Coupling Synthetic Antibodies with Mass Cytometry Allows the Development of a Highly Tailored, Multi-Parametric Single Cell Analysis Strategy

by

Amy Hu

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Molecular Genetics
University of Toronto

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University of Toronto
2017

Abstract

Increased appreciation for the inherent heterogeneity in cell populations has prompted a demand for more parameters at the single cell level. Synthetic antibody technology allows the rapid development of monoclonal antibodies with exquisite specificity towards any cellular epitope of interest, surpassing traditional hybridoma techniques. A new technology termed mass cytometry (CyTOF) improves on conventional fluorescence detection by labeling antibodies with metal isotope tags, significantly increasing the parameters that can be distinguished simultaneously. In this thesis, the convergence of synthetic antibody technology with mass cytometry is proposed. A comprehensive set of synthetic antibodies towards the Eph Receptor family is amended and tested for use in CyTOF. After validation, this toolset is applied to the study of cancer heterogeneity in Glioblastoma Multiforme. Subsequent analysis by ViSNE and SPADE unearths a putative brain tumour initiating cell population, illustrating an example of the potential afforded through combining these technologies.
Acknowledgments

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Most importantly, I would like to thank my family for supporting me, encouraging me and inspiring me. This thesis is dedicated to my beloved sister Aleana.
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<th>Description</th>
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<tbody>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BTIC</td>
<td>Brain tumour initiating cell</td>
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<tr>
<td>C</td>
<td>Constant domain</td>
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<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
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<td>CH</td>
<td>Constant heavy chain</td>
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<td>CyTOF</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
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<td>PBS/-</td>
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<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
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<tr>
<td>Sickkids</td>
<td>The Hospital for Sick Children</td>
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<td>SN</td>
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<td>SPADE</td>
<td>Spanning tree progression analysis of density-normalized events</td>
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<td>TIC</td>
<td>Tumour initiating cells</td>
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<td>TRAC</td>
<td>Toronto Recombinant Antibody Centre</td>
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<td>V</td>
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Chapter 1
Introduction

1 Introduction

1.1 Antibodies

A true workhorse of scientific research, antibodies or immunoglobulins (Igs), are indispensible tools in numerous fields due to their sensitivity and specificity for particular epitopes. The most common type of antibodies are Immunoglobulin G (IgG) molecules (Figure 1), which recognize and bind to pathogens in the body, playing an important role in the immune response.

Comprised of two heavy (H) and two light (L) polypeptide chains arranged in a Y-like shape, IgGs have two antigen-binding arms joined together by a tail region known as the Fc. Each arm is composed of a variable (V) domain at the amino terminus (V\(_H\), V\(_L\)) followed by a constant (C) domain (C\(_H\), C\(_L\)). A Fab fragment is a single free antigen-binding arm, prepared via proteolytic or recombinant approaches, which can function as an independent antibody (Karu, Bell & Chin, 1995). However, due to the presence of only one antigen-binding site, Fabs have lower avidity than IgGs.

![Figure 1. The Structure of an IgG1 Antibody.](image)

Figure 1. The Structure of an IgG1 Antibody. IgGs consist of an Fc region with two Fab arms. Hinge region disulfide bonds for reduction (required for labeling with CyTOF metal-isotope tags) are depicted. Adapted from Nelson, Lehninger & Cox (2008).
A natural component of the body’s defense system, IgGs can be generated towards antigens of interest via animal immunization and hybridoma technology. However, polyclonal antisera that is produced via animal immunization often lacks specificity and is prone to batch-to-batch variability. The advent of hybridoma technology allows the production of monoclonal antibodies of invariant specificity with virtually unlimited supply (Siegel, 2002). However, this technique also has several limitations. The process is highly expensive, there is a limit to the number of substances that is immunogenic in mammals and most importantly, it does not allow any modification of the antibodies produced.

1.2 Synthetic Antibody Technology

Advances in the understanding of antibody structure, DNA modification and bacteriophage replication have led to the development of recombinant or synthetic antibody technologies (Karu et al, 1995). Phage display is a core technique behind synthetic antibody development (Figure 2), where genetic engineering of bacteriophages allows the presentation of antibody fragments on the phage surface (Hammers and Stanley, 2014).

The process begins with preparation of a large antibody library. Unlike natural antibody libraries, which are derived from the genes of antibody-producing B cells; synthetic repertoires are engineered from scratch, a process that enables precise control over sequence diversity (Rajan & Sidhu, 2006). Synthetic libraries are created by introducing degenerate oligonucleotides into regions coding the complementarity determining regions (CDRs) of the VH and VL domains of antibodies (Sidhu & Fellouse, 2006). The resulting recombinant DNA is then cloned into an appropriate phagemid vector, where they are expressed in a bacterial host. Infection by bacteriophage results in acquisition of the phagemid, allowing the encoded antibody to be expressed as a coat protein, establishing a direct link between genotype and phenotype (Paschke, 2006). One advantage of phage display is the incredible molecular diversity that can be achieved by a single library, which can reach up to $10^{10}$ variants (Bazan et al, 2012). Thus, repeated rounds of antigen-guided selection or “phage panning,” is required in order to isolate...
antibodies of the desired specificity. In this procedure, the library is screened for phage binding to an immobilized antigen of interest through its surface expressed Fab. Unbound phage is washed off, whereas bound phage is eluted, propagated and re-panned. This cycle is repeated several times, allowing the enrichment of only the most specific binders. After final selections, the desired clones are isolated and their DNA is decoded. Further propagation and expression of the Fab DNA allows inexpensive and renewable production of functional monoclonal antibody. Though recombinant antibodies are usually produced as fragments such as Fabs (Ohara et al, 2006), they can also be reformatted into full-length IgGs as necessary via further genetic modification and expression in mammalian cells (Jostock et al, 2004).

Figure 2. The Phage Display Cycle. DNA manipulation creates a library of Fab variants that are displayed on the surface of phage particles. In an in-vitro selection process known as “phage panning”, phage-Fabs are screened for binding to immobilized antigen with a series of incubation, wash, elution and amplification steps. The phage-Fabs remaining after this process represent the tightest binders. Adapted from Hoogenboom et al (1998).

With protein engineering techniques, a multitude of other antibody formats can be designed, including those that recognize multiple antigens (Holliger et al, 2005). An example of this is the bispecific IgG, which unlike a traditional IgG, has two unique Fab arms with different specificities. Bispecific antibodies can be designed to bind different epitopes on the same antigen or even bind to two completely different antigens. Advantages to this modality may include increased binding efficiency to cellular targets by recognizing more epitopes (Fan et al, 2015). In addition, bispecific molecules hold great promise as therapeutic agents; their dual
specificity allows unique possibilities such as direct linking of immune cells to targets of interest and simultaneous targeting of different signaling pathways (Fan et al, 2015).

Overall, synthetic antibody design bypasses the limitations of natural immune systems in terms of diversity and functionality, allowing the development of extremely specific human antibodies towards virtually any target of interest that may have been otherwise unattainable (Miersch & Sidhu, 2012). Furthermore, they can be easily overexpressed and purified in a range of hosts and can be conveniently modified to alternate formats as necessary.

1.3 Multi-Parameter Single Cell Analysis

A major objective in biomedical research has been the search for biomarkers, substances that are representative of a specific cell type or disease state, such as proteins, metabolites, and post-translational modifications (Kierny, Cunningham & Kay, 2012). Biomarkers have broad utility in research, drug development and disease diagnosis. Cellular proteins are of particular interest as biomarkers, due to their integral role in numerous biological functions. The ability to obtain broad protein profiles on the single cell level would provide valuable information on the phenotypic and functional differences amongst cell types and between normal and diseased cells (Gedye et al, 2014).

Although a wide range of proteins can be measured using gene expression data or mass spectrometry based proteomics, there are certain limitations to these methods. For instance, gene expression does not always correlate with protein expression (Gygi et al, 1999, Chen et al, 2002), potentially rendering such readings unreliable. It is also technically challenging to isolate and measure cell surface proteins in mass spectrometry, limiting the utility of this technique in cell surfaceome characterization. Importantly, both of these techniques result in an averaged measurement of proteins over the entire sample, preventing the study of individual cell differences in heterogeneous samples (Gedye et al, 2014).
Fortunately, antibodies are an immensely useful reagent in the detection of biomarker expression in complex mixtures and tissues. By labeling antibodies with measurable tags, the presence of corresponding antigens on cell populations can be accurately detected. With techniques such as flow cytometry and CyTOF, such measurements can be studied on the single cell level.

For several decades, flow cytometry was unparalleled as a multi-parameter single cell analysis technology. In this technique, a panel of antibodies labeled with fluorophore tags is used to probe a cell sample of interest. Laser interrogation and simultaneous measurement of the fluorescence emission of the sample prepared in a single cell suspension allows the detection of antigens associated with each individual cell. Although flow cytometry is an invaluable tool, overlap of the fluorophore tags impedes the amount of parameters that can be easily detected simultaneously (Cheung & Utz, 2011).

The recent advent of a new technology termed mass cytometry (CyTOF), utilizes the same principle of antibody-based detection, but greatly expands the amount of markers that can be studied at once (Bandura et al, 2009). In CyTOF, fluorophore labels are replaced with highly purified, stable, heavy metal-isotope tags that have minimal overlap. Metals of the lanthanide series are most commonly used due to their virtual nonexistence in biological cells. At the moment, 35 different tags are commercially available for analysis; however, the CyTOF machine possesses the capability to read over 100 probes simultaneously (Cheung & Utz, 2011), which would far exceed current possibilities in flow cytometry. Instead of interrogation by lasers, the varied metal-isotope tags are distinguished via time-of-flight mass spectrometry (Figure 3). In this process, stained cells are nebulized into single cell droplets and introduced into the CyTOF machine where they are vaporized, atomized and ionized by inductively coupled plasma. Overly abundant ions from cellular material are filtered out by a quadrupole, leaving only the heavier ions that consist primarily of the reporter metal isotopes. Ion clouds representing single cells are accelerated through an electric field, where each ion’s time-of-flight is dependent on its mass to charge ratio. The presence of signals associated with metal isotope tags can then be correlated with the presence of its respective antigen on the single cell level (Figure 3).
Figure 3. Mass Cytometry (CyTOF). Antibodies labeled with heavy metal-isotope tags are used to probe a cell sample of interest. Labeled cells are nebulized into single-cell format and vaporized, atomized and ionized by inductively coupled plasma (ICP). A quadrupole filters out ions of lower mass, leaving mostly only the heavy reporter ions, which are analyzed via time-of-flight mass spectrometry. This data is recorded in single cell format and can be analyzed via traditional cytometric methods. Adapted from Bendall and Nolan (2012).

Mass cytometry is a powerful tool for studying biological systems. In a landmark paper, Bendall et al. (2011) effectively utilized highly multi-parametric analysis by CyTOF to visualize hematopoiesis as a continuum. Instead of defined cell subsets, the use of 18 simultaneous markers allowed the visualization of more nuanced states in hematopoietic development that have been otherwise unobservable with fewer parameters.

Although currently most often used in the field immunology, CyTOF is also immensely promising for the study of cancer. Tumour heterogeneity, referring to the existence of subpopulations of cells with varying phenotypes, poses a significant challenge to cancer research and therapeutic development. However, despite being seemingly random and chaotic, the heterogeneous nature of cancer cells implies the existence of a system and perhaps a potential order (Bendall and Nolan, 2012). In order to effectively decipher this network and potentially unveil a cancer’s weakness, CyTOF’s unique ability to perform vastly multiplexed single cell analysis may be of enormous use.
1.4 Mass Cytometry (CyTOF) Data Analysis Strategies

CyTOF data can be examined via traditional flow cytometry data analysis programs, where single cell data is usually visualized by two parameters at a time on a scatterplot with gates manually drawn. However, with the number of parameters used in a typical CyTOF experiment, the number of pairwise combinations can not only quickly become unmanageable but also result in the inability to view meaningful multivariate relationships. Thus, the highly complex, multi-dimensional data generated from CyTOF will often necessitate the use of specially designed software. These may include analytical programs SPADE and/or viSNE.

Spanning-tree progression analysis of density-normalized events (SPADE) is an analytical tool that extracts a hierarchy from multi-parametric cytometric data (Qui et al, 2011). SPADE views data as a high-dimensional point cloud of cells, uses topological strategies to decipher its geometry, and ultimately reduces it to an easily visualized 2D branching tree structure by operating in four computational modules (Figure 4). First, SPADE performs density dependent downsampling, making abundant and rare cell types equally represented. Second, SPADE performs agglomerative clustering to cluster cells into groups (nodes) based on similarities in phenotype. Third, SPADE constructs a minimum spanning tree that connects all nodes with minimum total edge length. Finally, SPADE performs upsampling, by mapping cells from the original data set to the node in the tree in which it is most similar. The resulting SPADE trees allow the properties of each group of cells to be visualized. For example, each cluster of cells in the tree can be coloured based on the median expression intensity of a marker used in the panel, allowing visualization of the intensity changes of that marker through the entire heterogeneous cell population.
**Figure 4. SPADE Analysis.** (i) A simulated two-parameter cytometry data set. (ii) The result of density dependent downsampling of data. (iii) Agglomerative clustering. (iv) Minimum spanning tree connecting clusters. (v) SPADE trees. Nodes represent groups of cells with colour correlated to median marker expression intensity and size correlated to number of cells contained within. Adapted from Qiu et al (2011).

ViSNE, based on the t-Distributed Stochastic Neighbor Embedding (t-SNE) algorithm, is another computational tool that allows the visualization of highly multi-parametric single cell data in a two dimensional format (Amir et al, 2013). Unlike SPADE and other computational tools, which cluster cells into groups resulting in the loss of single-cell resolution, viSNE provides a visual representation of single cell data while preserving high-dimensional relationships (Figure 5). In viSNE, each individual cell is viewed as a point in high dimensional space, where every coordinate represents one of the parameters (i.e., the expression level of a protein marker) used in the experimental panel. The t-SNE algorithm searches for a projection of the points from high dimensional space to low dimensional space, while best preserving pairwise differences (Amir et al, 2013). The resulting map is visualized as a two dimensional scatterplot with cell locations reflecting their proximities in all of the original dimensions, interactively coloured according to the expression intensity of a single marker (Amir et al, 2013).
Figure 5. ViSNE Analysis. (A) A simplified example where viSNE projects a one-dimensional curve embedded in three dimensions (left) onto two dimensions (right). The colour gradient demonstrates that points close in proximity in three dimensions remain in close proximity when condensed into two dimensions. (B) A typical viSNE graph. Each point represents a single cell. The colouring of the map represents the expression intensity of one marker. Axes are in arbitrary units. Adapted from Amir et al (2013).

ViSNE and SPADE both allow unsupervised clustering, eliminating the inherent human bias as well as the requirement for prior knowledge in traditional manual gating strategies. Thus, both approaches are excellent candidates for the study of less understood systems, such as cancer. Whereas viSNE is more qualitative, in allowing the visualization of individual cells, SPADE is more quantitative, in enabling more statistical measurements, such as median node intensity values, to be extracted. However, by displaying the heterogeneity in expression of a variety of markers and deducing the similarities in phenotypes based on all assessed markers simultaneously, both viSNE and SPADE permit the visualization of relationships, subpopulations and developmental progression on any cell population of interest.
1.5 Combining Synthetic Antibody Technology with Mass Cytometry

With developments in recombinant antibody technology and CyTOF, an obvious next step was to attempt to combine these technologies into a synergistic alliance. The ability of synthetic technologies to develop antibodies towards any desired antigen allows incredible flexibility in antibody panel design. The capacity of CyTOF to measure an unprecedented number of markers permits deep profiling on virtually any cell population of interest. Coupled together, a truly powerful workflow could be developed where curated libraries of synthetic antibodies are applied to probe biological populations with single cell resolution via CyTOF, allowing the generation of the most comprehensive understanding of cell phenotypes, function and signaling pathways.

1.6 Thesis Rationale and Objectives

We hypothesize that recombinant antibodies can be readily modified and applied for use in CyTOF. The overall goal of my thesis is to successfully develop this technology and demonstrate its potential application in the study of a biological system.

In the second chapter, a synthetically designed set of antibodies towards a complete subfamily of the receptor tyrosine kinases, the Eph Receptors (EphRs), is amended for use in CyTOF via the addition of heavy metal isotope tags. A series of experiments are described where these recombinant antibodies are validated for use in CyTOF.

In the third chapter, the recombinant metal isotope labeled EphR antibody set is applied to a biological problem: studying tumour heterogeneity and brain tumour initiating cells (BITCs) in Glioblastoma Multiforme (GBM). Using CyTOF, a potential BTIC subpopulation enriched in EphR expression is detected. Furthermore, a therapeutic EphA2/EphA3 bispecific antibody is qualified for CyTOF and utilized to explore targeted subpopulations.
Chapter 2
Technology Development: Coupling of EphR Synthetic Antibodies with Metal Isotope Tags for Use in Mass Cytometry.

2 Technology Development

2.1 Introduction

2.1.1 Eph Receptors

Due to their easy accessibility and importance in numerous biological functions, cell surface proteins are excellent candidates as biomarkers and/or targets for therapeutic intervention. The receptor tyrosine kinases (RTKs), a family of cell-surface receptors, are important mediators of critical cellular processes, such as proliferation, differentiation, and cell survival (Lemmon and Schlessinger, 2010). The Eph receptor (EphR) family represents the largest subset of the RTKs, composed of 14 members in humans (Figure 6). They are further subdivided into 9 EphA receptors and 5 EphB receptors, depending on their extracellular sequence homology and the class of ephrin (efn) ligands that they bind to (Figure 6). Unlike other RTKs, EphRs participate in bidirectional signaling with efns where ligand activation does not typically lead to activation of transcription factors, but instead results in modulation of the cytoskeleton, ultimately affecting processes such as cell migration and adhesion (Pasquale, 2005; Pasquale, 2010). Overall, EphR signaling can result in a wide array of biological effects such as neural development, cell morphogenesis, tissue patterning, angiogenesis and neural plasticity (Lisabeth et al, 2013).

![Figure 6. The EphR Receptor (EphR) Family. Comprised of 9 EphA receptors and 5 EphB receptors that bind to efnA and/or efnB ligands.](image-url)
The EphR family has also been heavily implicated in cancer, where EphR expression is frequently altered compared to the tissue of origin (Genander & Frisen, 2010). Although multiple EphRs are often expressed in many cancer cells (Hafner et al, 2004), both increased and decreased expression levels have been correlated with cancer progression (Genander and Frisen, 2010). Upregulation in several EphRs have been linked to tumour initiation, angiogenesis and metastasis in a variety of cancers (Surawaska, Ma & Salgia, 2004; Chen et al, 2012). One example is EphA2, whose overexpression in a variety of tumor types including ovarian cancer, breast cancer and GBM has been correlated with increased malignancy and lower patient survival (Chen et al, 2012).

Thus, there has been considerable interest in EphRs as therapeutic targets. However, EphR signaling is highly complex. EphRs are capable of signaling independently from their efn ligands, functioning in a kinase-independent manner and engaging in crosstalk with other RTKs involved in cancer (Lisabeth et al, 2013, Chen et al, 2012). Furthermore, different EphRs are known to heterooligomerize into signaling clusters (Janes et al, 2011), while the functional redundancies and differences among the various EphRs have yet to be well elucidated (Pasquale, 2005). Due to the complexities and paradoxes plaguing our understanding of the EphRs, a holistic systems level approach may be required in order to develop a comprehensive understanding of this family.

2.1.2 Comprehensive Set of Synthetically Raised EphR Antibodies

Using phage display, the Sidhu Lab generated the first comprehensive set of antibodies towards all 14 members of the EphR family (see Figure 7). Octet® data performed by Nick Jarvik demonstrates that each antibody is highly specific towards its antigen of interest and possesses minimal cross-reactivity with other members of the EphR family (Figure 7). This toolset grants us the novel ability to profile the entire EphR family on any population of human cells. By simultaneously assessing EphR co-expression patterns with single cell resolution, deeper insight on EphR family dynamics may be revealed. As the EphR family is large, developing a panel with all 14 antibodies for use in flow cytometry would be challenging. On the other hand, CyTOF
would be an ideal, due to its capability for highly multi-parametric single cell analysis. Therefore, we sought out to modify this set of synthetic antibodies for use in CyTOF.

![Table Image]

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**Figure 7. Affinity Measurements of Synthetic EphR Human Fabs to EphR Protein.** Response units at 200 seconds of 100nm EphR Fab measured on the Octet® Platform are displayed. TRAC IDs for original Fab clones are listed on the far left. Most of the EphR Fabs displayed here (with the possible exception of EphA5) are highly specific to their respective EphR antigen, with minimal cross-selectively to other EphRs.

### 2.1.3 Metal Isotope Labeled Antibodies for Use in CyTOF

Although there is currently a selection of metal-isotope antibodies tested for use in CyTOF that are commercially available, these are limited to mostly immunological targets. Fortunately, the availability of Fluidigm’s metal-labeling kit allows direct conjugation of metal isotope tags to IgGs. Although IgGs can be purchased commercially from a variety of vendors, as these are most often produced through traditional means, there may be issues regarding antibody performance, specificity and availability. For instance, a full set of antibodies towards all 14 EphRs has not been yet made commercially available, whereas our lab successfully raised a complete collection synthetically in a relatively fast and inexpensive fashion. Thus, it would be highly advantageous to be able to apply recombinant antibodies for use in CyTOF. To our knowledge, this has never been attempted before. In order to make synthetically produced antibodies amenable for use in CyTOF via the commercially metal-isotope labeling protocol, it must be in an IgG format. This is due to the fact that gentle reducing conditions are used to reduce disulphide bonds, which are significantly more accessible in IgGs than in Fabs. The resulting free thiol released on the hinge region of the IgG (Figure 1) is then allowed to react with the maleimide group on a metal-isotope loaded polymer (Figure 8), creating a stable
carbon-sulfur bond. As a result, the IgG molecule is successfully conjugated to a metal isotope tag and can be used to stain cell populations of interest.

2.1.4 Chapter 2- Rationale and Objectives

The primary goal of this chapter is to test and validate the premise of using synthetic antibodies in CyTOF. First, the possibility of using antibody fragments (Fabs) directly in CyTOF without converting to full-length IgGs is explored. After deciding on converting all Fabs to IgGs, a complete set of EphR IgGs is labeled with metal isotope tags, and used to probe human ovarian cancer cell line A2780cis. To validate this strategy, EphR expression profiles by CyTOF are compared to a similar experiment performed by flow cytometry. Next, SPADE and viSNE analysis is conducted for the visualization of EphR positive populations. Finally, a positive control using antigen coated latex beads to assess antibody cocktail quality is described.

2.2 Materials and Methods

2.2.1 Antibodies Towards EphR Family

A complete set of recombinant antibodies towards all 14 members of the EphR family were created via phage display by Dr. Jarrett Adams of the Sidhu Lab. A comprehensive protocol is available by Lee et al. (2004). Briefly, a naïve synthetic library termed Library F (Persson et al, 2013) was utilized where the Fab DNA was randomized, creating a combinatorial library of phage-Fab with approximately $10^{11}$ unique clones. In-vitro selections were performed on immobilized EphR antigen of human source when available or their highly similar murine homologs (>90% similarity). Although multiple unique clones towards each antigen were derived via this method, the most specific binder with the highest affinity per EphR, as determined by analysis on the Octet® platform (performed by Nick Jarvik), was chosen for the CyTOF panel. Fab DNA from selected phage-Fabs were cloned into appropriate expression vectors for Fab or IgG1 production by Dr. Jarrett Adams and/or staff members of the TRAC.
All EphR IgGs utilized in experiments were produced and provided by the TRAC. A comprehensive protocol is available by Jostock et al. (2014). Briefly, Fab DNA from selected clones was cloned into an IgG1 Expression vector. The IgG DNA was then transiently transfected into 293F cells and purified by protein A.

### 2.2.2 Fab Production

EphR Fab DNA was cloned into an IPTG inducible vector and provided by Dr. Jarrett Adams. Fab DNA was expressed in BL21 cells. Single cell colonies were selected and grown overnight in 3mL 2YT/Carb at 200rpm 37°C. The following day, the overnight starter cultures were used to inoculate 25mL of Magic Media/Carb and were again incubated overnight at 200rpm 37°C. The next day, confluent cultures are pelleted at 7000rpm for 15 minutes. After decanting the SN, the pellet was resuspended in 10mL of PBS and frozen at -80°C for 1 hour. The solution was then thawed and 100µL each of TritonX-100, MgSO4 and 50ug/mL lysozyme was added. Each sample was then neutated for 1 hour at 4°C and spun at 19,000xg for 30 minutes. The resulting supernatant was poured into a fresh tube containing 300µL of Protein A resin and neutated for at least 1hr at 4°C allowing Fabs to bind. Subsequently, a wash step was performed, where the tube was centrifuged at 250xg for 5 minutes, the supernatant decanted, and 40mL of PBS was added. After a final spin step, the visible Protein A beads (with captured Fabs) were collected via aspiration by pipette and added to a Poly-Prep column. Impurities were removed from the beads solution by allowing 10mL of PBS to flow through each column. To collect Fabs, 2.5mL elution buffer (50mM NaH2PO4, 100mM H3PO4 and 140mM NaCl, pH2.5) was added to each column. The eluate was captured in 1 mL of elution buffer (1M Tris, pH 8) and the buffer exchanged into PBS via protein concentration spin tubes.
2.2.3 CyTOF Antibody Conjugation

A panel of 20 different metal isotopes was selected for the CyTOF panel using Fluidigm’s MAXPAR Panel Designer (Fluidigm et al, 2014), based on lowest possible signal overlap. Each unique metal isotope tag was to be linked with a specific IgG, in order to allow detection of its marker of interest during CyTOF analysis. In the case of anti-Fab IgGs for pre-clustering experiments, different metal isotope tags were labeled with aliquots of the same anti-Fab IgG. Subsequent pre-clustering of the resulting complex with Fabs of interest as described in 2.2.5 could then allow measurement of the antigen of interest. The Maxpar Antibody Labeling Kit was used to conjugate IgGs with metal isotope tags for CyTOF analysis. The detailed protocol is published by DVS Sciences Inc (2013).

![Figure 8. CyTOF Antibody Labeling.](image)

(A) Polymers are loaded with metal isotopes. (B) TCEP is used to partially reduce IgGs. (C) The freed thiol of the IgG reacts with the maleimide group of the lanthanide-loaded polymer, creating a metal isotope labeled IgG ready for use in CyTOF. Adapted from DVS Sciences Inc (2013).

2.2.4 Positive Beads Control

Aldehyde/Amidine latex beads were diluted in MES buffer to a 2% concentration (20mg/mL). Antigens of interest were diluted to 1mg/mL in MES buffer. Latex bead solution was then added to each homogenous antigen solution at a 20ug to 1ug ratio. The mixture was incubated with gentle mixing at room temperature overnight. On the following day, the antigen-bead solution was washed 3 times to remove unbound protein by centrifuging at approximately 3000xg for 5minutes, decanting the SN and re-suspending in 10mL PBS. The resulting antigen-bead solution was then diluted to 1mg/mL in stain buffer (1µL estimated to be approximately 200,000 events). Appropriate amounts of each antigen-bead solution were then combined to create a heterogeneous mixture (totaling approximately 1-3 million events) ready for CyTOF staining.
2.2.5 Pre-Clustering Fab: anti-Fab IgGs

For flow cytometry, fluorophore labeled anti-fab IgGs were allowed to bind with Fabs of interest at a 3:1 molar ratio. Estimating that IgGs have a molar mass of 150,000 g/mol and Fabs have a molar mass of 50,000 g/mol, equivalent amounts (grams) of anti-Fab IgGs are mixed with Fabs in order to ensure saturation of the IgG binding arms. The binding reaction was protected from light and incubated for 30 minutes at room temperature. After the incubation procedure, the pre-clustered Fab:IgG molecule can be diluted to the working concentration or used directly. Although unbound Fab molecules are not removed, they should not significantly impact antigen binding due to their lower avidity. For pre-clustering Fabs with anti-Fab IgGs for use in mass cytometry, an identical procedure would be used; however, the anti-Fab IgGs would be labeled with metal isotope tags as described in 2.2.3 instead of fluorophores.
2.2.6 Cell Culture

Commercially available ovarian cancer cell line A2780cis was cultured in RPMI 1640 supplemented with 2mM glutamine, 10% FBS and 1X Antibiotic/Antimycotic at 37°C under sterile conditions. Cells were seeded at approximately $5 \times 10^4$ cells/cm$^2$. Confluent cultures were passaged 1:6 with 0.025% trypsin and treated with 1uM cisplatin every 2-3 passages. When harvesting cells for flow or mass cytometry, cultures are incubated with warm 10mM EDTA in PBS-/- for 5 minutes in order to best preserve surface epitopes. After dissociation, cells are washed with at least 10mL of PBS-/-, and spun down by centrifugation at 350xg for 5 min to remove the buffer. The cell pellet is then resuspended in 1mL PBS-/- and filtered to remove clumps and obtain a single cell suspension.

2.2.7 Cell Surface Staining for Flow Cytometry

Dissociated cells as described in 2.2.6 were resuspended up to 1 million per test in PBS-/- with each test placed in a separate tube or well of a deep well plate. Cells were washed by adding 1mL of PBS per test, spinning at 250xg and decanting the supernatant. Cell pellets were gently vortexed to resuspend cells in residual fluid. 50µL of 1X Live/dead red stain was added to each test, vortexed and incubated at room temperature protected from light for 20 minutes. Cells were then washed with 1mL of stain buffer (1% BSA, 0.1% sodium azide in PBS-/-). In the primary stain step, 50µL of Fab diluted to an appropriate working dilution in stain buffer was added to each well (unique Fab for each test) and incubated at 20min at 4°C in the dark. Cells were washed again followed by addition of an AF488 conjugated anti-Fab secondary antibody. After two more wash steps, cells were fixed with 1.4% paraformaldehyde in PBS for 20min in the dark. Cells were washed a final time and resuspended in 50µL of stain buffer for acquisition. Samples were acquired on a Beckman Coulter Cytoflex machine.
2.2.8 Cell Surface Staining for CyTOF

Dissociated cells as described in 2.2.6 were resuspended up to 10 million/mL in PBS-/- and filtered for single cells. Cell-ID Cisplatin was added to the cell solution at a final concentration of 5mM, gently vortexed and incubated at room temperature for 5 minutes. Immediately after, the staining reaction was quenched by adding five times the volume of stain buffer. The solution was then spun down and the SN removed. For each test, 1-3 million cells were aliquoted in a volume of 50µL stain buffer. 50µL of the surface stain metal-isotope labeled antibody cocktail at 2X concentration is added per test, resulting in a final volume of 100µL. After a gentle vortex, cells are stained for 30 minutes at 4°C and subsequently washed 3X with stain buffer. Cells were resuspended and incubated overnight at 4°C in a Fix/Perm buffer with Cell-ID intercalator added to a final concentration of 125nM. The following day, cells are washed 2X with stain buffer and 1X with DI water. Immediately prior to acquisition, pelleted cells are resuspended in DI H2O, filtered and adjusted to an appropriate concentration. EQ bead standards were added to the sample for normalization (Finck et al, 2013). CyTOF acquisition was performed on CYTOF2 or HELIOS machine using standard settings (Fluidigm, 2014; Fluidigm, 2017) by technologists at The Peter Gilgan Centre for Research and Learning.

2.2.9 CyTOF Analysis

Normalized data (Finck et al, 2013) in FCS format was uploaded to cloud-based platform Cytobank. Data was gated for event length, DNA content, singlets and viability (Figure 9). EQ beads were gated out (Finck et al, 2013). Post-gated live, singlet data was then analyzed via computational software SPADE (Qiu et al, 2011) or ViSNE (Amir et al, 2013). For viSNE, Channels selected for analysis were the markers used to stain the cell samples. Proportional event sampling was selected with desired total events set to 100,000. For SPADE, clustering channels chosen were typically all markers assessed during the experiment. Target number of nodes was generally set to 200, with the downsampled events target at 10%.
Figure 9. CyTOF Gating Strategy for Live Cell Singlets. (A) Event length (signal duration of ion cloud) vs DNA content (iridium nucleic acid intercalation) is examined to select cell populations. (B) Iridium isotopes 191Ir and 193Ir denoted as “DNA1” or “DNA2” detect single nucleated cells from doublets. (C) EQ beads are gated out. (D) A cisplatin-based viability stain is used to discern live cells from dead cells.
2.3 Results

2.3.1 Slight Cross-Reactivity Between Pre-Clustered Fab:Anti-Fab Complexes

Since antibody fragments are much easier to produce synthetically than IgGs, we explored the possibility of using Fabs directly in mass CyTOF without having to clone them into IgGs, while maintaining avidity. As explained in 2.1.3, Fabs do not have easily accessible disulphide bonds that can be reduced to create the free thiols necessary for attachment of the elemental isotope tags. Thus, another molecule that can mediate between both the Fab and the lanthanide-loaded polymer is required. For simplicity, we utilized anti-Fab IgGs, which are specific for any Fab molecule. For each Fab to be used in the panel, an aliquot of anti-Fab IgG is conjugated with a unique metal-isotope label. The labeled anti-Fab IgGs are then incubated with their corresponding Fabs at a 1:3 ratio, allowing binding to occur. The result is a pre-clustered labeled anti-Fab IgG:Fab complex (Figure 10) that is ready for cell staining.

![Figure 10. Pre-Clustering Fabs to Anti-Fab IgGs.](image)

(A) Anti-Fab IgGs are conjugated with a polymer loaded with unique metal isotopes, creating a metal isotope labeled anti-Fab IgG. (B) Synthetically produced Fab molecules specific towards an antigen of interest are allowed to bind with the metal isotope labeled anti-Fab IgG produced in (A) resulting in a pre-clustered complex that can be directly used for cell staining and analysis by CyTOF.
Although the Fabs are incubated with the anti-fab IgG at saturating levels, there remains a concern that not all antigen-binding arms of the IgG will be bound to a fab and/or the complex may dissociate. This presents a potentially problematic scenario, as cross-reactivity may occur between different complexes during the staining process, leading to erroneous results. A flow cytometry experiment was carried out to address this issue. Although CyTOF could have been used, flow cytometry is much more inexpensive to carry out. Fabs towards EphA1 and EphA5 were each pre-clustered with anti-Fab IgGs conjugated with a different fluorophore. APC and AF488 were selected due to their negligible spectra overlap. Preclustered Fabs were then used to stain A2780cis cells, which are characterized by extremely high EphA5 and minimal EphA1 expression. In the ideal scenario, where no switchover of Fabs occurs between fluorophore conjugated IgGs, a mixture of both pre-clustered complexes would not result in any signal shift of the fluorophore channel associated with EphA1. However, it appears that a minor amount of cross-reactivity does occur, as a very slight signal shift was observed in the pre-clustered mixture compared to negative controls (Figure 11). Despite this, it should be noted that a commercial polyclonal anti-fab IgG was used in the pre-clustering experiments. It is possible that the lower affinity antibodies in the polyclonal mixture are responsible for fab cross-reactivity due to higher antigen dissociation rates. Replacement with a highly specific monoclonal anti-Fab IgG, commercial or synthetic, may mitigate these cross-reactivity effects. However, this strategy may still be useable in mass cytometry; due to the lower sensitivity level compared to flow cytometry (Bendall et al, 2012), the minute amounts of cross-reactivity may not actually be detectable by CyTOF. Overall, the pre-clustered method discussed here allows quick use of easily produced synthetic Fabs in mass cytometry; but is not ideal due to potential cross-reactivity effects between antibody-antigen complexes. Due to these results, all synthetic antibodies used in subsequent experiments were converted from Fabs to IgGs.
Figure 11. Minor Cross-Reactivity Observed Between Pre-Clustered Complexes.
Fabs raised toward EphA5 (A5) were incubated with anti-Fab IgGs conjugated with AF488 creating and A5 Fab: AF488 IgG pre-clustered complex. Similarly, Fabs specific toward EphA1 (A1) were incubated with anti-Fab IgGs conjugated with APC, resulting in an A1 Fab: APC IgG pre-clustered molecule. A mixture of both pre-clustered complexes were then used to probe ovarian cancer line A2780cis, which is characterized by extremely high EphA5 and negligible EphA1 expression. (A). APC channel demonstrates slight shifting of the A5-AF488; A1-APC pre-clustered condition compared to negative controls A1-APC and APC only controls, indicating that a minute amount of A5 Fab crossed over to the APC conjugated anti-Fab IgG. However, there is an entire log difference between the cross-reactivity and positive controls A5 APC at both 20nm and 40nm conditions. A1 and A5 Fab simultaneously mixed with both AF488 and APC conjugated IgGs (not preclustered) is also significantly shifted, demonstrating the effectiveness of pre-clustering. (B). AF488 channel shows a significant shift of the A5-AF488; A1-APC pre-clustered and not pre-clustered conditions at the same level of positive controls A5-AF488 at both 20nm and 40nm strengths. This is evident compared to negative controls A1-AF488 and AF488 only.
2.3.2 Mass Cytometry Yields Comparable Results to Flow Cytometry

To test the validity of our metal-labeled isotopes in CyTOF, we utilized our EphR profiler on a cell line that has been well characterized in our laboratory. Using flow cytometry, the expression levels of each EphR were measured on ovarian cancer cell line A2780cis (Figure 12A). This cell line was characterized with extremely high EphA5 levels, a moderate amount of EphA3 and relatively low amounts of all remaining EphRs. The same cell line was then probed with metal-isotope labeled antibodies towards each member of the EphR family via CyTOF (Figure 12B). Similar results were obtained with very strong EphA5 expression, some EphA3 expression and low levels of most other EphRs. Although EphB3 appeared to be relatively higher in the CyTOF results, these differences may be due to inevitable issues such as batch variation and difference in cell line passages. Overall, the high degree of similarity between the two methods supports the established scientific literature (Bendall et al, 2012) that demonstrates mass cytometry yields comparable results to flow cytometry.

![Figure 12. Analysis of EphR Family Expression Congruent Between (A) Flow Cytometry and (B) CyTOF.](image)

(A) A2780cis cells were stained with Fabs towards each of the 14 members of the EphR family and probed with a fluorochrome labeled secondary antibody. Median Fluorescence Intensity plotted demonstrates extremely high EphA5 expression, moderate EphA3 expression and low expression of all other EphRs. (B) A2780cis cells were stained with metal-isotope labeled antibodies towards all 14 EphRs. Median intensity plotted shows extremely high levels of EphA5 and some expression of EphA3 and EphB3.
2.3.3 ViSNE and SPADE Analysis Allow Visualization of EphR Positive Populations

As discussed in 1.4, the high dimensional nature of CyTOF may often necessitate the use of specially designed software in order to fully appreciate the complex relationships between individual cells. The data gathered from CyTOF analysis on ovarian cancer cell line A2780cis described in 2.3.1, was analyzed via both viSNE and SPADE. ViSNE graphs are displayed in Figure 13, where each dot represents a single cell that is coloured according the expression level of its graph’s titular marker. It is important to note that the range of this expression intensity varies between graphs. The location of each cell represents its relationship to other cells in high dimensional space. Thus, cells that share more markers in common would be placed closer in proximity on the resulting viSNE graph than cells that share fewer markers in common. SPADE maps in Figure 14 are similar to viSNE graphs, where each map represents the expression intensity of one marker. However, SPADE groups cells into clusters or “nodes,” which are coloured according the median expression intensity of all cells contained within. The size of the node correlates with the number of cells contained within. Both viSNE and SPADE graphs reflect the expression profile observed in the bar graphs of Figure 12 with high Eph5, moderate Eph3 and negligible EphA1 and EphB6 expression, demonstrating their reliability and comparability.

Unlike the bar graphs in Fig12, with SPADE and viSNE analysis we are able to see the specific subpopulations that exhibit EphR expression. For instance, even though the majority of cells express EphA5, there still remains a small subpopulation (the bottom tail-like region coloured in dark blue) that is completely negative for this marker. In addition, even though EphA1 expression is extremely low, there are still a few scattered cells that exhibit some positive expression of this marker. Such subtleties would be lost in other analysis methods, such as those that use median or averaged measurements of expression intensity. Thus, viSNE and SPADE are useful techniques for visualizing differences in expression among heterogeneous cell subpopulations.
Figure 13. ViSNE Analysis of EphR Expression of Cell Line A2780cis. Ovarian cancer cell line A2780cis was dissociated into single cells and stained with synthetic EphR antibodies labeled with metal-isotope tags. After CyTOF acquisition, the data was gated for live singlets before applying viSNE analysis. ViSNE displays data as individual cells coloured based on the expression intensity of titular marker. Maps for select EphRs displayed. A2780cis exhibits moderate levels of EphA3, extremely high levels of EphA5 and negligible expression of EphA1 and EphB6. Axes are arbitrary.

Figure 14. SPADE Analysis of EphR Expression on Cell Line A2780cis. Ovarian cancer cell line A2780cis was dissociated into single cells and stained with synthetic EphR antibodies labeled with metal-isotope tags. After CyTOF acquisition, the data was gated for live singlets before applying SPADE analysis (EphRs used as seed nodes). SPADE arranges groups of similar cells into nodes coloured based on the expression intensity of one marker. SPADE graphs shown are as follows: (A) EphA1 – low expression, (B) EphA3- moderate expression, (C) EphA5- high expression (D) EphB6- low expression.
2.3.4 Antigen-Coated Latex Beads as a Positive Control

After completing the metal-isotope labeling protocol, it is worthwhile to ensure that the conjugation was successful and that antibody efficacy is maintained. In order to test the quality of the CyTOF conjugated antibodies, I invented a strategy using latex beads homogenously coated with antigens of interest. Equal amounts of beads coated with each antigen were subsequently pooled, creating a heterogeneous mixture containing different EphRs (Figure 15). Acting as a substitute for a cell population, this bead mixture is then stained with the metal-labeled antibody cocktail to be tested. The beads were processed via CyTOF and viSNE as if they were a cell population. If all conditions are met, viSNE analysis should result in distinct islands concentrated in one antigen. This strategy tests the following: if antibodies have been successfully labeled with metal isotopes, if the antibodies are specific for their antigen of interest, and if antibodies cross-react. In our case, EphR antigens were adsorbed onto the surface of polystyrene beads with added aldehyde and amidine groups for colloidal stability. The coated beads were then stained with our metal isotope labeled EphR antibody cocktail. The resulting viSNE graph displays specific regions exclusive to each EphR antigen (Figure 16), demonstrating that our EphR Fabs are specific, labeled with metal isotopes successfully and that CyTOF is not sensitive enough to pick up any potential cross-reactivity effects between pre-clustered complexes. The results also validate the use of ViSNE analysis as a useful tool in clustering cells of similar phenotypes together. Thus, the strategy described here can potentially be utilized as an effective control test for CyTOF experiments.

Figure 15. Development of a Positive Control Using Antigen Coated Latex Beads. EphR proteins are homogenously coated onto aldehyde/amidine latex beads via passive adsorption. Different protein coated beads are then combined to simulate a cell population. The CyTOF antibody cocktail to be tested is then used to stain the coated beads mixture.
Figure 16. ViSNE Analysis of Homogenously Coated Antigen Beads Test. EphR proteins were homogenously adsorbed onto the surface of aldehyde/amidine latex beads and combined into a heterogeneous mixture, which was stained by a comprehensive set of lanthanide labeled IgGs towards the EphR family. CyTOF and subsequent viSNE analysis show mutually exclusive subpopulations with EphR Expression.
2.4 Discussion

With the power of protein engineering, antibody libraries can be developed towards entire families of cell receptors, as demonstrated by our comprehensive EphR set. Currently, mass cytometry (CyTOF) represents the only technology that has the capacity to measure a high variety of proteins simultaneously on a single cell level without loss of resolution due to compensation. Thus, attempting to combine the incredible diversity of synthetic antibody design with the deep profiling capabilities of CyTOF was an obvious next step.

In this chapter, I described a series of experiments validating the use of synthetic antibodies in CyTOF. Since it is considerably easier and faster to produce synthetic antibodies in a Fab format than a traditional IgG, efforts were made to determine whether Fabs could be used in CyTOF without having to be cloned into full-length IgGs. Because Fabs do not have easily accessible disulphide bonds for reduction into free thiols, a pre-clustering strategy with anti-Fab IgGs was attempted. Flow cytometry experiments demonstrated that a very low amount of cross-reactivity over between pre-clustered Fab:anti-Fab IgGs occurred using APC, a very bright fluorophore. Since CyTOF can be up to 20X less sensitive that the brightest flow cytometry fluorophores (Bendall et al, 2012), this level of cross-reactivity would likely be virtually undetectable by CyTOF. However, to err on the side of caution, full-length synthetic IgGs were labeled with metal-isotope tags for use in all subsequent CyTOF experiments.

To perform CyTOF analysis, a set of IgGs towards each of the 14 EphR were labeled with metal-isotope antibodies. This set was then used to profile A2780cis, an ovarian cancer cell that had been well characterized in our lab. The EphR expression profile as measured by CyTOF was comparable to results from flow cytometry experiment, with extremely high EphA5 levels and moderate EphA3 levels. There were some minor discrepancies, such as a low amount of EphA2 from the flow cytometry experiment that was not detected by CyTOF. This might be due to the fact that despite its many advantages, CyTOF is still not as sensitive as flow cytometry (Bendall et al, 2012). Thus, the EphA2 levels may have simply been too low to be registered. Another possibility is that since the experiments were not run in parallel (the CyTOF experiment was
performed much later), differences in number of cell passages and/or time in culture may have affected protein expression levels.

CyTOF experiments using the EphR profiler were performed on ovarian cancer cell line A2780cis. SPADE and viSNE graphs both show extremely high expression of EphA5, moderate expression of EphA3 and negligible expression of the other EphRs displayed, as expected from the flow cytometry data. Whereas viSNE is more qualitative in allowing visualization of individual cells, the node system in SPADE allows for more statistical information, such as median intensity values, to be extracted. However, both analytical tools allow for the detection of subpopulations in complex heterogeneous cell populations with highly congruent results.

Finally, a positive control test using homogenously coated latex beads was developed to test for metal isotope labeled antibody quality. Antigens towards panel antibodies were passively adsorbed onto latex beads and tested via the CyTOF staining protocol. Discrete subpopulations of beads concentrated in one antigen confirmed that the elemental isotope tags were successfully conjugated, and that the synthetic antibodies successfully target their antigens of interest. Although some low amounts of potentially non-specific staining can be observed, these may be improved by optimization of the protocol. For instance, the number of wash steps could be increased. Another potential reason may be due to aggregated beads coated with different antigens. Although the latex beads used in this procedure have high colloidal stability, this control test would benefit greatly by the addition of embedded metal isotopes similar to the beads described in Finck et al. (2013), which would allow singlet beads to be more accurately gated. Future efforts can be directed towards developing specially designed latex beads for this purpose. Overall, the positive beads test provides further evidence that synthetic antibodies can be successfully utilized in CyTOF analysis to a high degree of accuracy.

Overall, the experiments outlined in this chapter demonstrate that our synthetic EphR antibodies labeled with metal-isotope tags can be accurately used to measure EphR levels on cell populations via CyTOF.
Chapter 3

Technology Application: Detection of Brain Tumour Initiating Cells (BTICs) in Glioblastoma (GBM).

3 Technology Application

3.1 Introduction

3.1.1 Glioblastoma Multiforme (GBM)

In recent years, cancer heterogeneity has been increasingly implicated as a major driver of treatment failure in a variety of tumour types. This is particularly evident in Glioblastoma multiforme (GBM), the most common malignant primary brain cancer, and one of the deadliest human cancers overall with a median survival time of under 15 months (Stupp et al, 2005). GBM is characterized by high levels of inter- and intra-tumoural heterogeneity, containing combinations of cells with a myriad of different phenotypes (Bao et al, 2006). This diversity in cell type is believed to be a major reason behind GBM’s dismal prognosis, as the selective pressure from treatment strategies confers an evolutionary advantage to certain cell types, resulting in evolution of the tumour (Burrell et al, 2013). Although an aggressive combination of therapies is usually used to combat GBM, disease recurrence is virtually inevitable (Adamson et al, 2009).

Mounting evidence supports the existence of a tumour initiating cell (TIC) population distinct from the majority of tumour cells (Figure 17), which possess the propensity to escape standard treatments and is able to give rise to new tumours (Chen et al, 2012). Believed to be a rare subpopulation of cells that may possess stem-like properties such as quiescence and self-renewal abilities (Reya et al, 2001; Li and Neaves, 2012), TICs have been identified in a variety of tumour types, including GBM (Singh et al, 2013), and are increasingly implicated as the source of cancer relapse and treatment resistance (Diehn et al, 2009; Meacham & Morrison, 2013). As a result, new treatment strategies have been
increasingly geared towards targeting TICs. Importantly, TICs have been shown to display distinctive markers, which vary by tumour type (Gupta et al, 2009). The ability to identify TICs by such biomarkers can pave the way for the development of efficient targeted therapies, which may ultimately eradicate the cancer.

**Figure 17. BTIC Model.** Conventional chemoradiotherapy eliminate tumour bulk cells while glioma stem-like cells such as GBM BTICs escape, resulting in tumour recurrence. Thus, in order for successful therapeutic intervention, the development of therapies directly targeting BTICs may be required to successfully eliminate the cancer. Adapted from Tabatabai and Weller (2011).
3.1.2 Brain Tumour Initiating Cell (BTIC) Markers in GBM

The first evidence for a brain tumour initiating cell (BTIC) in GBM was detected by Singh and colleagues in 2003. Brain tumour cells were sorted for the presence of surface glycoprotein marker CD133. Cells that were positive for CD133 showed higher rates of self-renewal and proliferation compared to cells that were negative for CD133. In culture, CD133+ cells were able to differentiate into tumour cells that phenotypically resembled the original patient tumour. However, later evidence suggested that some cells negative for CD133 might also possess BTIC-like properties (Beier et al, 2007). Furthermore, not all tumours express CD133 (Joo et al, 2008), necessitating the search for additional BTIC markers. Fortunately, after this initial discovery, many other putative BTIC markers were also identified in GBM, several of which are described below.

GBM cells expressing CD15, a fucose containing trisaccharide expressed on many types of stem cells (Son et al, 2009), exhibited self-renewal, multi-differentiation and the ability to recapitulate the original tumour (Mao et al, 2009). CD15+ CD133- cells isolated from tumour spheres demonstrated properties of BTICs, indicating that CD15 can be used as a marker for tumours that do not express CD133. Similar to CD133, CD15 does not enrich for a BTIC population in every tumour.

ITGA6 has also been identified as a BTIC marker (Lathia et al, 2010). As a member of the integrin family, ITGA6 plays an important role in the crosstalk between the cell and its surrounding stroma (Tabatabai et al, 2010). ITGA6 was found to mark the perivascular niche, co-express with CD133 and enrich for BTICs in GBM (Lathia et al, 2010). Targeting of ITGA6 inhibited self-renewal, proliferation and tumourigenicity. Furthermore, ITGA6 has also been found to be involved in radioresistance of BTICs (Stanzani et al, 2014).
Bmi1 is a member of the polycomb group proteins, which form large multimeric structures involved in the repression of gene expression through modifications in chromatin organization. Bmi-1 is expressed in normal neural stem cells, where it is required for self-renewal, but less important for progenitor proliferation (Molofsky et al, 2003). Bmi-1 has been found to be overexpressed in GBM, and highly enriched in tumour initiating CD133+ stem cells, where it helps maintain BTICs in an undifferentiated state (Abdouh et al, 2009).

Sox2 is a transcription factor involved in sustaining growth and self-renewal in several stem cell types, including neural stem cells. Sox2 expression has been found in several malignant tissues, including gliomas, where it is expressed in a highly variable percentage of cells (Schmitz et al, 2007). Silencing of Sox2 in GBM BTICs resulted in cessation of proliferation and loss of tumourigenicity (Gangemi et al, 2009).

FoxG1, a transcription factor belonging to the forkhead protein family, is an important regulator of neural stem cells. FoxG1 is commonly expressed in GBM, where it activates transcriptional programs involved in BTIC-initiated tumour growth (Verginelli et al, 2013). Knockdown of FoxG1 results in downregulation of neural stem markers, increased differentiation and decreased tumourigenesis. (Verginelli et al, 2013).

Although a multitude of prospective BTIC markers have been suggested by the literature, the markers discussed here represent some of the most compelling in GBM. Although there is evidence for the co-expression of a few of these in BTICs, a simultaneous assessment of all six has not been previously examined.
3.1.3 Eph Receptors (EphRs) in Glioblastoma (GBM)

Recently, two members of the EphR family, EphA2 (Binda et al, 2012) and EphA3 (Day et al, 2013), were identified as BTIC markers in GBM. Both EphA2 and EphA3 were shown to actively maintain GBM cells in a stem-like state by negatively regulating the MAPK pathway (Day, Stringer & Boyd, 2014). These results raise the intriguing question of whether or not these EphRs are co-expressed in the same cells, and if they overlap with expression of other known BTIC markers. Other members of the EphR family, such as EphA7 (Wang et al, 2008), EphA4 (Tu et al, 2012) and EphB2 (Wang et al, 2012), have also been shown to drive GBM progression.

Overall, numerous studies have implicated EphRs in GBM (Day, Stringer and Boyd, 2014); however, all of the literature to date have only examined individual EphRs at a time and have not addressed the potential complex effects of multiple EphR family members working in concert.

3.1.4 Chapter 3 Rationale and Objective

In this chapter, my objective is to demonstrate that our toolset of synthetic EphR antibodies amended for CyTOF can be applied to the study of a biological problem. The entire EphR family was profiled simultaneously on a GBM cell line, along with several putative BTIC markers in a 20 parameter CyTOF experiment. ViSNE and/or SPADE analysis were employed to examine potential BTIC subpopulations and EphR co-expression patterns. Additionally, we applied a therapeutic bispecific antibody developed by the Sidhu Lab to the same GBM sample in an effort to determine potential targeted populations. With these experiments, we aim to illustrate the benefit of using a wide array of synthetic antibodies in CyTOF analysis for examining highly heterogeneous, complex cell samples.
3.2 Methods

3.2.1 Cell Culture

At least 1 million live cells per test were grown for all CyTOF experiments.

For GBM cells, a complete Neurocult medium consisting of 1X NeuroCult Proliferation Supplement, EGF (20ng/mL), bFGF(10ng/mL), Heparin (0.0002%w/v) and 1X Antibiotic/Antimycotic was used. Patient derived recurrent GBM cell line BT241 was provided by the Singh Lab at McMaster University, quick thawed at 37°C and resuspended in complete NeuroCult medium. After a wash in PBS, GBM cells were seeded at approximately 2x10⁴ cells/cm² in complete NeuroCult medium. Cells were incubated at 27°C in a 5% CO2 humidified incubator with the medium changed every few days. When they reached 100-150µm in diameter, Neurospheres were pelleted, resuspended in 0.2mL medium and mechanically dissociate by pipette. This was not considered a passage. For cell staining, neurospheres were chemically dissociated into single cells with 0.2U/mL Liberase™ TM in PBS/- for 5 minutes at 37°C. Only such chemical dissociations were considered as a cell passage.

A2780 cells were cultured in the same manner as A2780cis (described in 2.2.6) but without addition of cisplatin.

3.2.2 CyTOF Antibody Conjugation

Commercial monoclonal antibodies to putative BTIC markers CD133, CD15, ITGA6, Bmi1, FoxG1, Sox2, a late neuronal marker MAP2 and a synthetically developed bispecific antibody were conjugated to metal isotope tags as described in 2.2.3. As the bispecific antibody was in an IgG format, it was directly conjugated and did not require any additional steps.
3.2.3 Intracellular/Intranuclear Staining for CYTOF Acquisition

Metal isotope tagged EphR IgGs created as described in 2.2.3, were combined with IgGs towards BTICs and/or MAP2 and/or bispecific IgG conjugated to metal tags as described in an antibody cocktail. GBM cells were prepared as described in 3.2.1. Surface cell staining was performed as described in 2.2.7. For intracellular and intranuclear staining (required for FoxG1, Bmi1, Sox2), the BD Transcription Buffer set was used. Briefly, samples were fixed and permeabilized for 45 minutes. After permeabilization, all spin steps were increased to 600gXg for 5 minutes. Cells were washed 3X with PermWash and stained with the intracellular/intranuclear stain cocktail for 45 minutes. After this, cells were washed 2X with stain buffer, 1X with DI water, and resuspended to an appropriate concentration with DI water for CyTOF acquisition, which was performed on a HELIOS machine by Sickkids technologists.
3.3 Results

3.3.1 Potential BTIC Subpopulation Identified in GBM Cell Line BT241.

A patient derived recurrent GBM cell line termed BT241 was kindly provided by the Singh Lab (McMaster University). As this is an extremely fast growing line, it was grown entirely in neurosphere format, and did not require adherent conditions for rapid expansion. According to a recent paper by Rahman et al. (2015), neurosphere and adherent conditions are equivalent for growing glioma cell lines and maintaining TICs in culture. Neurospheres were chemically dissociated into single cells and stained with a cocktail of metal isotope tagged antibodies towards the 14 EphRs as well as 6 putative BTIC markers (CD133, CD15, ITGA6, Bmi1, FoxG1, Sox2). A cisplatin-based viability stain and iridium DNA intercalator were added to detect live singlet cells. CyTOF and subsequent viSNE analysis demonstrated a small distinct subpopulation of cells distinct from the bulk that has heightened expression of virtually all BTIC markers assessed (Figure 18a). Unexpectedly, this population also exhibits positive expression of all 14 EphRs, at varied intensities (Figure 18b) Although pre-gated for live cells, low viability stain (Figure 18b) in the BTIC and EphR enriched subpopulation demonstrate that it is a live cell population. In order to lessen any concerns that patterns observed may be artefactual or a product of the CyTOF analysis system, a control cell line was run in parallel to GBM line BT241. Ovarian cancer cell line A2780 was stained with the same antibody cocktail and analyzed with CyTOF (Figure 19). A2780 does not appear to exhibit a similar subpopulation with enriched expression of all markers. Expression is very low for most EphRs and appears to be mostly scattered throughout the population rather than concentrated in specific subsets (Figure 19).
Figure 18. ViSNE Analysis of GBM Cell Line BT241. Patient derived GBM cell line BT241 grown in neurosphere format was dissociated into single cells at P7 and stained with a metal-isotope antibody cocktail (14 EphRs, 6 putative BTIC markers). After CyTOF acquisition, the data was gated for live singlets before applying viSNE analysis. Resulting viSNE graphs for all 14 members of the EphR family and the viability stain are displayed. A distinct subpopulation (directed by arrow) representing 1.3% of the total cells displayed, exhibits positive expression of all putative BTIC markers (A) and all 14 EphRs (B) at varied intensities. Although pre-gated for live cells, low viability stain in this subpopulations confirms its live status. Axes for viSNE graphs are arbitrary. EQ denotes a channel shared with EQ beads, which are gated out.
Figure 19. ViSNE Analysis of Control Cell Line A2780. Ovarian cancer cell line A2780 was dissociated into single cells and stained with a metal-isotope antibody cocktail (14 EphRs, 6 putative BTIC markers) for use as a control. After CyTOF acquisition, the data was gated for live singlets before applying viSNE analysis. Although there are areas with enriched co-expression of several BTIC markers (A), no subpopulation with universal expression is observed. Expression of all EphRs (B) is relatively low, with expression scattered throughout the population. Axes for viSNE graphs are arbitrary. EQ- denotes a channel shared with EQ beads, which are gated out.
3.3.2 BTIC Subpopulation is Low in Differentiation Marker Expression

Due to the unique phenomenon of the subpopulation in Figure 18 being positive for all markers assessed, it was imperative to repeat this experiment and show that the universal co-expression was not simply a result of an experimental artifact. Furthermore, in order to provide additional evidence that the cells in the distinct subpopulation are potentially BTICs, it is necessary to show that these cells are in an undifferentiated state. In an effort to satisfy both issues, I added a differentiation marker, MAP2 to the CyTOF panel. MAP2 is a late neuronal marker that is often expressed in GBM (Gunther et al, 2008; Rieske et al, 2009), and should not be present on a stem-like or tumour-initiating population. A commercially sourced monoclonal MAP2 antibody was labeled with a metal isotope tag for inclusion in the CyTOF panel. BT241 cells were re-grown and dissociated for CyTOF single cell analysis. Cells were stained with antibodies towards putative BTIC markers, EphRs and MAP2 for CyTOF analysis. ViSNE maps demonstrate a subpopulation along the fringes of the cell population (Figure 20) that displays heightened expression of all BTIC markers and EphRs expressed. Unexpectedly, the expression of MAP2 also appears to follow the same pattern. This is concerning, as co-expression of MAP2 with BTIC markers would be inconsistent with the theory that this subset of cells represents an undifferentiated, BTIC population. However, since viSNE displays data as single cells, it is exceedingly difficult to determine the co-expression status of each individual cell. MAP2 expression, in particular, is scattered throughout the region. It is possible that MAP2 is expressed by EphR positive cells, causing its localization along the fringe, and not necessarily because MAP2 is co-expressed with BTIC markers. Thus, I re-analyzed the same data with SPADE, a CyTOF analysis program that clusters similar cells together. MAP2 was selected as a seed node, allowing MAP2 positive cells to be grouped together. As a result, co-expression patterns, or lack thereof, can be much more easily deciphered. In Figure 21, a distinct subpopulation can be observed (circled in red) that is high in expression for all BTIC markers, and most EphRs including EphA2 and EphA3. Importantly, this subpopulation is low in MAP2 expression, as would be expected for a potential BTIC subpopulation.
Patient derived GBM cell line BT241 grown in neurosphere format was dissociated into single cells at P9 and stained with a metal-isotope antibody cocktail (6 putative BTIC markers, 12 EphRs and putative differentiation marker MAP2). After CyTOF acquisition, the data was gated for live singlets before applying viSNE analysis. Resulting viSNE graphs for (A) BTICs, MAP2 and (B) EphRs are displayed. A distinct subpopulation (outlined in red) exhibits positive expression of BTICs and EphRs at varied intensities. Unexpectedly, MAP2 appears to follow a similar pattern. Axes for viSNE graphs are arbitrary. EQ- denotes a channel shared with EQ beads, which are gated out.
Patient derived GBM cell line BT241 grown in neurosphere format was dissociated into single cells at P9 and stained with a metal-isotope antibody cocktail (6 putative BTIC markers, 12 EphRs and putative differentiation marker MAP2). After CyTOF acquisition, the data was gated for live singlets before applying SPADE analysis (BTIC markers and MAP2 were applied as seed nodes). Resulting SPADE graphs for (A) BTICs, MAP2 (B) and EphRs are displayed. A distinct subpopulation (outlined in red) exhibits positive expression of BTICs and EphRs at varied intensities. Importantly, this subpopulation is negative for MAP2 expression.

Figure 21. SPADE Analysis of GBM Cell Line BT241 Restained with MAP2.
In order to more effectively target BTIC subpopulations in GBM for therapeutic purposes, it would be advantageous to target multiple BTIC markers simultaneously. As observed in Figure 18, many EphRs are co-expressed in a possible BTIC subpopulation, including known BTIC markers EphA2 and EphA3. Thus, in an attempt to target this subpopulation, a therapeutic triselective bispecific antibody was designed by Max London of the Sidhu Lab. This recombinant antibody possesses one fab arm that binds to EphA2 and another that recognizes either EphA3 or EphA7. Through antagonizing the action of its targeted EphRs, this bispecific antibody may theoretically be able to hinder GBM tumour growth and/or initiation. Therefore, we sought out to determine whether or not this bispecific antibody is able to target the potential BTIC subpopulation observed in Figure 18. After labeling with a metal-isotope tag, this synthetically designed antibody was added to the panel and used to stain BT241 cells, now at a later passage. The EphR epitopes recognized by the bispecific IgG were designed to be different from the single EphR probes used in the rest of the panel, avoiding any potential competition in binding. CyTOF and viSNE analysis show a subpopulation of cells (indicated by arrow in Figure 22) that displays heightened expression of several BTIC markers (Bmi-1, Sox2, FoxG1 shown). This subpopulation also exhibits positive expression of EphRs (EphA2, EphA3 and EphA7 shown). In addition EphA2, EphA3 and EphA7 have concentrated expression in other adjacent subpopulations (circled in red) on the viSNE map. According to the results displayed in Figure 22, the therapeutic bispecific antibody appears to target the BTIC-enriched subpopulation as well as additional subsets concentrated in EphA2, EphA3 and EphA7. Thus, it seems that the bispecific antibody may broaden therapeutic coverage of GBM cell line BT241 targeting. These results demonstrate that other synthetic antibody formats, such as bispecific IgGs, may also be adapted as probes in CyTOF analysis. The highly multi-parametric nature of CyTOF could allow the simultaneous observation of therapeutic antibodies in addition to numerous subset-identifying markers, making it a highly useful technique in the search for effective therapeutics.
Figure 22. ViSNE Analysis of Subpopulations Targeted by Bispecific Antibody.
Patient derived GBM cell line BT241 grown in neurosphere format was dissociated into single cells at P10 and stained with a metal-isotope antibody cocktail (select EphRs, BTIC markers and therapeutic triselective bispecific IgG). After CyTOF acquisition, the data was gated for live singlets before applying viSNE analysis. Resulting viSNE graphs for EphA2, EphA3 and EphA7 show unique subpopulations with high expression for each (circled in red). ViSNE maps for BTIC markers Bmi1, FoxG1 and Sox2 demonstrate a subpopulation with enriched expression for all (directed by arrow). The therapeutic triselective bispecific antibody selective for EphA2 and EphA3/EphA7 appears to target all subpopulations described (outlined in red). Axes for viSNE graphs are arbitrary. EQ- denotes a channel shared with EQ beads, which are gated out.
3.4 Discussion

Glioblastoma multiforme (GBM) is universally feared as one of the deadliest human cancers. Marked by high levels of tumour heterogeneity, this disease has proven extremely challenging to treat. The discovery of potential brain tumour initiating cell (BTIC) populations in GBM holds great promise for therapeutic targeting; however, further understanding of this rare and elusive population is required. A number of studies have identified a variety of putative BTIC markers, such as CD133, CD15, ITGA6, FoxG1, Sox2, and Bmi1 as well as two members of the EphR family, EphA2 and EphA3, in GBM. Other members of the EphR family have also been implicated in GBM pathogenesis. Since EphR signaling is marked by substantial levels of functional redundancy (Edwards & Mundy, 2008; Zhang & Hughes, 2006) we sought out to study the EphR family as a whole in GBM, in an effort to determine the subsets of EphRs that drive oncogenesis. This was possible due to our novel set of metal-isotope labeled synthetic EphR antibodies validated for use in CyTOF as described in Chapter 2.

Patient Derived GBM cell line BT241 was analyzed with a 20 parameter CyTOF strategy. ViSNE analysis confirms the highly heterogeneous nature of GBM with numerous cells of different phenotypes. In addition, a small subpopulation of cells representing 1.5% of the total number of live cells assayed was discovered, which exhibits enriched expression of all 6 BTIC markers as well as unexpectedly, all 14 EphRs. Since EphA2 and EphA3 are both BTIC markers, it is not unexpected that they would be coexpressed in this subpopulation. However, since EphRs have been shown to have opposing roles in cancer, it was surprising that all EphRs were inclusively expressed in this group of cells. Although there is no evidence in the literature that refutes the possibility of BTICs expressing all EphRs, more evidence was required to show that this was a true occurrence and not simply the result of an experimental artifact. With the addition of differentiation marker MAP2, we were able to negatively stain for the BTIC-positive subpopulation, demonstrating its undifferentiated status. This evidence provides support to the notion that the observed subset of cells may genuinely represent a stem-like subset of cells. A possible explanation for this universal expression is Zipori (2004)’s
A major benefit of synthetic antibody design is the ability to generate novel therapeutic antibodies in a variety of formats. The Sidhu Lab designed a therapeutic triselective bispecific antibody to EphA2 and EphA3/EphA7. Since EphA2 and EphA3 are known BTIC markers, this therapeutic antibody is geared towards targeting BTICs. As EphA7 has also been implicated in the poor prognosis of GBM, the triselective design may prove advantageous in targeting several EphRs at once. By labeling the bispecific IgG to a metal-isotope tag, targeted subpopulations could be determined via CyTOF analysis. The highly multi-parametric capability of CyTOF was what allowed simultaneous assessment of the bispecific antibody in addition to the multiple BTIC and EphR markers necessary to delineate the subpopulation. ViSNE analysis demonstrated that the bispecific antibody successfully targeted subpopulations high in EphR and BTIC marker expression.

Although the same cell line BT241 was used in all GBM CyTOF experiments, the BTIC enriched subpopulations in the resulting viSNE maps exhibited some visually observed differences between experiments. These disparities are likely due to lengthy culturing in-vitro, resulting in evolution of the cell line. Since BT241 was grown solely as neurospheres, which grow at different rates and do not require regular chemical dissociation for continued expansion, passage number is somewhat arbitrary and may not properly reflect time spend in culture. It would be ideal to perform experiments on lower passage GBM cells. However, despite the potential differences accumulated from time spent in in-vitro culture, a BTIC/EphR enriched subpopulation was repeatedly observed upon CyTOF analysis. It is important to note that in some repeats of this experiment on later passages of BT241 (data not shown), a potential BTIC subpopulation
(CD133+/EphA2+/EphA3+) did not always exhibit universal EphR expression, demonstrating that BTIC EphR expression may be dynamic and/or affected by long periods in culture.

In this chapter, synthetic antibody probes amended for use in CyTOF were used to discover a potential BTIC subpopulation in GBM cell line BT241. In addition, a bispecific antibody was also utilized in CyTOF analysis to examine the range of its therapeutic coverage. Overall, the experiments outlined in this chapter indicate the utility of applying a panel of synthetic antibodies in CyTOF analysis to the study of a biological system.
Chapter 4

Conclusion

4 Conclusion

4.1 Summary

The experiments outlined in this thesis describe the first efforts, to our knowledge, that synthetic antibodies have been implemented for use in mass cytometry (CyTOF). As multiple Eph Receptors (EphRs) have been known to be involved in glioma pathogenesis, including the recent discovery of EphA2 and Eph3 as BTIC markers, it was of tremendous interest to perform a complete assessment of all EphRs in Glioblastoma Multiforme (GBM). However, being the largest subset of the receptor tyrosine kinases, the EphR family could not be easily studied all at once. Firstly, an entire set of antibodies towards all 14 members was not commercially available. Secondly, traditional fluorescence-based cytometric methods would not easily be able to measure over 14 markers without considerable signal overlap and lowered resolution. Synthetic antibody technology and CyTOF were able to successfully remedy both issues respectively.

In the second chapter, efforts were made to validate the use of synthetic antibodies in CyTOF. By conjugating with elemental tags, a set of antibodies towards the EphR family was used to profile a cell sample. Resulting expression profiles matched those obtained from an analogous flow cytometry experiment, verifying the accuracy of our CyTOF probes.

In the third chapter, our set of CyTOF-ready synthetic EphR antibodies was applied to the study of GBM. Along with antibodies towards a selection of putative BTIC markers, CyTOF and subsequent SPADE/viSNE analysis confirmed the heterogeneity of the GBM line and even unveiled a minute subpopulation of cells enriched in both BTIC marker and EphR expression. This subpopulation was low in differentiation marker MAP2.
expression, and was targeted by a therapeutic synthetic bispecific antibody. These results suggest that our high parameter CyTOF strategy may have identified an elusive BTIC population in a GBM cell line, and perhaps even a way to therapeutically target it.

The work discussed here demonstrates the advances afforded by combining two powerful technologies, synthetic antibody production and mass cytometry (CyTOF) and provides an example of the wealth of data that can be accumulated and analyzed by such a coalition.
4.2 Future directions

4.2.1 Technology Improvement

Although we have demonstrated that synthetic IgGs can be easily utilized in CyTOF, the ability to directly use Fabs without having to first convert to full IgGs would result in a considerably simpler, faster and more streamlined workflow. Unfortunately, our pre-clustering strategy discussed in 2.2.5 was not ideal due to cross-reactivity effects of between Fabs and lanthanide labeled anti-Fab IgGs. However, other prospective strategies may exist. One possibility would be to exploit the high affinity between streptavidin and biotin and utilize metal-isotope labeled streptavidin as CyTOF probes. An advantage with synthetic antibody technology is the ease in which Fabs can be modified by modifications to its genetic code. By cloning in an Avi-Tag, Fabs of interest could be easily biotinylated. Although streptavidin does not have the disulphide bonds necessary for the addition of the metal-isotopes, a modified streptavidin with the addition of a thiol tail such as utilized by Newell and colleagues (2013) can be applied for this purpose. Despite its many advantages, CyTOF is still not as sensitive as flow cytometry. However, modifying the conjugation protocol to load more metal tags onto a single streptavidin might have the ability to increase CyTOF’s detection sensitivity. Furthermore, by creating fab tetramers through the binding to streptavidin, avidity of the antibody complex would also be improved, thus potentially further increasing sensitivity. Although this method is highly promising, considerable and costly optimization would be required to develop and validate it successfully.
4.2.2 Biology Application

The results gleaned from Chapter 2 have the potential to expand into many other avenues of research. The CyTOF analysis of GBM cell line BT241 suggests the existence of a small subpopulation of cells high in EphR and BTIC marker expression but low in differentiation marker expression. In order to determine whether or not this subset of cells represents a BTIC subpopulation, further studies are required. As previously mentioned, the universality of EphR expression in late passages of BT241 was not always observed in some later experiments (data not shown). Thus, future CyTOF studies can be conducted to examine the potential dynamic remodeling of EphR expression through tumour evolution. However, co-expression of CD133, EphA2 and EphA3 was consistently detected, suggesting the possible importance of these markers in BTIC maintenance. However, to validate any identified subpopulations as BTICs, they would be need to be isolated and tested for tumourigenic potential in vivo by engraftment into NOD-SCID mice. Thus, an immediate future experiment could be to isolate BT241 cells for positive co-expression of CD133, EphA2 and EphA3 via fluorescence activated cell sorting (FACS), and comparing their tumourigenicity to cell populations with negative expression for these markers.

Our CyTOF panel could also be applied to additional GBM cell lines as well other cancer types to observe whether similar patterns in EphR expression can be found. Furthermore, synthetic antibodies developed towards additional proteins, such as other members of the RTK superfamily, could also be assessed to gain a more comprehensive understanding of the cancer proteomics. The Sidhu Lab has since developed additional therapeutic antibodies towards the EphRs that can also be tested via CyTOF for specificity in targeting GBM BTICs. Naturally, pre-clinical testing of these candidates as well as the therapeutic EphA3/EphA3 bispecific antibody tested in 3.3.3 must be performed in order to assess their efficacies in the treatment of human GBM. In addition, phosphoproteomic studies, potentially using a novel CyTOF phosphoflow strategy (Fernandez and Mæecker,
2015), can also been used to elucidate the dynamic signaling events that occur upon EphR modulation and/or treatment with therapeutic antibodies.

Overall, the biological implications of unearthing a BTIC subpopulation in GBM are immense. Since BTICs are theorized to be the main culprit in GBM treatment failure recurrence, the ability to successfully target this elusive cell population may be the key to ultimately eradicating the cancer.

4.3 Concluding Remarks

Coupling synthetic antibody development with complementary techniques such as CyTOF can broaden the scope of applications for both technologies. By combining the power of phage display in designing recombinant antibodies with the highly multi-parametric single cell analysis ability of CyTOF, endless research possibilities may be realized.
References


