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Single-cell omics technologies — Fundamentals on how to create single-cell looking glasses for reproductive health

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Abstract

Over the last decade, in line with the goals of precision medicine to offer individualized patient care, various single-cell technologies measuring gene and proteomic expression in various tissues have rapidly advanced to study health and disease at the single cell level. Precisely understanding cell composition, position within tissues, signaling pathways, and communication can reveal insights into disease mechanisms and systemic changes during development, pregnancy, and gynecologic disorders across the lifespan. Single-cell technologies dissect the complex cellular compositions of reproductive tract tissues, providing insights into mechanisms behind reproductive tract dysfunction which impact wellness and quality of life. These technologies aim to understand basic tissue and organ functions and, clinically, to develop novel diagnostics, early disease

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biomarkers, and cell-targeted therapies for currently suboptimally-treated disorders. Increasingly, they are applied to pregnancy and pregnancy disorders, gynecologic malignancies, and uterine and ovarian physiology and aging, which are discussed in more detail in manuscripts in this special issue of AJOG.

Here, we review recent applications of single-cell technologies to the study of gynecologic disorders and systemic biological adaptations during fetal development, pregnancy, and across a woman's lifespan. We discuss sequencing- and proteomic-based single-cell methods, as well as spatial transcriptomics and high-dimensional proteomic imaging, describing each technology's mechanism, workflow, quality control, and highlighting specific benefits, drawbacks, and utility in the context of reproductive medicine. We consider analytical methods for the high-dimensional single-cell data generated, highlighting statistical constraints and recent computational techniques for downstream clinical translation. Overall, current and evolving single-cell "looking glasses", or perspectives, have the potential to transform fundamental understanding of women's health and reproductive disorders and alter the trajectory of clinical practice and patient outcomes in the future.

Keywords

multi-omics; proteomics; reproductive biology; single-cell; transcriptomics

Introduction

Reproductive health disorders account for over 12% of the global burden of disease.[1] By the time a patient presents with signs or symptoms of disease, dysregulations that began as a series of complex cellular interactions have amplified into multisystem dysfunctions across multiple biological compartments. Understanding the intricacies of disease pathogenesis at the cellular level is an essential step towards developing effective diagnoses and treatments for reproductive health disorders. Single-cell technologies enable investigation of biological systems with unprecedented cellular resolution, allowing a deeper look into patient-specific pathobiological mechanisms of complex disease processes. By uncovering predictive biomarkers for the diagnosis or prognosis assay development and identifying therapeutic targets for the design of precision medicine clinical trials, these technologies hold immense clinical potential for the personalized care of patients with reproductive health disorders worldwide.

Recent applications of single-cell technologies to study reproductive diseases and infections have been promising. These studies have advanced our understanding of the underlying immunological mechanisms of disease and honed in on specific cell phenotypes and functional attributes that can be translated into potential clinical biomarkers.[2–4] For example, these approaches have enabled unravelling the dynamic role of natural killer (NK) cells in the uterine mucosa in responding to specific placental infections, such as Listeria, and the role of NK cells in the modulation of spiral artery remodeling and trophoblast migration in early gestation.[4] These technologies were also employed to study patients with endometriosis and allowed identifying specific macrophage attributes contributing to endometriosis pathogenesis and potential cellular targets for the development

of novel therapies for endometriosis.[5] In several areas, exciting discoveries driven by single-cell approaches are at the cusp of benefitting clinical practice in reproductive medicine by informing the clinical development of novel diagnostic and therapeutic strategies.[6–8] For example, single-cell genomic and transcriptomic profiling technologies have enabled the discovery of novel biomarkers for breast cancer subtypes, prognosis, and treatment response,[9,10] ovarian reserve,[11] endometrial receptivity,[12] and HPV-related cervical oncogenesis.[13] In addition, single-cell flow and mass cytometry technologies have been leveraged to detect fetal red blood cells in stillbirth cases caused by feto-maternal hemorrhage[14] and have been used to identify predictive biomarkers of ovarian cancer progression as well as pregnancy complications such as preeclampsia and fetal growth restriction.[13,15–17]

In this review, we discuss how advances in single-cell technologies have transformed our ability to study complex, multicellular biological systems, providing “single-cell looking glasses” to investigate cell-specific and clinically relevant mechanisms underlying human reproduction and reproductive disorders (Figure 1). Our aim is to provide the clinically oriented reader with practical, technical, and analytical information necessary to understand and evaluate the potential for these technologies to translate into precision medicine tools that can improve the diagnosis and treatment of patients with reproductive diseases. We first review a number of single-cell profiling technologies (Table) including sequencing-based approaches (Figure 2) such as single-cell sequencing and spatial transcriptomics as well as proteomics-based approaches (Figure 3) such as flow cytometry, mass cytometry, and high dimensional proteomic imaging such as multiplexed ion beam imaging (MIBI) and imaging mass cytometry (IMC). For each technology, we discuss the general aim of the technology, the mechanism and workflow, sample preparation, quality control, and the final sequencing, imaging, or mass spectrometry analysis as relevant. We further discuss the benefits and drawbacks of each approach (Figure 4) as well as future directions—all in the context of reproductive health applications. We end with a discussion of the analytical framework, tools, and considerations for downstream data analysis and data integration.

Single-cell sequencing

Aim of the technology

Single-molecule sequencing has emerged as a novel method to directly analyze nucleic acids from biological samples. This includes RNA sequencing (RNA-seq), which maps messenger RNA (mRNA) sequences to a reference genome to identify their gene of origin and measure their abundance (ie, the transcriptome), providing insights into cellular functions at specific points in time or under certain conditions.[18,19] RNA-seq has been an essential tool to gain insights into the complex mechanisms by which cells and tissues carry out their functions and how small changes in gene expression can lead to cellular and tissue dysfunction resulting in the pathogenesis of various diseases. Nevertheless, it is important to note the poor ability to analyze cell types in greater detail, such as analysis of rare cell subtypes and different cell states of the same cell type or cell-cell interactions within and across cell types. This limitation motivated the development of single-cell RNA-seq. In 2009, scientists conducted the first RNA-seq experiments on single cells opening the door to

single-cell transcriptomics.[20] Since then, advances have been made that have exponentially increased the scalability of single-cell transcriptomic techniques from analysis of just a few cells to hundreds of thousands of cells.[21] This transcriptomic analysis of thousands of single cells has revealed the extreme heterogeneity of cellular gene expression within tissues and even within cell populations of the same lineage, giving rise to novel findings on existing cellular subtypes and states and revealing just how important it is to be able to analyze the transcriptome on a single-cell level.[22] For example, single-cell transcriptomics has further characterized the heterogeneity of cervical cancer by identifying 4 molecular subtypes based on gene expression profiles and cell type frequencies, with subtypes having differing levels of immune infiltration indicative of response to immune checkpoint blockade therapies.[23] This technology has also allowed a multitude of researchers to explore the role of endocrine-disrupting chemicals in reproductive health in a variety of contexts, such as proper development of the female reproductive system.[24]

Additionally, new assays have been developed that allow for parallel analysis of the transcriptome, proteome, and epigenome from a single cell. These advances have contributed to further profiling of the cellular and transcriptomic landscape of a given tissue or disease state and expanded the ability of researchers to explore the various factors at play in disease pathogenesis at a single-cell level by identifying important disease-associated cell types or states. For example, transcriptomic comparison of different tissue sites and disease states in endometriosis revealed endometriomas and peritoneal lesions are distinct entities with different immune cell profiles[6] (and manuscript 10, this AJOG issue), and analysis of endometrial transformation across menstrual cycle timepoints revealed 1 phase of transformation exhibits abrupt activation of a set of genes in specific cell types that correlate with the window of implantation[7] (and manuscript 3, this AJOG issue). Results from these studies present an avenue for better recognition of the ideal time to perform embryo transfer in in vitro fertilization cycles based on single-cell profiles as opposed to bulk RNAseq in current commercial tests for endometrial receptivity.[25] Additionally, comparison of term and preterm placental samples showed differences in single-cell gene signatures, providing a potential method for noninvasive prediction of pregnancy complications (discussed in manuscript 6 in this issue). These are just a few examples of the promise of single-cell approaches to aid in the generation of novel diagnostic and therapeutic approaches.

Step-by-step approach (workflow)

In general, the workflow of single-cell RNA sequencing (scRNA-seq) is quite similar to that of conventional RNA-seq—isolation of mRNA from cells followed by reverse transcription into complementary DNA (cDNA) which is subsequently fragmented and size selected to ensure the molecules are suitable for sequencing. The main difference is that in scRNA-seq, these steps occur in a manner that allows for the resulting cDNA molecules to be tracked back to a single-cell rather than the pool of cells from which the RNA is isolated. In some cases, it may be necessary to perform single-nucleus RNA sequencing (snRNA-seq) rather than scRNA-seq, such as the use of flash frozen tissues from which viable cells cannot be isolated or from tissues containing large or sensitive cell types which may not be suitable for scRNA-seq applications. It is important to note, however, that the processes of cell isolation and nuclei isolation result in different cell type enrichments, thereby influencing

the outcomes. Additionally, depending on the cell type, certain RNAs are rapidly exported to the cytoplasm, while others remain continuously in the nuclei, contributing to further differences. Nevertheless, both snRNA-seq and scRNA-seq can be quite informative and which method to choose depends highly on the tissue being studied. In 1 study, both snRNA-seq and scRNA-seq were utilized to investigate changes to the endometrium and track uterine cell states over the time course of the menstrual cycle.[26]

This technique requires a high-quality single-cell or single-nuclei suspension from the sample of interest which can include whole blood, stimulated or cultured cells, or tissue specimens. From whole blood, leukocytes can be isolated via red blood cell lysis or peripheral blood mononuclear cells can be isolated via density gradient centrifugation. From tissue specimens, cells or nuclei can be isolated via mechanical or enzymatic dissociation protocols which may need to be optimized based on tissue type and downstream single-cell assay.[27] The maternal fetal interface is particularly interesting when studying reproductive biology, which is why isolation of single cells from placental tissues has been optimized for use in a variety of single-cell experiments, as described by Garcia-Flores et al[28]

Sorting isolated cells and nuclei allows for the removal of unwanted cell populations or the selection of specific ones. This step is crucial for achieving a high-quality cell suspension, especially in cases with a high concentration of dead cells or low-quality nuclei. These compromised cells and nuclei can lead to the leakage of RNA into the suspension, referred to as ambient RNA. The presence of ambient RNA can “blur” the true transcriptomic signal of the biological question and lead to misleading results. Taking advantage of single-nucleotide polymorphisms, natural genetic variations in the human genome, has allowed researchers to pool and process samples from different individuals together in the same experiment.[29,30] Moreover, various barcoding techniques, such as oligo-conjugated antibody or lipid tagging, allow for the tagging of samples before pooling. This enables the subsequent pooling of cells from different samples or patients, a process also known as multiplexing. This has substantially lowered costs and minimized batch effects in single-cell experiments, expanding the capacity for cell analysis.

Finally, historically, single-cell transcriptomic methods have been limited to the use of cells or nuclei from fresh samples; however, recent advances now allow the use of fixed cells and nuclei isolated from fresh, flash-frozen, or formalin-fixed paraffin-embedded (FFPE) tissue sources.[31] This provides many benefits including locking cells/nuclei in their biological states, preserving fragile cell populations, streamlining single-cell workflows, and reducing experimental variability. Fixed sample workflows typically utilize probe-based methods rather than whole transcriptome mRNA capture, providing another method of multiplexing samples from different individuals with the ability to use a unique probe set for each sample. Recently, this advancement has been used in a combined approach of single cell and spatial transcriptomics exploring the tumor microenvironment of a single human breast cancer FFPE tissue block, allowing for integrated and in-depth analysis of this tumor at the cellular and molecular level.[32] The ability to use FFPE tissues with these technologies will allow them to become much more applicable to clinical practice as FFPE tissues are abundantly available in pathology departments.

After obtaining a high-quality cell or nuclei suspension, the next crucial step is to isolate single cells or nuclei for capturing mRNA in preparation for transcriptomic analysis. This technology's success relies on the ability to isolate single cells. Methods to achieve this include microfluidics-based, plate-based, or in-situ techniques. Microfluidics employ precise pressure control in micro-channels, isolating cells in oil droplets.[33] Plate-based methods utilize well separation in plates, isolating individual cells.[34] In-situ methods use the single cell itself as an isolated environment, often employing combinatorial barcoding for single-cell resolution.[35] In these isolated environments, mRNA is reverse transcribed into cDNA. Each cDNA molecule is tagged with a unique molecular identifier (UMI) and a single-cell barcode. The specific barcoding method depends on the platform used. For example, with the 10x Genomics platform, cells are encapsulated in oil droplets with a unique barcode sequence, resulting in cell-specific barcoding. Alternatively, platforms like Parse Biosciences fix cells or nuclei to complete barcoding steps in situ, adding unique barcodes through combinatorial ligation. This method also creates a unique cell-specific barcode. Following mRNA capture and barcoding, cDNA molecules undergo amplification, fragmentation, and size selection to form a library that can be sequencing using short read sequencing methods. In analysis, these unique barcodes are used to identify cDNA molecules from the same cell. Single-cell transcriptomics has been instrumental in gaining a better understanding of the reproductive system in health and disease. For instance, a small study using scRNA-seq showed extensive transcriptomic differences between endometriomas, eutopic endometriosis, and peritoneal endometriosis suggesting that these conditions may require distinct diagnostics and treatments and highlighting the relevance of single cell transcriptomics in further elucidating the cellular and molecular differences between endometrial conditions in future studies.[6]

Quality control

A variety of biological factors can impact the quality of resulting single-cell transcriptomics data and impacting the researcher's ability to interpret the data in a meaningful way. For example, presence of dead/dying or low-quality cells and nuclei can result in the release of ambient RNA which can contaminate other cells and nuclei or it can lead to clumping of cells and nuclei together resulting in loss of single-cell resolution. Both cases would result in parts of the data that cannot be used. While it is important to try to mitigate these factors during the experiment, it is equally important to account for these factors in analysis of the data.

After generating and aligning the data against a reference genome in single-cell transcriptomics, several quality control checkpoints are crucial. Firstly, examining the total UMI counts or the total number of expressed genes in a single cell helps filter out low-quality cells and potential doublets. Extremely low UMI/feature counts indicate poor-quality or noncellular entities (empty droplets with ambient RNA). Conversely, very high counts may suggest doublets or multiplets (droplets containing more than 1 cell). These thresholds vary by sample type and often require iterative filtering.[36] Additionally, a high proportion of mitochondrial or ribosomal genes expressed in a cell can signal poor-quality or unhealthy cells. Again, the cutoffs for filtering may vary by sample type and may require iterative

adjustments. Beyond these methods, software tools have been developed to help identify and exclude ambient RNA or multiplets in the dataset.[37,38]

Additional modalities measured by single-cell sequencing methods

Single nuclear ATAC-seq: Motivated by the importance of post-transcriptional and epigenetic regulation in biological processes, scRNA-sequencing has been rapidly adapted to enable the measurement of additional cellular features at the single cell level.[39] Single-cell transcriptomics observes the mRNA in a cell, providing information about the current activity of that cell. However, depending on the research question at hand, it may be of interest to explore other cellular characteristics as well (Figure 2 Top Panel, Table). The assay for transposase accessible chromatin (ATAC) allows researchers analyze open chromatin regions in single cell DNA. This assay utilizes the Tn5 transposase, which targets open chromatin, fragmenting DNA, and adding NGS sequencing adaptors in a process called transposition.[40] Transposition is followed by isolating single nuclei and barcoding DNA fragments with unique cell identifiers and UMIs. ATAC-seq uses a single-nuclei suspension as input, which can be derived from a single-cell suspension or whole tissue specimen. For single-cell suspension, cell lysis and wash steps are needed for nuclei isolation, with parameters optimized by sample type.[41] Prior to ATAC-seq, nuclei quality is checked using a viability dye and microscopy, cell counter, or flow cytometer. This technology provides insights into gene expression programs of single cells through chromatin accessibility, shedding light on cell-cell interactions and disease influences.

Multimodal single-cell sequencing is the new frontier in single-cell analysis, empowering researchers to investigate multiple cellular characteristics from the same cell providing additional layers of information necessary to make novel biological discoveries.[42–44] In recent years, researchers have developed methods for profiling both the transcriptome and epigenome from a single cell, combining single-cell ATAC- and RNA-seq into 1 assay.[45] This multiomic assay has been optimized so that sample preparation is no different than that of ATAC-seq alone, rather there are slight differences in reagents used and downstream steps; both the DNA fragments from open chromatin regions and the mRNA within the nucleus are captured and 2 separate libraries generated as a result. Although underutilized, study of the epigenome on a single-cell level provides a unique opportunity for researchers to explore changes in DNA in an unbiased way allowing for the identification of key regulatory elements, generation of new hypotheses, and development of novel diagnostic and therapeutic tools. For example, in one study utilizing multimodal ATAC- and RNA-seq, integration of transcriptomic and epigenomic information from ovarian and endometrial tumor samples identified cancer-specific distal regulatory elements providing insights into the epigenetic mechanisms of tumor pathogenesis.[46] These assays can also be of particular interest to the field of obstetrics and gynecology, where epigenetic regulation via DNA histone modifications may be essential to many important processes related to the cyclical changes of the female reproductive system.

Single-cell CITE-seq: Another example of multiomics is cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), which provides both transcriptomic and cell-surface proteomic data from a single-cell.[47] It involves staining a single-cell

suspension with oligo-conjugated antibodies, each with a unique nucleotide sequence for identification during sequencing. This generates 2 libraries—1 for gene expression and 1 for protein expression. Fortunately, CITE-seq utilizes the same library construction as unimodal scRNA-seq allowing for similar gene capture and library diversity between the 2 assays. CITE-seq does require a higher sequencing depth to account for the protein modality; however, this does not impact the sequencing depth of the RNA modality. Biolegend offers pre-mixed antibody panels for comprehensive cell-surface profiling in both human and mouse samples. Additionally, individual oligo-conjugated antibodies are available for specific markers. After staining, cells are partitioned, and cDNA libraries are created from both mRNA transcripts and antibody-derived tags. This enables precise classification of cell populations and states using both gene and protein expression data, such as in the case of further characterizing decidual MAIT cells at the maternal-fetal interface, a cell population thought to be implicated in maintaining the sensitive immune balance.[48]

Each technique in single-cell transcriptomics involves sequencing the cDNA libraries, resulting in raw data files (eg, FASTQ or FASTA) with nucleic acid sequences. These sequences are then aligned to a reference genome to identify genes, and cell-specific barcodes are matched to create a matrix file of cells and their associated genes.

Limitations and looking forward

Single-cell transcriptomics has revolutionized our ability to explore cellular and tissue transcriptomes at an unprecedented resolution. However, these technologies come with notable limitations. The extensive manipulation required, including tissue dissociation, cell sorting, and lysis, can stress the cells or nuclei under study. Depending on the dissociation method, certain cell populations may be preferentially selected, potentially skewing the representation of the tissue's cellular landscape. Rare cell populations are often lost, and there are constraints on the number of cells that can be analyzed. Furthermore, the single-cell nature of these assays may lead to lower capture efficiency and potential bias towards specific genes, causing dropout of others.[22] Additionally, medium and small labs often struggle with limited computational resources when tackling scRNA-seq data, which can pose challenges in terms of storage, processing power, and the capacity to handle large datasets effectively because they cannot access to a high-performance computing cluster or cloud-based services. While single-cell omics enable detailed analysis and predictive modeling of cell-cell interactions, the process of creating a cell suspension may lead to the loss of organizational and spatial information at the tissue level. This information is crucial, especially when studying specific cell functions or intercellular interactions in disease contexts.[49] While it is crucial to acknowledge these limitations, the field of single-cell transcriptomics is witnessing continuous progress in addressing them. A prime example is the emergence and refinement of spatial transcriptomics, which effectively surmounts the challenges of spatial context loss and preferential cell type capture often encountered in conventional single-cell transcriptomic approaches.

Spatial transcriptomics

Aim of the technology

Recent advancements in single-cell techniques have enabled comprehensive profiling of transcriptomes, proteomes, and epigenomes in suspended single cells across diverse biological contexts and diseases. However, the reliance on cell suspensions leads to the loss of spatial information and can sometimes miss crucial, sensitive, or rare cell populations. In situ hybridization (ISH) methods offer a spatially contextual RNA analysis, with fluorescence ISH (FISH) being a notable technique. While it provides spatial transcriptomic information, it has limited throughput and is mostly qualitative in gene expression quantification.[50]

Recent years have seen advances in FISH with the use of combinatorial fluorescent barcodes to increase throughput.[51] Yet, this method is still constrained by the number of detectable genes due to optical limitations and requires prior knowledge of the target genes.[50] In situ sequencing methods, utilizing a set of RNA probes complementary to mRNA sequences combine to sequencing chemistry, offering spatial transcriptomic analysis through sequencing rather than fluorescent readout.[52] This approach provides greater flexibility in the number of analyzed genes.[53]

Today, new technologies have been developed for high throughput spatial transcriptomics enabling the analysis of hundreds to thousands of genes, highly and lowly expressed. This ability to survey gene expression at the spatial level has been used to identify ligand-receptor interactions of fibroblasts in the tumor microenvironment, which correlated with survival rates in patients with advanced high-grade serous ovarian cancer.[54] These technologies also allow for the capture of multiomic spatial information, including transcriptome, proteome, and epigenome.[55] Employment of paired spatial transcriptomics, snATAC-seq, and snRNA-seq from the same placental and decidual tissue specimens has further delineated trophoblast differentiation trajectories and their role in successful pregnancy as compared to pregnancy disorders.[56] This multimodal approach of integrating spatial transcriptomic and single-cell transcriptomic information has also been used to create a comprehensive delineation of uterine leiomyomas, including the surrounding pseudocapsule and normal myometrium, which has both validated previous findings on leiomyoma dynamics and provided new findings of cell-cell interactions that may contribute to disease onset.[57] These advancements empower researchers to investigate cell-cell interactions, explore cellular neighborhoods, and profile tissue organization across various contexts, enhancing our understanding of disease pathogenesis from the subcellular to tissue level.

Step-by-step approach (workflow)

Current spatial transcriptomics technologies encompass imaging-based and sequencing-based approaches compatible with both fresh-frozen and FFPE tissues, as well as 2D and 3D cell and organoid cultures.[58,59] When choosing a spatial transcriptomics approach, key considerations include desired resolution, sensitivity, and throughput. Technologies like Visium from 10x Genomics offer whole-transcriptome coverage but lack single-cell resolution. In contrast, Xenium from 10x Genomics or CosMx from Nanostring can achieve

subcellular resolution but are limited in the number of targeted genes and proteins. Whole-transcriptome approaches suit discovery-focused experiments, while panel-based approaches excel in analyzing specific genes or proteins at high resolution. The available imaging-based spatial transcriptomics platforms vary in throughput, detecting 100 s to 1000 s of genes and 10 s to 100 s of proteins simultaneously. Whole-transcriptome approaches tend to be less sensitive, whereas imaging-based methods offer high sensitivity, with a trade-off between throughput and sensitivity.[60]

For fresh-frozen tissues, the initial steps involve sectioning, fixation, and permeabilization. Some platforms may require tissue embedding in optimal cutting temperature compound for structural preservation. FFPE tissue, already fixed and permeabilized, requires rehydration, sectioning, and tissue deparaffinization and decrosslinking. Most platforms offer specific tissue slides for their protocols, and the tissue section is transferred to these slides. A recommended step is Hematoxylin and Eosin (H&E) staining to assess tissue quality and identify regions of interest for spatial transcriptomic analysis. In some cases, H&E staining may follow spatial transcriptomic analysis. Subsequent steps depend on the chosen platform. For sequencing-based methods like Visium, the slide contains spatially barcoded reverse transcription primers for mRNA capture. This involves reverse transcription, second-strand synthesis, cDNA amplification, library construction, and sequencing. In imaging-based methods like Xenium or MERFISH, RNA-specific and sometimes antibody-specific probes are hybridized to the tissue section. These probes can be commercial panels or custom-made. Some platforms involve probe ligation to create circular DNA probes, followed by probe amplification. The platforms then automate cyclic in-situ hybridization, imaging of fluorescent markers onto hybridized probes, cleavage, and fluorophore washing. This process is iterated, generating a unique fluorescent barcode for each target. The instrument consolidates multiple images into a single representation of the tissue section and captured gene transcripts.

Quality control

Prior to completing a spatial transcriptomics run, H&E staining can be used to qualitatively assess the tissue to be analyzed. Tissue processing and sectioning artifacts (improper fixation, squeezing or crushing of the tissue, hemorrhaging, or oxidized blood, etc.) may interfere with the quality of the data downstream and can indicate whether a tissue section should be used for a spatial transcriptomics experiment. After completion of a spatial transcriptomics run, much of the quality control for the data comes from filtering (removal of background signal and noise), segmentation (defining cell boundaries), normalization (statistical transformation to account for differences in capture rate), and deconvolution (identifying the contribution of separate cells to 1 capture spot).[50]

Spatial transcriptomics represents the cutting-edge advancement in single-cell transcriptomics, enabling the simultaneous examination of transcriptomic, proteomic, and epigenomic factors within a spatial framework. These innovative technologies empower researchers to probe both proteins and genes, explore intricate cell-cell interactions, and unveil the cellular landscapes of tissue sections. This deeper insight holds the potential

to revolutionize our comprehension of disease mechanisms, opening new vistas for the development of prognostic and therapeutic tools.

Limitations and looking forward

However, it is important to note that, being a relatively nascent technology, spatial transcriptomics comes with its share of significant limitations. The process of conducting spatial transcriptomic experiments is notably resource-intensive, demanding substantial investments in both sample preparation and instrument runtime. This financial and temporal commitment inherently restricts the number of samples that can feasibly be analyzed.

Moreover, existing spatial transcriptomic technologies may not exhibit the same level of sensitivity as their single-cell sequencing counterparts. This can result in diminished signal strength for certain markers and lead to fewer reads per cell and in some cases, even a complete absence of reads, making accurate gene expression quantification a challenging endeavor.

Given the novelty of these methodologies, the capacity to effectively analyze the data also presents a limitation. While a range of software tools are available for analysis, there is currently a lack of standardized pipelines for data cleanup and quality assurance. Addressing these challenges will be crucial in harnessing the full potential of spatial transcriptomics.

Flow cytometry

Aim of the technology

Flow cytometry and fluorescence-activated cell sorting (FACS) are single-cell technologies for the proteomic analysis (flow cytometry) and sorting (FACS) of cells in suspension. Flow cytometry was developed in the late 1960s[61] and since, advances in fluorochromes, lasers, and instrument specifications have made the technology readily available. Flow cytometry can be used to characterize cellular heterogeneity in cells from blood or digested tissue, which can be instrumental in understanding health and disease. These technologies are based on fluorescently labeled probes (usually antibodies) for analysis and sorting through electrostatic flow. In flow cytometry, cells in suspension are stained with fluorophore-conjugated antibodies, after which the sample is run through a flow cytometer, which incorporates fluidics, optics, and electronic systems to analyze cells. Flow cytometry allowed for the development of great advances in immunology, drug discovery, oncology, and many other fields.

Flow cytometry has proven instrumental to the identification and characterization of cells in the female reproductive tract. This includes advances from the characterization of innate immune cells in the uterus and placenta, to cross reproductive tract comparisons, to the use of flow cytometry in characterizing vaginal microbiome dynamics in HPV infection.[2,3,62–64]

Step-by-step approach (workflow)

Prior to the experiment, a fluorophore-conjugated antibody panel must be designed to not only detect all cell types of interest but also to ensure that the minimum spectral overlap is achieved. Due to this technology’s dependence on the electromagnetic spectrum, panel

design is key to ensure interpretable results. Fluorophore-conjugated antibodies can be acquired in ready-to-use kits from some biotechnology companies. For scientists that are interested in in-house customization of panels, unconjugated antibodies can be purchased separately from fluorophores for in-house conjugation. Additionally, some biotechnology companies offer customized sets of conjugated antibodies. Standard flow cytometers can detect over 14 parameters, with recent optimization of a 28 parameter protocol using a five-laser flow cytometer.[65–67]

Flow cytometry workflow is summarized in Figure 3. Flow cytometry requires that the analyte to be studied is in a single-cell suspension. Most commonly, the analytes are single cells that can be either fixed or unfixed. Thus, cells already in suspension, such as blood, can be easily processed for use in flow cytometry via a lysis step to remove red blood cells from the suspension and resuspend the remaining lymphocytes in a buffer such as phosphate buffered saline. Cells from tissue culture can also be suspended and run with flow cytometry. Cells in solid tissue must be mechanically disaggregated and/or enzymatically digested into single-cell suspensions for staining. Additionally, flow cytometry experiments can benefit from multiplexing through fluorescent cell barcoding, in which unique samples are labeled with dyes or fluorochrome-conjugated antibodies and pooled into 1 cell suspension.[68–70] Barcoding reduces the requirement for antibody and reduces error across samples.

Once a cell suspension is prepared, the suspension can be directly stained with the prepared antibody cocktail for extracellular protein targets. The cells in suspension must undergo permeabilization to stain intracellular targets. After staining, cells are resuspended and can be immediately run through a flow cytometer or stored for future analysis or sorting.

Quality control

Flow cytometry instruments require regular standardization and calibration using reference particles, which can be general or designed to be spectrally reflective of the experimentally utilized fluorophores. In the flow cytometer, the cell suspension is streamed into a swiftly moving fluidics system which hydrodynamically focuses the cells into a single stream and promotes separation between cells.[71] Subsequently, the stream is vibrated to create droplets containing single cells which are guided into an analysis space. The single-cell droplets in the stream are passed through a light source that excites the fluorophores on or in the cells. The light source is scattered by the cell and also the fluorophores emit light. Both the scattered and emitted light is read by detectors. These optical signals are converted into digital signals which can then be analyzed. Relative cell size and granularity are reflected by forward and side scatter signals, respectively, while cell phenotypes can be assigned based on fluorescent signals. The emissions of the excited fluorophores coating the cell are filtered and sent to detectors, which then transmit data on the spectral information to a connected computer for signal processing and output. A key component of flow cytometry data processing is compensation, where spectral overlap from other filters is accounted for via subtraction of a fraction of the overlapping signal from the other.[72] With more complex panels, compensation is calculated computationally.[73]

If the experimental goal includes cell sorting, either fluorescence-based or electrostatic force–based sorting can be used. For fluorescence-based sorting, the fluorescence and scatter

signals are selected by the researcher based on the population desired to be separated. If the particle meets the criteria, the suspension in which it flows is charged at the break-off point, which is when the particle separates from the stream into an individual droplet. The charged particle droplet is then deflected by an electrostatic field and is collected in a separate tube from the nonselected particles.

Post collection, cell populations and phenotypes are identified via the process of gating. Manual gating involves visual evaluation of cell population in a 2 dimensional or three-dimensional plot and selecting populations of interest based on their signal of the interrogated markers. Selected populations are the subject of analysis and comparison. While flow cytometry data are largely gated manually, immunophenotyping via automated gating methods have been developed and offer an important complementary approach to manual gating and are particularly useful for large datasets, with artificial intelligence and machine learning–based analyses making their way to multiparameter flow cytometry for both gating and postgating phenotypic analysis.[74–76]

Limitations & looking forward

Conventional flow cytometry instruments are limited in potential parameter detection by spectral overlap of fluorochromes. However, several strategies to overcome this limitation have been developed. For example, fluorescent nanocrystals with a narrow emission spectrum can be used in conjunction with fluorochromes in order to maximize the analysis possible with 1 experiment.[77–79] Additionally, technologies building on conventional flow cytometry have emerged to expand parameter detection limitations. Spectral flow cytometry expands on conventional instruments by using a series of detectors to measure the full spectral emission of a fluorophore as opposed to solely the peak emission detected by single target detectors in conventional instruments.[22] Given that each fluorophore has a unique spectral emission signature, spectral flow cytometry requires algorithmic distinction between fluorophores.[80] Spectral flow cytometry expands possible parameters to over 40 fluorescent parameters.[81–83] More recently, Infinity Flow was developed by Becht et al to analyze large series of flow cytometry experiments, deemed massively parallel cytometry experiments, with overlapping panels. These overlapping panels include key “backbone” antibodies consistently and varying “exploratory antibodies.” Post acquisition, the expression levels of these exploratory antibodies are imputed using a supervised machine learning approach based on levels of the backbone antibodies. Infinity flow has been employed in varying immunological and cancer studies, with potential for use in reproductive studies.[84–86] Additionally, flow cytometry has been employed to isolate and characterize a more recently described cell type, the uterine innate lymphoid cell.[87]

Mass cytometry

Aim of the technology

Mass cytometry, also known as cytometry by time of flight (CyTOF), is a mass spectrometry–based solution for the high dimensional flow cytometry analysis of single cells in suspension. Mass cytometry deeply phenotypes cells in suspension by employing rare earth metal isotope-labeled antibodies, which probe protein targets of interest and are

detected by mass spectrometry by time of flight. The rare earth metal isotopes used for mass cytometry have stable atomic masses which can be detected and analyzed without the requirement for compensation. Mass cytometry enables measurement of both the presence and signal intensity of over 50 unique targets in a single experiment.[88] While fluorescence-based technologies (eg, spectral flow cytometry or infinity flow cytometry) have been developed to allow a similar degree of multiplexing, in practice, the near lack of spectral overlap of mass spectrometry greatly facilitates the design and implementation of high-dimensional mass cytometry panels. Mass cytometry analyzes cells in liquid suspension, which makes the platform particularly useful for peripheral blood mononuclear cells (PBMCs), whole blood samples, or digested tissues. The high dimensionality afforded by mass cytometry can be leveraged not only for the precise phenotyping of multiple cell subsets base on surface protein expression but also for the simultaneous analysis of cellular functional states based on the detection of intracellular markers, including post transcriptional modification of signaling proteins, intracellular cytokine expression, cell cycle states, and epigenomic regulatory states.[42,89–93] Additionally, prior to cell fixation in suspension, the immune cells can be stimulated with known immune stimulants such as bacterial lipopolysaccharide or cytokines to evaluate immune responses in samples from different patients or persons with different conditions represented in the study.[91,93] By investigating activation markers, an additional dimension of activation potential as opposed to static cell state can be appreciated. Furthermore, recent development in mass cytometry now allows the codetection of cellular DNA or RNA transcripts together with proteomic epitopes, encouraging further links between cell transcriptional activity and phenotype.[94]

The application of mass cytometry in the female reproductive tract has largely centered on peripheral blood signatures associated with reproductive pathologies for the purpose of predictive modeling. This includes peripheral blood correlates that could give insight into pre-term birth and induction of labor.[95,96] However, mass cytometry has also been used to evaluate cells collected from the FRT, including evaluation of HIV-infected endometrial T cells and the description of NK cell subsets in the decidua.[97,98] These methods can be combined, as they were in an endometriosis study where immune cells from both peripheral blood and digested endometrial tissue were analyzed in parallel with mass cytometry.[99] Mass cytometry can also be employed in analyzing solid ovarian tumor cancer cells, with a published protocol available.[100]

Step-by-step approach (workflow)

Workflow of mass cytometry is similar for the analysis of whole blood samples, PBMCs, or other cell suspension preparations and is summarized in Figure 3. Detailed protocols are available, with the aim to standardize mass cytometry workflows.[101] We will describe a workflow routinely used in our laboratory for the analysis of whole blood samples with CyTOF for exploratory studies aimed towards biomarker discovery or hypothesis-driven studies focused on detecting specific phenomena. Whole blood samples collected through venipuncture into heparinized tubes are either left unstimulated for the detection of endogenous cell states or stimulated with a panel of receptor-specific ligands within 30 to 60 minutes of phlebotomy. Poststimulation, the samples are fixed using a paraformaldehyde-based fixative buffer (eg, SmartTube Prot1 stabilizer) prior to storage at −80°C where

samples are stable for several years. After thawing and red blood cell lysis, samples can be used immediately or frozen again in a cell staining media containing bovine serum albumin and sodium azide for short-term storage.

For large mass cytometry experiments, multiplexing (ie, analyzing multiple samples simultaneously) can be employed via barcoding, for the simultaneous acquisition of multiple samples. The most common barcoding method currently in use is the palladium-based barcoding,[102] which employs six isotopes of palladium, a combination of which is used to stain each sample. With palladium-based barcoding, data for up to 20 samples can be collected simultaneously, and the samples can be computationally separated after collection of the data. Just as in flow cytometry, sample barcoding decreases antibody requirement and instrument time and substantially reduces experimental variability. Once barcoded, samples are pooled and stained with a panel of metal-conjugated antibodies. Many antibodies are commercially available preconjugated to various metal isotopes, with some antibodies sold together in pre-made panels for ease of use (Standard BioTools, formerly Fluidigm). Alternatively, antibodies can be conjugated in-house for greater flexibility in panel design using established protocols.[103] When intracellular antibodies are included in a panel, the staining occurs in 3 steps: first, extracellular protein antibody staining is completed. Subsequently, the sample is permeabilized, typically with methanol, for intracellular protein staining. Finally, the DNA of each cell is stained with an intercalator and the mass cytometry run can then begin.

Each mass cytometry run typically begins with calibration of the machine using a solution of metal-labeled beads for which standard detection expectations are set. The detector of the mass spectrometer can then be assessed to ensure that the isotopes in the sample will be accurately detected. Once the detector voltage and acquisition parameters have been calibrated, acquisition can begin. The stained cell samples are resuspended in a normalization bead buffer, which contains beads with specific isotopes and the detection of these isotopes are used at the end of the acquisition to normalize the strength of event detection across the total acquisition time, as detector effectiveness decreases during the span of the acquisition time. The sample is then run through the machine, which aerosolizes the cell suspension little by little into a single-cell stream, and vaporizes the aerosol droplets, so that they can then be ionized. It is then that the ions that enter the time-of-flight chamber where they are separated by mass-to-charge ratio as they approach the detector. In the end, the software collects information on detection events and the masses detected at each event. Each event typically corresponds to a single cell, although this is dependent on the rate of sample flow through the system. The acquisition data is then exported into an fcs (flow cytometry standard) file.

After acquisition, postprocessing involves normalizing the signal across the acquisition time using the normalization beads and debarcoding to demultiplexing samples into separate fcs files. These files can then be exported into gating software for further analysis. Once cell types are gated, median detection values for each cell type for each protein stained for can be exported for further analysis, which can involve utilizing machine learning algorithms such as LASSO and its variations, ElasticNet, or STABL for the generation of predictive models from mass cytometry data.[104–108]

Quality control

Given that mass cytometry collects large number of datapoints on a relatively small n (in many cases) quality control and attention towards reproducibility is of utmost importance. Unstimulated and stimulated samples are used for quality control. First, numbers of cells acquired per debarcoded sample can be analyzed to better understand if certain samples were of poorer quality and had a higher number of dead cells or fewer cells acquired, making conclusions made regarding rare cell types less strong. Stimulated samples are also useful in quality control, as certain immune stimulants have canonical immune responses that should be present in all samples. For example, stimulation with LPS should lead to a response in MyD88/MAPK markers in classical monocytes. This information can be useful in understanding how successful stimulation was. Panel design should consider the incorporation of canonical responses for quality control. Kleinstauber et al point towards rigorous evaluation of reference samples in comparison to study samples, in order to QC for antibody staining, evaluate for the presence of bath effects, and develop a gating strategy supported by the appropriate positive and negative controls.[101]

Limitations and looking forward

A main limitation of mass cytometry is that the sample is destroyed in order for collection to occur. Therefore, no further analysis of the sample is available. Additionally, sample loss can occur at each step of preparation, acquisition, and analysis. This includes sample loss during blood lysis, barcoding, staining, and most significantly, acquisition.[109] Additionally, a percentage of cells are lost during the computational processing of the data, particularly in the process of debarcoding and excluding doublets from analysis, leading to a final analysis of about 1/3 of the immune cells that were in an initial whole blood sample.[110,111]

Mass cytometry continues to be promising as a tool to characterize immune and stromal cell populations in health and disease through not only protein expression but also analyzing the epigenomic landscape with EpiTOF.[90] Coupled with advancing computational methods for analysis and increasing availability of the technology, mass cytometry remains a promising tool for analysis of the female reproductive tract.

High-dimensional proteomic imaging

Aim of the technology

While flow and mass cytometry characterize single cells in suspension, high-dimensional proteomic imaging (HDPI) technologies enable the incorporation of spatial information, ranging from cell-to-cell communications, to neighborhood-level interactions, to tissue organizational information. Spatial information provides meaning and context to proteomic data, as the complex relationships between single cells and tissue architecture undergird disease histopathology. Additionally, multiparametric assays incorporating spatial components have the potential to improve development and applicability of machine learning and artificial intelligence models for the development of predictive models and the identification of treatment targets.

HDPI is currently emerging as a powerful tool to investigate the female reproductive tract, both solitarily and in conjunction with single-cell transcriptomic and epigenomic tools. Multiplexed ion beam imaging by time of flight was employed in conjunction with spatial transcriptomics to describe the maternal-fetal interface with both spatial and temporal resolution.[112] Additionally, the previously underestimated role of myeloid cells at the maternal fetal interface, likely due to cell loss with tissue digestion, has been investigated and characterized recently with IMC.[113] Combining IMC with single cell transcriptomics has allowed researchers to further characterize the pathophysiology of eutopic and control endometrium vs peritoneal and ovarian endometrial lesions, providing clinically relevant findings which can be used for the development of more effective early diagnosis and therapeutics.[5] These examples point towards more widespread use of high-dimensional proteomic imaging in the reproductive space.

Step-by-step approach (workflow)

Sample type for HDPI is typically FFPE tissues, although cryosectioned tissues, cultured cells, and whole-mount tissues can also be used in some technologies. Conventional immunohistochemistry and immunofluorescence imaging is limited by spectral overlap. HDPI overcomes this limitation.[114]

HDPI modalities employ 2 broad methods for the detection of protein at subcellular resolution using predominantly antibody probes: fluorescence-based imaging and metal-isotope–based imaging. Technologies that are fluorescence-based include both technologies that directly use fluorophore-tagged antibodies (eg, MACSima)[115–121] and other technologies that use DNA-tagged antibodies that are subsequently detected by hybridization of the DNA-tagged antibodies to fluorescent probes (eg, CODetection by indEXing, CODEX).[109,122] Just as discussed in the flow cytometry section, spectral overlap and compensation concerns affect these fluorescence-based technologies. Mass cytometry has thus also been developed for use in imaging, with the use of metal isotope-tagged antibodies (IMC, MIBI).[123,124] Just as with metal-conjugated antibodies for mass cytometry, antibodies for MIBI and IMC can either be purchased commercially or can be conjugated within in house with the same protocols available for mass cytometry antibodies.[103] Additionally, as in mass cytometry, metal isotope–based HDPI technologies enable detection of a variety of target types, including RNA detection.[125] Finally, mass spectrometry imaging is a nonbiased high-dimensional imaging method that can detect varying molecule types in biological samples, including proteins, peptides, lipids, and metabolites, by generating a mass spectrum for each molecular species ionized.[126,127]

HDPI workflows vary most significantly in the method of staining and imaging. Fluorophore-tagged antibody-based technologies employ cyclical staining, where samples are stained with antibody probes for a subset of the total markers to be captured, imaged by the microscope system, and undergo probe removal in preparation for the next cycle of staining. This method can allow for over 90 probes to be used.[128] Thus, staining and imaging are both cyclical in these methods and in principle, extends the method of traditional fluorescence microscopy.[116] DNA-tagged antibody-based technologies enable staining of all targets of interest at once and cyclical imaging with the cDNA probe-

conjugated fluorophores.[129] Finally, metal isotope-tagged imaging enables both staining of all targets at once and imaging of all targets at once.

Preparation for a high-dimensional imaging study includes the identification of protein targets that are of interest based on the study question. Many panels include markers for tissue-specific features, stromal and vascular cells, and immune cells within the tissue. The usage of antibodies in HDPI requires optimization for utilization on a new tissue type. This applies particularly in cases where the antibody is conjugated for the first time in-house but also for the usage of new antibodies that are commercially acquired.

Flow of high-dimensional imaging begins with sample preparation. These imaging modalities are typically used with sectioned FFPE tissues onto positively charged slides, although cryosection tissues, whole mount tissues, and cultured cells can be used with altered protocols. The slides containing the tissue sections undergo dewaxing, rehydration, antigen retrieval, Fc blocking, and finally antibody staining. We will describe a workflow typically used in our lab for imaging mass cytometry analysis of FFPE-preserved tissues, which is summarized in Figure 3. The acquisition procedure begins with warming up and tuning the machine according to manufacturer protocols. The slides to be imaged are then scanned and regions on the tissue to be imaged are chosen using high-resolution images generated with the machine's camera. Once regions of interest are chosen and their size determined, the isotopes to be detected are input into the accompanying software and acquisition can begin. The imaging mass cytometer ablates a 1 μm^2 square area at a time and sends a plume containing the isotopes through the time-of-flight mass spectrometer, which allows for the recording of the present isotopes. 1 square millimeter of tissue requires 1 hour and 40 minutes to acquire.

Quality control

Post-acquisition, imaging data is exported for further analysis, beginning with image segmentation based on nuclear and surface markers, as well as cohort-wide synchronicity analyses, including consistency in cell coverage of tissue regions of interest (ROI) and frequencies of detected markers across all acquired ROIs. Each segmented cell is then phenotype based on the surface and cytoplasmic markers included in the panel. Single-cell phenotyping can then be used to inform spatial analyses, including cell-cell interactions, neighborhood analyses, directional information, and other spatial/morphological considerations, all of which can be leveraged by the aforementioned machine learning algorithms to develop predictive models.

Limitations and looking forward

HDPI technologies vary in accessibility. Many require rigorous optimization of antibody clone/DNA probe and concentration, which must be replicated for new tissue types or tissue preservation methods. Additionally, given the elevated acquisition time, larger cohorts require significant time investment for acquisition, particularly for cyclical staining and imaging methods.

Looking forward, three-dimensional imaging mass cytometry–based modeling has recently been achieved by acquiring serially sectioned tissue samples and assembling the images post

acquisition.[130] This method could be applied to other high-dimensional imaging modalities to offer an understanding of cells in 3D space, with promise for application in reproductive tract tissues.

Analytical considerations

Now that we have introduced a number of both proteomics and sequencing-based technologies to measure biological signals at a single-cell level; herein, we present several analytical steps and considerations that are useful for deriving biological insights from the resulting, high-dimensional single-cell data. Analytical approaches are further discussed in the next chapter by Llera-Oyola et al in the context of the single-cell sequencing technology, but here we provide an overview of shared principles across data modalities discussed above.

Data preprocessing: batch correction, normalization, and filtering

Raw data from the sequencer, cytometer, or relevant tool requires careful examination and standardized processing for the data to be in such a form that it can be successfully parsed for biological signals. This varies highly by data modality though will typically include a combination of batch correction, normalization steps, quality filters, and demultiplexing in cases where samples are multiplexed. Fortunately, workflows and tools are abundant for the discussed modalities, though these approaches are ever evolving. For mass cytometry data, typical preprocessing includes bead-based normalization to account for variance and decay in machine sensitivity.[131] Subsequent steps include single-cell debarcoding should samples be multiplexed[91,102] and manual gating for viable singlets. Should there be multiple batches run, batch correction may be run using algorithms such as CyCombine and CytoNorm.[132,133] Flow cytometry includes similar preprocessing steps though must include compensation of fluorescence based on spectral overlap and autofluorescence.[134]

Single-cell sequencing data requires a series of steps that are discussed in detail in the next chapter. In brief, data must be aligned to the genome with tools such as CellRanger (10X Genomics), followed by quality filtering to remove dying cells, contamination, and empty droplets. Additional tools may be run to remove doublets,[135] though should data be multiplexed, a large proportion of doublets will be identified and removed as part of the demultiplexing process.[29] Filtering scRNA-seq data and downstream analysis below can be performed using the R tool Seurat[43] and python's SCANPY.[136] Batch correction methods such as Harmony and CCA are additionally applied to account for technical variation while attempting to preserve true biological variation.[137,138] Other modalities such as snATACseq have specific tools such as ArchR[139] for other needs such as peak calling and modality-specific filtering. Similarly, spatial transcriptomic technologies discussed above have additional processing tools including Space Ranger and Xenium Ranger (10X Genomics).

Identifying cell populations: clustering, gating, annotation

A key step in the analysis of single-cell data is the grouping and identification of cell populations of interest. This can be pursued in an unsupervised manner using clustering

approaches or by using prior knowledge such as using manual gating in the case of cytometry data. Clustering, or the task of grouping cells in such a way that similar cell observations are placed in the same group (called a cluster), is often 1 of the first steps in the analysis of high throughput data. A series of clustering algorithms is routinely applied to high-dimensional single-cell data that are reviewed in detail elsewhere.[140,141] Some of the most common include flowSOM, Phenograph, and CLARA in cytometry data[142,143] and Louvain and Leiden clustering that are community-based detection algorithms used frequently in scRNA-seq data.[144,145]

Cell type annotation, whether based on proteomic or transcriptomic markers, follows initial clustering or gating of the data to identify cell types of interest such as immune or epithelial cells. There are both automated[146] as well as manual approaches to carry out cell annotation which further allows exploration of the data for individual cell types of interest.

Dimensionality reduction

Dimensionality reduction, or the transformation of data from a high-dimensional space into a low-dimensional space so that the low-dimensional representation retains some meaningful properties of the original data, is often used to both visualize and in some cases to derive additional shared signal from the types of data described. For example, standard practice for scRNA-seq data is to perform principal component analysis on the gene expression data followed by a graph-based dimensionality reduction method such as t-SNE or UMAP to visualize the data in a two-dimensional representation that preserves higher dimensional distances.[147] Dimensionality reduction can also help identify covarying features in the data such as gene modules or programs from transcriptomic data, using approaches such as WGCNA and NMF-based algorithms.[148–150]

Differential analysis

Robust approaches of pre-processing and population identification allows for further analysis of these populations across sample groups of interest. These comparisons can be differential abundance, that is, the relative number of cells of a given cell type in samples from 1 group versus another or differential levels of another measured property such as gene expression (scRNA-seq) or protein level (cytometry). This allows us to compare groups of interest and identify disease-associated signals. These cell-type–specific features can be further used as inputs in machine learning models for outcome prediction or other related questions. Features that are identified as differentially expressed can be additionally assessed using tools for pathway enrichment[151] on a cell-specific level.

Data integration

Data integration has become of increasing interest as single-cell datasets and multimodal measurements expand. Several types of integration goals exist, which then dictates the type of approach to use for the integration. Horizontal integration refers to the integration of datasets from the same type of measurement, such as multiple scRNA-seq datasets. This type of integration could be used for example to integrate several studies that for example profile endometriosis vs healthy controls using scRNA-seq, thereby increasing your power for detection of disease-specific signal. Then there is integration of multiple modalities

derived from the sample. This can be vertical integration or “paired” integration, meaning the measurements are derived from the same cells such as CITEseq (transcriptomic and proteomic measures from the same cells). Alternatively, diagonal or “unpaired” integration refers to different measures from different cells from the same sample or patient. An example of this would be if a patient sample was aliquoted for the run on a series of different modalities such as some of it used for mass cytometry, some used for scRNAseq. Approaches for analysis across each of these scenarios have been reviewed[152] and can tremendously powerful for leveraging these complex datasets for further understanding of disease.

Summary

Single-cell technologies have revolutionized biological research, delving into cellular heterogeneity at an unprecedented level—spanning proteomics, transcriptomics, genomics, and epigenetics. They play a pivotal role in unraveling intricate biological systems and have significantly advanced reproductive sciences. This review delivers an exhaustive examination of current single-cell technologies, detailing their fundamental principles, methodologies, and constraints. In today’s single-cell research landscape, it is crucial for scientists to identify and utilize the most apt technologies for their studies. Aiding in this decision-making, we have classified these technologies according to 3 principal factors: the extent of features captured, cell processing throughput, and accessibility, which includes the robustness of the analysis pipeline (Figure 4). For example, flow cytometry (represented by green dots) is characterized by its high throughput and ease of access, yet it has limitations in the range of features it can capture. On the other hand, sc/snRNAseq (indicated by red dots) is proficient in detecting a wide spectrum of features and remains highly accessible, but it falls short in processing a high volume of cells. As the complexity of biological systems unfolds, single-cell technologies progress towards concurrent measurements within individual cells. Yet, the incorporation of orthogonal measurements through complementary assays imparts robustness to scientific findings. Hence, it remains crucial to acknowledge that technologies with fewer captured features, but higher throughput, remain indispensable in the researcher’s toolkit.

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GLOSSARY OF TERMS

Ambient RNA
Cell-free mRNA that is released from dead, dying, or low-quality cells and nuclei.

Batch correction
A method which aims to remove technical variation from the data preventing this variation from confounding downstream analysis.

Batch effect
When non-biological factors in an experiment cause changes in the data produced.

Demultiplexing
The process of computationally separating individual samples which were pooled together in an assay.

Epigenome
The record of the chemical changes to the DNA and histone proteins of an organism, often assessed by analysis of chemical modifications of the genome.

Flow cytometry
A technique used to detect and measure the characteristics of cells or particles.

Fluorochrome/Fluorophore
Chemical molecules that can absorb light of a certain wavelength and then re-emit light at a longer wavelength.

High-dimensional
Data whose dimension is larger (relative to the number of datapoints) than typically considered in classical multivariate analysis.

High-performance computing (HPC)
The ability to process data and perform complex calculations at high speeds uses super-computers and computer clusters to solve advanced computation problems.

IVF
In vitro fertilization.

Mass cytometry
A mass spectrometry technique based on inductively coupled plasma mass spectrometry and time of flight mass spectrometry used for the determination of the properties of cells.

Microfluidics
A system that manipulates a small amount of fluids using small channels (10–100 μM).

mRNA
Messanger RNA.

Multimodal

Relating to, having, or utilizing more than 1 mode or modality.

Multiplexing
Combining of multiple samples into a single pooled sample in which downstream assays can be performed.

Proteome
The set of proteins expressed by an organism, at a specific time, or in a specific cell type.

RNA-sequencing
A technique that uses next-generation sequencing to reveal the presence and quantity of RNA molecules in a biological sample, providing a snapshot of gene expression.

Sequencing
Laboratory technique for determining the exact sequence of nucleotides, or bases, in a DNA or cDNA molecule.

Spectral overlap
The phenomenon when a fluorochromes exhibits fluorescence that “spills over” into a detector channel where it is not expected to show up.

Transcriptome
The set of all RNA transcripts, including coding and non-coding, in a sample.

Transposase
A class of enzymes capable of binding to the end of a transposon and catalyzing its movement to another part of a genome, typically by a cut-and-paste mechanism or a replicative mechanism, in a process known as transposition.

Transposon
A transposable element in DNA that can change its position within the genome.

t-SNE
t-distributed stochastic neighbor embedding is a statistical method for visualizing high-dimensional data by giving each datapoint a location in a 2 or three-dimensional map.

UMAP
A dimension reduction technique that can be used for visualization similarly to t-SNE, but also for general non-linear dimension reduction.

UMI
Unique molecular identifiers, a type of molecular barcoding that provides error correction and increased accuracy during sequencing.

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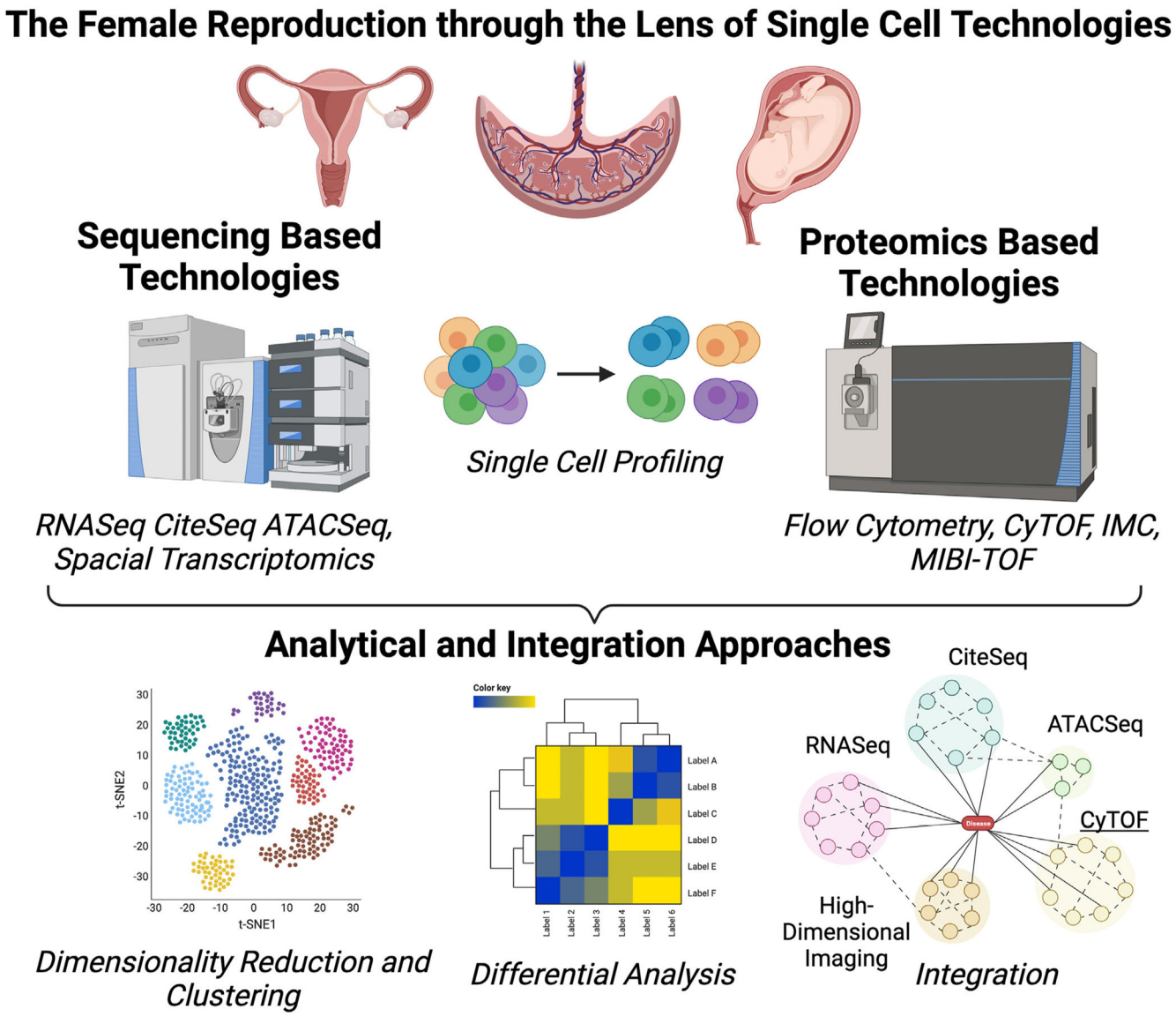

FIGURE 1. Overview

Here, we present a number of single-cell profiling technologies including sequencing-based approaches such as single-cell sequencing and spatial transcriptomics and proteomics-based approaches such as flow cytometry, CyTOF, high dimensional imaging approaches with examples of applications in reproductive health. We further discuss the benefits and drawbacks of each approach and end with a brief discussion of computational considerations for downstream data analysis and integration.

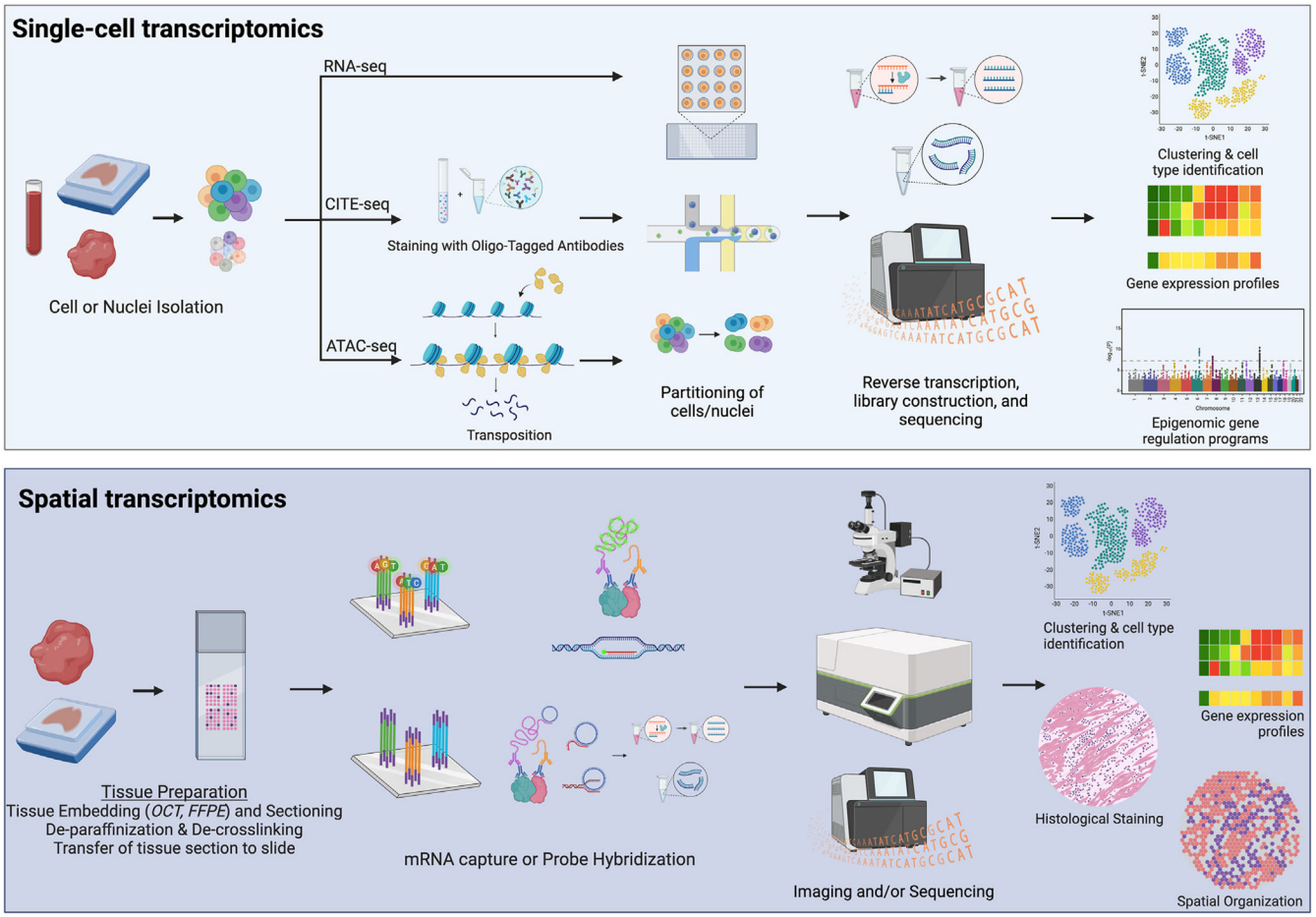

FIGURE 2. Summary of sequencing-based single-cell profiling technologies

Workflow for single-cell transcriptomics and spatial transcriptomics. Single-cell transcriptomics require isolation of either individual cells or individual nuclei for further processing and data collection. Subsequently, data analytical methods analyze cell genotypes or epigenotypes. Spatial transcriptomics requires preparation of preserved tissue for data collection involving mRNA capture or probe hybridization for subsequent sequencing and analysis of gene expression for either single cells in situ or regions of tissue.

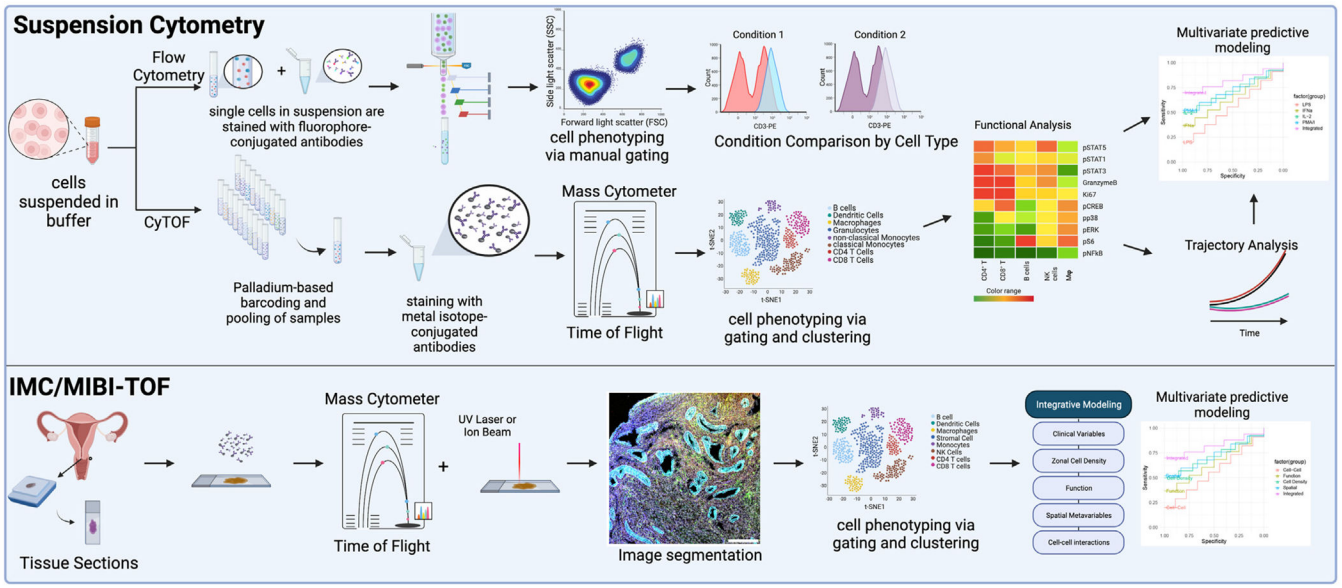

FIGURE 3. Summary of proteomics-based single-cell profiling technologies

Workflow for suspension cytometry and imaging mass cytometry (IMC) and multiplexed ion beam imaging by time of flight (MIBI-TOF). Each technology involves processing and staining of cells or tissues with either fluorophore-labeled or metal isotope-labeled antibodies, acquisition of data with a flow cytometer or a mass cytometer, and analysis, which can include phenotyping, dimensionality reduction, and machine learning–based modeling. Integrative modeling with mass cytometry can include immune stimulation conditions which influence functional data, while integrative modeling with IMC/MIBI can include spatial factors as well as functional data.

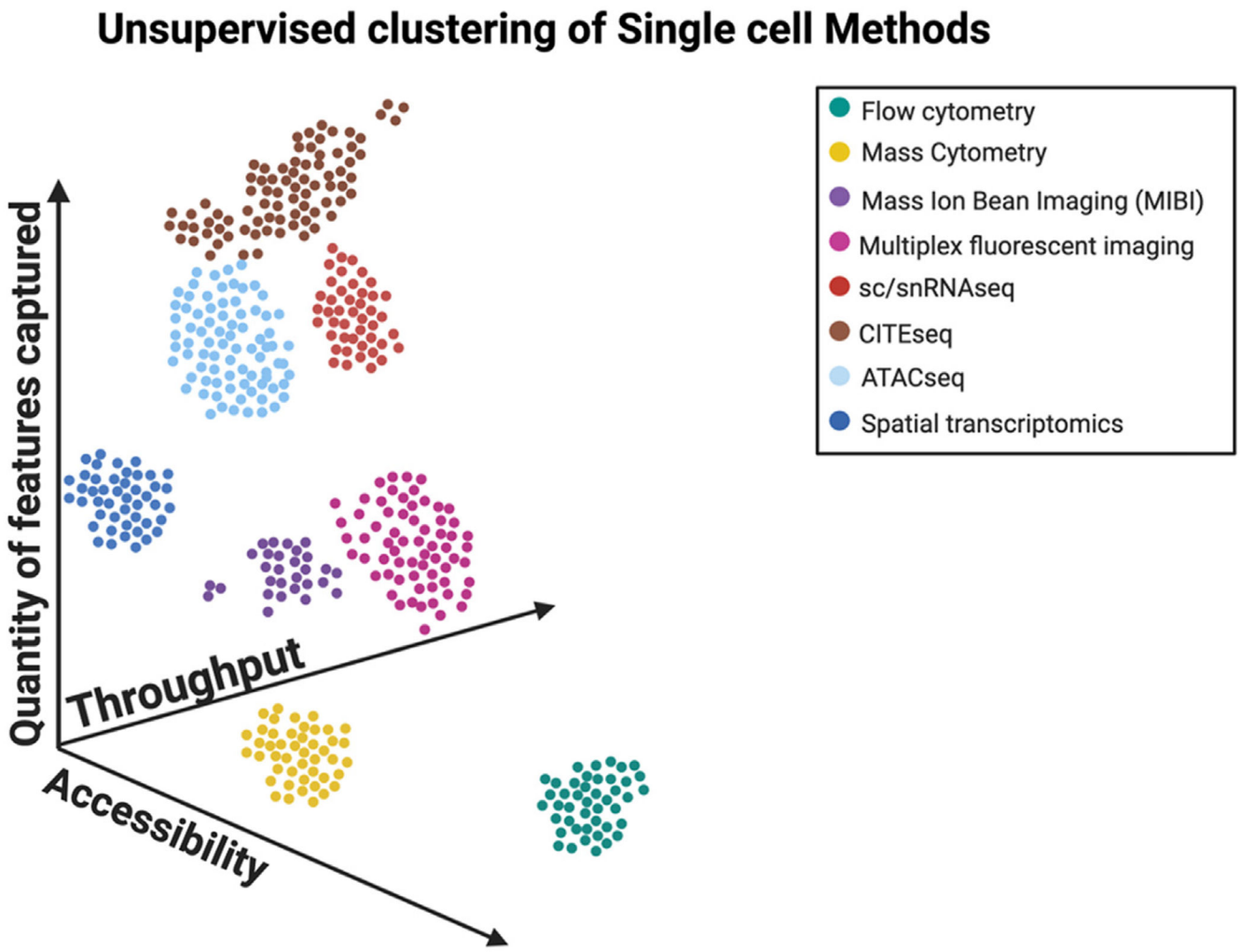

FIGURE 4. Unsupervised clustering of single-cell profiling methods

Here, we categorize the technologies based on 3 key criteria: the number of features captured, cells throughput, and accessibility, which also incorporates the robustness of the analytical pipeline. For instance, flow cytometry (*green* dots) offers high throughput and accessibility but captures a limited range of features. In contrast, sc/snRNAseq (*red* dots) excels in capturing a vast array of features and maintains high accessibility, yet it is limited by a lower throughput in terms of the number of cells measured.

TABLE

Benefits and caveats of single-cell proteomic and sequencing-based technologies

Technology	Input	Output	Benefits	Caveats
Flow cytometry	Fixed or live cells in suspension	Single cell spectral output → protein expression	• Well-developed and widely available • Cells can be recovered and sorted	• Spectral limitations • Reliance on compensation
Mass cytometry	Fixed cells in suspension	Single cell isotope reading → protein expression	• High level of detail per sample (40–60 markers) • Collection of up to 20 unique samples at once • 10^6 cells per hour acquired	• Cells cannot be recovered • High optimization time • Low sample yield
High-dimensional proteomic imaging	Sectioned preserved tissues or organoids	250 nm^2-1 μm^2 isotope reading → spatially resolved subcellular protein expression	• High level of detail per sample (40–60 markers) • Use of archival tissue • Single cell spatial resolution	• High optimization time • High acquisition time • Analytical pipelines in development
RNA-seq	Fixed or live cells or nuclei in suspension	Single cell mRNA capture→ gene expression	• Widely available • High throughput • Whole transcriptome • Wide range of sample origin • Multiplexing capabilities	• No spatial information • No proteomic information • No epigenomic information
ATAC-seq	Freshly isolated nuclei in suspension	Single nucleus accessible chromatin fragment capture → epigenetic information	• Single-cell resolution • High throughput • Epigenomic analysis • Whole genome • Multiplexing capabilities	• No spatial information • No transcriptomic information • No proteomic information
CITE-seq	Fixed or live cells or nuclei in suspension	Single cell mRNA and oligo-conjugated antibody capture → gene and protein expression	• Single-cell resolution • High throughput • Whole transcriptome • Cell-surface protein analysis • Multiplexing capabilities	• No spatial information • No epigenomic information
Spatial transcriptomics	2D or 3D cell cultures Sectioned preserved tissues or organoids	50 nm^2 to 1 cm^2 tissue imaging/ sequencing → spatially resolved gene and protein expression	• Spatial analysis of tissues • Can have subcellular resolution • Cell-surface protein analysis • Epigenomic analysis	• High cost • Low throughput • Limited gene/protein detection • Time-consuming • May not always have single cell resolution • Analytical pipelines in development