

The Choices Cells Make: Single-Cell Techniques for Stem-Cell Research

Stem-cell gene expression and differentiation

The ability for humans and other organisms to develop specialized cells that compose diverse tissues such as muscle, liver, brain, and others, relies on their development from a small pool of undifferentiated cells, called stem cells.¹ Despite their essential role in development and homeostasis, stem-cell populations are typically small in number and are intermixed with differentiated and intermediate cell types within a specific tissue, creating a heterogeneous distribution of cells.² In addition to this spatial heterogeneity within a tissue, there is also a temporal heterogeneity that occurs as stem cells turn on and off gene expression patterns as a function of time and enter the beginning, intermediate, and final stages of differentiation.²

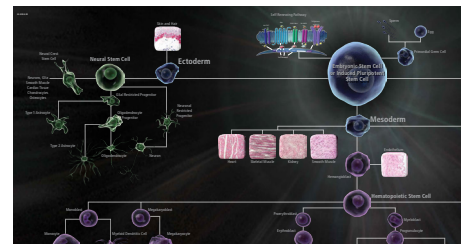


Figure 1: [Markers of self-renewal and differentiation](#)

Studying heterogeneity in stem-cell biology

Understanding the patterns of stem-cell gene expression is one of the central goals for stem-cell biologists and one that holds great therapeutic potential. The ability to understand how a pool of stem cells becomes a liver, heart, or brain cell could allow researchers to grow organs and tissues in a laboratory, eliminating the necessity for organ transplants.²

Studying these patterns has proven difficult as tools for analyzing gene expression in rare cells have been limited



to bulk analysis of spatially and temporally heterogeneous cell populations, like those found in tissues.² Bulk analysis of gene expression averages data across an entire population of cells, making it impossible to deduce what rare stem cells, that can make up less than 0.01% of a cell population, are doing at the transcriptomic or proteomic level.^{2,3}

Over the past decade, single-cell “omics” techniques have advanced, making it possible to collect single-cell data at the genomic, transcriptomic, proteomic, and epigenomic level.² More recently, the use of single-cell sequencing in stem-cell biology has allowed the analysis of multiple types of molecules from a single cell, in parallel.⁴ These techniques have incredible potential for uncovering previously unappreciated cell diversity, tracing cell lineages, identifying new cell subpopulations, and uncovering novel regulatory mechanisms.⁴

Here, the value that single-cell techniques have already provided to advancing the stem-cell field will be reviewed and the potential that multi-omics techniques hold for furthering stem-cell research is discussed.

Single-cell sequencing in stem-cell biology

Over the years, specific subpopulations of stem cells in various stages of differentiation have been identified. These subpopulations express distinct genes that can be used as biomarkers to denote various stages along the path of differentiation. Stem-cell researchers have used these markers, through detection at the RNA or protein level, to analyze and/or isolate these specific cell sub-types in bulk. However, even these “pure” cell types can be heterogeneous due to their existence in discrete microenvironments within a tissue, cell-cycle differences, and stochasticity. In addition, there may be more distinct populations of cells within these subpopulations that are difficult to detect without the use of single-cell sequencing technologies.²

Single-cell RNA-seq and stem cells

In the past decade, single-cell RNA-seq techniques have been used to study many types of cells, including embryonic, developing, and adult tissue-specific stem cells. For instance, several studies with stem cells from different tissues of origin have revealed developmental intermediates between what were thought to be two distinct cell types, allowing researchers to trace stem-cell lineages and reconstruct a more accurate pathway of how stem cells differentiate. In addition, single-cell RNA-seq studies have also revealed the role that cell cycle plays in tissue-resident stem cells making the choice to differentiate into specialized cells or not.²

Single-cell proteomics and stem cells

While single-cell RNA-seq has been powerful for understanding the transcriptome of stem cells, the most direct and primary

effectors of cellular function are proteins.⁵ Understanding how they are expressed, modified, and change the phenotype of a cell is critical to stem-cell research.⁵ Therefore, being able to quantify protein expression in individual cells on a large scale, sensitively and quickly, is important for advancing research.⁵

Flow cytometry

One of the tried-and-true methods for characterizing, analyzing, and isolating single stem cells based on protein markers is flow cytometry. Historically, researchers have discovered characteristic cell-surface biomarkers and intracellular biomarkers to distinguish certain types of stem cells and their differentiation into specialized cell types. Flow cytometry uses antibodies conjugated to fluorophores for detecting cells that are expressing these specific, distinguishing biomarkers.

This method is high-throughput and can be used to isolate live cells through fluorescence-activated cell sorting, or FACS™. Based on the detection of a fluorescent signal, cells can be deposited in bulk or as single cells, making this method amenable to the use of downstream single-cell sequencing techniques.

Use of FACS™ in stem-cell research is widespread and with good reason: this method is preferable for the isolation of very low frequency cell types as a large number of cells can be sorted in a relatively short period of time. With the discovery of new subpopulations and stem-cell markers, the need for high-parameter analyses has become critical for stem cell research. Addressing this need, flow cytometry has evolved in recent years allowing researchers to utilize and analyze as many as 30 parameters, simultaneously.^{5,6}

Mass cytometry

Another method of protein profiling, called mass cytometry, has been used to analyze stem cells and identify unique cell subtypes.⁷ This method has been used to analyze muscle stem cells and hematopoietic stem cells, which give rise to all the cells in the blood.^{3,7,8} The principle of this method is similar to flow cytometry, though instead of using fluorescent dye-conjugated antibodies, they are conjugated to heavy metal ions, allowing antibody binding to be detected with the precision of mass spectrometry.⁸

One of the major benefits to this method is that it can potentially allow the multiplexing of over 100 antibodies.^{5,8} In practice, however, between 30 and 45 parameters can be measured in a single mass-cytometry experiment without having to do any additional data correction.⁸ Single cells that are studied using mass cytometry are atomized and ionized, making the recovery of live cells for downstream analysis, like single-cell RNA-seq, an impossibility.⁸

The power of single-cell multi-omics

Combining multiple single-cell “omics” techniques holds a lot of potential for researchers in the stem-cell field. The ability to simultaneously analyze genomics, epigenomics, transcriptomics, and/or proteomics, in a single cell, with one workflow could provide an incredible amount of data to uncover novel biological processes, gene-regulatory mechanisms, and cell subpopulations. Ultimately, this could lead to the ability to gain a deep understanding into the link between cell genotype and phenotype and to track individual cell lineages with exquisite resolution, a major goal of developmental and stem-cell biology.^{2,4}

While integration of all of these “omics” workflows may seem decades away, methods for simultaneous coupling of high-parameter protein profiling with unbiased, high-throughput sequencing methods like single-cell RNA-seq, have recently been developed. These methods overcome many of the limitations to high-parameter protein profiling discussed above by using antibodies conjugated to DNA oligonucleotides.⁵

Protein profiling of cell-surface markers using antibody-DNA conjugates

Using antibody-DNA conjugates, Adam Abate’s group at the University of California, San Francisco, developed a method called Abseq, that could theoretically allow for the profiling of greater than 100 proteins.⁵ The use of antibody-DNA conjugates allows researchers to use a unique “barcoding” sequence for each unique antibody.⁵ Thus, the use of a 10-nucleotide sequence can create greater than 1,000,000 unique sequences, allowing for extremely high-parameter protein profiling, on a scale that is far greater than the number of total human proteins.⁵

The use of DNA tags can also help researchers detect low-abundance surface proteins which may be difficult to detect using other techniques. DNA tags can be amplified by PCR and thus, hypothetically, it would be possible to detect a single copy of a distinct surface protein.

In a proof-of-principle experiment, Abate’s group showed that this method can accurately profile immortalized T cells and B cells in a single-cell sample using antibody-DNA conjugates that bind to two cell-surface biomarkers.⁵ While not explicitly tested or validated, the use of high-throughput sequencing as a method for quantifying protein expression gives this new method the potential to integrate with high-throughput single-cell RNA-seq workflows.⁵

CITE-seq and REAP-seq

Months following Abate’s publication, two methodologies for integrating both mRNA and protein expression profiling into a single workflow were published.^{9,10} The first, a method called CITE-seq, was developed by laboratories at the New York Genome Center and NYU Center for Genomics and Systems

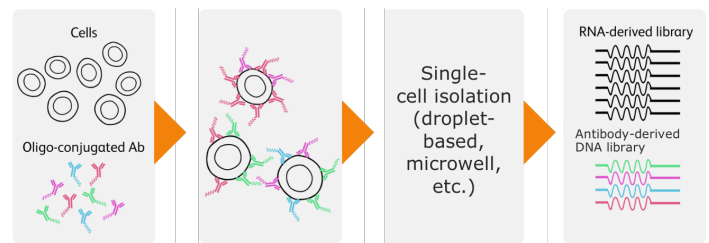


Figure 2: General workflow for single-cell profiling of both transcripts and proteins using antibody-DNA conjugates. Cell sample is stained with antibody-DNA oligonucleotide conjugates. After cells are stained using protocols similar to those used in flow cytometry, single-cell isolation, cDNA library preparation (labeled RNA-derived library), and extension of antibody-derived DNA oligonucleotides can be carried out with a previously validated single-cell RNA-seq workflows.

Biology.⁹ The DNA oligonucleotides conjugated to antibodies contain a unique sequence for each distinct antibody and a common sequence that can be used for amplification by PCR (often called a PCR handle).⁹ After cells are labeled with antibody and single cells are isolated, cDNA library preparation and extension of antibody-derived DNA can be done simultaneously using validated single-cell RNA-seq workflows.^{9,11,12}

CITE-seq was able to identify immune cell subpopulations and quantify differences in cell surface protein expression.⁹ Furthermore, using 13 monoclonal antibodies against cell surface antigens typically used for cell profiling, CITE-seq was able to accurately differentiate specific cell subpopulations with greater resolution than single-cell RNA-seq analysis alone.⁸

REAP-seq uses a nearly identical approach to that of CITE-seq, though REAP-seq allowed for the quantification of 82 distinct cell surface proteins and >20,000 genes in a single workflow.¹⁰ This demonstrated the power of REAP-seq to measure high number of parameters in a way that can easily integrate with single-cell RNA-seq workflows.

The major advances of CITE-seq and REAP-seq for stem cell biology are their capability to measure a high number of parameters that can integrate with single-cell RNA-seq protocols. Additionally, these methods enable simultaneous and high-parameter examination of RNA and protein in a single workflow.

Synergies of FACS™ and single-cell multi-omics

The successful profiling of mRNA and protein in a single workflow holds extreme promise for answering many unanswered questions in stem-cell biology and could help researchers understand new mechanisms of regulating gene expression and identify subtle, yet important, steps in stem cell differentiation.⁵ In combination with FACS™, which can be used to enrich for low-abundance stem cell populations before single-cell sequencing, these multi-omics techniques can facilitate the identification of rare cell types and hold the

potential to gain insight into novel gene regulatory mechanisms involved in stem-cell differentiation. Conversely, the discovery of new biomarkers that come with single cell multi-omic analysis shows promise in streamlining flow cytometry panel design. Future breakthroughs in multi-omics may allow additional characterization of the genome and epigenome in single stem cells.⁵ With the current multi-omics techniques in use, and for those in development, tracking the flow of information in individual stem cells has direct applicability to identifying the role that stem cells play in the development of tumors, in discovering how embryonic and tissue-resident stem cells can be used therapeutically for regenerative medicine, and in figuring out how differentiated cells can be reprogrammed into pluripotent states. As additional methods for measuring mRNA and protein expression are developed, the barriers for implementation into basic research will be lowered and these techniques may become more widely used to understand complex, heterogeneous biological systems.

BD's continuing commitment to cutting-edge multi-omics technology

BD's commitment to making innovative and transformative research tools available to the broader, global research community includes a portfolio of trusted antibodies and demonstrated success in providing platforms for single-cell analysis. One of these innovations, the newly available BD™ AbSeq assay, utilizes antibody-oligonucleotide conjugates. When used in combination with the BD™ Rhapsody single-cell analysis platform provides researchers with an accessible, off-the-shelf, system solution that can simultaneously analyze RNA and proteins in thousands of individual cells and develop a more complete picture of the role genes and proteins play in biological systems.

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23-20898-00

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The BD AbSeq assay combines genomics and immunology technologies to facilitate even deeper single-cell analysis insights. Utilizing the BD AbSeq technology with mRNA analysis can help elucidate complex biological systems with distinct clustering of different cell subsets. The assay enables high-parameter interrogation of cell-surface protein markers in a single panel.

BD offers a full single-cell multi-omics system solution including a catalog of assays (mRNA-seq, protein detection, and multiplexing) for library preparation, secondary analysis, and data visualization.

Summary

As single-cell analysis becomes more widely used in basic research, the appreciation for the heterogeneity of cells in biological systems will continue to grow, leading to the potential for development of new diagnostics and treatments for a number of disease states. BD's current offerings for single-cell analysis, from flow cytometry to multi-omics, can help researchers advance their understanding of cell heterogeneity by offering the right research tools for a broad range of experimental questions.

Disclaimer

The method developed by Adam Abate's laboratory⁸ at the University of California, San Francisco has been named Abseq but is not to be confused with BD AbSeq technology. The two methods were developed independently and have significant differences in overall methodology. Discussion of Dr. Abate's work within this paper are included and discussed to provide the reader with appropriate background, context, and understanding of current multi-omics techniques being used for single-cell profiling.

