possible; precise cell handling, nanolitre chamber volumes and the sensitivity of TIRFM. The latter of which detects fluorescent signal with high resolution and thereby permits detection of proteins from individual cells. This chapter presents several cell experiments where cells are lysed directly on-chip. Unfortunately, this did not result in single cell sensitivity but does show a logical progression, working to overcome the obstacles to single cell protein detection.

7.1.1 Single cell proteomics in microfluidic devices

The development of soft lithography in the late 90's made fabrication of devices with nanoscale features easily and readily available. The technique involves creating a mask on silicon wafers and using that to mould the elastomer, polydimethylsiloxane (PDMS). Development of microfluidic devices for RNA sequencing and single cell PCR is well underway in the field of single cell biology (Buganim et al. 2012; White et al. 2011). Microfluidic devices offers small volumes, facilitated cell handling and limits reagent dilution; thereby reducing sample loss which consequently facilitates the detection of target molecules.

The underlying idea of using microfluidic devices in this study is to minimise the diffusion distance of the proteins and thereby establish equilibrium quickly. In addition, a smaller volume drives the binding reaction toward the bound state as it increases the relative concentration of printed antibody to antigen. This in turn, means it is able to capture more protein and thereby increase assay sensitivity (see chapter 6 for more detail). With respect to cells, these chips facilitate cell handling and restrict the effect of dilution on the lysed cells.

The single cell microfluidic chip in this study consisted of a main channel, through which to flow cells, with 30 cell chambers branching off (Figure 7.1). The chamber dimensions are $300\ \mu\text{m}^2$ by $50\ \mu\text{m}$ depth, giving a volume of $4.5\ \text{nl}$. This is a reduction from the $10\ \text{nl}$ volume of the original lysate chips. At the time of study, this was the smallest physical dimensions for the cell chip that could accommodate the antibody spots. The small volume reduces the total diffusion time, allowing equilibrium to be established within 10-20 minutes (Salehi-Reyhani et al. 2013; Salehi-reyhani 2011), together with nanomolar affinity antibodies, the total capture percentage will be further increased beyond that of the lysate chips.

7.2 Methods

7.2.1 Microfluidics for cell handling and on chip lysis

The design of the microfluidic devices for the cell experiments differs from that of the lysate experiments and was developed by other members of the group (Salehi-Reyhani et al. 2013). Instead, they have a large central channel through which to flow cells. Branching off of this are 30 chambers, in which cells are lysed (Salehi-reyhani et al. 2011). These have the dimensions of $300\ \mu\text{m}^2$ by $50\ \mu\text{m}$ in height giving a volume of $4.5\ \text{nl}$ each (Figure 7.1). These chambers are separated by a small access channel, this separation is to reduce diffusion into and out of the cell chamber, minimising contamination from the bulk cell solution and loss of sample out of the chamber.

Optical trapping was used to confine cells in to separate chambers of the microfluidic device. Cells were then lysed and protein pull-down subsequently occurred in these defined, small volume chambers, using the antibody sandwich systems for pull down and detection. The benefit of this setup is that the solution containing cell debris remains separate and does not come into contact with the printed antibody. Thus, this reduces the possibility of contamination from free Gata-4 protein in the cell suspension.

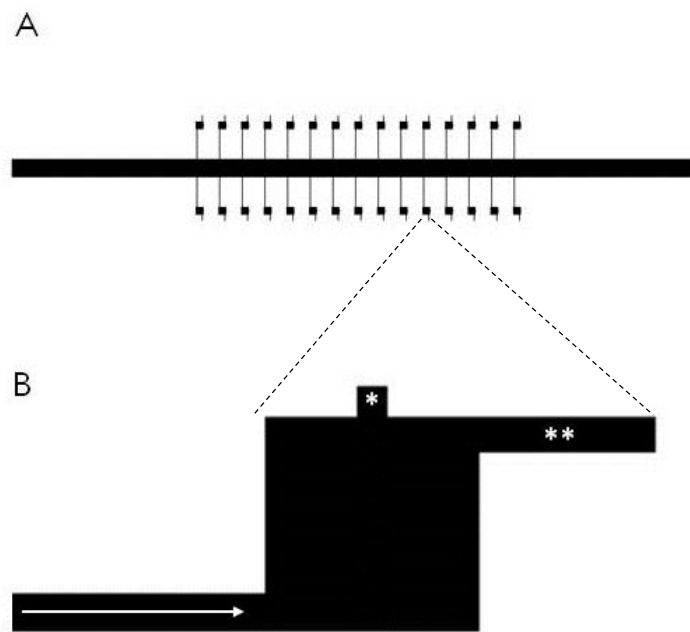


Figure 7.1. Diagram of the microfluidic design used for single cell experiments. (A) is the design of the whole chip, which consists 30 square chambers branching off of the central channel, through which the cells are flown. (B) is a close up of the $300\ \mu\text{m}^2$ cell chamber which, when cast in PDMS, has a volume of 4.5 nl. The white arrow marks the input channel, where cells are introduced into the chamber. Cells are placed in the small square outcrop (*), ready for laser lysis. There is an extra reserve channel (**) for retaining air bubbles that are not desiccated out of the device.

7.2.2 Cell handling

Cells were cultured as described in the methods in Chapter 2. Cells were detached using trypsin EDTA 0.05% and kept on ice in L15 medium with 10%FBS. L15 medium is designed for cells kept out of the incubator for long periods of time. Cells were spun down and diluted to appropriate cell number in L15 medium containing 10% FBS, 4% BSA, protease inhibitors and secondary antibody diluted to the appropriate concentration.

7.2.3 On-chip cell lysis methods

Two lysis methods were used in the development of this single cell proteomic assay; laser lysis and chemical lysis. Laser lysis had the benefit of being a precise and quick method for lysing single cells. It works by using a pulsed laser to produce a cavitation bubble near the cell, the force of which causes

the cell to rupture. Whilst this hypothetically also disrupts the nuclear envelope, the degree of this is unclear (Salehi-reyhani 2011).

The method of chemical lysis employs non-ionising surfactants to disrupt the cell integrity. With the current design (Figure 7.1) this solution can only be introduced by diffusion which takes about 50 minutes (appendix, figure C). Despite this limitation, this method is of benefit when there are many cells in one chamber. In this scenario laser lysis is very challenging as the force of the cavitation bubble causes the cells to move around. This means that one has to chase the cells in x, y and z directions in order to lyse them with the laser, which is very time consuming.

7.2.4 Large volume cell experiments

Coverslips were printed as previously described (see Chapter 2, section 0). Coverslips were then aligned with perfusion chambers (Sigma Aldrich) of 8 μ l in volume. Cells were loaded at either 1778, 889, 178 or 89 cells per chamber. The loading volume was 4 μ l, with an addition of 4 μ l of 1x lysis solution (Cell signaling, #9803) to lyse the cells. The chambers were then sealed with film to prevent dehydration. Antibody spots were imaged before cell lysis and for 90 minutes afterwards.

The number of cells loaded in the 8 μ l chambers was determined based on the volume of the MAC chip chamber. In the MAC chip set-up, if the assay had single cell sensitivity then we would be able to detect 1 cell per 4.5 nl volume, or 0.22 cells/ μ l. Scaling this up to the large volume format of 8 μ l would equate to 1778 cells per chamber. Therefore, the large volume format is equivalent to testing cells in the range of 1 cell to 0.1 cell per MAC chip chamber.

7.3 Results

7.3.1 Single cell experiments with GFP-tagged Gata-4 expressing cells

In order to test the efficacy of this assay at the single cell level HeLa cells transiently transfected with a GFP tagged Gata-4 expression vector were used. This permits Gata-4 binding to the printed antibody to be directly detected due to its fluorescent tag. Whilst it does omit the secondary antibody, it does give an indication of whether the assay can detect protein at the single cell level in the microfluidic chip, illustrated in Figure 7.1.

HeLa cells were harvested 36 hours after transfection and cell chambers were loaded with either 1, 2, 3, 5 or 10 cells. Successfully transfected cells were identified by their fluorescence using epifluorescence microscopy and loaded into cell chambers using an optical trap. Cells were lysed on chip by diffusion of lysis solution.

In the raw data, very distinct binding to the Gata-4 antibody was observed in comparison to the isotype or no cell control. However, after processing there was little significance between binding to the Gata-4 antibody and the controls in chambers loaded with 0, 2 or 3 cells (

Figure 7.2). There was only significant binding of Gata-4 protein above background with 1 cell and 5 cells per chamber (

Figure 7.2).

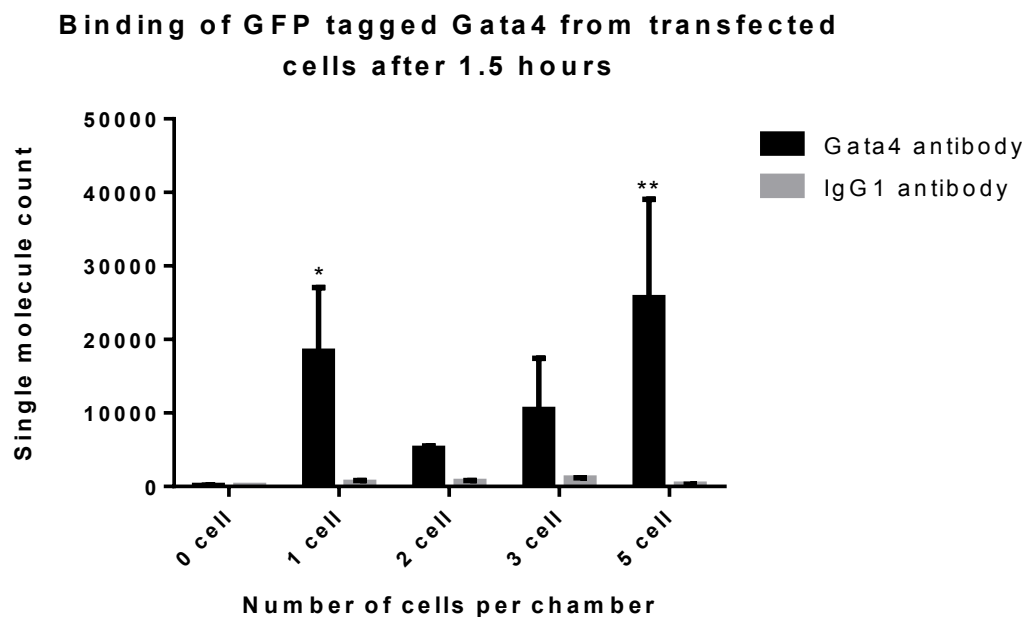


Figure 7.2. Titration of cells transiently transfected with GFP tagged Gata-4 expression vector. Graph shows the binding observed after 140 minutes after cell loading, or 90 minutes after effective cell lysis with lysis solution (*, $p < 0.05$; **, $P < 0.01$).

7.3.2 Single cell analysis of cardiac progenitor clone, SP3

Initial experiments with lysate and with Gata-4 purified protein (see section 6.3.4) indicated that this assay has the potential to detect protein at the single cell level. In order to test this with real cells, the microfluidic device illustrated in Figure 7.1 was used, loading cells into the chambers using an optical trap and then lysing them on chip with a laser pulse. Five chambers containing either printed IgG1 or anti-Gata-4 antibody were loaded with 1 cell, 2 cells or no cells.

Two conditions were tested, first laser lysis (Figure 7.3) and chemical lysis (Figure 7.4); lysed cells were subsequently incubated on chip over 1.5 hour period. In the first experiment with laser lysis (Figure 7.3) an overall increase in signal was observed with 2 cells per chamber and was significantly

higher than with 1 cell or no cell per chamber. However; there was no distinguishable difference in binding to the Gata-4 antibodies over the isotype control (Figure 7.3).

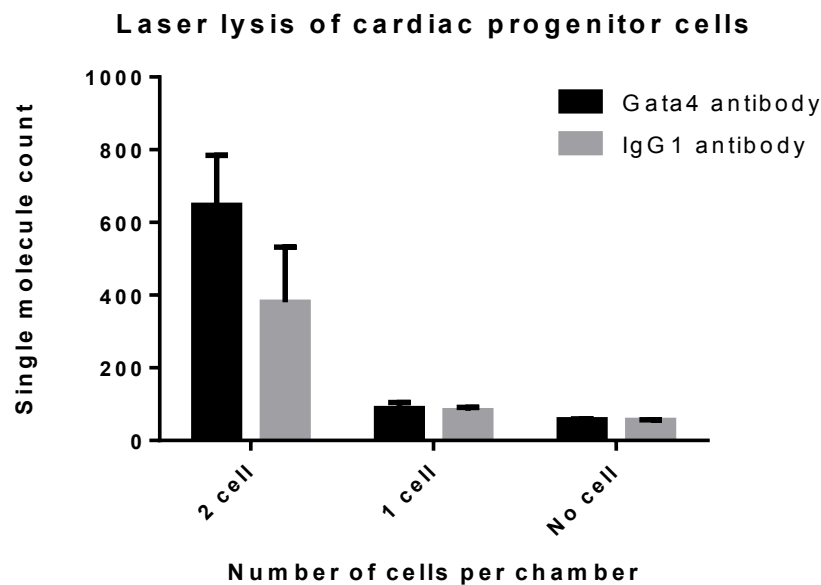


Figure 7.3. Analysis of Gata-4 protein expression after laser lysis of cardiac progenitor clones. Cells were loaded into chambers containing either printed IgG1 or Gata-4 antibody, at 1, 2 or 0 cells and binding measured 1.5 hours after cell lysis. No significant difference was observed.

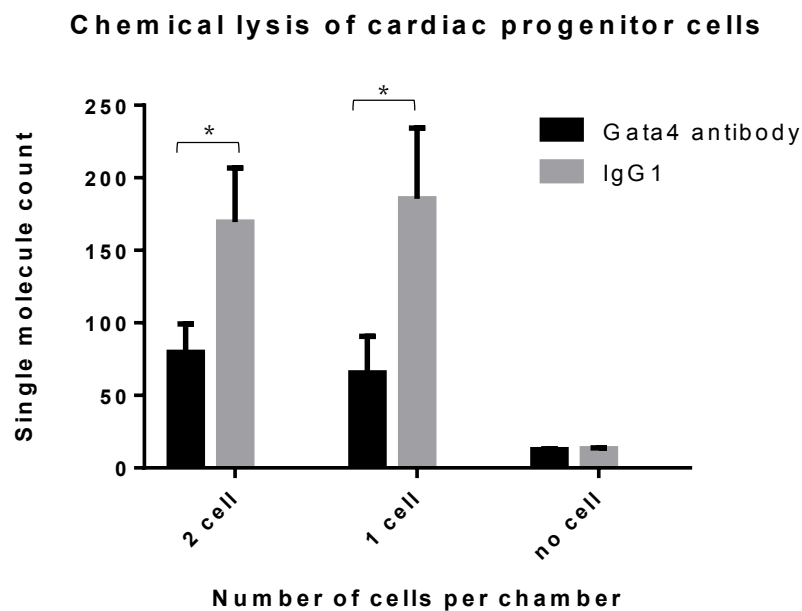


Figure 7.4. Chemical lysis of cardiac progenitors in microfluidic chips. Lysis solution was diffused into cell chambers containing either printed IgG1 or Gata-4 antibody and loaded with 1, 2 or 0 cells. Binding was measured 1.5 hours after cell lysis, where a statistical difference was observed between IgG1 and Gata-4 antibody chambers containing either 1 or 2 cells.

From the raw data, it could be seen that the fluorescence remained localised in the vicinity in which cells were lysed (Figure 7.5.) and did not diffuse across the chamber to the antibody spot as expected. This can be seen from the tile scan images in Figure 7.5, a sequence of images taken across the cell chamber and stitched together. This set of images compares the control chamber containing printed IgG1 with that of anti-Gata-4 each containing either 1, 2 or 0 lysed cells.

Subsequently, chemical cell lysis was tried in place of laser lysis (Figure 7.4). The cell experiment was carried out as previous, but allowing diffusion of lysis buffer from the main channel to the cell chamber to lyse the cells (Figure 7.4). Overall, the single molecule count for all antibody spots was low, both Gata-4 antibody and isotype control. No statistical significance was observed between isotype control and the Gata-4 antibody with 0 cells per chamber. There was larger binding to the isotype control in both 1 cell and 2 cells per chamber.

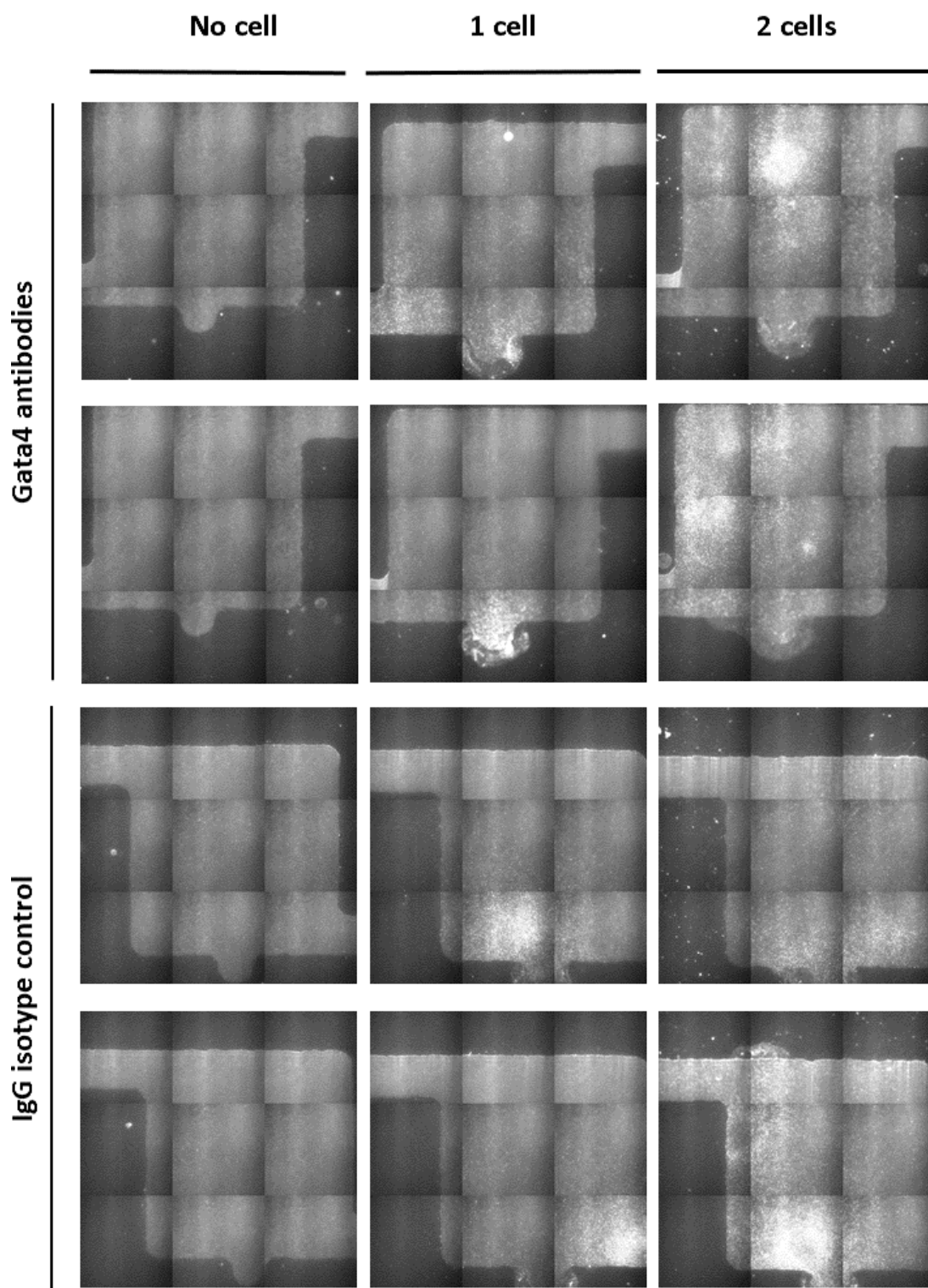


Figure 7.5. Tile scan images of single cell chambers 2 hours after laser lysis. Printed antibody spots (BD) are located in the central image tile. Typically cells are loaded and lysed in the cell loading chamber (semi-circle outcrop on right hand side of chamber). Images show that fluorescence does not accumulate on antibody spot but rather remains in the area where cells were lysed and does not diffuse throughout

the chamber after 2 hours of incubation. Here, the cardiac progenitor cells, clone 3 was used and the fluorescently labelled Gata-4 antibody (MAB) was used.

7.3.3 Limit of detection in large volume chambers

Since the experiments with cells in the microfluidic chips were not successful, I turned to the large volume assays to try to determine the problem. The possible issues were either: inefficient cell lysis, limit of antibody sensitivity, or the antibodies were no longer active. To begin the assessment, I decided to repeat the experiment on a large scale format, lysing cells directly on chip by pipetting on lysis solution.

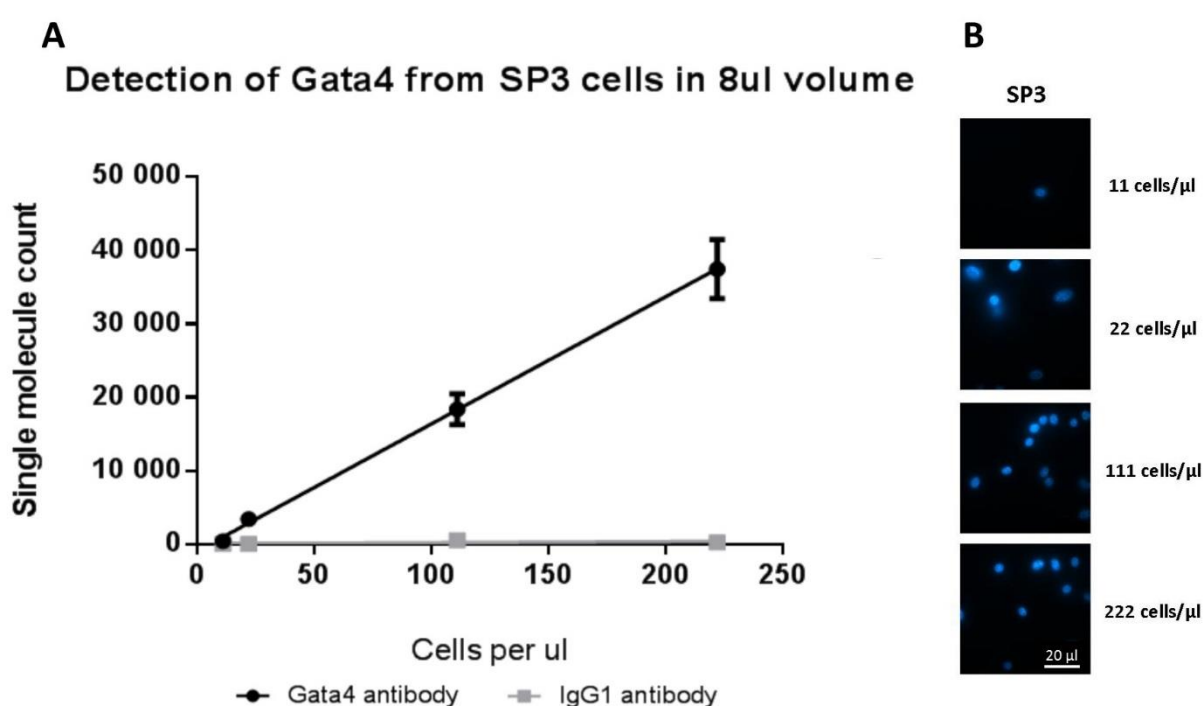


Figure 7.6. A) Direct cell lysis of cardiac progenitor cells, SP₃, in microlitre volume chambers. Cells were loaded into 8 μ l chambers with both printed anti-Gata-4 antibody (black) and IgG1 control (grey). Binding can be detected with as few as 111 cells/ μ l, which is equivalent to 0.5 cells in a microfluidic chip chamber. There is no significant difference between anti-Gata-4 and the IgG1 control at 22 or 11 cells per μ l. Error bars represent standard error of the mean of three replicates, each containing 4 capture spots of each antibody. B) Cells were stained with propidium iodide and imaged post lysis. Images show presence of cells in chambers containing different concentrations of cells. Data are presented as means of 3 independent experiments using the same cell line $\pm$ SEM.

The assay was conducted in 8 μl chambers, with two of the cardiac progenitor clones: SP₃ which expresses Gata-4 protein and SP₁₆ which does not express Gata-4. Cells were diluted to either 1778, 889, 178 and 89 cells per chamber. This equated to between 1 to 0.1 cell of the MAC chip chamber.

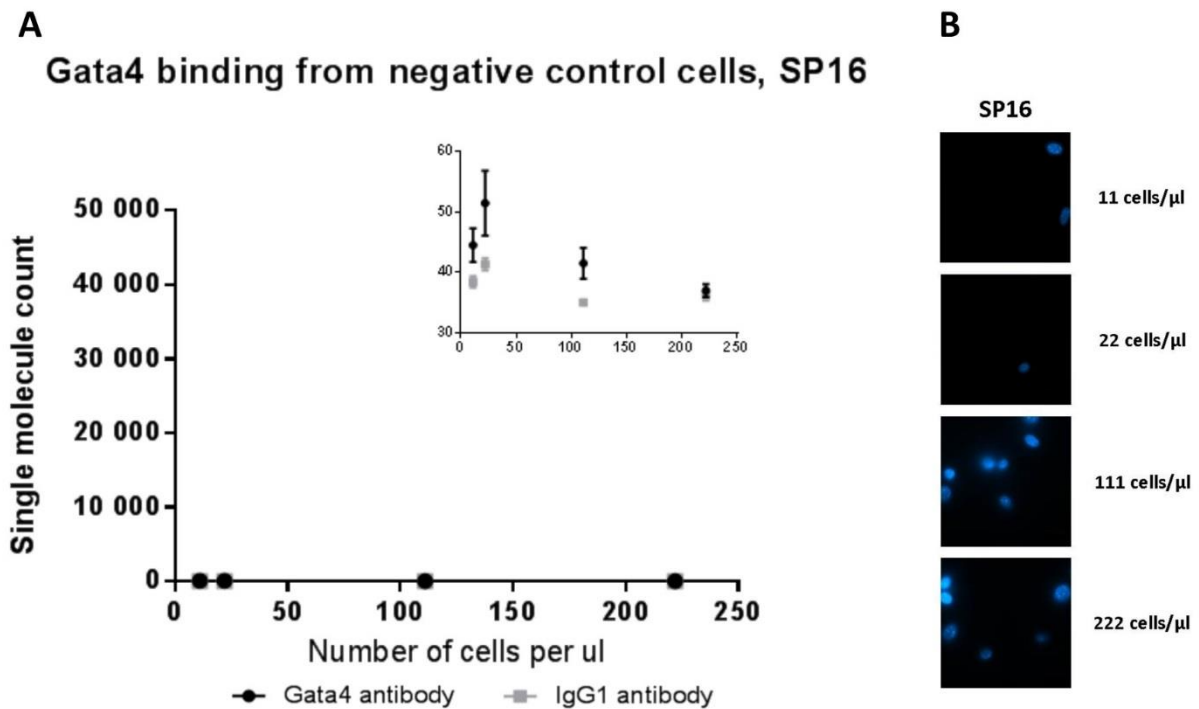


Figure 7.7. A) Lysis of control cardiac progenitor cells, which do not express Gata-4 protein, in microlitre chambers. The graph shows negligible binding of Gata-4 from SP₁₆ negative control cells to either Gata-4 (black) or IgG₁ (grey) control. B) Cells were stained with propidium iodide and imaged post lysis. Images show presence of cells in chambers containing different concentrations of cells. Data are presented as means of 3 independent experiments using the same cell line $\pm$ SEM.

According to this data set, the assay can detect Gata-4 protein at a level equivalent to the single cell sensitivity in the microfluidic chip (Figure 7.6). The lowest limit of detection tested here was 11 cells per microliter. There is good linearity between the number of cells loaded and the signal produced, with the R^2 value of 0.743.

Testing the assay with a negative control cell line, SP₁₆, showed that there was little detectable Gata-4 in these cells (Figure 7.7). This agrees with PCR data which shows SP₁₆ does not express Gata-4 (see figure 4.5).

7.3.4 Limit of detection in the microfluidic antibody chip

Since experiments with single cells or two cells per chamber yielded no result, we set out to find the limit of detection of this single cell set-up. The lysate calibrations previously conducted (chapter 5), along with cell experiments in larger volumes (section 7.3.3), suggested that this antibody sandwich system could detect Gata-4 protein at the single cell level. However, this did not correlate with experiments with real single cells. Hence, we set out to find the true limit of detection for this assay in the microfluidic volumes.

To investigate this, 3 chambers of the single cell chip, containing either printed anti-Gata-4 or IgG1 antibody, were loaded with 3, 5, 10, 15 or 0 cells. Cells were then lysed by diffusion of 10x lysis solution into the cell chambers. Diffusion from the main channel to the cell chambers takes approximately 60-90 minutes and the binding plateau is reached at approximately 150 minutes (see Appendix, Figure C).

After 4 hours incubation on chip, observed binding to the Gata-4 antibody is higher than to control antibody (Figure 7.8). However, it is only significantly higher with 15 cells per chamber and is not significantly different for chambers containing 0, 3, 5 or 10 cells.

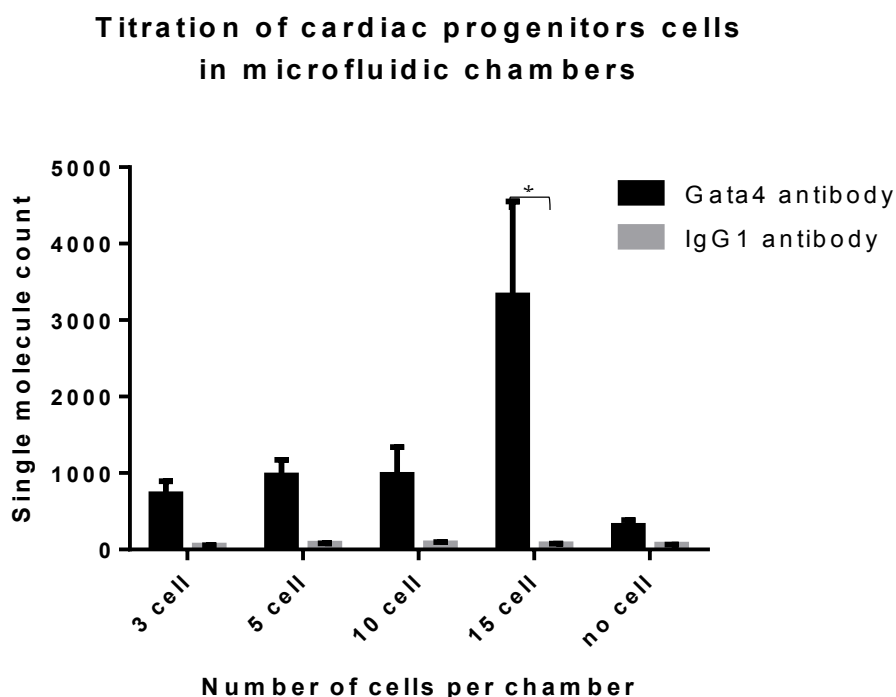


Figure 7.8. Titration of cardiac progenitor cells in microfluidic chambers and chemically lysed. Chambers were loaded with either 3 cells, 5 cells, 10 cells, 15 cells or no cells. A significant increase in binding to Gata-4 antibody (black) above the IgG control (grey) was only observed at 15 cells per chamber.

7.4 Discussion and limitations

7.4.1 Variable expression of Gata-4 in GFP tagged Gata-4 transfected cells

There were a few caveats with this experiment, the first being that transfection can stress the cells as it forces cells into overproduction of protein. Cell viability was calculated from trypan blue, and the addition of propidium iodide helped to exclude selection of dying cells on-chip. The second limitation is that the amount of protein produced per cell cannot be controlled. Whilst fluorescent intensity might give a good indication of protein content, it can also be misleading as GFP takes time to mature (Evdokimov et al. 2006), and may not be functional or simply misfolded. In addition, the intensity can be affected by cell size and cellular location, though in this instance, fluorescence was nuclear.

Whilst in (

Figure 7.2) there is a clear difference between isotype control and the Gata-4 antibody, the lack of significance could be an artefact of processing. Some of the fluorescent cells could be seen in the field of view, giving a high fluorescent background. This in turn would increase the single molecule count due to the increased fluorescent intensity in the images. It is a challenging task to process out these artefacts without losing real single molecules.

In general, there was a large variation in the single molecule count for the Gata-4 antibody; whereas there was a very small variation for the isotype control. This possibly reflects the different Gata-4 expression levels in the transfected cells. However, this experiment was limited to 3 samples per antibody, per cell number; thus sample size would have to be increased in order to see the true variation.

Another factor to consider is the degree of cell lysis and the lack of mixing in the chambers. Further still, the PDMS is inherently sticky and despite passivation with BSA, protein may be adhered to the chamber walls and hence not able to bind to the antibody.

Despite these caveats, this data suggests that this assay, at least in part, is capable of detecting protein at the single cell level. Albeit, this is testing an over expressing cell line and not endogenous expression.

7.4.2 Challenges with single cell proteomics with cardiac progenitor clones

Lysing CPCs in the microfluidic chip (Figure 7.3 and Figure 7.4) yielded no signal at either 1 or 2 cells per chamber. Two different lysis methods were tried but this made no improvements. In the first experiment, a generalised increase in signal was seen with 2 cells per chamber, and was significantly higher than 1 or no cells per chamber. However, the isotype control produced an equivalent signal. It is possible that this is due to the location of the cells, being closer to the antibody spot (see Figure 7.5.). With laser lysis, minimal diffusion of the Gata-4 protein away from the cells was observed. Thus, the cells being present near the antibody spots can increase the overall intensity in the field of view and thereby increasing the single molecule count.

In contrast to chambers containing 2 cells, the signal in the 1 cell chambers is constrained to one image (one field of view) and does not interfere much with the central field of view, where the printed antibody spot is located (Figure 7.5). This might contribute to the reduced signal in the 1 cell chambers, which would consequently have much reduced fluorescent intensity.

Whilst there was more binding to the isotype control with chemical lysis (Figure 7.4), this was generally quite low, especially when considering the lysate calibrations (Chapter 6). The signal produced was similar to background levels expected, and is most likely due to non-specific binding.

Although one might be cautionary with the use of lysis solution in a protein assay, preliminary data with lysate showed that lysis solution did not hinder the assay (see Appendix, Figure D). However, from the results obtained here chemical lysis produced meager binding when compared to the laser lysis technique. Although the laser technique seemed to release more protein, the signal remained in the location of cell lysis and perhaps requires on chip mixing.

7.4.3 Directly lysing cells in large volumes indicates single cell sensitivity

Initially, this experiment was conducted to see if there was a problem with the chemical lysis solution. To help replicate the conditions on chip, it was necessary to minimise any mixing; loading cells into chambers and directly lysing on chip by careful addition of lysis solution, avoiding any adverse mixing.

The cells were diluted so that the cells per volume were equivalent to that found in the microfluidic chips. A linear correlation was observed between the number of input cells and the single molecule count, as would be expected. In essence, this experiment showed that the assay works and

demonstrates a propensity for single cell sensitivity. However, this doesn't correlate with the single cell experiments conducted in the microfluidic chips.

One possible reason for this is that the lack of mixing might create a high concentration of protein local to the printed antibody spot. Since the cells settle at the bottom of the chamber due to gravity, any cell lysis will occur in this area. Without adequate mixing, the proteins released will be at a higher concentration in this region, relying on diffusion to equilibrate the solution. Since the printed antibodies are also in this region, the very high local concentration of Gata-4 would favour increased Gata-4-antibody binding (illustrated by Figure 7.9).

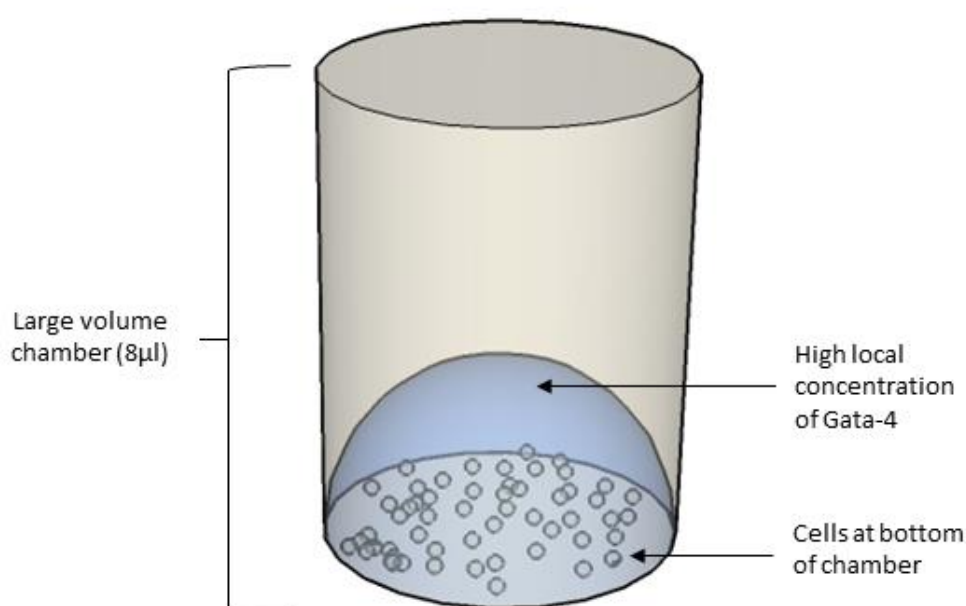


Figure 7.9. Illustration of high local concentration of Gata-4 protein in region of printed antibody. Increased Gata-4 signal observed due to higher concentration of the protein (shaded blue area) in lower part of chamber. Cells (small circles) loaded into the chambers sink to the bottom. Upon addition of lysis solution, cells are not mixed and are lysed in the vicinity of the printed antibody spots. On release of intracellular proteins, diffusion occurs within the local area. Time to reach homogenous concentration throughout the 8µl volume is much longer than the 1.5 hour incubation time.

This experiment does not give information on the number of Gata-4 molecules per cell. The percentage capture in this large 8µl volume is miniscule in comparison to the microfluidic chips (see calibrations in Chapter 6). According to the Gata-4 calibration curves, the percentage capture is as low as $4 \times 10^{-7} \%$ in the large volume chambers (Chapter 6). In this large volume experiment, the printed antibody patches detect on average 20 Gata-4 molecules per cell. Based on the theoretical copy number of Gata-4 per cell calculated in Chapter 6, the capture in these experiments is on the

order of 10^{-4} %. This is still in the ambient analyte regime but the capture is 3 orders of magnitude higher than that experimentally determined in Chapter 6. This could possibly be explained by the theory of high local Gata-4 concentration, as this would favour more Gata-4-antibody binding.

Whilst there was limited mixing in this large scale experiment, there is still potentially more mixing than occurs in the microfluidic chips. This extra agitation, however slight, might contribute to the positive signal seen in the 8 μ l chambers. Thus, it is conceivable that the cells in the microfluidic chips are not adequately agitated in order to release nuclear protein.

Another factor to notice about this experiment is the control conducted with the Gata-4 negative cells, SP16 (Figure 7.7). These gave virtually no non-specific binding, with non-specific binding between 30-60 single molecules. This indicates that on chip these antibodies are truly specific to their target protein. Further still, the direct cell lysis produces low non-specific binding and thus the limit of detection might be lower than calculated in Chapter 6.

7.4.4 Improving the lysis methods on the MAC chip

There are a couple of limitations with the current microfluidic chip design, and it's possible that a few adjustments may increase the sensitivity. One of the issues not resolved here is that of cell lysis. It is not certain that the cell lysis methods, either by using laser or chemical lysis, was effective enough to release the nuclear localised transcription factor, Gata-4. Whilst work in the group has shown that laser lysis is efficient at releasing the transcription factor P53 (Salehi-reyhani et al. 2011), it did not work with Gata-4. In contrast to P53, Gata-4 is constrained to the nucleus and may not be as abundant. It might require high salt buffers in order to elute the nuclear proteins and DNase to break down the DNA. Although the salt concentration of 150mM seemed effective in the large volume experiments, this could be increased to release proteins on chip.

Normally, when making lysate in bulk, one uses a combination of mechanical force with detergents to release nuclear proteins. In the case of on-chip cell lysis, no mechanical force is used. It is possible that the application of on-chip mixing would release more nuclear proteins, freeing them to bind to the antibodies.

With chemical lysis, the nucleus of cells in the microfluidic chips remains intact (Figure 7.6 and 7.7). This is seen by incubating the cells with propidium iodide (data not shown). Whilst the nuclei may be chemically perforated, there is no mechanical force to disrupt the membrane physically.

In contrast laser lysis does physically disrupt the cell nucleus. However, without the use of detergent it is foreseeable that the Gata-4 protein remains attached to the DNA. One solution could be to use laser lysis in combination with chemical lysis; lysing cells first with a cavitation bubble and then

diffusing in the lysis solution. The draw back here is that diffusion of the lysis solution takes approximately 1 hour.

This raises another drawback to the current system of chemical lysis: the diffusion time. It takes approximately 1 hour for lysis solution to diffuse from the main channel to the cell chambers (see Appendix, Figure C). During this time cells can begin to undergo apoptosis, thereby destroying proteins and reducing the amount of Gata-4 available to be detected. However, all solutions do contain protease inhibitors, although this will not inhibit intracellular protein degradation prior to cell lysis.

It would be better to introduce the lysis solution so that cells were lysed instantaneously. This could be done by using a two layer microfluidic device, but this was beyond the scope of this project. Another option would be a “flip chip” design, which has been trialed by members of our group. This entails having a grid of chambers on which you pipette a cell solution, diluted so that there is approximately 1 cell per chamber. A cell lysis solution would then be applied on top and the grid sealed. This would save a huge amount of time in both cell loading and cell lysis; thereby reducing cell stress and degradation. The two main disadvantages are that of cell loading and antibody-chip alignment.

In this flip-chip system there is no control over how many cells are loaded per chamber. However, this could be sidestepped by using bright field imaging to count the number of cells in each chamber. The second is the problem of aligning the chip on to a printed coverslip. The problem of alignment arises due to the delicate nature of the grid. It would be open topped and have a maximum height of 50 μM , in order to retain small chamber volumes. Thus, the thinness of the grid would make it challenging to manipulate, unlike the current PDMS microfluidics.

7.4.5 Reducing the cell chamber volume

The volume of the chambers in the microfluidic chips is 4.5 nl. Diffusion time across the chamber is reported to be within 30 minutes (Salehi-reyhani 2011; Salehi-reyhani et al. 2011). Hence, diffusion time is not the limiting factor at this volume. Instead, the assay is limited by the binding kinetics, LOD and sensitivity. The volume of 4.5 nl is large enough that antibodies are in sampling mode, as defined by Ekins’ microspot theory (Ekins 1998), detecting only a small proportion of the proteins present. Reducing this volume would push the proteins more towards the bound state, thereby increasing sensitivity. Simulations from our group indicates that this is true (Salehi-reyhani 2011).

However, there are technical caveats with reducing the chamber volume even further. The first is that the chambers need to be sufficiently deep to manipulate and load the cells. Another issue is that

of alignment. Presently we align the printed slides and microfluidic devices using a hand-operated jig. This can be very challenging, even at the present chamber sizes. Reducing the cell chamber sizes would require a more precise, perhaps automated, alignment jig. Without this the device, output would be significantly reduced.

7.5 Summary

- The experimental limit of sensitivity achieved here was 15 cells, a very small population of cells.
- Experiments with cells transfected with GFP-tagged Gata-4 suggests that this assay has single cell sensitivity.
- In addition, large volume cell experiments indicates the assay has single cell sensitivity.
- The assay might be limited by lysis methods, antibody kinetics or chamber dimensions.
- The data presented here shows the TIRFM assay is a promising technique for single cell proteomics, although working with transcription factors proves challenging.

Chapter 8

8. DNA Capture of Gata-4 protein as an alternative to antibodies

8.1 Overview of aptamers as protein capture agents

Whereas DNA microarrays have excelled in recent years, application of protein arrays is vastly limited particularly in the area of single cell studies. One of the bottle necks in protein array technology is that of the antibodies themselves; limited by their kinetics, sensitivity, specificity and stability.

Manufacturing good antibodies is also a constraint: it is costly, time consuming and the inherent inconsistency leads to batch-to-batch variability, requiring functional assessment of each batch. Research into alternative capture agents has led to the development of aptamers, affibodies, peptide binders and molecular imprinted polymers (MIPs) (Even-Desrumeaux et al. 2010; Germer et al. 2013; Gunneriusson et al., 1999; Jing et al. 2011; McKeague et al. 2012; Patel et al., 1997; K.-M. Song et al. 2012; Vasapollo et al., 2011).

Improvements in antibody technology itself has seen the rise of Fab fragments, ScFv, as well as different antibody species such as chamelid antibodies (Nelson, 2010). The benefit of these is that the *in vitro* manufacturing yields more control, reducing costs and giving more flexibility for modifications. However, like antibodies they are still relatively thermally unstable, being generally less stable than the full length antibodies (Nelson, 2010). Recent exploration of Chamelid antibodies offers an interesting alternative, though not yet commercially available. These antibodies contain no light chain, consisting of a single chain these are thus easier to produce *in vitro*. Coupled with the streptavidin biotin immobilisation, they become very sensitive protein detection tools, with a subnanomolar limit of detection (Even-Desrumeaux et al., 2010).

Antibody development has taken a few different avenues in the past few decades, with many having great success and applicability in the clinic. The simplest was the modification of monoclonal antibodies into Fab fragments, essentially cleaving off the Fc region leaving only the binding region, the Fab fragment (Nelson, 2010). A derivative of this is the short chain variable domain fragment (scFv), where the variable regions of both the light and heavy chain are manufactured by prokaryotes, and fused together by a linker peptide (Nelson, 2010). The advantage of these is that

the lack of Fc region reduces nonspecific binding from this region, although this is more relevant in direct cell staining than immunoassays. The major benefit of scFv is that manufacturing can be less time consuming and less costly.

Application of phage display technology to antibody production offers an automated production system, accelerating the synthesis of high affinity, high specificity antibodies. Libraries containing all the variable (V) genes of antibodies permits *in vitro* selection by incubating with the antigen of interest (Schirrmann et al. 2011; Winter et al. 1994). To attain the antibody fragment of interest the phage libraries are subjected to several rounds of selection, amplification and mutation (Wild, 2001). This process of increasing the affinity by mutation has had varying degrees of success with one study achieving picomolar affinity for fibronectin (Pini et al., 1998) and others claiming up to a 4 fold increase (Winter et al., 1994). The winning phage can then be sequenced and V genes used to make Fab, scFv fragments or even full length antibodies (Cai et al. 2013; Schirrmann et al., 2011).

8.1.1 Alternatives to antibodies

RNA has many functions in the cell, from transcription and translation to expression regulation. One of their inconspicuous roles is their protein binding capabilities. First identified in the 1980s from research in HIV, viral RNA was shown to bind proteins with high specificity and affinity (K.-M. Song et al., 2012). Single stranded RNA molecules can form intricate tertiary structures that allow protein binding. In addition, these molecules are thermally stable, reforming after heating, and are stable at a range of pH (K.-M. Song et al., 2012).

The use of single stranded DNA or RNA molecules, termed aptamers, have been in development since the 1990s when two research groups (Ellington et al. 1990; Tuerk et al. 1990) simultaneously developed a selection process that specifically identified protein binders. This involved using a library of aptamers and selecting the most specific to the protein of interest. This process, termed Systematic Evolution of Ligands by EXponential enrichment (SELEX), involves several cycles of aptamer-protein binding, followed by washing away of unbound aptamer, eluting the protein and amplifying the aptamers of interest by PCR. The products then go through several repeat rounds to produce the aptamer with highest specificity (Germer et al. 2013; K.-M. Song et al., 2012; Tombelli et al. 2005). There has even been some clinical success with RNA aptamers with the first RNA-binding drug, Pegaptanib, being approved for Macular Degenerative Disorder in 2004 (Jing et al. 2011).

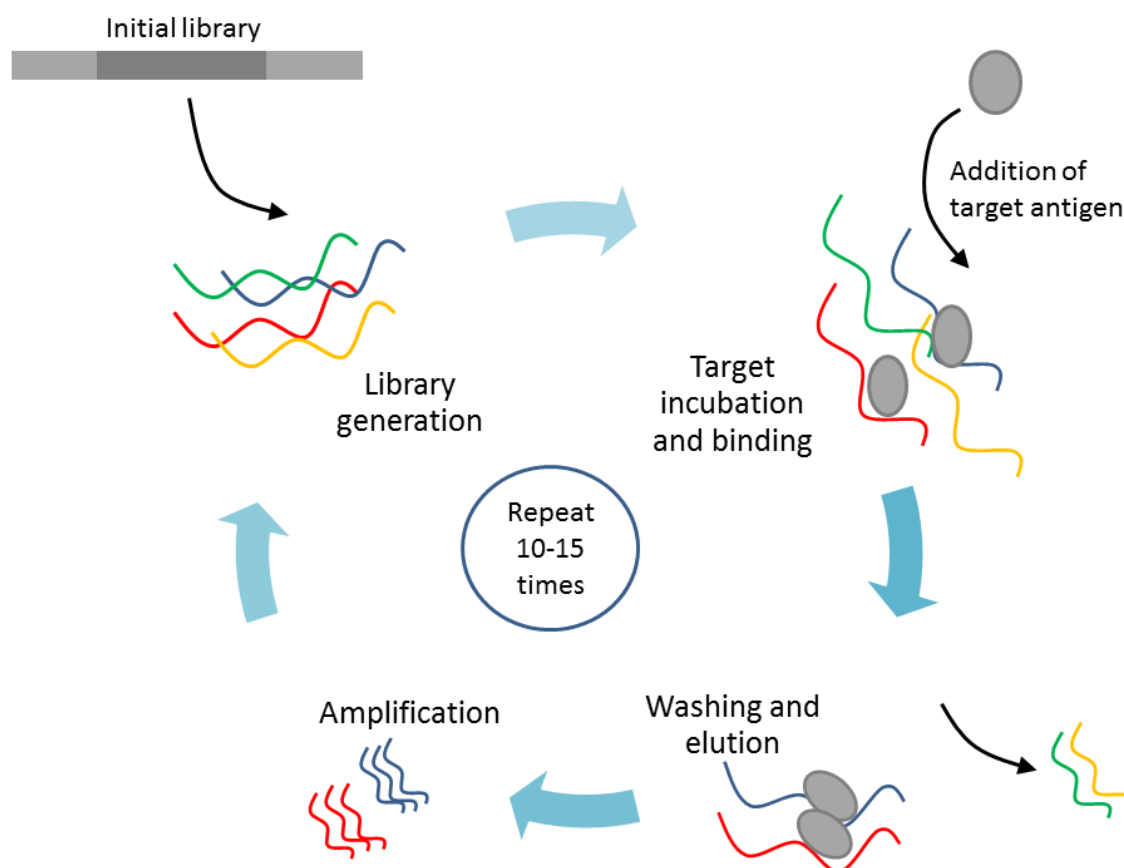


Figure 8.1. Generation of RNA aptamers by the technique of Systematic Evolution of Ligands by EXponential enrichment (SELEX). A library containing random nucleotide sequences is synthesised and then incubated with target antigen. This is then washed to remove unbound RNA, and the bound RNA is eluted and amplified. The amplified library is then subjected to further rounds of selection in order to identify the most specific protein binders. *Adapted from Tombelli et al. 2005; Song et al. 2012.*

Whilst there are many strategies to produce high affinity, high specificity protein binders, development and validation is very time consuming and was outside the scope of this project. Instead, I decided to use Gata-4's quality as a transcription factor and used double stranded DNA containing the consensus sequence as a method of protein capture.

Double stranded DNA hasn't been conventionally developed for protein capture on microarrays. However, protein-binding DNA microarrays have been used to determine transcription factors sequence specificity (Berger et al., 2006; Bulyk et al., 2001; Mukherjee et al., 2004). These studies suggested that the DNA binding quality of transcription factors can be harnessed for protein capture, more specifically using double stranded DNA as the bait to capture Gata-4.

8.1.1.a Gata-4 binding specificity for DNA

The original premise of using DNA as a capture agent is that it is cheap, has high affinity (Merika et al. 1993) and is easy to manipulate on glass surface with high level of activity. In addition, it is anticipated that the DNA will provide a more natural binding conformation that would also allow detection of protein-protein interactions. Finally, zinc finger transcription factors have a good reputation for conferring specificity and picomolar affinity (Moore et al. 2001). By substituting only a few amino acids in the DNA binding domain and adding more zinc fingers in modular fashion, the class I zinc fingers have been harnessed for targeting specific genes (Moore et al., 2001).

There are 6 members to the Gata family, each having varying yet sometimes overlapping roles. Gata1 and Gata2 are both found in the developing haematopoietic system; including erythrocytes, mast cells and megakaryocytes. Gata3 is more restricted to T lymphocytes and brain cells (Merika et al. 1993). Gata-4 specifically is involved in the developing endoderm and heart, having overlapping expression with Gata5 and 6 (Holtzinger, Rosenfeld et al. 2010; J D Molkentin, 2000; Singh et al., 2010; van Berlo et al., 2010; Zhao et al., 2008).

Members of the Gata family each contain two zinc fingers which belong to the class IV structure (Gronenborn, 2005). The DNA binding core consists of two antiparallel beta sheets and an alpha helix, stabilised by four cysteine's that coordinate with central zinc atom (Omichinski et al., 1993). These Gata zinc fingers typically recognise 8 nucleotide bases (Omichinski et al., 1993) for which the consensus sequence is A/T GATA A/G (Merika et al. 1993). The Gata enhancer that is found in the promoter regions of target genes varies considerably from the consensus sequence. A study by Merika and Orka (1993) showed that Gata1 and 2 have quite lax binding specificity, whereas Gata3 is more restricted. It's postulated that the differential recognition of similar but not identical Gata binding sequences might convey the differential gene activation of the Gata factors (Merika et al. 1993). This is important considering they are often co-expressed in the same cell.

In general the Gata factors have demonstrated high affinity for their binding sequences (Merika et al. 1993). Specifically, a binding study of Gata-4 and Gata-6 showed that these both bound the ANF promoter with nanomolar affinity (Charron et al., 1999). However, the K_d of the two Gata members differed depending on whether they bound the distal or proximal enhancer. This differential affinity presents a possible method of discriminating binding from unwanted Gata family members. Development of DNA aptamers began by looking at a database of Gata-4 consensus sequences, selecting those which are more specific for Gata-4.

8.2 Methods

The methods used are as described in Chapter 6. The 10 nl lysate chips were used, with lysate from either endogenous Gata-4 expressing cells or transient transfected cells (see Chapter 6). Lysates were incubated at 7.5 pg/nl or 75 pg/nl, equivalent to 1 or 10 cells per chamber. This was calculated on the basis of cells containing on average 75 pg of protein (Chapter 6 for more detail).

8.2.1 Design of the Gata-4 Capture agents

Information on Gata 4 binding elements was collected from the Transfac database, which provides reference material of published binding sequences. Sequences were chosen on the basis of how many of the Gata family members bound the sequence, preferring only Gata-4. At the time, longer sequences were selected as oppose to length similar to 6 base consensus sequence, hoping that it would confer higher specificity.

DNA oligo	Forward sequence	Reverse sequence
Gata4	TATAT GATAGCAGT GATATATAT AAGTCAATCGGTCC	*GGACCGATTGACTTATATATATC ACTGCTATCATATA
ANF	TATAG GATCTCCCAGGA AAGATA ACCAAGGACT CGTATATATAAGT CAATCGGTCC	*GGACCGATTGACTTATATATACG AGTCCTTGGTTATCTTCCTGG GAGATCTATA
Mutant	TATAG GATCTCCCAGGA ACCTAA CCAAGGACT CGTATATATAAGTC AATCGGTCC	*GGACCGATTGACTTATATATACG AGTCCTTGGTTAGGTTCTCTGG GAGATCTATA

Table 8.1. List of DNA sequences used as protein capture agents. Forward and reverse strands listed in the direction 5' to 3'. Bold sequences identify the binding sequence; otherwise the sequence is the spacer used to lift the DNA off of the glass surface. The reverse sequence was conjugated at the 5' end with and amino linker (*).

The sequences used are listed in Table 8.1. The DNA oligos included an extension sequence (Bulyk et al., 2001), whose aim was to lift the DNA off of the glass surface to prevent steric hindrance (Shchepinov et al. 1997). To attach the DNA to the glass surface, the 5 prime sequence of the forward was modified to contain an amine group separated from the DNA by a 6 carbon spacer. This single amine group thus permits orientation of the double stranded DNA.

8.2.2 Surface modification for printing

Glass coverslips were functionalised with GPTS solution for amine attachment of the modified DNA. GPTS forms a neutral surface coating (Carré, Birch, et al. 2007; Guiseppi-Elie et al. 2005) which is good for reducing unwanted protein-surface interactions. The epoxide is very reactive due to the strained nature of the three atom cyclic ring (et al. 2005). Nucleophilic attack by a primary amine, opens the ring allowing the now charged oxygen molecule to bind its nearest hydrogen and the free nitrogen to covalently bond the carbon molecule (Figure 8.2; Guiseppi-Elie et al. 2005). Coverslips are prepared by cleaning in ethanol with sonication, followed by sodium hydroxide, rinsed in water and then air dried and with a final activation of the hydroxide groups by plasma treating for 1 minute. The SiOH activated slides are immediately incubated in 2.5% GPTS solution in Ethanol and 5% acetic acid.

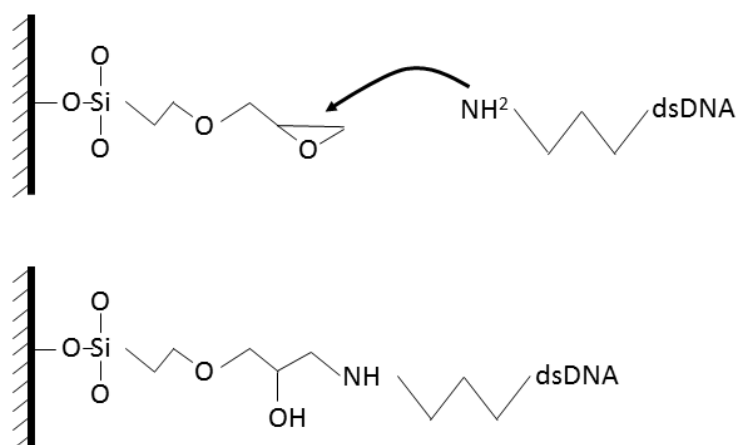


Figure 8.2. Immobilisation of DNA onto GPTS functionalised glass surface. DNA is modified with an amine linker, such that it contains one binding site per double stranded DNA molecule. This amine group is then able to react with cyclic carboxyl ring on the epoxysilane, forming a covalent bond. Adapted from Guiseppi-Elie & Lingerfelt 2005

8.3 Results

8.3.1 Optimisation of DNA concentration

DNA microarrays is a well-established field, having been in development since the 1980's and 90's. Here the printing buffer of 3x SSC with 1.5M betane was used to prevent spot drying and give optimal signal (Diehl et al., 2001). In addition spacer sequences were included to reduce steric hindrance which arises due to the interaction of DNA with the electrostatic charges on the glass surface (Peterson, 2001; Shchepinov et al., 1997). Protein-DNA microarrays are not a common technique and whilst some studies suggest the use of between 10-20 μM of DNA (Bulyk et al., 2001) the effect of DNA density on protein binding is not well studied. Too little DNA will result in reduced signal, too much can result in inhibition of binding, as has been seen in traditional DNA microarrays (Peterson, 2001). Here, I optimised the concentration of printed DNA for protein binding.

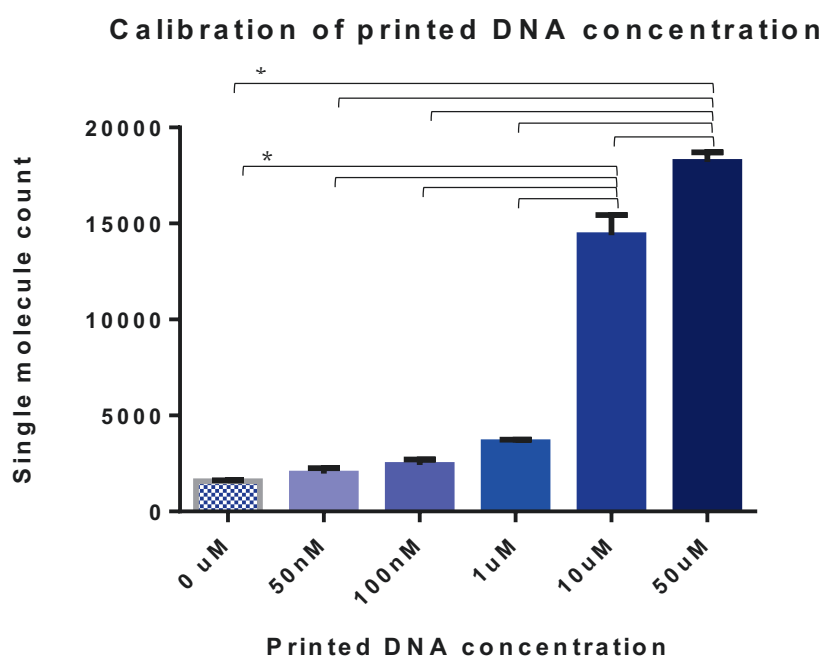


Figure 8.3. Titration of printed Gata-4 DNA oligo concentration. DNA capture agent incubated with 5 $\mu\text{g/ml}$ Gata-4 purified protein for 30 minutes on an open chip assay. $P < 0.01$

DNA was diluted from 50 μM to 50 nM and incubated with purified Gata-4 protein. Minimal binding was observed between 0 to 1 μM of printed DNA, with no significant difference observed (Figure 8.3). Gata-4 protein bound significantly higher to DNA at 10 μM and 50 μM , than at any other concentration. The concentration of 50 μM was significantly higher than 10 μM . Thus DNA was printed at 50 μM in all subsequent experiments.

8.3.2 DNA probe development

Gata-4 binds many enhancer sequences, here I used the distal ANF enhancer which is known to have nanomolar affinity for Gata-4 and yet has a 3 fold lower affinity for Gata-6 (Charron et al., 1999). The ANF oligo was compared with the Gata-4 consensus sequence and a mutated ANF sequence. DNA oligos were incubated with either HEK Gata-4 transfected or HEK untransfected cell lysate. Figure 8.4 shows that whilst there is significantly higher binding to the ANF oligo compared to the other two oligos, there is also significantly higher non-specific binding of the untransfected lysate. There is significant signal with the HEK-Gata-4 lysate with both the Gata-4 consensus sequence and the mutated sequence in comparison to incubation with untransfected lysate.

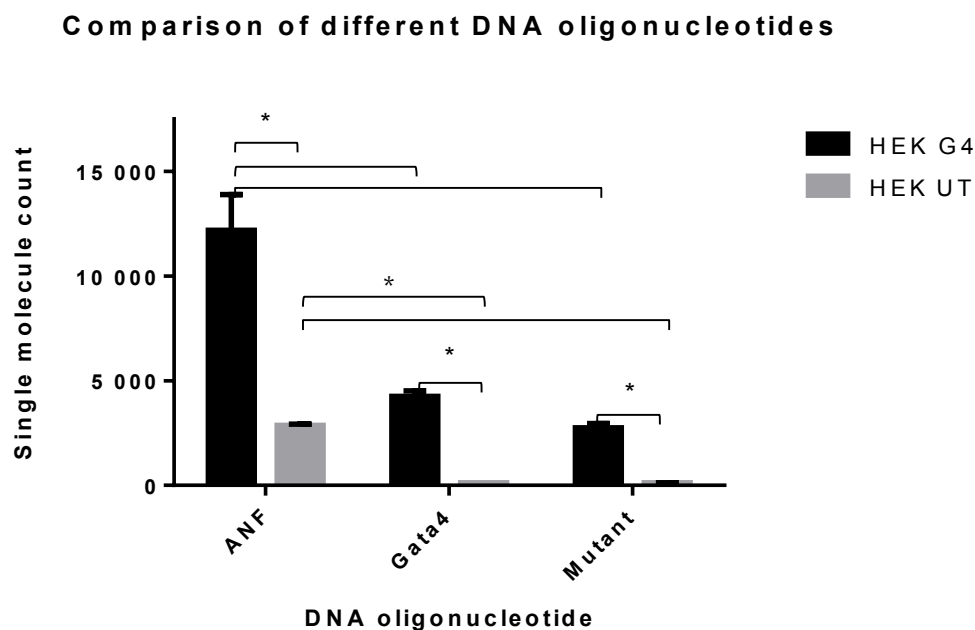


Figure 8.4. Compares Gata-4 binding to three different oligonucleotides, the Gata-4 ANF enhancer (ANF), Gata-4 consensus sequence (Gata-4) and the mutated ANF sequence (Mutant). Oligonucleotides were incubated with either HEK Gata-4 transfected lysate (HEK G4) or HEK untransfected lysate (HEK UT). The ANF oligo bound both the transfected and untransfected lysate to high degrees. Whilst no significant differences were observed between the mutant oligo and the Gata-4 oligo, neither oligo bound protein from the untransfected lysate control. The ensuing experiments were pursued with the Gata-4 consensus oligo.

8.3.3 Gata-4 protein pull down with printed oligonucleotides

Ability of DNA oligos to capture Gata-4 protein was assessed in a microfluidic chip with HEK lysate transfected with Gata-4. Lysate was diluted to either 7.5 pg/nl or 75 pg/nl, which equated to 1 cell per 10 nl assay chamber and 10 cells per chamber respectively. The untransfected lysate acted as an

additional control to the mutant oligo. There was significantly higher binding to the Gata-4 oligo with the HEK Gata-4 transfected lysate than the untransfected control lysate. In addition there was a significant difference between the Gata-4 oligo and mutant oligo at 7.5 ng/nl of lysate. However, this was not the case at the higher lysate concentration of 75 ng/nl.

Binding of Gata-4 to DNA oligos in microfluidic volumes

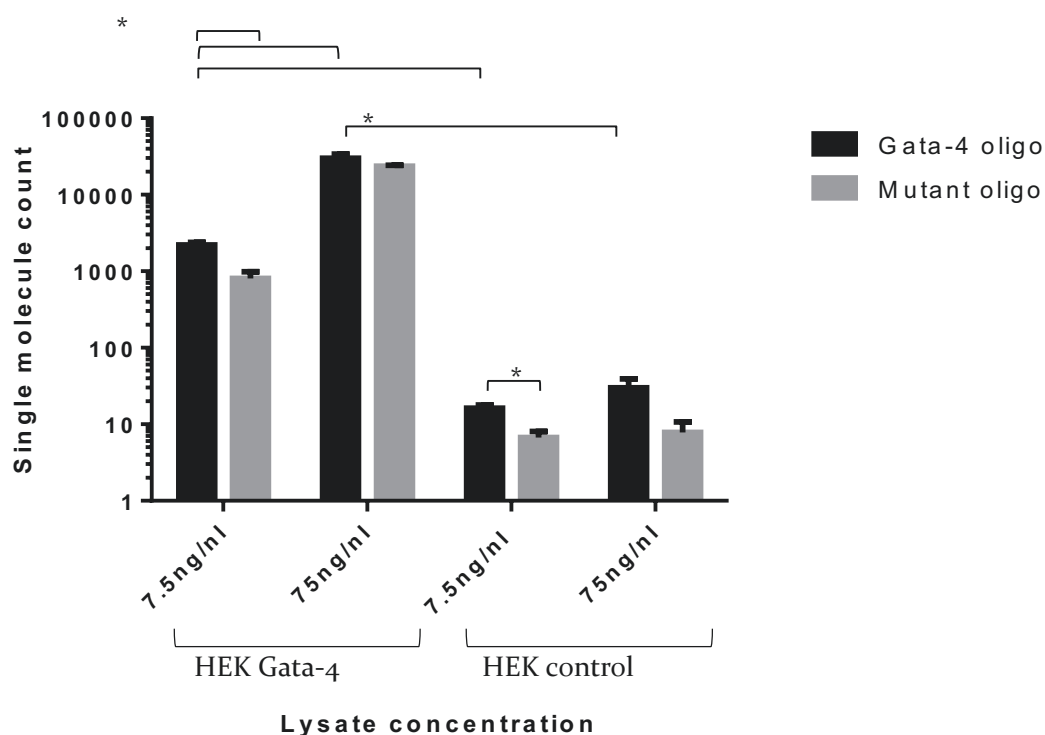


Figure 8.5. Testing the DNA capture sensitivity, incubating with lysate at either 7.5 or 75 ng/nl; this equates to 1 cell and 10 cells per chamber respectively. DNA oligos were incubated with either HEK Gata-4 transfected lysate (HEK Gata-4) or untransfected lysate (HEK control). There was significantly higher binding to Gata-4 oligos incubated with Gata-4 transfected lysate than untransfected lysate control lysate. However at 75 ng/nl no difference was observed between the Gata-4 and mutant oligo when incubated with transfected lysate. A difference in binding between mutant and Gata-4 oligos was observed at 7.5 ng/nl for both lysates. Students t-test, $p < 0.01$

8.4 Discussion

8.4.1 Optimisation of printing density

A range of printing concentrations were tested using purified protein. The research groups that had developed the protein-binding DNA arrays had suggested between 10-20 μM of DNA (Bulyk et al., 2001; Linnell et al., 2004). This data certainly shows that any less than this is ineffective at pulling

down protein. Concentrations higher than 50 μM of DNA were not tested here, as the starting concentration was 100 μM and the printing solution required 1:1 mixing.

8.4.2 DNA oligonucleotides for Gata-4 pull down

Three different oligos were tested for Gata-4 pull down (Figure 8.4); the ANF enhancer, the Gata-4 consensus sequence and the mutated ANF sequence. The ANF oligo had the highest level of Gata-4 binding. Whilst there was a significant difference between the positive and negative control lysate for ANF, the level of binding from the untransfected control was high. This level of binding, at just below 3000 single molecule counts, would be enough to mask any signal from single cell. This signal seen with ANF negative control could be endogenous Gata-4 from the untransfected cell lysate. However, HEK untransfected lysate was negative for Gata-4 in Western blot analysis (Chapter 4).

The Gata-4 and the mutant oligo generated similar signals from the HEK transfected lysate. This was unexpected as the mutant oligo had a mutated sequence that should inhibit Gata-4 binding. However, in contrast to the ANF oligo, the Gata-4 and mutant oligo had much less signal from the untransfected control. This indicates that the Gata-4 and mutant oligo are binding Gata-4 protein specifically, but suggests that the mutant oligo is not a suitable negative control.

The Gata-4 consensus oligo was used for further investigations. Whilst it had reduced signal in comparison to ANF, it had minimal binding from the HEK control lysate, indicating it is more specific than the ANF probe.

8.4.3 Does DNA capture provide single cell sensitivity

At first sight the DNA capture agents appear unsuccessful, with Gata-4 binding to both the positive and negative control oligo (Figure 8.4 and Figure 8.5). This is deceptive as testing this against a negative control cell lysate shows that there is no binding in the absence of Gata-4 protein (Figure 8.4 and Figure 8.5).

Given this data, it is likely that the mutant oligo (Mt) is in fact pulling down Gata-4 protein rather than non-specific protein and that all the specificity is conferred by the Gata-4 antibody. An alternative to this is that the mutant oligo is picking up protein interactions, with the Gata-4 protein binding other proteins in the lysate which subsequently bind to the oligo. However, the level of binding is similar to that for the Gata-4 oligo and further still, experiments with other negative control oligos shows that these also bind Gata-4 (see Appendix, figures F-H).

As noted in Chapter 5, the non-specific binding increases upon addition of lysate. Here the LOD when incubated with negative control lysate was negligible at, 3.7 and 9.2 single molecule counts for lysate concentration of 7.5 ng/nl and 75 ng/nl respectively. The single molecule count for Gata-4

protein at single cell concentration is approximately 1000 molecules (Figure 8.5). This is well above the limit of detection, therefore indicates that the DNA capture has single cell sensitivity.

Whilst the DNA capture shows an impressive limit of detection with very limited antibody-DNA cross talk, the dose-responsiveness of the assay still remains to be tested. This DNA assay was not pursued further due to the major limitation with negative control oligos, which bind Gata-4 protein.

8.4.4 Transcription factors inherently bind nonspecific DNA

The fact that we see Gata-4 binding to supposedly non-specific DNA may be no surprise. Since the 80s much research has gone into how transcription factors find their specific binding sites. There are four main mechanisms proposed (see Figure 8.6); 3D diffusion, 1D sliding along DNA, intersegmental transfer and sliding with intrasegmental 'hopping' (Givaty et al., 2009; Halford et al. 2004; von Hippel et al. 1989).

The action of 3D diffusion, means that the process of a protein finding its binding site is random, fuelled simply by Brownian motion. This passive process places an upper limit on the rate of association of 10^8 Ms^{-1} (Halford et al. 2004). In many instances, protein DNA binding has been experimentally determined to exceed this. So what are the driving forces behind this increased association?

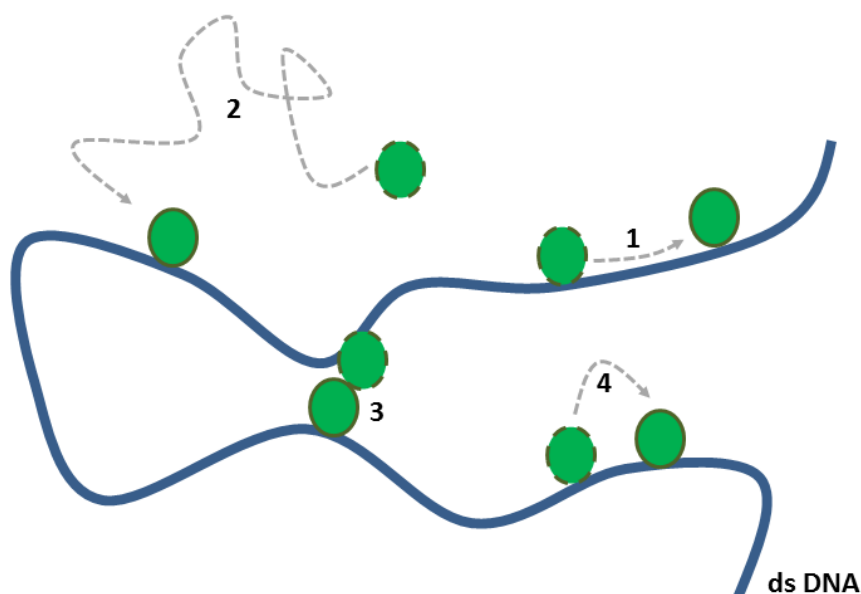


Figure 8.6. Illustration of transcription factors searching for DNA binding sequence. This is achieved either through sliding along the DNA (1); through 3D diffusion within the nucleus (2); intersegmental transfer whereby protein simultaneously binds to two distant DNA sequences (3); or by hopping between various local DNA sites. The degree of non-specific binding may depend on

which mechanism is prominent, although it is currently thought a combination of these mechanisms are used. *Adapted from Givaty & Levy 2009.*

An alternative mechanism is the theory of facilitated diffusion. This is where transcription factors bind DNA non-specifically and search for their specific targets by a process of one dimension diffusion along the DNA (Halford et al. 2004; von Hippel et al. 1989). This is measured to be three orders of magnitude quicker than the passive process of 3D diffusion, (Gao et al. 2009).

A few factors increase the likelihood of the non-specific binding; the first is the inherent stickiness that occurs when two macromolecules collide, the collision not being as elastic as micromolecules (von Hippel et al. 1989). The second is the electrostatic forces between the two molecules. Salt concentration is the final factor with low concentration favouring binding and high concentration, as occurs *in vivo*, impeding binding by masking the local charges on the protein (Halford et al. 2004).

Together, this indicates that transcription factors do indeed bind DNA non-specifically. Reportedly they have a low affinity of anywhere between 10^3 - 10^6 M (Jen-jacobson, 1997; Jolman et al. 2011) and when considering the mechanisms at play searching for target sites, there is probably high rate of association and dissociation.

Further to this, a specific study on Gata binding specificities showed that the members of this family do bind sequences unrelated to the core A/T GATA A/G consensus sequence (Merika et al. 1993). Whilst this study was not specifically on Gata-4, it did show that Gata-1 and 2 are more flexible in terms of their binding sequence, whereas Gata3 was more restricted (Merika et al. 1993). Whether Gata-4 is as liberal as Gata-1 and 2 is still unanswered, and if this is the case it may be difficult to find a suitable negative control.

As mentioned earlier, salt concentration is a crucial factor to protein-DNA binding; too low and the protein remains tightly bound, whereas too high a concentration impedes associations (Givaty et al. 2009). Standard PBS buffers were used in these experiments, giving a salt concentration of 137 mM. According to a computational study by Givaty et al. (2009) this would favour 3D diffusion over the 1D sliding. Perhaps this could be manipulated to further decrease binding, but this might cause knock on effects with antibody-protein binding and other protein-protein interactions.

8.4.5 Further development of negative controls is required

Whilst many studies have used a mutated binding sequences to show specificity (Charron et al., 1999; Hautala et al., 2001; Ip et al., 1997) these sequences do not necessarily fully abrogate binding. In this chapter, a mutated ANF sequence (Charron et al., 1999) was used as a control but the mutation did not appear to completely block Gata-4 binding. This could be due to the ability of transcription factors to bind DNA non-specifically, or it could be that the sequence requires further

mutation. The DNA oligos present an alternative to antibody capture, and are limited by the lack of a negative control in this study. Further development of the controls should be considered, using either a non-related enhancer sequence, random non-specific sequence, or by adjusting the buffer compositions to weaken non-specific interactions. Despite the problem of control oligonucleotides, the DNA capture agents provide binding with high affinity and this could be applied to other proteins when there is limited availability good antibodies.

8.5 Summary

- In the absence of a good antibody pair, DNA capture agents offer an alternative for capture of transcription factors
- Coupled with antibody detection, DNA oligonucleotides are able to pull down Gata-4 protein
- The antibody confers good specificity in this instance, as seen by the negative control lysate
- Negative control DNA oligonucleotides appear to be the limiting factor
- Confers advantage of low non-specific binding, giving a superb limit of detection, as defined by background binding of negative control lysate

Chapter 9

9. Discussion and conclusion

Here, a sensitive assay was developed that can detect Gata-4 protein from a small population of fifteen cells. Many challenges have been faced in developing this immunoassay; these include identifying suitable antibodies, followed by their subsequent immobilisation and labelling for use in this immunoassay. Limitations to the sensitivity of the assay is highly dependent not only the antibody affinity, but the limit of detection as defined by non-specific background and also the physical dimensions of the assay. This chapter discusses the main findings and challenges to single cell proteomics using this assay, and closes with the future work resulting from this study.

9.1 Antibody validation and optimisation

The primary limitation of any antibody sandwich assay is the availability of antibodies. They need to be specific, have good affinity for their targets and each antibody in the pair needs the ability to recognise different epitopes. In this thesis 6 monoclonal antibodies were tested by Western blot for suitability in this assay. Of these, three proved to be specific, antibodies MAB2606 (R&D systems), Sc (Santa Cruz Biotechnology) and BD (BD Biosciences; Figure 4.2), with the rest having intermittent results.

The Gata family has 6 members, of which Gata-5 and Gata-6 are the most similar to Gata-4, sharing 45% and 33% identity respectively (as determined by NCBI BLAST analysis). To ensure specificity to the protein of interest, the antibodies, BD and MAB2606, were tested against HeLA cells transfected with either of the three closest members of the Gata family, Gata-4, Gata-5 or Gata-6. Immunofluorescent staining showed that these antibodies were specific to the protein of interest (figure 4.6).

This together with the clean profile shown by western blotting suggested that these antibodies have the specificity required for the TIRF assay. In addition these antibodies recognised different epitopes, MAB2606 in the N-terminus and the BD antibody in the C-terminus (Figure 4.1) suggesting that they are suitable for use in a sandwich assay format. Whilst the antibodies have shown promising results with regard to specificity, it is worth noting that the success of the antibodies in the microfluidic

immuno assay is also highly dependent on the affinity of the antibodies for their target and must be at least 1 nM for the assay to work (Salehi-Reyhani et al. 2013).

A third antibody, Sc, may also have been a good candidate for the TIRF assay (figure 4.2), however, this was not rigorously explored. Whilst preliminary TIRF experiments with this antibody suggested this was not a suitable (data not shown), this may have been confounded by the long difficulties faced with protein stability, and thus led to this antibody being overlooked. Protein stability in general may be a shortcoming of the assay. Indeed, it is known that printed antibodies remain largely non-functional (Kusnezow et al. 2003a) and conducting experiments at room temperature to aid kinetics (Goodrich et al. 2007) may be detrimental to protein stability. Whilst steps were taken to mitigate the latter factor, such as protease inhibitors and keeping all reagents on ice prior to the experiment, this assay was marred by problems with protein stability. Great effort was put into determining the cause of the problems experienced, this included changing printing buffers, testing fresh reagents, different labelling techniques and different antibody combinations. Ultimately, the issue of protein stability in combination with other factors, such as the physical failure rate experienced with the microfluidic chips, severely hindered development of this assay.

9.1.1 Antibody immobilisation directly affects their activity

Inherent to the success of all immunoassays, is the antibody immobilisation technique, which directly impacts the antibody's functionality through steric hindrance, denaturation and orientation effects. There have been many thorough reviews of surface immobilisation (Seurynck-Servoss et al. 2007; Angenendt et al. 2003; Kusnezow et al. 2003; Vijayendran & Leckband 2001), and whilst these strategies increase the maximal signal and produce high signal to noise ratios, they also increase the background noise, without much improvement in the lower detection limit. In addition, these surface chemistries are not applicable to all antibodies and would need to be optimised specifically for each antibody.

An interesting approach that avoids antibody-specific surface chemistries is the antibody-DNA barcode strategy. By conjugating antibodies to a single stranded DNA oligonucleotide, they can be immobilised by annealing to the printed complementary strand. This therefore maximises antibody functionality as they are lifted off of the glass surface. The Heath group (California Institute of Technology, USA), who developed the technique, subsequently showed single cell sensitivity using an epifluorescent scanner for detection (Shin et al. 2010; Wei et al. 2013; Shi et al. 2012). The caveat

to this method is that the preparation of DNA-antibody conjugates results in antibody loss. This method was therefore not pursued in this thesis.

In this thesis, immobilisation using protein G was trialled due to the minimal loss of antibody and its effectiveness at pulling down antibody. However, the printed protein G was not sufficiently saturated with pull-down antibody, despite the range of concentrations trialled (Figure 5.6). This resulted in unwanted binding of the fluorescently conjugated antibody to the protein G. This background binding was as high as 4600 molecules which is too high for this application, as it would mask any signal from a single cell.

Buffer evaporation is another stressful factor for printed antibodies. This has the effect of rapidly increasing the spot concentration, the ionic strength and changes the pH, all of which can cause protein denaturation. Additives can be included in the printing buffer to help resolve this. Some improvements have been seen with trehalose (Kusnezow et al. 2003), PVA (Wu & Grainger 2006) and glycerol, the latter of which completely inhibits evaporation (MacBeath & Schreiber 2000).

9.1.2 Antibody conjugation

This assay uses detection with fluorescently conjugated antibodies, thereby avoiding genetic modifications to the proteins of interest. Whilst antibody conjugation can reduce antibody activity there are a few different methods available to help circumvent this. The most common method labels the antibody at the primary amines or at the cysteine residues in the disulfide bridge, located in the hinge region. Labelling the cysteine residues is a more targeted approach than labelling the primary amines which can be located anywhere in the antibody, including the antigen binding region. However, cysteine labelling involves breaking the disulfide bridges which can make the antibody less stable.

Labelling via Fab fragments was also tested in this thesis; however, this approach yielded high levels of background binding (Figure 5.4 and 5.5). Thus, labelling via primary amines was used as the conjugation method of choice. Although there was a lack of control over the location of the antibody labelling, the degree of labelling could be controlled, and the antibodies were finally assessed, by measuring the concentration of fluorescence.

9.2 Assay kinetics and calibration

9.2.1 Assay kinetics sufficient for single cell sensitivity

The avidity of the assay was measured as $6 \text{ nM} \pm 2.2 \text{ nM}$ (Figure 6.4). This assay required the use of antibodies with nanomolar affinity, in order to achieve single cell sensitivity. Whilst the affinity for each antibody was not determined, the measured avidity represents the strength of interaction across the three interacting molecules and suggested that the assay had the sensitivity for single cell protein analysis.

The benefit of microfluidics is that they offer nanolitre assay volumes. This means this assay operates in the mass sensing regime. This has the benefit of capturing more protein, which is particularly useful for low protein concentrations. Ideally, we would like to bind all the protein expressed in cells and count the absolute protein expression. However, in the current format, this assay can only capture a maximum of 9% of the expressed protein, a percentage which decreased as the protein concentration increased (Figure 6.5).

Improvement in mass sensing is not greatly increased by better affinity antibodies. This is because with high affinity antibodies most sites are already occupied (Wild 2001). Therefore, one has to increase the number of active antibody molecules printed on the surface. Currently, it is estimated that only 0.14% of the printed antibody is active. Whilst this number might seem low, it could be explained by the steric hindrance, denaturation and the orientation effects that can occur with immobilisation onto a solid surface (Saviranta et al. 2004; Wild 2001). Many studies have assessed surface chemistries to improve the reduced antibody functionality seen with immobilisation (see section 5). However, improvements in activity are generally quite limited and often coincide with a decrease in the amount of antibody deposited due to the antibody loss that occurs during modifications.

Other factors affecting the antibody affinity include: denaturation due to extreme buffer pH, the buffer ionic strength which impacts the binding reaction, and the incubation temperature. The latter is important to the binding stability with low temperatures stabilising binding by reducing the energy of water (Reverberi & Reverberi 2007).

9.2.2 Sensitivity and limit of detection

The limit of detection (LOD) is the signal above the non-specific binding, for which it can be 99.7% confident that the single molecule count is due to Gata-4 binding. The LOD in this assay is

approximately 1810 molecules. A calibration from cell lysate indicates that there are between 3500 and 5000 detectable Gata-4 protein molecules per cell (Figure 6.7 and Table 6.1), which corresponds to approximately two times the LOD. These numbers indicate that the assay should be capable of detecting Gata-4 from single cells.

However, the addition of lysate seems to have a higher non-specific binding than the antibody alone (Figure 6.7, time zero represents antibody alone). At single cell concentrations there is not a significant difference between the test antibody and the control, suggesting that the assay is at its limit with respect to single cell analysis.

This high non-specific binding seems to be specific to lysate, as cells lysed directly on-chip has much lower levels of non-specific binding. This could be due to the lysate preparation conditions. The chemical lysis buffer used in the cell experiments was commercial, and was similar to the non-denaturing lysis buffer used to make the lysate, with both containing 1% Triton X-100. The non-specific background is likely due to ionic and electrostatic interactions between non-specific proteins in the lysate and the antibodies. The overall charge on any given protein is determined by its isoelectric point (pI). The pI of antibodies can range between 5.8 and 8.5 and so at physiological pH, antibodies can be positive, negative or neutral. Therefore they are attracted to negatively charged, positively charged or hydrophobic proteins respectively.

Although blocking with BSA can minimise these non-specific interactions, non-specific binding from both antibody-antibody cross-reactivity and non-specific interactions with lysate were observed in the TIRF assay. Other blocking reagents could be used, such as casein, which was reported to be particularly efficient at blocking hydrophobic interactions (Boenisch et al. 2001). However, it was not tested here. Other passivation methods include using detergent such as tween-20, adjusting the buffers pH or ionic strength, or coating with the copolymer poly ethylene glycol (PEG) (Boenisch et al. 2001). The properties of PEG make it particularly attractive for surface passivation; its amphiphilic quality gives it solubility in water whilst retaining its hydrophobic properties which blocks non-specific protein adsorption (Jonkheijm et al. 2008).

PEG passivation was tested in this thesis. Whilst it produced a high signal to noise ratio, there was a large variation in positive single in comparison to the BSA passivated surface (Figure 5.7 and Figure 5.8). This suggested that the PEG surface is not stable for antibody printing and cannot give reproducible single molecule counts, particularly at the higher lysate concentration. PEG can be combined with biotin-streptavidin to aid antibody immobilisation. However, highly concentrated

antibody spots are required in this assay and printing biotin-conjugated antibodies on a layer of diffuse biotin-streptavidin might not yield the concentration required.

A technical caveat that should be mentioned is the leakiness of PEG microfluidic devices. PEG being a non-stick surface means that often microfluidic devices will fail. In order to circumvent a detrimental device failure rate, and in favour of increasing over all signal output, BSA passivation was chosen. Whilst these surfaces have high backgrounds, this scales with the lysate concentration and is adequately low at low lysate concentrations. In addition, at the theoretical single cell level of 7.5 pg/nl, there was no difference between PEG and BSA passivated surfaces as neither distinguished signal between the Gata-4 antibody and the IgG isotype control. At the opposite end of the scale, the PEG surface seems to saturate at higher concentrations of lysate, suggesting that inadequate amounts of antibody is immobilised on the surface.

A final point to raise on passivation is the inherent stickiness of PDMS (Huang et al. 2005). In the experiments conducted in this thesis, PDMS was passivated by BSA and the issue of non-specific binding was not explored any further. It is possible that protein is depleted by adsorption to the PDMS, and one study has suggested that n-dodecyl-beta-D-maltoside can reduce this (Huang et al. 2005). This would need to be considered in the future in order to improve assay sensitivity.

9.2.3 Sensitivity

Sensitivity is defined by the responsiveness of the assay to the increasing concentration of protein. Ideally the sensitivity of the assay would lie in the linear, most responsive, region. However, detecting such low concentrations means the assay is working in the sublinear region, as defined by the Gata-4 calibration curve in Chapter 6 (Figure 6.4). Despite this, data showing a linear increase in single molecule count with lysate concentrations indicates the assay is sufficiently responsive at low concentrations (Figure 6.7). This implies the assay is sensitive with small numbers of cells and that the overall sensitivity is limited by the LOD as previously discussed.

Nonetheless, pushing the assay sensitivity into the linear range would be beneficial. This could be achieved by increasing the quantity of capture antibody and its activity. As mentioned earlier (Section 5) antibody activity is directly impacted by immobilisation. Various chemistries have been tested to help improve this. However, the antibody preparation steps required in order to achieve this often results in antibody loss. Whilst the activity may be increased, the overall antibody concentration is reduced.

Another solution could be to simply print higher concentration of pull down antibody. A study of microarray production parameters showed that a maximum signal can be achieved with up to 1 mg/ml of antibody printing solution, but higher concentrations adversely affects the signal (Kusnezow et al. 2003). A caveat to this is that excess antibody will be washed away. In the case of the closed chambers, excess antibody will freely float in solution and consequently bind antigen, thereby reducing overall signal. A final solution could be to reduce the chamber volume, which is discussed in section o.

9.2.4 Variable expression of Gata-4 in GFP tagged Gata-4 transfected cells

In order to determine the sensitivity of the assay, cells were transfected with GFP-tagged Gata-4 expression vector, and protein pull down was conducted on cells lysed *in situ*. The results from this study were promising, as a significant difference was observed for one and five cells per chamber. However no difference was observed for two or three cells per chamber (Figure 7.2). One possible explanation for observing a significant difference at one cell and not two or three cells is the variable expression of protein between cells. It is possible that the one cell had higher expression than the others. Another possibility may be that the signal variation between chambers with two and three cells was too large for the statistics to determine a statistical difference.

In general, there is large variation in the single molecule count for the Gata-4 antibody; whereas there is very small variation for the isotype control. This is likely a reflection in the different Gata-4 expression levels in the transfected cells. However, there are only 3 samples per antibody, per cell number; n would have to be increased to see the true variation.

Despite these caveats, this data suggests that this assay is capable of detecting protein at the single cell level. Albeit, this is testing an over expressing cell line and not endogenous expression.

9.2.5 Single cell sensitivity indicated by on-chip cell lysis in microlitre volumes

Cells were serially diluted and lysed directly in large volume chambers. Initially, this experiment was conducted to investigate the usability of the chemical lysis solution. However, the results suggested that the assay had single cell sensitivity, in addition to exceptionally low background binding with the fresh cells (Figure 7.6 and Figure 7.7).

The cells were diluted so that their concentration would be equivalent to that found in the microfluidic chips. A linear correlation was observed between the number of input cells and the single molecule count, and thus demonstrated single cell sensitivity. However, this did not translate to the single cell experiments conducted in the microfluidic chips (Figure 7.3, Figure 7.4 and Figure 7.8).

Contributing to this observation might be the lack of mixing in the large volume chambers. To replicate conditions in the microfluidic chips, the large volume chambers were deliberately not mixed. Since the cells settle to the bottom due to gravity, lysis without mixing would likely create a high concentration of protein local to the printed antibody spot. Increased local protein concentration would therefore favour increased Gata-4-antibody binding (Figure 7.10).

Another noticeable factor in this experiment is the control conducted with the Gata-4 negative cells, SP16. These gave negligible non-specific binding, with a low single molecule count of between 30 and 60 (Figure 7.7). This indicates that on chip these antibodies are truly specific to their target protein.

9.2.6 Challenges with single cell proteomics with cardiac progenitor clones

The cardiac progenitor clone, SP3, which is known to express Gata-4, was tested in the single cell experiment set up. However, testing at both one and two cells per chamber yielded no signal. Two different lysis methods were tested but this made no improvement (Figure 7.3 and Figure 7.4). In the first experiment, a generalised increase in signal was seen with two cells per chamber, above one or no cells per chamber. However, an equivalent signal was observed with the isotype control. Looking at a tilesan of the raw data in Chapter 7 (Figure 7.5), negligible on-spot binding is observed; instead the fluorescence seemed more diffuse. Consequently, a different lysis method was tested.

The concern of using chemical lysis in an antibody assay was addressed in preliminary experiments, where no detrimental impacts were seen on the outcome. However, chemical lysis showed no improvement in the assays ability to detect protein from cells.

9.3 Future work and improvements

9.3.1 Improving the lysis methods on the MAC chip

There are a couple of limitations with the current microfluidic chip design, and it is possible that a few adjustments could increase the sensitivity. One of the challenges which were not resolved here

is that related to the cell lysis. It is not certain that cell lysis, either laser or chemical, was effective enough to release the nuclear localised transcription factor Gata-4. Whilst work in the group has shown that laser lysis is efficient at releasing the transcription factor P53 (Salehi-reyhani et al. 2011), it was not found to be as successful here with Gata-4. In contrast to P53, Gata-4 is constrained to the nucleus and may not be as abundant as P53 is in the cytoplasm. Therefore, it might require high salt buffers in order to elute nuclear proteins and DNase to break down the DNA.

Further to this, nuclear staining of cells in the MAC chip indicated that the nucleus remains intact after chemical lysis. Whilst the nuclei may be chemically perforated, there is no mechanical force to disrupt the membrane physically.

In contrast, laser lysis physically disrupts the cell nucleus. However, without the use of detergent it is possible that the Gata-4 protein remains attached to the DNA. One possible solution could be to use laser lysis in combination with chemical lysis. This process would first lyse cells with a cavitation bubble and then diffuse in the lysis solution.

The diffusion time is a current limitation of using chemical lysis alone. It takes approximately 1 hour for lysis solution to diffuse from the main channel to the cell chambers. During this time cells can start apoptosis, thereby destroying proteins and reducing the amount of Gata-4 available to be detected.

It would be better to introduce the lysis solution so that cells were lysed instantaneously. This could be done by using a two layer microfluidic device. Or by using a “flip chip” design, which has been trialled by members of our group. This design entails having a grid of chambers onto which a diluted cell solution is applied, so that there is approximately 1 cell per chamber. A cell lysis solution would then be applied on top and the grid sealed. This would be much more time efficient with regards to both cell loading and cell lysis, and therefore also reduce cell stress. The two main disadvantages are that firstly, there is no control over how many cells are in each grid. Although, this could be sidestepped by using bright field imaging to count the number of cells in each chamber. The second is the challenge of aligning the chip onto a printed coverslip. The grid would be open topped and in order to retain small volumes the height would be restricted to 50 μM . Therefore, the device would be inherently flimsy and challenging to align.

Normally, when making lysate in bulk, one uses a combination of mechanical force with detergents to release nuclear proteins. In the case of on-chip lysis, no mechanical force is used. It is possible that with the use of on-chip mixing, more nuclear proteins would be released and able to bind the antibodies .

Another advantage of mixing is that the system becomes less restricted by mass transport. Mass transport arises due to depletion of analyte in the reaction zone, and insufficient mixing means binding is gradient driven (Cretich et al. 2014). Making the solution homogeneous would make the reaction kinetically driven (Cretich et al. 2014). However, chamber size helps to overcome the limit of mass transport as small volumes reduce the time to establish equilibrium.

9.3.2 Reducing the cell chamber volume

The volume of a MAC chip chamber is 4.5nl. The diffusion time across the chamber is reported to be 30 minutes (Salehi-reyhani 2011). Hence, the diffusion time is not the limiting factor at this volume. Instead, the assay is limited by the binding kinetics. The volume of 4.5nl is large enough that antibodies are in sampling mode, as defined by Ekins' microspot theory, and is only detecting a small proportion of the proteins present. Reducing this volume would push the proteins towards the bound state, thereby increasing sensitivity (Salehi-Reyhani et al. 2013).

However, there are some technical caveats with reducing the chamber volume even further. The first is that the chambers need to be sufficiently deep to manipulate and load the cells. Another challenge is that of alignment; presently we align the printed slides and microfluidic devices using a hand-operated jig. This can be very cumbersome, even at the current chamber sizes. Reducing the cell chamber sizes would make alignment more difficult and reduce the output of what has already a limited throughput.

9.3.3 Development of alternative capture agents

Gata-4 can bind its DNA enhancers with nanomolar affinity (Charron et al. 1999), and this makes DNA-protein capture an attractive proposition for the TIRF assay in development here. At first sight the DNA capture agents appeared unsuccessful, with Gata-4 binding to both the positive and negative control DNA oligo (Figure 8.4). However, testing this against a negative control cell lysate shows that there is no binding in the absence of Gata-4 protein (Figure 8.5).

Given this data, it is likely that the mutant oligo (Mt) is pulling down Gata-4 protein rather than non-specific protein and that all the specificity is conferred by the Gata-4 antibody. A potential alternative process is that the mutant oligo is picking up protein interactions. Gata-4 protein could be binding other proteins in the lysate and that this complex is then binding to the oligo. However, the level of binding observed is similar to that for the Gata-4 oligo (Figure 8.4 and Figure 8.5), and experiments with other negative control oligos show that it binds these also (Appendix, figure F-H).

An important benefit of the DNA capture system is that the limit of detection for this set up is very low at less than 10 single molecules. This is much lower than with the antibody sandwich assay which as a LOD of 1800. Since the signal for single cell is estimated to be at least two orders of magnitude higher than the LOD, the DNA capture assay might be more suitable for single cell studies.

9.3.4 Transcription factors inherently bind non-specific DNA

Transcription factors have affinity for non-specific DNA sequences, although this might be very low in comparison to specific sequences. This a consequence of the searching mechanisms which include; 3D diffusion, 1D sliding as well and intra- and inter-segmental hopping ' (Halford & Marko 2004; von Hippel & Berg 1989; Givaty & Levy 2009).

Other factors that increase the likelihood of the non-specific binding include macromolecule inertia, electrostatic forces and buffer compositions (von Hippel & Berg 1989; Halford & Marko 2004). Further to this, other Gata members are known to be less stringent in their binding specificities (Merika & Orkin 1993). Whilst in this thesis Gata-4 was observed to bind non-specific DNA sequences, the exact stringency of Gata-4 remains unknown.

Despite the problem of control oligonucleotides, the DNA capture agents provided a promising solution for single cell protein analysis. This is particularly advantageous where suitable antibodies are in short supply. Development of DNA controls could be explored further, starting with the buffer conditions which are known to affect the molecular interactions. Additionally, other non-related DNA sequences should be tested in order to find a suitable negative control.

9.4 Limitations

9.4.1 True concentration of pull down antibody unknown

One particular limitation with this system is that the exact amount of pull down antibody which is immobilised remains unknown. Printing with a micro-contact printer enables antibodies to be printed at high density, maximising the number of capture sites, which is therefore beneficial to this assay. However, the actual volume of printed antibody is not defined.

In measuring the affinity, the assay must be conducted in the ambient analyte regime and the concentration of the pull down antibody must be lower than the expected affinity. According to the manufacturer, the delivery volume is estimated to be around 200 pL. Based on this information, the effective concentration of printed antibody in a 10 μ L volume would be approximately 56 pM. Given that immobilised antibody usually has very low activity of around 1%, the effective antibody

concentration is likely to be much lower than this. The average monoclonal antibody is estimated to have nM affinity for its target protein. Therefore, in this setup the effective pull down antibody concentration will be about 100-fold less than the K_d , and should be suitable for measuring affinity (Goodrich & Kugel 2007).

In the microfluidic chips, the pull down antibody should be at a high enough concentration to push the assay into the mass sensing regime. With the current assumption of printing 200 pL, the maximum concentration of antibody would be 56 nM, although the effective concentration could be much lower due to limited antibody activity.

9.4.2 Antibody affinity is estimated

Although the affinity of the two antibodies was not measured individually, the affinity obtained is a good representation of the assay strength as a whole. Uncertainties lie in the unknown amount of pull down antibody deposited, the antibody functionality, and the efficacy of saturating the antigen with the secondary antibody.

It is possible to attain affinities of the individual antibodies using label free techniques such as chromatography, isothermal titration calorimetry and surface plasmon resonance (Hearty et al. 2012). The limitation of the first two techniques is that they require large quantities of protein and antibodies (milligrams) and would therefore not be suitable here.

Surface plasmon resonance is a powerful, label-free technique which can measure effectively antibody-antigen affinity in a bimolecular system. It uses small volumes and flow to assess the binding at equilibrium whilst avoiding the limitations of mass transport. In addition, measurements in real time mean that it can even provide kinetic data (Hearty et al. 2012). This technique was not pursued in this study due to the lack of access to a Biacore facility and its related cost. In effect, the TIRF system can perform a similar experiment with the only limitation of fluorescently labelling for detection purposes.

The TIRF setup can be arranged so that it measures the affinity in a bimolecular system, either by printing the antigen and detecting with a fluorescently labelled antibody or alternatively by immobilising the antibody and detecting with a fluorescently labelled antigen. The latter method would require fluorescently tagging the antigen which could adversely affect antigen-antibody binding. Additionally, printing Gata-4 directly onto a glass surface may give spurious affinity readings as the binding epitopes might be obscured.

9.4.2.a Activity of purified protein unknown

Ligand activity may also pose a limit; activity of purified protein was not assessed prior to experiments. The actual activity may be much lower than its concentration. Therefore the affinity constant attained provides an upper limit.

As a transcription factor, the activity of Gata-4 is defined by how well it binds to DNA. In theory this can be assessed by using an electromobility shift assay to determine how much of the Gata-4 is unbound under saturating conditions (Goodrich & Kugel 2007). Unfortunately, it is hard to determine this without labelling the protein and it requires high sensitivity that only radio-labelling can provide.

9.4.3 Limited input data

A big draw back with assessing the sensitivity and LOD is the number of data points. Because the microfluidic chips only contained five independent lines, only five different concentrations could be tested at any one time. It would be ideal to have more data points to populate the standard curve.

9.4.4 Estimating cellular protein concentration for calibration

In order to assess the capability of this assay, cell lysates were tested at various concentrations. However, estimating true cellular protein concentration depends on the cell activity and the cell size. The SP3 cells are quite small, having a radius between 4 and 9 μ M. Therefore the volume approximates to between 0.2-3.3 pL. Reported values of protein content per cell volume is approximately 0.2g/ml (Albe et al. 1990), giving a range of 40-660 pg per SP3 cell. A lower estimate of 75pg per cell was used, as it is better to under-estimate the assay capability. In addition, this calibration suggested an estimated number of Gata-4 proteins per cell, although how much this affects the true number is limited by the true value of protein concentration in these cells.

9.5 Applications of TIRFM single molecule counting to cardiac progenitor cells

Single molecule counting using TIRFM offers a multitude of possibilities in cell biology. Ideally, this system would support a panel of antibodies, to observe protein expression across the board in cardiac progenitor cells. This panel would include the core cardiac transcription factors; Tbx5, Mef2c, Nkx2.5, SRF and NFATc, as well as the chromatin regulators such as Baf60c, the class II HDACs and the HAT, p300 as well as cardiac structural genes. However, this relies on the availability of

antibodies that are highly specific and have high affinity for their targets and requires two antibodies that bind to different epitopes with high affinity.

Another issue with multiplexing is the antibody-antibody cross-reactivity. Whilst other studies have reported use of up to 11 proteins at a single time (Shi et al. 2011), in this thesis we have seen antibody cross talk with only two or three antibodies used.

One interesting approach would be to study the protein-protein interactions from single cells. Co-immunoprecipitation is the standard method for detecting protein-protein interactions. Typically, these studies are conducted in bulk and not at the single cell level. Another caveat with this method is that it can only detect strong protein interactions. Using the sensitive system of TIRFM, interactions could be visualised in real time and it is possible that the weaker interactions could be detected. In addition, this would only require one antibody per protein.

Other protein interaction studies use techniques such as FRET. The close proximity of proteins labelled with a fluorophore confers an energy transfer between the donor and acceptor fluorophores. Whilst this implies an interaction it is not entirely reliable and can give no information about the affinity of the interaction. Furthermore, it relies on genetic modification of the proteins of interest which can produce other artefacts.

Another facet would be to investigate the distribution of expression within a population of single cells. In recent years, there has been a lot of interest in the role of stochastic expression in stem cell differentiation. (Enver et al. 2009; Wang et al. 2011; Raj & van Oudenaarden 2009)

Compelling work by Paulkin and Vallier has shown that stem cell differentiation is also highly dependent on the stage of the cell cycle and not only on gene expression (Paulkin & Vallier 2013). They showed that endoderm and mesoderm formation was preferentially formed by cells in early G₁ phase, and neurectoderm by those in late G₁. This highlighted a method by which many stem cell lineage choices could be controlled. With regard to cardiac progenitor cells it could be interesting to look at the cell cycle phase in correspondence to cardiac gene expression.

In order to determine if cardiac gene expression was dependent on cell cycle, TIRFM could be applied to measure the cardiac protein expression. This could have implications in how best to differentiate the SP cells from an arrested state to the cardiac cell fate.

9.6 Conclusion

This thesis shows the steady progression toward single cell proteomics in cardiac progenitors. Whilst single cell sensitivity was not achieved here, the complexity of the setup means there are many avenues for improvement. These include modifications to microfluidic chip design, reducing the chamber volumes, different passivation compounds, more effective cell lysis methods and alternative capture agents.

An increasing number of studies are demonstrating single cell sensitivity (Shin et al. 2010; Shi et al. 2011), showing that the immunoassay is an effective tool for single cell proteomics. However, these have not achieved absolute quantification to date. Further still, many of the proteins in these studies are involved in oncogenic signalling pathways and are highly studied proteins, having a huge repertoire of antibodies available. Ideally, a panel of antibodies would need to be validated in order to conduct insightful studies on the cardiac progenitor cells. However, this is severely limited by the availability of suitable antibodies.

The development of novel protein capture agents could be a viable alternative to antibodies. They offer the benefits of high affinity, although from this study the stringency of the specificity is not yet fully understood. The results here suggest that Gata-4 inherently binds to non-specific DNA and the challenge of developing good quality negative controls still remains. Despite this, discrimination of non-specific binding could be achieved with the combination of DNA capture with a highly specific antibody for detection.

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Appendix

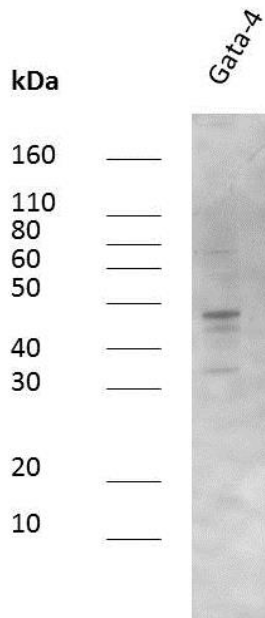


Figure A. Polyacrylamide gel electrophoresis of Gata-4 purified protein under denaturing conditions. The most prominent band is at the height expected for Gata-4 protein. Lower bands are faint and indicate slight protein degradation. There are a few faint bands at a higher height, these are unlikely to be Gata-4 aggregates due to the denaturing conditions used. These thus indicate a small amount of contaminant protein in the Gata-4 protein sample.

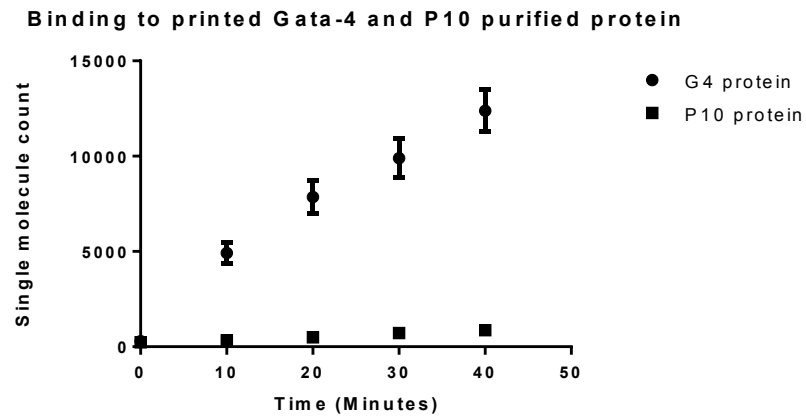


Figure B. Binding of Gata-4 antibody to printed purified protein, Gata-4 and P₁₀, over a period of 40 minutes. Binding to Gata-4 protein increases over time whilst binding to P₁₀ control protein remains low, demonstrating the high specificity the antibody has for its target.

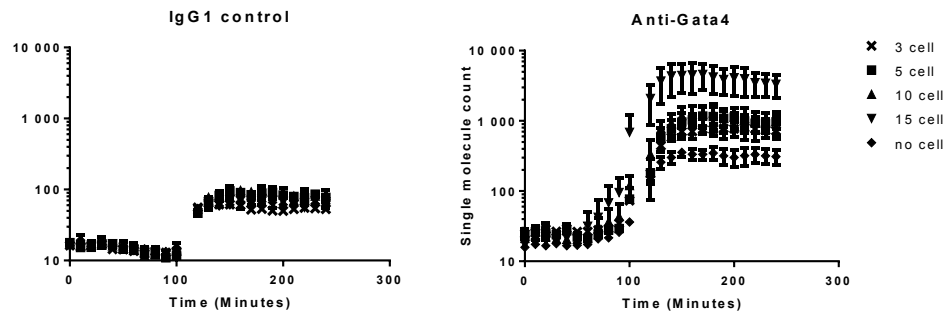


Figure C. is a cell experiment, where chambers are loaded with 3, 5, 10, 15 or 0 cells. Binding of Gata4 protein to either the isotype control (left) of the Gata4 antibody (right) is observed over 4 hours of incubation. At time zero, cells are loaded into chambers and not lysed. Lysis solution is added before the next time point of 10 minutes. Diffusion of the lysis solution from the main channel to the cell chambers appears to take approximately 60-90 minutes. This is indicated by the steady increase in signal from 60 minutes incubation onward.

Comparison of solutions: lysis solution versus BSA

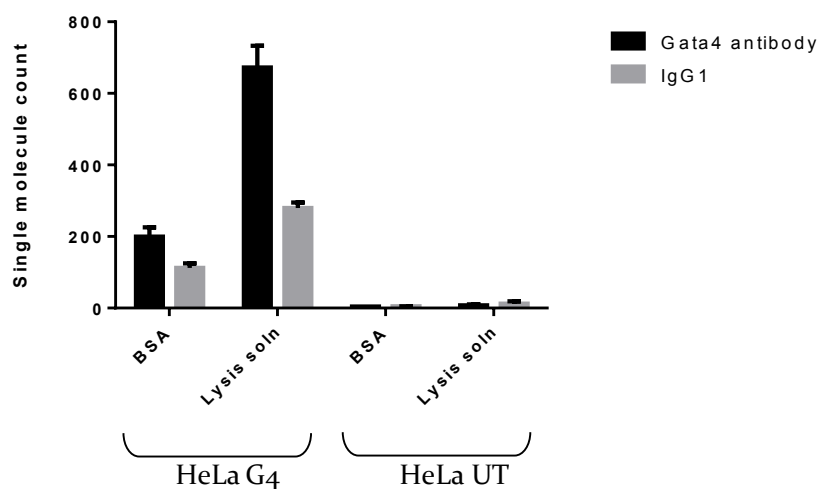


Figure D. As a priori to conducting cell experiments with lysis solution, the assay was tested by comparing lysate in BSA only with Lysate in 1x lysis solution. This was in order to see if lysis solution degraded the assay. The two solutions were compared with both HeLa Gata-4 (G4) transfected lysate with untransfected lysate (UT) as the negative control. No signal was observed with the HeLa untransfected cells. With the transfected lysate a slight improvement was seen with the lysis solution in comparison to BSA solution.

Binding of HepG2 lysate to Gata4 oligo over 60 minutes

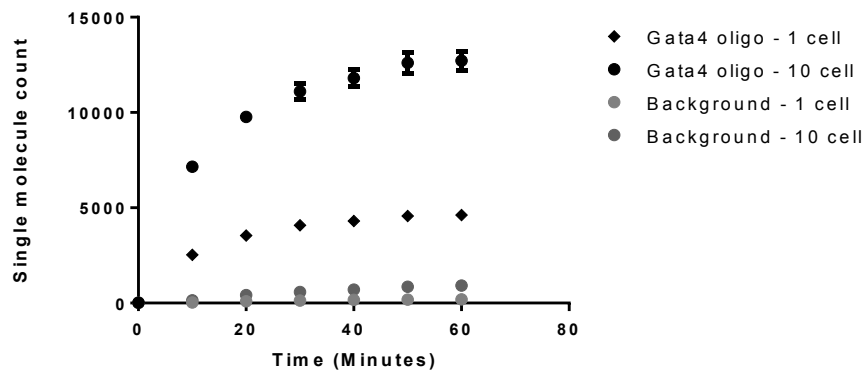


Figure E. DNA-protein binding assay with cell lysate that endogenously expressed Gata4. The one cell concentration gives a similar single molecule reading to that of the SP₃ lysate in the antibody-antibody sandwich assay. This might suggest that the two assays are equally powerful in detecting Gata4 protein from single cells.

**Comparison of different DNA oligonucleotides,
tested with positive and negative control cell lysate**

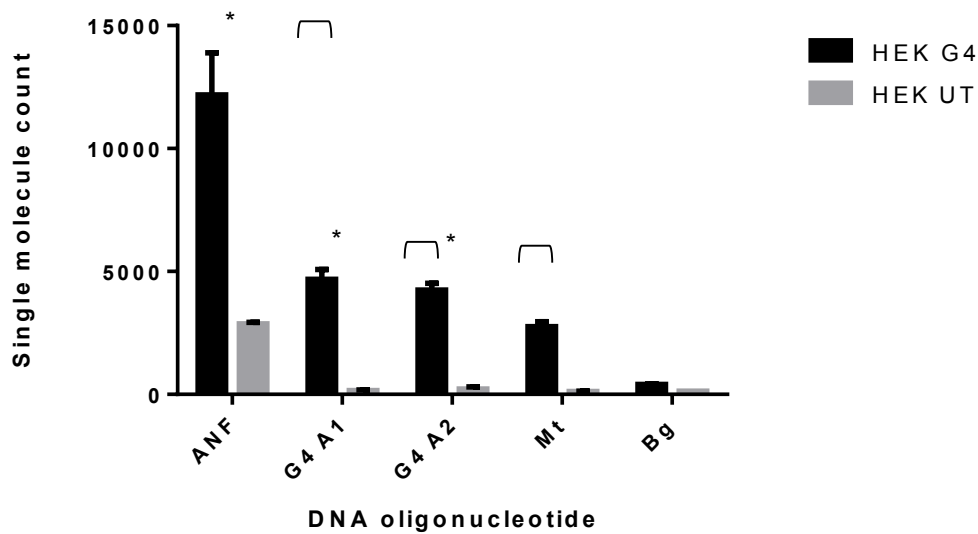


Figure F. Open chip comparison of different DNA oligonucleotides to capture Gata4 protein, incubation with either HEK Gata4 transfected or untransfected cell lysate. ANF represents a putative Gata4 binding sequence found in the promoter for the atrial natriuretic peptide. Gata4 A1 and A2 are similar sequences known to bind Gata4, the only difference being in the spacer sequence. Mt is the mutated ANF sequence and Bg is simply background binding to plain glass. ANF shows exceptional binding of Gata4 however, the binding of the negative cell lysate control is also quite high. Despite there being significant difference between them, this background binding will be too high for single cell sensitivity. G4 A1 and A2 display similar binding and are both significantly higher than the negative lysate control. Although there is no significant difference between Mt with positive and negative cell lysate, this is also not different to the Gata4 oligos A1 and A2 when incubated with HEK lysate expressing Gata4.

Comparison of Gata4 oligo with negative control oligo

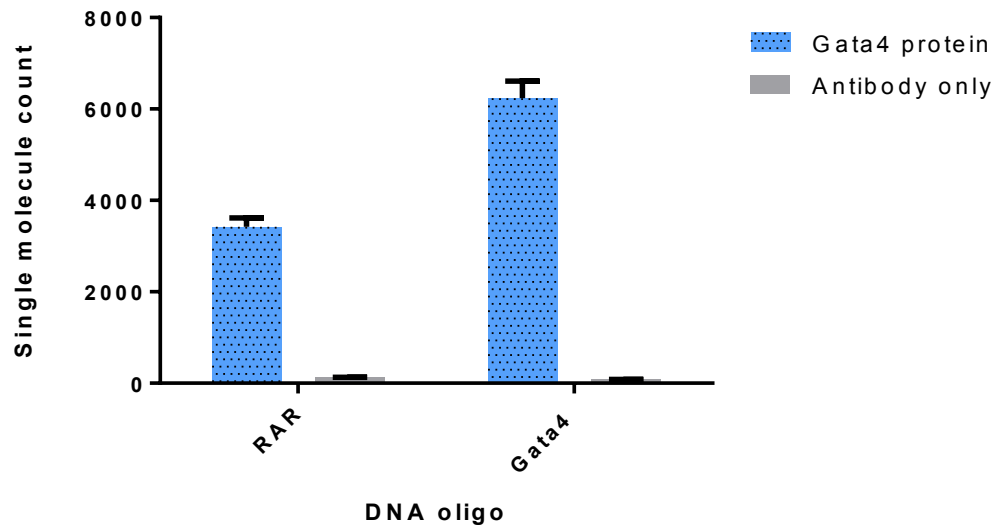


Figure G. Comparison of binding to two different DNA oligos, the Gata4 binding sequence (Gata4) and the retinoic acid receptor response sequence (RAR). Higher binding is observed to the Gata4 oligo but still significant binding to the RAR oligo, which gives a signal well above the LOD 194 and 101 for Gata4 and RAR oligo respectively.

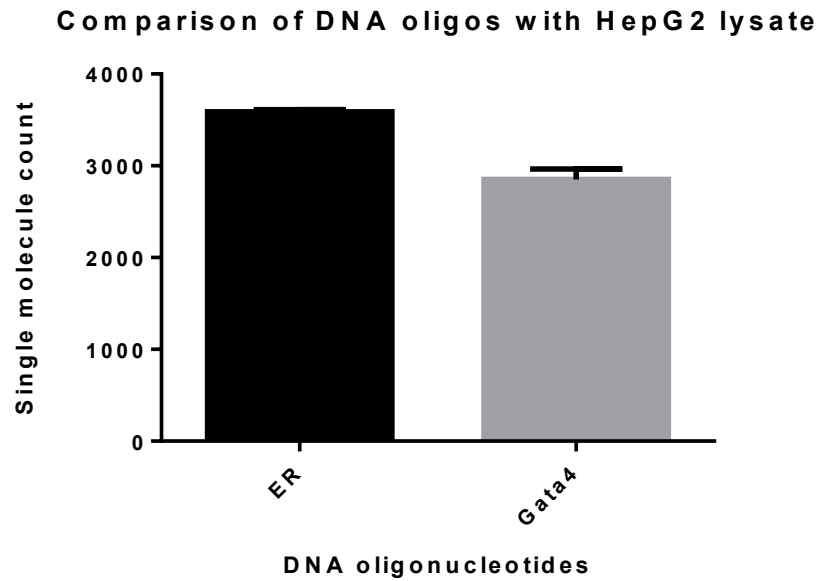


Figure H. Here Gata binding was compared between a Gata4 oligo and that of the oestrogen receptor (ER). The oligos were incubated with Hep G2 lysate that endogenously expresses Gata4, equivalent binding was observed to both oligos suggesting ER is not a suitable negative control

Technology	Detection method	Number of different proteins detected	Operating volume (l)	Sensitivity	Materials	Refs
TIRF	Antibodies- fluorescence	3	5.00E-05	LOD of 600zM, Linear range 800zM-37aM	TNF alph purified protien and clinical saliva sample	Lee et al. 2009
TIRF	Antibodies and YFP +mCherry tagged proteins	6	1.00E-04	10 cells	Cell lysate	Jain et al. 2011
Single cell mas cytometry	Antibodies- conjugated to metal tag	22	5.00E-03	Single cell, semi quantitative	leukaemia cell lines and clinical samples	Bandura et al. 2009
Single cell mas cytometry	Antibodies- conjugated to metal tag	31	1.00E-03	Single cell, relative expression	human haematopoietic cells	Bendall et al. 2011
Fluorescent scanner	Antibodies immobilised with DNA bar code, detection via fluorescent antibodies	12	3.00E-09	Lower limit of 100-1000 (protein dependent) to 10e6 molecules/chamber	Secreted cytokines from single cells	Ma et al. 2011
Fluorescent scanner	Antibodies immobilised with DNA bar code, detection via fluorescent antibodies	10	2.00E-09	Single cell sensitivity, relative expression	Single cancer cells, lysed on chip	Shin et al. 2010, Shi et al. 2011

Epifluorescent microscopy with separation by electrophoresis	Fluorescent antibodies	labelled	1	1.00E-12	Single cell, detect between 2000-60,000 proteins per cell	Single cells transduced with BzAR	Huang et al. 2007
Epi fluorescence	FISH and proteins	YFP tagged	1	NA	as low as 10 proteins per cell	YFP tagged protein library in bacteria	Taniguchi et al. 2010
Epi fluorescence with flow	Fluorescent antibodies		1	1.00E-04	LOD 10-100pg/l	Plasma proteins: cytokines or biomarkers	Todd et al. 2007
MS in microfluidics	No labelling		3 peptides	1.00E-04	2 peptide released from single cell, 2 peptides from 5 cells	Secreted peptides from single neurons	Jo et al. 2007
Mass spectrometry	No labelling		5 peptides	1.00E-04	attomolar sensitivity	Vesicles, 1uM in diameter	Rubakhin et al. 2000
Mass spectrometry with capillary electrophoresis	No labelling		100 chemical molecules	1.00E-04	low nanomolar	Single neurons	Lapainis et al. 2009

Table A: summary single cell techniques and their limits of detection