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REVIEW

Integrating Micro and Nano Technologies for Cell Engineering and Analysis: Toward the Next Generation of Cell Therapy Workflows

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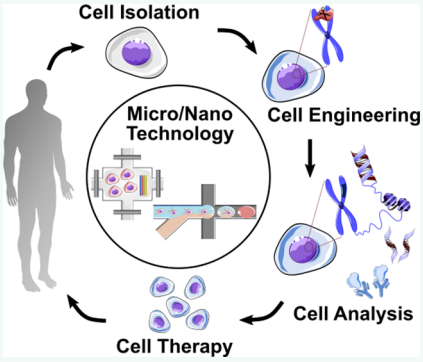
ABSTRACT: The emerging field of cell therapy offers the potential to treat and even cure a diverse array of diseases for which existing interventions are inadequate. Recent advances in micro and nanotechnology have added a multitude of single cell analysis methods to our research repertoire. At the same time, techniques have been developed for the precise engineering and manipulation of cells. Together, these methods have aided the understanding of disease pathophysiology, helped formulate corrective interventions at the cellular level, and expanded the spectrum of available cell therapeutic options. This review discusses how micro and nanotechnology have catalyzed the development of cell sorting, cellular engineering, and single cell analysis technologies, which have become essential workflow components in developing cell-based therapeutics. The review focuses on the technologies adopted in research studies and explores the opportunities and challenges in combining the various elements of cell engineering and single cell analysis into the next generation of integrated and automated platforms that can accelerate preclinical studies and translational research.



KEYWORDS: *cell therapy, cell sorting, cell engineering, intracellular delivery, single cell analysis, bioinformatics, multi-omics, spatial-omics, integrated workflows, clinical translation*

INTRODUCTION

The use of cells as therapeutics offers major advantages over conventional drug therapy as they can simultaneously and dynamically perform complex biological functions based on their ability to home to specific organs, perform cell−cell interactions, and secrete bioactive factors. Cell therapies have the potential to treat a diverse array of diseases, and available therapeutic targets include a spectrum of cell types, cellular functions, and processes. To realize the therapeutic potential of cells, measuring the abundance of all molecular classes and their spatiotemporal interactions from single cells, in healthy and diseased tissues, has been a long-standing goal. This is critical not only for understanding the mechanisms of diseases but also for developing effective therapeutic interventions. However, obtaining this extent of information from a single experiment is still impractical. Nevertheless, recent advances in micro and nano technologies, molecular biology, and bioinformatics methods have enabled the investigation of the molecular composition, structure, and function of individual cells in great detail, bringing us closer to realizing this goal. Over time, these technological advances have evolved our understanding of heterogeneity in molecular abundance and distribution at the cellular level, giving us insights into its critical role in physiological processes, diseases, and response to treatments. The rapid development of these fields has ushered in an era of "omics" scale analysis. Researchers have frequently adopted single cell technologies to examine the genome, transcriptome, epigenome, proteome, and their combinations at high resolution for investigating disease progression and designing mitigative strategies.[1−8] At the same time, micro and nano technologies have enabled the precise engineering of cells, leading to opportunities in developing cell-based therapeutics.

The concomitant progress in cellular engineering and single cell analysis technologies has enabled high-throughput study

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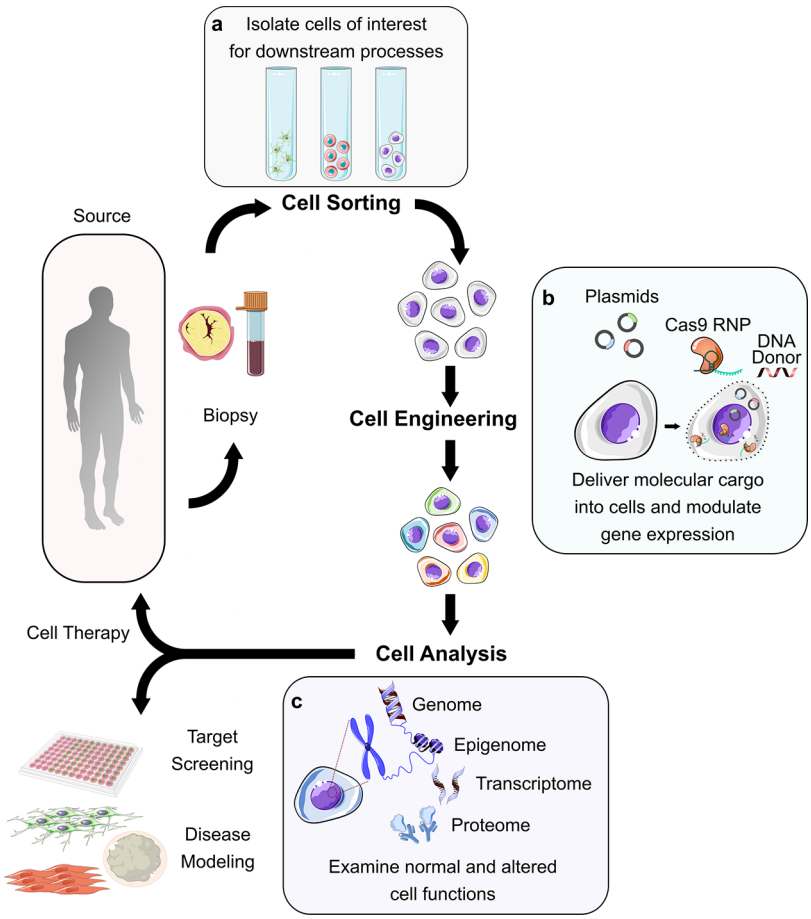

Figure 1. Cell Therapy Development Workflow. (a) Sorting and isolating specific cell types to be studied from a heterogeneous population is an important step in the cell therapy development workflow. For example, it may be required to isolate tumor infiltrating lymphocytes (TILs) from a tumor biopsy to characterize their phenotype or hematopoietic stem cells (HSCs) from whole blood for correcting a disease-causing mutation. (b) Engineering isolated cells is often another key process in the pipeline. This is achieved by the intracellular delivery of exogenous molecules that alter the gene expression of cells. For instance, a study may involve the introduction of a Cas9/gRNA plasmid library to perform a pooled knockout screen. Alternatively, it may be required to introduce a specific CRISPR/Cas9 RNP to treat a monogenic disorder and generate therapeutic cells. (c) Analyzing the phenotype of the isolated or engineered cells is another important step in the cell therapy development process. Applications for this step can range from screening genetic drivers of diseases to validating the results from a corrective therapy.

designs that span a broad spectrum of the cell therapy development workflow (Figure 1), ranging from target discovery and validation in diseases to determining the efficacy of therapeutics in clinical trials. Often these workflows involve three key processes: (1) isolating the cells of interest, (2) intracellular delivery for cell engineering, and (3) performing the single cell assays and their bioinformatics analyses that quantify the target molecular species in the engineered cells. Several recent studies addressing various aspects of cell therapy development, have workflow designs involving these three key processes.[9−14] Micro and nano technologies have played a significant role in developing the single cell analysis methods. Additionally, micro and nano technologies have been instrumental in developing cell engineering and isolation systems that could potentially replace some of the conventional techniques essential to these workflows. In this review, we discuss how micro and nano technologies have enabled the next generation of cell engineering, cell isolation, and single cell analysis methods and focus on some of the most promising platforms that are increasingly being employed in studies targeted toward developing cell-based therapeutics. We then look at the prospects of incorporating key elements from current technologies into the next generation of integrated platforms for precision cell engineering, followed by high-throughput and high content analysis that can aid in biomarker discovery and designing effective therapeutics.

CELL SORTING AND ISOLATION

Isolating the cells of interest from complex tissues is integral to most biological workflows involving cell engineering and analysis. Single cell isolation constitutes two steps for solid tissues (e.g., solid tumors). The first step involves mincing and disaggregation of the tissue, enzymatic digestion, and finally filtration to remove debris and extracellular components, which results in a heterogeneous cell suspension. This heterogeneous mixture is sorted to obtain the desired cell population in the second step. Single cell isolation in the case of liquid biopsies (e.g., blood samples) involves only the second step of cell sorting. A variety of microfluidic platforms for disaggregation, digestion, and filtration of solid tissues have been implemented, increasing single cell recovery, minimizing stress on cells, improving processing times, and reducing manual labor compared to conventional methods.[15−17] However, these technologies have not matured enough yet to be employed

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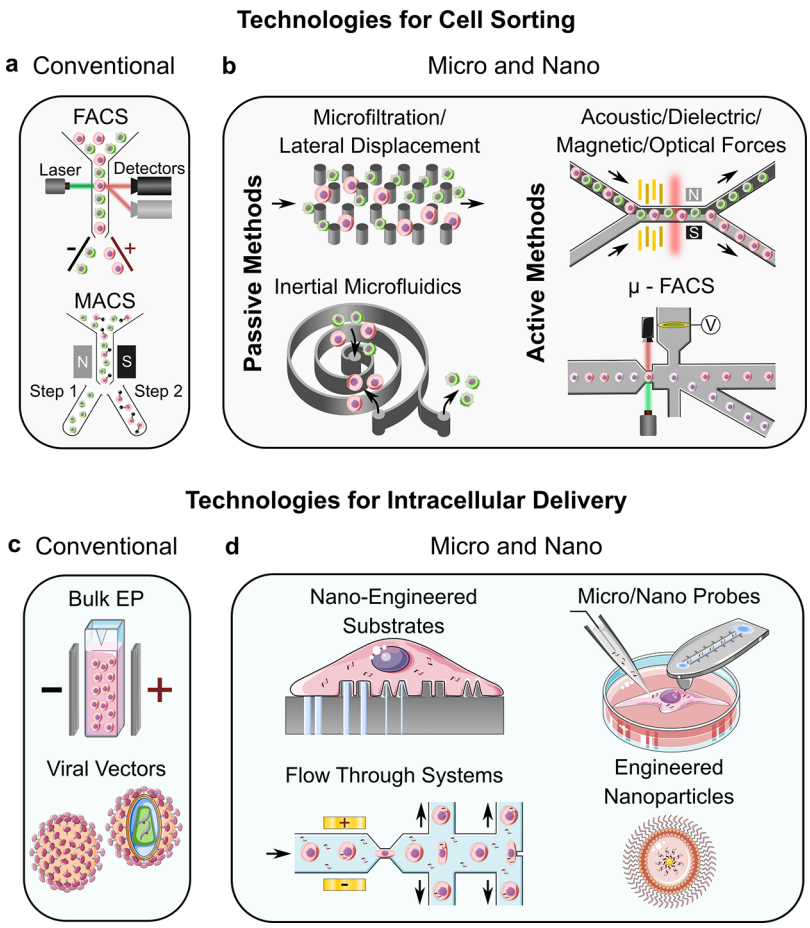

Figure 2. Technologies for cell sorting and intracellular delivery. (a) Conventional sorting methods include Fluorescence Activated Cell Sorting (FACS) and Magnetic Activated Cell Sorting (MACS). In FACS an electric field deflects cells in electrostatically charged droplets into different bins based on optically determined phenotype. In MACS cells are labeled with antibodies attached to magnetic beads and separated using a magnetic field. (b) Microfluidic cell sorting methods can be active or passive. Passive methods use flow obstructions or hydrodynamic forces such as inertial flows for cell separation. Active methods involve external forces of which acoustic, dielectric, magnetic, and optical forces are common. *μ*FACS is another emerging active method where sorting is performed in a microfluidic channel with gentle actuation forces that causes much less cell stress compared to conventional FACS. (c) In cell engineering applications, bulk electroporation and viral vectors are the commonly used intracellular delivery methods. (d) Microfluidic platforms for intracellular delivery can be categorized into nanoengineered substates, flow through systems, probe-based methods, and engineered nanoparticles.

in widely adopted commercial solutions. Hence, in this review we will restrict ourselves discussing of technologies used for the second step, i.e., sorting cells from heterogeneous suspensions. Cells can be sorted in a label-free manner based on a range of intrinsic properties or via labeling specific cell surface markers using antibody tags. In this regard, Fluorescence Assisted Cell Sorting (FACS) and Magnetic Assisted Cell Sorting (MACS) continue to be the gold standard and are widely used for cell sorting. Another commonly used method for cell sorting is density gradient centrifugation. Cells are suspended in a density gradient medium (e.g., Ficoll solution) before centrifugation such that cells of different densities equilibrate in different layers of the solution and can be separated. It is important to note that many single cell analysis methods use next-generation sequencing (NGS) as their readout and sorting cells before analysis can be a necessary part of the protocol (e.g., for SmartSeq[18,19]) or could be used to enrich the sample for the target population. This ensures that rare target subpopulations are adequately represented in a data set without sequencing many nontarget cells, thus reducing sequencing cost. Common usage scenarios include studies involving a rare cell type (e.g., effector T cells) within a heterogeneous cell sample or screening and lineage tracing studies where only the cells that have been correctly engineered to exhibit a specific phenotype need to be analyzed. Moreover, enriching the target population may improve the overall outcome of cell engineering in therapeutic applications. For example, hematopoietic stem cells (HSCs), defined by the markers $CD34^+CD90^+CD45RA^-$, can be enriched by immunostaining and cell sorting. A similar level of therapeutic effect has been demonstrated using highly enriched HSCs instead of hematopoietic stem and progenitor cells (HSPCs) while allowing a 10-fold reduction in the number of cells that need to be edited and transplanted along with the requirement for editing reagents.[20] Sorting steps can also provide cytometry data to complement downstream sequencing for multiomic analysis.[21] This has been especially useful in studying immune cell populations that can be classified based on well-defined surface markers. Additionally, certain cell types (e.g., granulocytes) are difficult to process on many single cell sequencing platforms due to their low RNA and high RNase content, and cytometry data can highlight these missing or underrepresented cells in the biological problem under

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Table 1. Summary of Technologies for Cell Sorting and Isolation

Separation Technique	Working Principle	Advantages	Disadvantages	Commercial Technologies
		Conventional		
Fluorescence Activated Cell Sorting (FACS)	Optical detection of cell properties—morphology, antibody labels. Electrostatic separation of cells within charged droplets.[243]	High-throughput (20,000–100,000 cells/s). Cell sorting based on multiple parameters. Imaging-based flow sorters provide intracellular information and morphometry.	Requirement of complex, specialized equipment and trained personnel. Possibility of excessive stress or cell damage. Recovery is 70–80%, which may not be ideal for rare cell types.	Several FACS solutions are provided by instrumentation companies like BD Biosciences, Thermo Fisher Scientific and Agilent Technologies.
Magnetic Activated Cell Sorting (MACS)	Labeling of cell surface markers with antibodies tagged to magnetic beads. Separation of labeled cells using external magnetic field.[244]	High-Throughput (>100,000 cells/s). High specificity and purity (>90%) enable separation of subtle phenotypic differences. Relatively simple to use.	Reliant on surface markers and high-quality antibody labels. Effect of labeling on cell phenotype not totally known.	MACS (Miltenyi Biotec), Dynabeads (Thermo Fisher), Mojosort (BioLegend)
		Micro and Nano Technology - Passive		
Filtration Mechanisms	Exclusion based on size, shape or deformability.[26,27]	Simplicity of process and low cost. Label-free. Generally high throughput ($\sim 10^5$–10^6 cells/s).	Clogging issues. Cell size/shape dependent device design. Cell recovery may be low (~50%) depending on design.	Parasortix (Angle), CellSieve (Creatv MicroTech), ScreenCell (ScreenCell)
Deterministic Lateral Displacement (DLD)	Difference in hydrodynamic forces on cells of different sizes and shapes, when flowed passed geometric arrays.[28,29]	Simplicity of application. Label-free process.	Medium throughput (~10,000 cell/s). Clogging issues and cell type dependent device design. Purity ranges from 50 to 90%, depending on design and cell type.	Curate Cell Processing System (Curate Biosciences)
Inertial Microfluidics	Balance of lift and drag forces or generation of secondary vortices to separate cells into different streamlines.[32,33]	High throughput ($\sim 10^5$–10^6 cells/s). Label free. Simple implementation. High purity (>90%) for investigated cell types has been reported.	Individual cells are difficult to analyze. Some designs may be prone to clogging and lower recovery (~50%).	ClearCell FX1 System (Biolidics Limited), Vortex (Vortex Biosciences)
		Micro and Nano Technology - Active		
Magnetic Forces	Separation of cells labeled with magnetic particles using external magnetic field.[37,38]	High throughput (>100,000 cells/s). High specificity and purity (>90%).	Magnetic labels can alter cell properties. Reliant on distinct surface markers and availability of good antibodies.	IsoFlux (Fluxion Biosciences), LiquidBiopsy (Cynvenio Biosystems)
Dielectric Forces	Cells separated based on their polarizability and localization in different regions of a nonuniform electric field.[39,40]	Label-free. Easy to integrate with other modalities. Generally high purity (>80%).	Throughput can be low for some designs (100–5,000 cells/s). Electric fields can damage cells.	ApoStream (ApoCell)
Acoustic Forces	Cells separated using acoustic pressure waves.[41,42]	Label-free and reduced cell perturbation. Reported purities are >80%.	Throughput can be low (1,00–5,000 cells/s). Excessive heating may be an issue.	AcouTrap/AcouWash (AcouSort)
Optical Forces	Cells are manipulated using optical radiation force.[43,44]	Low invasiveness. High separation resolution.	Complex and costly instrumentation. Throughput can be low (~1000 cells/s).	Beacon (Berkeley Lights)
μ-FACS	Separation principle similar to FACS but in a microfluidic channel. Actuation can be piezoelectric, pneumatic, optical or electrostatic.[245,246]	In addition to the advantages of conventional FACS, perturbation on cells is lower in μ-FACS.	Instrumentation can be complex. Throughputs are lower than conventional FACS (100–10,000 cell/s).	WOLF Cell Sorter (NanoCellect), Hana/Pala (Namocell), Gigasort (Cytonome), On-Chip Sort (On-Chip Bio), Single Cell Dispensers (Cytena)
Integrated Methods	Combination of microfluidic methods to leverage respective advantages.[47,48]	Combination of methods can provide higher throughputs (up to 10^7 cells/s) and purity (>95%).	Complexity of implementation. Due to differences in flow rates and architectures, integration may be challenging.	TellDx (TellBio) - Inertial and Immunomagnetic

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investigation.[22,23] Despite their widespread use, commercial FACS platforms have significant drawbacks, including high cost, specific sample requirements (e.g., specialized buffers and cell density or number), and sophisticated, bulky instrumentation requiring dedicated facilities with in-house technical expertise. This may also pose logistic challenges while integrating with downstream single cell analysis experiments since the transfer of samples across platforms and facilities increases the risk of contamination or degradation. Moreover, FACS exerts excess fluid shear stress on cells that can affect cell viability and function especially in the case of sensitive primary immune and stem cells,[24,25] which may confound the single cell analysis results. On the other hand, centrifugation-based methods require large sample volumes, can be challenging to integrate with downstream cell engineering and analysis steps without extensive manual intervention, and are often limited to the separation of blood cell types, which exhibit significant differences in density.

To overcome the limitations mentioned above, researchers have actively pursued microfluidic sorting approaches (Figure 2). Most research has been focused on developing label-free microfluidic sorting methods that harness hydrodynamic or external force fields. Passive microfluidic cell separation methods that rely on hydrodynamic forces include filtration mechanisms (e.g., using micropores or pillars),[26,27] deterministic lateral displacement (DLD),[28,29] and pinched flow fractionation (PFF).[30,31] These methods rely on the physical properties of cells (e.g., size, shape, and deformability) and the characteristics of the flow field through specific microfluidic geometries, for separating cells. Hydrodynamic systems have been shown to offer a range of throughputs and sorting efficiencies/purities for different cell types (Table 1). However, one critical limitation of these systems is that the device design (e.g., pore diameter for filtration or pillar dimensions and pitch for DLD) needs to be tailored to the cells under investigation, making them less generalizable for different sorting applications. Another class of hydrodynamic methods uses inertial forces generated due to channel curvature[32] or undulations,[33] which determine the equilibrium position of particles in the microchannel cross-section, to separate cells of different sizes. By tuning the geometric and flow parameters in inertial microfluidic devices, sorting different cell types has been possible.[34−36] Inertial microfluidic systems are simple to implement and provide high throughputs but require sample dilution to prevent cell−cell interaction. Despite these advancements, most hydrodynamic sorting technologies are still at proof-of-concept stages, and not many have been successfully commercialized. One major reason is that most of these methods employ cell type specific designs which are not universally applicable like FACS. Other drawbacks include device clogging or cell aggregation issues, lack of industrial scalability and low throughput. The hydrodynamic methods, however, have found niche applications such as isolation of circulating tumor cells (CTCs), with several emerging commercial products like the microfiltration-based CellSieve (Creatv MicroTech), and the Dean's flow-based ClearCell FX1 System (Biolidics Limited), to name a few.

Among the active microfluidic cell separation methods that utilize external force fields, magnetophoretic-,[37,38] dielectrophoretic-,[39,40] acoustophoretic-,[41,42] and optical force[43,44]-based separation are the most common (Table 1). As the system parameters in active methods are tunable, it is possible to adjust the same device for sorting different types of cells, thus providing greater flexibility than hydrodynamic methods. However, some of these systems are limited by their medium to low throughput. Hybrid devices have also been implemented to combine the advantages of hydrodynamic and external force fields, which have yielded improved throughputs and efficiencies while providing greater versatility across cell types.[45−48] For example, a recently reported method, microfluidic affinity targeting of infiltrating cells (MATIC), combined immunomagnetic sorting with hydrodynamic forces to demonstrate the isolation of tumor infiltrating lymphocytes (TILs) expressing moderate levels of CD39 with high purity (up to 98%) and yield (up to 30-fold higher).[47] The TILs isolated using MATIC were self-renewing and exhibited enhanced *in vivo* tumor killing potential in a mouse model. Another platform, the CTC-iChip combined DLD, inertial focusing, and magnetophoresis to separate CTCs from the whole blood.[49] One class of active cell separation method, conceptually similar to FACS but in a microfluidic format (μFACS), is now being used in commercial cell sorting solutions. These μFACS methods manipulate small volumes of fluid containing the cells in microchannels using various actuation methods (e.g., piezoelectric, pneumatic, optical, and electrostatic) for gentle cell sorting. They provide several advantages over conventional FACS, such as reduced shear stress, low cell damage, and handling small sample sizes. Moreover, μFACS methods can be combined with droplet microfluidics for cell encapsulation, cell sorting, and downstream omics analysis in integrated workflows.[50,51] Functionally, droplet encapsulation can enable additional readouts such as protein secretion (e.g., antibody secretion by B lymphocytes), metabolite levels and cell proliferation for sorting, which is not compatible with conventional FACS. Some of the commercially available sorters that use μFACS principles include the Hana single cell dispenser (Namocell), the WOLF cell sorter (NanoCellect), and the On-chip Sort System (On-chip Biotechnologies). These sorters are also compact benchtop units that can eliminate sample handling challenges present with the usage of FACS in shared facilities. Finally, sorting based on imaging cytometry is gaining traction,[52,53] which when combined with deep learning enhanced analysis on these microfluidic platforms, can provide advanced sorting functionalities and high-dimensional information about cell morphology.[54]

Apart from microfluidic chip-based single cell sorting and isolation techniques, there have been advances in the design and implementation of miniaturized robots for the direct capture, transportation and manipulation of cells that have the potential for use in precision single cell biopsy from tissues or tumors.[55−57] One recent example is the use of untethered thermoresponsive grippers for the capture of single live cells or the extraction of a few cells from a clump.[58] The gripper can be guided through *ex vivo* tissue using a directed magnetic field. Other commonly used actuation mechanisms for these soft micro robots include pneumatic control, electrostatic interactions, and shape memory materials, which have been discussed in detail elsewhere.[59] Although these technologies show great potential for single cell capture and manipulation, high throughput cell type agnostic solutions need to be further explored for their integration with downstream cell engineering/analysis platforms and adoption in clinical settings.

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Table 2. Summary of Technologies for Intracellular Delivery and Cell Engineering

Delivery Technique	Working Principle	Advantages	Disadvantages	Commercial Technologies
		Conventional		
Viral Vectors	Viral Particles encapsulating nucleic acids that can infect the target cells to express the foreign gene.[66]	Works for both *in vitro* and *in vivo* settings. High throughput (~10^8 cells per run). High efficiency. Works for most cell types including post mitotic cells.	Limited to nucleic acid delivery and restricted payloads. Difficult to spatially confine the delivery. Can trigger an immune response. Dosage control is difficult.	Viral vectors (lentivirus, adeno-associated virus etc.) are widely available from companies and distributors such as Takara Bio and AddGene
Bulk Electroporation	Cell membrane is permeabilized for cargo delivery by exposing the cells to a strong electric field for a short duration.[66]	High throughput (~10^7–10^9 cells per run). High efficiency for most cell types when optimized. Can deliver wide variety of molecular cargo.	High cell death for sensitive cell types (>80% cell loss reported in primary and stem cells). Changes in temperature and pH can damage cells or alter their function. Dosage control is difficult.	Several Commercial Bulk Electroporators are available in the market – Nucleofector (Lonza), Gene Pulser XCell (Bio-Rad), Neon (Thermo Fisher)
		Micro and Nano Technology		
Nano-Engineered Substrates	Substrates with high-aspect ratio nanostructures such as nanochannels, nanostraws or nanoneedles are used to penetrate or locally electropermeabilized the cell membrane for cargo delivery.[75,78,80]	High-cell viability and efficiency reported in several studies. Superior dosage control compared to conventional methods. Has been shown to work with a wide variety of cells and delivery cargo.	Medium throughput (10^3 – 10^5 cells per run) which may not be sufficient for applications in biomanufacturing and cell therapy. May require extensive optimization of different cell types. Complex fabrication can be a hindrance to scaling up and widespread adoption.	Nanostraws (Navan Technologies), Celletto (Basilard Biotech)
Flow Through Systems	Cells are mechanically or electrically perturbed (using a constriction, an inertial flow or an electric field) as they are passed through a microchannel. This disrupts the cell membrane allowing for cargo delivery.[86,87,89]	High throughput (10^7–10^8 cells per run). Simple device architecture making it easy to scale up. Can work with different cargo types. High delivery efficiency, high cell viability and minimal cell perturbation has been reported in multiple studies.	Clogging issues. Cell type/size dependent device design. May require large volume of cargo. *In vivo* solutions do not exist.	CellSqueeze (SQZ Biotech, Roche), Flowfect (Kytopen)
Engineered Nanoparticles	Specially designed nanocarriers encapsulating the delivery cargo that can bypass biological membranes.[100,103]	Can be used for both *in vitro* and *in vivo* settings. Usually high throughput (~10^6–10^8 cells).	May be complex to synthesize. Potential cytotoxicity. Does not work with all cell types. Efficiency and dosage can be low/hard to control due to low endosomal escape for vehicles that rely on endocytosis.	Sophisticated nanoparticle vehicles are usually custom designed and synthesized in laboratories for specific applications. However, simpler liposomal or polymeric systems for cargo delivery like Lipofectamine (Thermo Fisher) and TransIT-X2 (Mirus Bio) are available commercially.
Micro/Nano Probes	Cell membrane is disrupted or electropermeabilized for cargo delivery using a solid or hollow probe (e.g., AFM tip or glass nanopipette).[91,93]	Cell selective cargo delivery which can be useful for cell–cell signaling studies. High-efficiency, high cell viability and precise dosage control.	Low throughput and scalability (10–100 cells per run). Complex instrumentation.	FluidFM (Cytosurge), Nanofountain Probe (Infinitesimal LLC)

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INTRACELLULAR DELIVERY FOR CELL ENGINEERING

Intracellular delivery of functional molecular cargo is critical in generating engineered cells with the desired phenotype. For instance, the combined usage of programmable nucleases like CRISPR/Cas for precise cellular engineering and single cell analysis has enabled experimental designs such as high-throughput and high-resolution genome-wide screens for targets implicated in diseases or having therapeutic potential.[60−62] Moreover, cells have been engineered to express molecular barcodes that enable tracking of clonal lineages in disease progression[1,63] and reconstitution of therapeutic cells *in vivo*.[64,65] This section will take a deeper look into the current technologies that enable precise engineering of cells and the upcoming micro and nano technologies that can succeed the existing methods.

Every delivery technique for cellular engineering applications has its own advantages and limitations. A few metrics to evaluate a delivery technique are minimum perturbation of normal cell health and function, scalability, the versatility of delivery in terms of cell types and cargo, delivery efficiency, dosage control, and cost. The two most used methods of delivery are viral vectors and bulk electroporation. Viral vectors allow for highly efficient and scalable cargo delivery even for postmitotic cells like neurons and are routinely used for *in vitro* and *in vivo* cellular engineering.[66] Not surprisingly, nearly all gene therapies currently available use one of three vector types: adeno-associated-virus (AAV) vectors, adenovirus vectors, or lentivirus vectors, with AAV being the most popular. Viral transduction has also been a method of choice for engineering hematopoietic lineage cells for therapeutic purposes.[13,67] Recently, viral vectors have been especially useful for randomly integrating a library of genetic modifications in a cell population, where individual cell perturbation and identity can be resolved using downstream single cell analysis. For instance, lentiviral transduction was used to label HSCs with heritable molecular barcodes for single cell lineage tracing.[68] Similarly, lentiviral transduction has also been used for combinatorial single-cell CRISPR screens by using perturbation libraries having multiple gRNA expression vectors.[69] However, viral vectors are limited by restricted payload, off-target expression, and immunogenicity; they require technical expertise to synthesize, making their implementation expensive and time-consuming.[70] Moreover, despite the advances in viral vector engineering, it is difficult to spatially confine the payload with viral particles, which may be important for certain *in vivo* applications. As a result, nonviral vector-based delivery has recently predominated over viral vector-based studies in investigations of gene-editing therapies. In particular, bulk electroporation, which exhibits high intracellular delivery efficacy in hard-to-transduce cells, has become a popular and safer choice for researchers. Especially in cellular engineering applications such as gene editing for screening and developing therapeutics, electroporation of gene editing machinery for transient expression is sufficient, potentially providing a safety advantage over viral delivery methods.[71] Despite its advantages and wide usage, bulk electroporation still lacks precise dosage control, leads to high cell death and has been reported to cause nonspecific cellular activation.[66,72] Therefore, there has been a need to develop delivery technologies that can confer safe, efficient, scalable and versatile intracellular delivery while conserving normal cell health and function. It is important to mention here that several other cell permeabilization techniques (e.g., thermal, optical and ultrasonic) have been developed for intracellular delivery. However, due to low throughputs, complex instrumentation and insufficient technological maturity, these methods are limited to proof-of-concept demonstrations and have not yet been widely adopted in biological workflows. The readers are referred to other reviews covering these methods in detail.[66,73,74]

Micro/nano technology-based delivery platforms provide an opportunity to improve upon the traditional delivery methods. The various micro/nano technologies that researchers have developed to circumvent some of the issues of viral and electroporation mediated delivery can be categorized into four groups: (1) Nano-Engineered Substrates, (2) Flow Through Systems, (3) Micro/Nano Probes, and (4) Engineered Nanoparticles (Figure 2 and Table 2). This subsequent section will look at the pros and cons of each of these methods and their potential integration into cell therapy pipelines.

Nano-Engineered Substrates. Substrate-based techniques use engineered biocompatible substrates with micro or nanoscale geometrical features to facilitate molecular delivery. A few prominent examples are high aspect ratio nanostructures such as nanochannels,[75−77] nanostraws,[78,79] and nanoneedles.[80,81] These platforms have been used to penetrate the cell membrane directly or apply a localized electric field and permeabilize the cell membrane to deliver molecular cargo into the cell cytoplasm or nucleus. The substrate-based methods have been reported to offer higher cell viability, greater uniformity, spatially targeted delivery, precise dosage control, and lower off-target cellular perturbation compared to traditional methods in several delivery studies.[76−80,82] As a result, these technologies are finding utility in engineering cells *in vitro* and *in vivo* for gene/cell therapy applications.[79,83−85] Thus, upcoming commercialized platforms based on these technologies may succeed bulk electroporation and viral delivery methods in cell therapy development pipelines. However, the throughput of substrate-based systems designed for *in vitro* delivery is limited to a moderate level (∼50,000 cells/device) which restricts their cellular engineering applications. For *in vivo* delivery, substrate-based methods have only been used for superficial tissues like the skin and muscles. Further technology development is necessary for delivery into other internal tissues and organs.

Flow Through Systems. Many promising flow-based cellular engineering techniques have emerged in the past decade. In these methods, cells flowing in a microchannel are subjected to a mechanical perturbation (e.g., constrictions and inertial flows)[86−88] or in combination with an electric field[89] to permeabilize them. These flow-based methods have been used to engineer various cell types, including primary immune and stem cells for therapeutic applications. Additionally, they have been shown to preserve cell integrity and function better than bulk electroporation.[72,90] The simplicity, scalability, and low cost of the flow-based methods make them ideal for in vitro applications and readily available to be incorporated into cell therapy workflows. Some of these platforms, such as the mechanoporation-based Cell Squeeze (SQZ Biotech) and the electroporation-based Flowfect (Kytopen), are already commercialized. However, the major limitations of these systems that may hinder adoption include cell type and size-dependent device design, microchannel clogging, and the requirement of a large volume of cargo at a high concentration for operation.

Micro/Nano Probes. Probe-based methods typically use nanopipettes or AFM tips to deliver cargo into the cells.[91−94]

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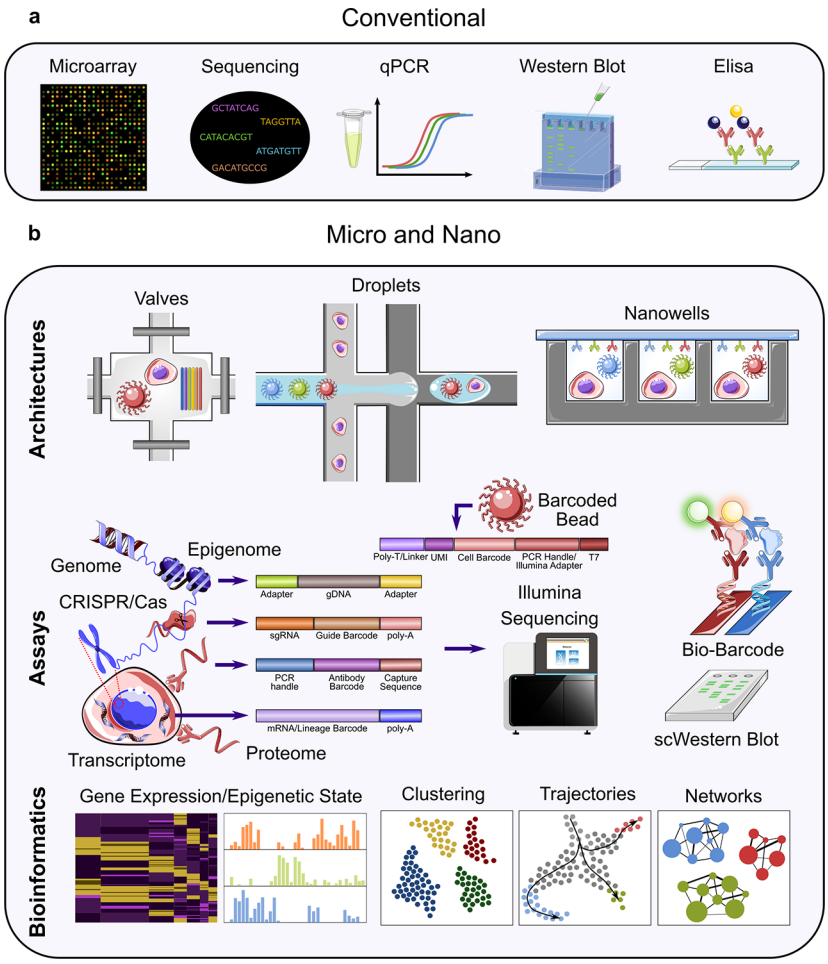

Figure 3. Technologies for cell analysis. (a) Conventional methods for genomic, transcriptomic, and proteomic analysis like microarrays, bulk-sequencing, qPCR, Western blots and ELISA do not provide single cell resolution. (b) Architectures: Pneumatic valves, droplets, and nanowells are the primarily used microfluidic architectures for compartmentalization of cells and miniaturization of molecular assays to provide single cell resolution. Assays: Engineering cells to express barcodes and capturing molecular species with barcoded beads has enabled multimodal molecular profiling while simultaneously capturing perturbation identity or lineage information from single cells using NGS. Non-NGS-based proteomic methods such as single cell biobarcodes and single cell Western blots are also employed for single cell profiling. Bioinformatics: Single cell omics data is high-dimensional and can be used to identify the different cell types involved in the biological process under investigation, look at their gene expression differences, arrange them along developmental trajectories, and build genetic networks to look at pathway interactions.

Some methods like the FluidFM (CytoSurge)[93] directly puncture the cell membrane and inject the payload inside the cell, while others like the Nanofountain Probe (NFP-E)[95,96] use localized electroporation for intracellular delivery. As the probes can be precisely controlled and positioned, these methods provide subcellular resolution over cargo delivery and are ideal for situations where cell-selective or spatially targeted cell engineering is necessary for a heterogeneous cell population. Probe-based methods have been used for engineering a wide variety of cell types *in vitro* and single cell targeted transfection *in vivo*.[97,98] However, one major limitation of probe-based methods is their serial nature and low-throughputs, which makes them unsuitable for integration into high-throughput workflows. Thus, their usage is restricted to single cell targeted cellular engineering and analysis tasks that cannot be addressed by other methods.

Engineered Nanoparticles. Due to advancements in nanotechnology and biochemical synthesis, intelligently engineered nanoparticles (NPs) have become promising candidates for cellular engineering applications both *in vitro* and *in vivo*. An important example is polymeric NPs, where the cargo is encapsulated in the NP core, entrapped in the polymer matrix, and chemically bonded to the polymer or the NP surface. For instance, charged polymeric NPs like poly-(amidoamine) (PAMAM) dendrimers that allow easy conjugation of charged cargo like nucleic acids serve as efficient vehicles for nucleic acid delivery.[99] Polymeric NPs are promising candidates for delivery as they are biocompatible, water-soluble, stable and chemically modifiable for targeted delivery.[100] Recently, a lipid nanoparticle (LNP) delivery system with liver tropism, encapsulating mRNA for Cas9 protein and a single guide RNA has been infused into patients with transthyretin amyloidosis to knockout the misfolded transthyretin (TTR) protein.[101] Another class of NPs is inorganic (gold, silica, iron oxide) NPs, with gold NPs being widely used due to their chemically inert, nontoxic nature, and easy attachment of different ligand species and chemical moieties for delivery.[102] For example, gold nanoparticles containing Cas9 RNP and donor DNA were used to efficiently correct the DNA mutation that causes Duchenne muscular

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dystrophy in a mouse model.[103] In summary, engineered NPs have many tunable features like size, shape, charge and surface properties which can be altered to optimize delivery for a specific application, with a few limitations being possible cytotoxicity and insolubility. Overall, engineered NPs provide a promising and safer alternative than viral transduction to accomplish cellular engineering tasks for screening and therapeutic applications both *in vitro* and *in vivo*.

SINGLE CELL ANALYSIS IN CELL THERAPY

We have seen how micro and nano technologies have led to increased precision cell sorting and cell engineering methods. This section will discuss how micro and nano technologies have enabled single cell assays that are gradually succeeding bulk assays like microarray analysis, bulk-sequencing, and enzyme-linked immunoassay (ELISA) in cell therapy development pipelines. In this regard, there have been two key advancements: (1) compartmentalization of individual cells using microfluidic architectures, which has miniaturized molecular biology assays to provide single cell resolution while greatly increasing throughput, and (2) barcoding the molecular contents of each cell, that has enabled the simultaneous acquisition of "omics" scale readouts and single cell identity from NGS (Figure 3). Before these developments, single cell "omics" scale assays were low-throughput and tedious processes carried out in well-plates.[104] Single cell compartmentalization using microfluidics (Table 3) has been achieved primarily using three architectures, namely (1) Elastomeric Valves, (2) Aqueous Droplets in a carrier oil, and (3) Nanowell arrays.[105,106] Here, it is important to note that for most "omics" assays and analysis, microfluidic methods have generally enabled the single cell library preparation step. The readout of these libraries is performed using NGS platforms such as those from Illumina. For developments and advances in NGS, readers are directed to other reviews.[107−109]

Several early studies implemented and established valves,[110,111] droplets,[112,113] and nanowells[114,115] as viable microfluidic technologies for cell encapsulation and library preparation for omics analysis. The fundamental operation principles of these microfluidics technologies have been covered in previous reviews.[116,117] In this review, we will focus our attention on how these architectures have been utilized for single cell genomic, epigenomic, transcriptomic, proteomic, and multiomic assays (Table 4 and Table 5) in cell therapy applications. We will look at the experimental designs and bioinformatics methods for handling single cell omics data and provide an outlook on the opportunities and challenges in integrating various technologies and data analysis pipelines.

Genomics. *Experiments.* Single cell genomics has been primarily utilized to reveal single nucleotide variants (SNVs) and copy number variants (CNVs) present in developmental anomalies, degenerative diseases, and cancers. More recently, it is finding applications in developing and evaluating gene/cell therapy. For example, clonal tracking through whole-genome sequencing (WGS) by employing spontaneous somatic mutations as unique clonal tags has allowed for the detailed mapping of the genomic landscape of patients for improved assessment of safety and potential leukemia-initiating events in the context of gene therapy.[118] During the early phases of single cell genomics, the uneven amplification of DNA starting from the small amount of genomic material (two copies of gene per cell) or allelic dropouts led to biases and presented significant challenges. To uniformly amplify genomic DNA

Table 3. Common Architectures for Single Cell Analysis

Classification	Working Principle	Advantages	Disadvantages	Commercial Technologies
Valves	Pneumatically actuated elastomeric valves are used to create compartments in microfluidic channels to capture cells and control reagent flow.	High degree of control over individual compartments. Suitable for temporal assays.	Throughput is limited (48−800 cells per run). Complex control systems necessary for operation especially for large footprint devices.	C1 (Genomics and Transcriptomics, Fluidigm), Iso-Light (Proteomics, Isoplexis)
Droplets	Aqueous droplets in carrier oil to encapsulate single cells, barcoded beads and reagents.	High throughput (500−10,000 cells per run). Simple to implement.	Long-term cell culture is difficult, and location of droplets cannot be registered making them unsuitable for temporal or multitime point assays. Capture efficiency of beads can be low (10−30%).	Chromium (Multiomics, 10X Genomics), Nadia (Transcriptomics, Dolomite Bio), Tapestri (Genomics and Proteomics, Mission Bio)
Nanowells	Microfabricated arrays of wells for single cell, barcoded bead and reagent capture.	Medium to high throughput (100−10,000 cells). Simple to implement and lower cost. Temporal assays possible.	Automation is difficult. For sequencing-based assays using barcoded beads, limitations are the same as for droplets.	Milo (Proteomics, Bio-Techne), CellRaft Air (Genomics, Cell Microsystems), Rhapsody (Multiomics, BD Biosciences), ICELL8 (Multi-Omics, Takara Bio)

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Table 4. Assays Developed for Single Cell Analysis[a]

Assays		Working Principle
		Genomics
MDA[119,127]		Hexamer primers and phi29 polymerase used for isothermal amplification.
DOP-PCR[247]		Degenerate primers are used for thermal cycling.
MALBAC[120]		Looping of amplicons implemented to reduce PCR bias.
		Epigenomics
DNA Methylation	scBS-seq[147]	Bisulphite treatment converts C to T, but 5mC remains intact. Readout is NGS.
	scAba-seq[248]	Glucosylation of 5hmC and digestion of glucosylated-DNA using AbaSI restriction endonuclease followed by sequencing.
	scRRBS[249]	Digestion with MspI restriction endonuclease and size selection prior to bisulphite sequencing. Readout is using NGS.
	CLEVER-seq[250]	Labeling 5fC with malononitrile as a result of which 5fC are read as T in NGS.
Chromatin Accessibility	scATAC-seq[134,251]	Tagmentation of open chromatin regions using Tn5 transposase followed by NGS.
	scDNase-seq[252]	Sequencing of DNase I hypersensitive sites using NGS.
Histone Modification	scCHIP-seq[140]	Digestion of open DNA using MNase, barcoding of nucleosomes and immunoprecipitation of modified histones followed by NGS.
	scChIC-seq[253]	Antibody directed MNase digestion of chromatin around nucleosomes. Readout is using NGS.
3D Chromatin Structure	scHi-C[254]	Cross-linking of chromatin, digestion, biotin labeling, ligation of fragments and library preparation to identify interacting regions of the chromosome. Readout is using NGS.
Chromatin-Protein Interaction	scDamID[144]	Fusing protein of interest with DNA adenine methyltransferase to introduce adenine methylation at the locations of chromatin−protein interaction. This is followed by DpnI enzyme digestion, adaptor ligation and NGS.
		Transcriptomics
Smart-Seq2 (Fluidigm C1 library prep.)[18]		Full length transcript libraries are prepared by priming, RT, template switching, tagmentation with Tn5 transposase, and PCR prior to NGS. UMIs are not used. Full length sequencing is beneficial for isoform identification.
CEL-Seq2 (Fluidigm C1 library prep.)[255]		3′ Counting that uses an RT primer with UMI, cell barcode, and Illumina sequencing adapters.
Drop-Seq (Dolomite Bio)[155] In-Drop[156] 10X Chromium Library Prep.[256]		Droplets-based protocols for cell encapsulation and library preparation. All the three methods use beads with poly-T sequences, cell barcodes and UMIs for poly-A mRNA capture, RT, amplification, and sequencing but have subtle differences that affect performance metrics.
Seq-Well[158] ICELL8 (Takara Bio)		Nanowell-based protocols for cell encapsulation and library preparation. Bead designs and library preparation steps are similar to the droplet-based methods.
		Proteomics
Flow Cytometry[257]		Staining cell proteins with fluorescently labeled antibodies (Abs) followed by optical detection. Often used for single cell sorting into well plates and combined with other sequencing-based single cell analysis methods. Can detect ∼15 different proteins.
Mass Cytometry[177]		Use of metal labeled Abs to tag cellular proteins followed by time-of-flight (TOF) mass spectrometry. Up to ∼100 proteins can be detected simultaneously on the Fluidigm CyTOF System.
Mass Spectrometry[258]		Direct analysis of proteins from individual cells using TOF mass spectrometry. Can detect several proteins (>500) but limited by throughput (∼10 cells).
Single Cell Barcode Assay (SCBC)[185]		Spatially encoded antibody arrays within valve-based microfluidic chips for capture of proteins from single cells. Multiplexed detection of up to 40 targets from each cell.
Microengravings[114]		Conceptually similar to barcode assay with different functionalization protocol and executed in nanowell arrays. Limited to detection of a few markers.
sc-Western Blot[181]		Western blot protocol executed after capturing single cells on a polyacrylamide gel chip with nanowell arrays. Can assay a panel of ∼12 proteins.
DropMap[259]		Capture of secreted proteins from cells using Ab coated magnetic beads followed by fluorescence readout. Can detect 4−10 secreted markers simultaneously.
Proximity Ligation Assay (PLA)[189] Proximity Extension Assay (PEA)[260]		Matched pair of Abs with unique oligo tags are used to label protein targets. The oligos hybridize and are used to generate DNA amplicons that can be detected using PCR or NGS. These assays have typically been used for multiomic profiling of mRNA and proteins from single cells. Number of markers that can be profiled depends on the available Ab panel.

[a]MDA, multiple displacement amplification; DOP-PCR, degenerate oligonucleotide-primed polymerase chain reaction; MALBAC, multiple annealing and looping-based amplification cycles; NGS, next generation sequencing; UMI, unique molecular identifier.

from single cells, researchers developed whole genome amplification (WGA) methods such as multiple displacement amplification (MDA)[119] and multiple annealing and looping-based amplification cycles (MALBAC),[120] which outperformed PCR. With the advancement of high-throughput microfluidics, it was possible to carry out these reactions at massive scales. The first microfluidic architectures that were developed for harnessing these methods were valve based, and they could handle enough cells such that the WGA products would saturate sequencing capacity. Valve-based architectures eventually led to Fluidigm's valve-based commercial solution, the C1 system that supports automatic single cell library preparations for whole genome and whole exome sequencing. Valve-based microfluidics has been utilized in unraveling the mechanisms of several diseases. For instance, Gawad et al. performed WGA of nearly 1,500 single cells on this platform to study the genetic events leading to childhood acute lymphoblastic leukemia (ALL).[121] However, with the

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Table 5. Multi-Omic and Spatial-Omic Assays

Assays		Working Principle
		Multi-Omics
Genome + Transcriptome	DR-seq,[261] TARGET-seq,[262] G&T-seq,[263] SIDR[264]	Genomic DNA (gDNA) and mRNA are isolated from individual cells before parallel sequencing for genome and transcriptome analysis. Note that these methods are implemented in well plates using traditional pipetting or FACS. Microfluidic implementation could be a future possibility.
Genome + Proteome	DAb-seq[265]	Oligo tagged Abs are used to label cell surface proteins. Droplets with beads are used for tagging Ab oligos and gDNA with unique cell barcodes prior to NGS.
Transcriptome + DNA Methylation	scMT-seq,[266] scM&T-seq,[267] sc-GEM[268]	gDNA and mRNA from single cells are separated out followed by parallel execution of transcriptomic and epigenomic (e.g., scBS-seq for scM&T-seq and scRRBS for scMT-seq) analysis protocols. Out of these, only sc-GEM uses the microfluidic C1 platform. scM&T-seq uses FACS while scMT-seq uses manual pipetting.
Transcriptome + Chromatin Accessibility	SNARE-seq,[213] sci-CAR,[269] scCAT-seq[270]	Combination of scRNA-seq and scATAC-seq protocols with differences in processing steps. SNARE-seq uses barcoded beads in droplets to capture both open chromatin fragments and mRNA. sci-CAR uses pooled combinatorial barcoding in well plates. scCAT-seq separates out the nuclei and mRNA for parallel processing after FACS of single cells.
Transcriptome + Proteome	CITE-seq,[190] REAP-seq,[212] PLAYR[271]	CITE-seq and REAP-seq use oligo tagged Abs for surface protein labeling followed by droplet-based library preparation and NGS to read mRNA sequences and Ab oligo tags. PLAYR uses PLA for mRNA followed by FACS or mass cytometry detection.
Transcriptome + Chromatin-Protein Interaction	scDam&T-seq[272]	Combination of scDamID and scRNA-seq protocols.
Chromatin Accessibility + DNA Methylation	scNOMe-seq[273]	Use of GpC methyltransferase (M.CviPI) to methylate GpC sites that are not bound by nucleosomes, in nuclei isolated by FACS. This is followed by scBS-seq protocol. DNA methylation sites and accessible chromatin (methylated GpC) are simultaneously detected.
Chromatin Accessibility + Proteome	ASAP-seq[214]	scATAC-seq protocol in droplets combined with oligo tagged Abs for surface protein readout using NGS.
DNA Methylation +3D Chromatin Structure	Methyl-HiC,[274] sn-m3C-seq[275]	Combination of the scBS-seq and scHi-C protocols.
Transcriptome + Lineage	TracerSeq,[276] CellTag,[277] LARRY,[278] scGESTALT,[279] LINNAEUS[280]	Use of heritable molecular barcoding strategies that can be read out by NGS. Lineage hierarchies can be constructed over single cell transcriptome maps using the barcode information to identify clonal populations. Some are CRISPR based (e.g., scGESTALT, LINNAEUS). Others use retrovirus (e.g., LARRY, CellTag)
Transcriptome + CRISPR perturbation	CRISP-seq,[281] Perturb-seq,[216] CROP-seq,[215] Mosaic-Seq[282]	Pooled CRISPR screens combined with scRNA-seq to simultaneously obtain transcriptome and gene perturbation information. Each CRISPR synthetic guide RNA (sgRNA) has an associated unique barcode that is read out using NGS.
Chromatin Accessibility + CRISPR perturbation	Perturb-ATAC[283]	Combination of CRISPR perturbation with ATAC-seq
Transcriptome + Chromatin Accessibility + DNA methylation	scNMT-seq[284]	Combination of scNOMe-seq and scM&T-seq protocols.
Genome + Transcriptome + DNA methylation	scTrio-seq[285]	Execution of scRNA-seq and scRRBS protocols in parallel after manually separating nuclei from mRNA.
Genome + Chromatin Accessibility + DNA methylation	scCOOL-seq[286]	scNOMe-seq combined with adaptor tagging post bisulphite treatment.
Transcriptome + Proteome + T cell receptor + CRISPR Perturbation	ECCITE-seq[219]	Combination of CITE-seq protocol with direct capture of sgRNA using barcoded beads to identify genetic perturbation along with transcriptome and proteome.
		Spatial-Omics
Next Generation Sequencing (NGS) based	Spatial Transcriptomics[222] (ST), Slide-seq,[223] DBiT-seq,[228] Seq-Scope[287]	Use of arrays of barcoded beads or microfluidics to capture and/or tag transcripts from tissues spatially followed by NGS. Commercial solutions such as Visium (10X genomics) and GeoMx (NanoString) have been developed.
In Situ Hybridization (ISH) based	MERFISH,[225] seqFISH[288]	Detection of transcripts by binding of complementary fluorescent probes.
In Situ Sequencing (ISS) based	FISSEQ,[224] Barseq,[289] ISS/hybISS,[290,291] ExSeq[292]	mRNA is reverse transcribed, amplified and sequenced within the tissue. A commercial solution based on FISSEQ and ISS will be made available on the Xenium Platform (10X Genomics).

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expansion of sequencing capacity, high-throughput droplet and nanowell-based platforms have become more popular for single cell library preparation with successful commercialization. For example, Mission Bio's droplet-based Tapestri platform has been used to study the genetic backgrounds of diseases such as acute myeloid leukemia (AML)[122] and cerebral cavernous malformations (CCMs).[123] Single cell DNA sequencing using the Tapestri platform can identify SNVs, indels, CNVs, loss of heterozygosity (LOH), and translocations from the DNA of thousands of individual cells to interrogate clonal architecture, rare subclones associated with tumor development and therapeutic resistance.[124] On the other hand, Cell Microsystems' nanowell array-based CellRaft technology has been used to investigate somatic mutations that accumulate in B lymphocytes over time and CNVs leading to complex karyotypes in human neocortical neurons across individuals of different ages.[125] With the rapid development of platforms which improve sequencing bandwidth, reduce amplification errors and automate library preparation, single cell genomics will likely be integrated more frequently in future studies. For example, the Tapestri platform has already been used to investigate multiplexed CRISPR-based genetic mutations that recur in chronic lymphocytic leukemia[126] and may find utility in other CRISPR-based cell engineering and single cell screening studies.

Data Analysis. As mentioned previously, single cell genomics studies primarily focus on detecting two types of mutations, namely SNVs and CNVs. A critical challenge in accurately identifying SNVs arises from the fact that single cell WGA methods suffer from artifacts introduced during sample preparation steps such as cell lysis, low genomic coverage due to the small amount of DNA present (~6 pg per cell) and amplification errors which can lead to dropouts or false positives in SNV calling. To overcome these, researchers have developed bioinformatics methods such as scCaller,[127] LiRA,[128] and Conbase[129] that account for artifacts and dropouts to improve the accuracy of single nucleotide polymorphism (SNP) calling from noisy data. Similarly, accurate CNV identification from single cell data also suffers from shallow coverage and amplification biases, leading to the development of algorithms such as SCNV102, CHISEL103 and SCOPE104 for improved accuracy CNV calling. Extensive research is still being done on WGA techniques that reduce amplification biases and errors and increase the depth and uniformity of coverage to improve the SNV and CNV calling accuracy. Additionally, work is being done to combine inferences from single cell and bulk sequencing data to provide more information regarding the origin of mutations and introduce statistical frameworks that accurately model amplification biases and errors to reduce calling uncertainties.

Epigenomics. *Experiments.* Epigenomics is concerned with studying how cellular identity is regulated by transcription factors, chromatin regulators, their accessibility/structure, DNA methylation, histone modifications, and various other factors that affect the genome topology. Traditional detection methods require large amounts of genomic material and their pooling to meet the mass requirement, resulting in the masking of cell−cell epigenetic heterogeneity. With the advent and progress in single cell sequencing techniques, high resolution single cell epigenomic assays have been developed.[130] Additionally, developments in microfluidic technology, improvements in automation and high-throughput operations have enabled the handling of ultralow sample volumes and subsequent processing, providing significant insights into cell heterogeneity brought about by epigenetic differences. Moreover, factors like simplifying protocols and reducing reaction costs due to lowering reagent volumes and reaction times have facilitated the screening of epigenome-based therapeutic targets for clinical application.[131] Here, we will discuss some of these developments.

Valve-based microfluidic platforms were the earliest reported architectures that enabled epigenetic assays with single cell resolution. For instance, Fluidigm's microfluidic systems were used to probe DNA methylation in single cells at multiple loci.[132,133] These approaches helped circumvent DNA degradation and loss by material transfer, allowing for single cell resolution. Valve-based chips were also used to detect and analyze chromatin accessibility in single cells by a technique known as single cell assay for transposase-accessible chromatin sequencing (scATAC-seq).[134] As scATAC-seq can capture the cell-type-specific chromatin accessibility landscape at a single cell resolution, it has been rapidly adopted by the scientific community. It has led to an investigation of the role epigenetic mechanisms play in cancer cell plasticity, invasiveness, and response to therapy.[135,136] Based on insights gained from single cell epigenomic studies, epigenome editing approaches have been developed to modify the epigenome of disease models toward a more "normal-like state".[137] Over time, droplet- and nanowell-based methods, which provide much higher throughputs than valve-based chips, have become more prevalent in single cell epigenomics assays; scATAC-seq has been adapted to both architectures in commercialized platforms.[138,139] Other epigenomic assays harnessing microfluidic technologies have also been developed. For example, the droplet-based scCHIP-seq was developed for detecting histone modifications.[140] Recently, scCHIP-seq was used to study chromatin states in a patient-derived xenograft model of breast cancer, which revealed the loss of chromatin mark H3K27me3 in resistant tumor cells.[141] It must be noted here that protocols for assessing aspects of the 3D chromatin structure such as chromatin conformation (scHI-C)[142,143] and chromatin−protein interactions (scDamID)[144] are well-plate based and have not been adapted to high-throughput microfluidic platforms yet, likely because of the complex processing steps involved. Nevertheless, the implications of 3D chromatin structure in disease mechanisms are becoming increasingly apparent and can prove to be potential targets for therapeutics.[145]

Data Analysis. For the analysis of epigenomic sequencing data, the first step is appropriate normalization and application measures to account for a high level of noise encountered in single cell data. Moreover, the sparse coverage of single cell epigenomic data presents another challenge for downstream analysis and requires resolution. Some developments like single cell Accessibility Based Clustering (scABC) have allowed the identification of open chromatin sites in single cells from scATAC-seq data.[146] scABC weighs cell data concerning sequencing coverage and uses the ranked data to cluster cells with similar epigenetic makeup, thus avoiding biases from highly overrepresented regions. Another analysis method aggregates data from adjacent regions or regions with similar regulatory elements.[147,148] For example, cisTopic[149] and SCALE[150] pool results from cell- and region-level aggregation for peak identification, while chromVAR[151] uses the above strategy for identifying open chromatin sites from scATAC-seq data. A third strategy is to use predictive computational models

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to gather missing information, which has been used for imputing single cell methylation data.[152] For the imputation of scHI-C data, the hypergraph representation learning-based Higashi algorithm has been recently reported to identify multiscale 3D genome features.[153]

Transcriptomics. *Experiments.* Transcriptomics is arguably the most mature and widely used single cell molecular profiling method. Important steps in the single cell transcriptomic assay include capturing polyadenylated (poly-A) mRNAs from single cell lysates, reverse transcription of the captured mRNA to cDNA, and using NGS to profile the amplified cDNA. Similar to single cell genomics and epigenomics assays, the earlier transcriptomic studies utilized valve-based microfluidic architectures to isolate, lyse and profile cells. Eventually, Fluidigm's C1 platform provided a commercial solution for single cell library preparation using the Smart-Seq protocol.[154] However, the key innovation of utilizing barcoded beads to capture and uniquely tag mRNAs originating from single cells allowed for pooled sequencing and computational demultiplexing of cell identity. As a result, droplet- and nanowell-based methods became more popular. Individual compartments trap a single cell and a barcoded bead simultaneously, allowing cell lysis, poly-A mRNA capture using oligo(dT) universal primers, barcoding, and reverse transcription to synthesize cDNA. These methods greatly simplified library preparation steps compared to valve-based systems, providing higher throughputs. Since the demonstration of droplet-based single cell library preparation protocols such as DropSeq[155] and InDrop,[156] many commercial systems have been developed, such as the Chromium (10X Genomics), Nadia (Dolomite), and InDrop (1CellBio). Similarly, nanowell-based demonstrations such as Microwell-seq[157] and Seq-Well[158] have been followed up by commercial platforms such as Rhapsody (BD) and ICELL8 (Takara Bio). The emergence of these technologies has greatly accelerated the application of transcriptomics in biology and led to the identification of rare cell types or gene regulatory pathways that can be immediately useful to disease research and gene therapy. For example, gene and cell therapy for cystic fibrosis previously focused on targeting ciliated cells until single cell RNA sequencing (scRNA-seq) identified pulmonary ionocytes as the target, which represent only 1% of airway epithelial cells but are the primary source of cystic fibrosis transmembrane conductance regulator (CFTR) activity.[159] Similarly, scRNA-seq of mouse kidney cells revealed transitions between cell states and potential target cells for kidney diseases such as metabolic acidosis.[160]

It is important to note that each platform has its advantages and disadvantages. For example, the C1 platform provides higher transcript capture efficiencies, full-length transcript libraries, and multiple sequencing runs on specific wells. On the other hand, droplet and nanowell platforms provide much higher throughputs, which alleviates some of the noise issues with lower efficiency transcript capture from single cells and shorter read lengths. Recently a hybrid platform combining the advantages of valves and droplets for deterministic single cell capture and sequencing, has also been reported.[161] These architectures are particularly beneficial for handling small sample sizes such as cells from organoids, without compromising on throughput. Ultimately the platform of choice depends on the application of interest and readers are referred to some excellent studies that provide a detailed comparison of the different platforms and guidelines for their selection.[162,163]

Data Analysis. The bioinformatics pipeline for single cell transcriptomics usually entails several steps. This includes alignment of sequencing reads to a reference genome, quality control, dimension reduction, clustering, and visualization of the data. With the rapid advancements in single cell transcriptomics, software tools like Seurat[164] and Scanpy[165] have been developed, combining many of these processes into integrated packages. Commonly used methods for these processes have been extensively reviewed, and the analysis pipelines are fairly established.[166−168] For example, dimension reduction and visualization are often performed using non-linear methods like t-distributed stochastic neighbor embedding (t-SNE) or Uniform Approximation and Projection method (UMAP), with UMAP preserving the underlying topology well and providing good scalability. Similarly, hierarchical clustering, k-means clustering and k-nearest neighbor-based algorithms are popular choices for segregating cells into groups.[169] Additional steps in the analysis pipeline may include trajectory inference to study dynamic processes, differential expression (DE) analysis to identify gene expression differences across conditions, and Gene Set Analysis (GSA) or Gene Regulatory Networks (GRNs) to examine affected pathways and gene−gene interactions. In trajectory inference methods, a pseudotime variable represents developmental time, concentration gradients or spatial positions, along which cells are arranged based on their gene expression profiles. A variety of trajectory analysis methods are available that have been benchmarked previously.[170] Out of these, Monocle, Wishbone and Slingshot are popular choices. Once cells have been arranged, grouped, and identified based on their gene expression, DE analysis is performed to find the list of genes whose expression varies across conditions. Although DE analysis is routinely performed for bulk sequencing data, it is much more challenging to perform for single cell data due to technical noise, dropouts and comparisons between distributions of gene expression instead of single values for each gene. Several methods have been developed to address these challenges, out of which MAST and weighted bulk DE analysis tools such as DESeq2 and EdgeR perform the best.[166,171] It must be noted that DE analysis only provides a list of significantly different genes between conditions, and it may be difficult to interpret how they interact and affect cellular function. As a result, GSA or GRNs are usually employed to provide a systems-level view of biological processes that are perturbed. This is done by investigating the expression of sets of related genes involved in a pathway (GSA) or groups of genes that work together, interact, and regulate gene expression (GRN). Gene Set Enrichment Analysis (GSEA) and sigPathway have been the most powerful among the used GSA methods. GRN methods that have been developed for single cell data include PIDC, GRNBoost2 and SCENIC with PIDC and GRNBoost2 performing the best in benchmarking tests.[172] Further improvements in experimental methods are likely to address low transcript capture efficiency, high technical noise and frequent dropouts. Additional developments in bioinformatics methods will help improve the resolution of identifying rare cell subtypes and the inferences that can be drawn from GSA and GRNs.

Proteomics. *Experiments.* Measuring and quantifying the proteome of the single cell is essential to forming a fundamental understanding of cellular mechanisms, as this diverse class of macromolecules carries out a multitude of functions, from regulation of gene expression to extracellular

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communication. However, no universal method exists for measuring the whole proteome in an analogous fashion to the NGS of nucleic acids. Furthermore, scRNA-seq cannot estimate protein abundance in single cells due to a lack of correlation between RNA expression and protein levels.[173] Instead, commonly used proteomic methods include affinity-based assays (e.g., flow cytometry,[174] ELISA,[175] Western blots,[176] mass cytometry[177]) and mass spectrometry.[178] Affinity-based methods are commonly used to measure protein abundance quantitatively, whereas mass spectrometry is used to measure protein composition.[179] The performance of quantitative assays can be characterized by sensitivity, dynamic range, multiplexing capacity, and throughput. Gold-standard affinity assays like ELISA and Western blot lack the sensitivity required for single cell measurements due to the dilution of analytes in the microliter-scale working volumes employed by commercial systems. Moreover, they can only detect a limited number of targets at a time. Recently, technologies have emerged capable of measuring increasing numbers of proteins with single cell resolution.

In many of these technologies, miniaturization of protein assays within microfluidic devices has enhanced the assay sensitivity down to single-cell resolutions due to the small volumes and higher analyte concentrations. For example, Love et al. developed the microengraving technique, which involves trapping single cells in an array of nanowells that is interfaced with an antibody-coated glass substrate used to capture proteins secreted from live cells.[114] Similar to ELISA, the readout of this assay is fluorescence based. In one study, microengravings were used to measure the dynamic secretion of multiple cytokines from isolated primary human T cells.[180] The results revealed that T cells predominantly secrete one cytokine at a time instead of a sustained release of all cytokines. Similarly, miniaturization of the Western blot immunoassay was reported by Hughes et al., which follows all the key steps of a traditional Western blot but with single-cells captured in nanowell arrays.[181] This method, referred to as scWestern, detects up to a dozen proteins from thousands of single cells.[181] Although these systems enable sensitive detection of proteins from single cells, the multiplexing capacity is limited due to spectral overlap. Approaches to increasing the multiplexing capacity of affinity-based systems include the tagging of antibodies with heavy metal isotopes for cytometry by time-of-flight[182] (cyTOF (Fluidigm): ∼100 unique markers), and spatial encoding of capture antibodies using single cell barcoded chips[183] (SCBCs (Isoplexis): ∼42 proteins). The SCBC method developed by Heath and co-workers consists of patterning a glass substrate with DNA-encoded antibodies in a barcode arrangement containing ∼15 stripes, thus providing spatial separation between capture antibodies and enabling fluorescent measurements of up to 42 proteins.[183,184] SCBCs have been interfaced with valve-based and microwell-based architectures to measure secreted proteins from live cells or intracellular proteins from lysed cells.[183,185] In a recent study aimed at investigating the antitumor response of simultaneously administering the prodrug NKTR-214 with adoptively transferred T cells (ACT) into melanoma bearing mice, mass cytometry was utilized to characterize the functional phenotype of immune cells in the spleen and tumors of mice after treatment. The study found that the treatment increased the proliferation of CD8 T cells in the spleen and tumor sites.[186] Furthermore, the SCBC was used in the same study to measure the secretion profile of the CD8 T cells, which revealed that the treatment increased the polyfunctionality of the T cells. This case study demonstrates that various proteomic methods can be employed simultaneously to leverage their respective advantages. If higher throughputs are required, droplet-based microfluidic systems provide a clear advantage over nanowell- and valve-based architectures as they can process millions of cells per hour.[187,188] Additionally, droplet-based systems have emerged that can be integrated with NGS to measure proteins via labeling with DNA-functionalized antibodies such as proximity ligation assay (PLA)[189] and CITE-seq[190] Valve-based architectures, on the other hand, have proved useful for long-term live-cell proteomics for studying temporal dynamics.[191,192] Recently, these microfluidic architectures have also been combined with ultrasensitive nanoplasmonic or surface-enhanced Raman spectroscopy (SERS)-based immunoassays that harness the interaction of light with metallic nanostructures for signal amplification. For example, Altug and co-workers have developed nanoplasmonic microarrays for the detection of single cell secretions.[193,194] Similarly, droplets have been used in conjunction with antibody conjugated metallic nanoparticles for cell secretion analysis.[195−197] Another method called single-cell plasmonic immunosandwich assay (scPISA) reported by Liu et al. used probe-based microextraction from single cells and plasmon-enhanced Raman scattering for ultrasensitive detection of low copy number proteins.[198] Notably, single cell proteomic analysis is becoming increasingly crucial for investigating the role of cellular heterogeneity in determining therapeutic efficacy. For instance, in ongoing LentiGlobin gene therapy clinical trials for treating sickle cell disease, the quantification of cells expressing pathogenic and/or transgene beta-globin enabled assessment of treatment efficacy by unmasking cellular heterogeneity in the gene therapy product at the protein level.[199] Thus, it is likely that single cell proteomics assays will only gain popularity in the next few years for therapeutic studies.

Data Analysis. Signal processing and data analysis workflows for the microfluidic single cell proteomic systems are similar across methods and consist of multiple steps. First, the acquired signal is mapped to each cell to generate an array of measured signals. In the microfluidic systems described, the cells in each array unit are imaged with a microscope and counted using image processing and computer vision tools. Deep learning algorithms, such as convolutional neural networks (CNNs) and fully convolutional networks (FCNs), are useful in identifying and counting cells.[200,201] Microarray scanners, such as the GenePix (Molecular Devices), are used in scWesterns, SCBCs, and microengraving platforms to measure the fluorescent intensity signal for each region of interest in the array in an automated manner. The measured signal is background subtracted and compared against calibration standards to quantify the protein amounts secreted or lysed from each cell. At this stage of the workflow, quality control steps are implemented to filter the data. For example, data corresponding to locations with zero cells, saturated signals, or measurement artifacts is removed. Next, the data is transformed (e.g., log or glog) and normalized using ensemble statistics or principal component analysis (PCA). Correlation matrices and metrics such as the signaling network activity index (SNAI)[202] are used to characterize protein interactions and the strength of protein signaling coordination. Dimension reduction methods like those utilized in transcriptomic analysis pipelines (e.g., t-SNE, UMAP, PCA) are often employed in

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multidimensional proteomic data sets and are useful for visualizing cells' clusters with differing phenotypes. Heatmaps combined with hierarchical clustering are also commonly used to visualize single-cell protein levels and compare protein levels to perturbations such as drug treatments. For immune cell secretome analysis, the polyfunctional strength index (PSI) is used to characterize the fraction of polyfunctional T cells in the population under study.[203] For NGS-based proteomics, the data analysis pipeline is similar to that of transcriptomics.

Metabolomics. Metabolomics constitutes the detection and measurement of products of cellular metabolism like glucose, calcium, and various other intermediates. High diversity of metabolites along with high variability of their relative numbers within cells makes single cell metabolomics (SCM) challenging. However, recent advancements in microfluidic technologies with regard to performance metric like single cell tracking, throughput and sensitivity have initiated and advanced SCM. There are primarily three approaches to SCM: droplet microfluidics-based fluorescence analysis,[204−206] electrochemical assays,[207−209] and mass spectroscopy. Droplet microfluidics enables confining single cells into small droplets that increases the analyte concentration, thereby enhancing sensitivity. For instance, high sensitivity (200−400 attomole) quantitative measurement of H_2O_2 secretion from single cells was obtained using a droplet-based fluorescence assay.[204] In another instance, a droplet-based system was used to detect as few as 10 circulating tumor cells (CTCs) among 200,000 white blood cells based on lactate secretion induced pH change signal given off by a ratiometric dye.[205] Droplet microfluidics combined with flow-based fluorescence methods allows for high throughput and increased sensitivity. However, multiplexing is still a challenge due to the need for more labels. Similarly, high sensitivity and label free electrochemical assays have been combined with microfluidic systems for detection of electroactive metabolites like glucose, calcium, lactate and oxygen.[207−209] Such microfluidic electrochemical assays are low cost and easy to fabricate but are confined to the detection of electroactive species. Additionally, the integration of microfluidics with mass spectroscopy (MS) has enabled high throughput and multiplexed analysis of single cells. For example, droplet-based microextraction was combined with mass spectroscopy to enable the detection of tens of different metabolites from single MCF-7 cells.[210] Matrix-assisted laser desorption/ionization (MALDI)-MS is one of the most common approaches for metabolic analysis owing to its high sensitivity, throughput, and compatibility with micro/nano well-based platforms. For instance, researchers have developed microarrays for MALDI-MS, that enables automated isolation of single cells and their subsequent analysis.[211] Although MS is better than fluorescence-based methods in terms of coverage, it is destructive for the cells analyzed, unless the analysis is limited to secreted metabolites. Therefore, with advancements in microfluidics and nondestructive cell analysis workflows, there is a need for technology that can obviate the current limitations in terms of multiplexing, throughput, and temporal cell analysis.

Emerging Omics Approaches. Individually the single cell assays are powerful and provide us with information that would not have been possible a few years ago. However, there are still limitations to these assays. For instance, profiling only a single molecular subtype may not provide sufficient information regarding cell state in diseases. Second, the lack of spatial context in tissues and the inability to obtain temporal information due to the destructive nature of the assays precludes the full spatiotemporal context of disease progression and therapeutic response. These limitations are already being overcome with micro and nano technology-inspired solutions.

Multi-Omics. Several single cell multiomic methods have emerged for simultaneous profiling of multiple molecular types from the same cell (Table 5). For example, the CITE-seq[190] and REAP-seq[212] methods use nucleic acid barcoded antibody libraries to label surface proteins and simultaneously obtain proteomic and transcriptomic data from single cell sequencing platforms like the 10X Chromium. Similarly, SNARE-seq[213] and ASAP-seq[214] enable concomitant profiling of chromatin accessibility and gene expression or chromatin accessibility and protein abundance, respectively. Multiomics solutions are already being provided by commercialized single cell analysis platforms and are being used for disease and cell therapy-related studies. For example, a recent study used the combination of CITE-seq and mass cytometry to reveal the characteristics of a persistent population of $CD4^+$ CAR-T cells that helped in the long term remission of chronic lymphocytic leukemia in patients, a decade after transplantation of the reprogrammed cells.[14] Researchers have also combined genetic perturbations with single cell assays, such as in CROP-seq[215] and Perturb-seq,[216] where pooled CRISPR screens are performed with single cell gene expression as the readout. Innovations such as cell hashing[217] and combinatorial cell indexing[218] have enabled even higher-throughput single cell perturbation screens at reduced sequencing costs. Such high degree of multiplexing is being used for the next generation of cell atlas projects. The technique ECCITE-seq[219] takes the concept of perturbation screens even further with pooled CRISPR-based genetic modifications combined with multimodal single cell measurement of gene expression, proteins and CRISPR edits. Frangieh et al. recently used this concept of pooled perturbations and multimodal single cell profiling to study resistance mechanisms to immune checkpoint inhibitors in a tumor cell−lymphocyte coculture model.[7] In these recent multiomics technologies, the critical innovations lie in the assay designs, with their miniaturization being supported by existing microfluidic solutions. However, combination of microfluidic architectures such as droplets with nanowells or valves with droplets are also advancing multiomic assays with examples being the simultaneous profiling of cell transcriptome and proteome.[220,221]

Spatial-Omics. Concurrent with multiomic methods, spatial-omics, particularly spatial transcriptomics, has also gained much traction. Several approaches have been adopted to provide positional context to single cell transcriptomic measurements from tissues (Table 5). Some techniques like Spatial Transcriptomics (ST)[222] and Slide-seq[223] use 2D arrays of barcoded primers to capture transcripts from tissue sections placed in contact with the array while others like Fluorescence *in situ* sequencing (FISSEQ)[224] and Multiplexed error-robust FISH (MERFISH),[225] sequence or label the transcriptome *in situ* to obtain spatial information. Some of these methods, such as ST (Visium, 10X Genomics), FISSEQ (ReadCoor; now part of 10X Genomics), MERFISH (Vizgen), and GeoMx Digital Spatial Profiler (GeoMx, Nanostring), are now available commercially and have been extended for simultaneous measurement of RNA and proteins. Not surprisingly, spatial transcriptomics is being integrated into workflows investigating disease pathophysiology and their treatments.[226,227] However, it must be noted that not all these technologies have attained

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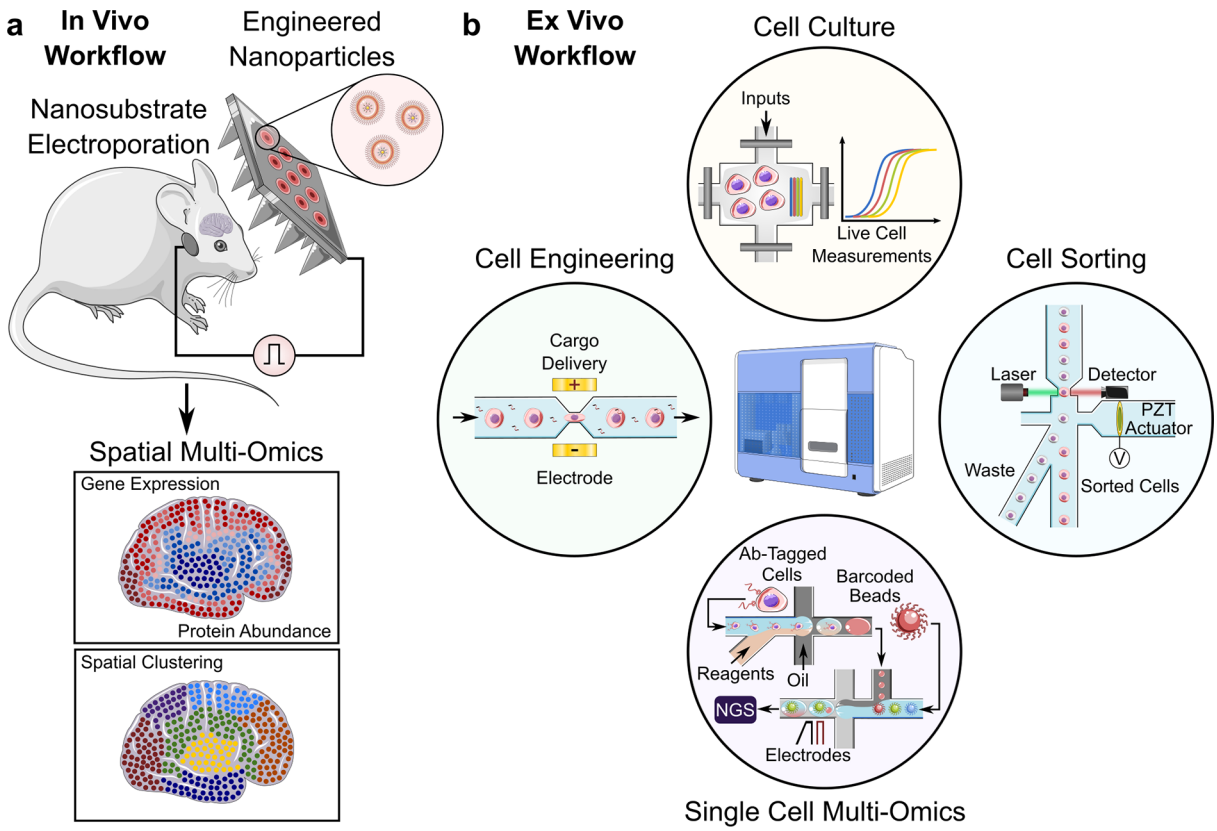

Figure 4. Integrated technologies for *in vivo* and *ex vivo* cell therapy development. (a) Hybrid micro/nano technology inspired devices can be employed for precise *in vivo* cell engineering. For example, in a mouse glioma model, *in vivo* cell engineering can be used to elicit an antitumor response. The efficacy of the therapeutic intervention can be scrutinized in detail using spatial multiomics platforms. (b) For *ex vivo* cell therapy development, cells isolation, culture, engineering, and multimodal profiling can be performed on an integrated platform.

single cell resolution or whole transcriptome coverage yet. To push these boundaries, a recent method called deterministic barcoding in tissue for spatial omics sequencing (DBiT-seq)[228] presented an important step toward spatial multiomic measurements at single-cell resolution. In this work, the authors used microfluidic channels to label cells in tissues with barcodes that help reconstruct the spatial distribution of post sequencing. Using this approach, the authors were able to query the transcriptome, proteome, or epigenome while retaining positional information.[228,229] This technique highlights the continued contribution of micro and nano technology in the evolution of single cell analysis.

Temporal-Omics. As most single cell omics assays are inherently destructive, we can only obtain snapshots of single cell states. However, reconstructing dynamic processes from single cell snapshots is inherently challenging. Nevertheless, technologies are being developed that enable the acquisition of temporal information from single cell omics assays (Table 5). The first class of methods implements heritable barcodes for sequencing-based prospective lineage tracing that can be used to infer clonal relationships from scRNA-seq data.[230] Most of these methods employ viral integration of barcode libraries or use CRISPR-Cas9 to generate random mutations at specific genomic target sites that serve as barcodes. With the advent of these technologies, lineage tracing with single cell omics is being widely used to investigate dynamic processes such as the evolution of tumor microenvironment[1] and the development of drug resistance.[6] The second class of methods relies on extracting intracellular contents from the same cells at multiple time points to obtain live-cell temporal dynamics.[231−233] The advantage of these methods is that they can provide real-time gene expression data and can be used to study postmitotic gene expression dynamics. This can be challenging with lineage tracing methods. However, high-throughput omics scale analysis with single cell resolution is still challenging with these approaches.

INTEGRATED WORKFLOWS USING MICRO AND NANO TECHNOLOGIES

Micro and nano technologies have greatly influenced the development of cellular engineering and analysis platforms over the past decade. So far, we have discussed the upcoming micro and nanotechnology platforms that can handle cell sorting, intracellular delivery, or single cell analysis tasks in isolation and replace some of the traditional technologies. However, there is a potential opportunity for integration of the discussed methods. Consider the study by Rodriguez-Fraticelli et al.[68] where the authors have used scRNA-seq-based lineage tracing to decipher the long-term regenerative capacity of HSCs. The authors used lentiviral transduction to introduce heritable barcodes into HSCs extracted from mice, then transplanted them back and harvested them a few weeks later. Barcoded cells with reporters were enriched using FACS, and scRNA-seq was used to characterize the molecular signatures of different HSC clones by simultaneously analyzing their lineage and transcriptome. This study revealed that the expression of a transcription factor Tcf15 promotes a self-renewing and quiescent HSC state with long-term repopula-

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tion potential, which could inform future therapeutic designs using engineered HSCs. Additionally, the authors used CROP-seq to validate their findings. In a very different study, Roth et al.,[234] designed a pooled CRISPR knock-in screen to identify effective immunotherapy constructs. They first electroporated a barcoded homology directed repair (HDR) template library into primary human T cells. This library introduced a specific T cell receptor (TCR) transgene (NY-ESO-1) into the cells along with another gene product that varied across the library. Then the authors used FACS to isolate the TCR expressing T cells and performed a scRNA-seq experiment. This enabled them to correlate the CRISPR genetic modifications to the cell's transcriptomic state and reveal the constructs that enhance fitness under immunosuppressive conditions *in vitro* and *in vivo*. The authors further performed arrayed protein expression and cancer cell killing assays to validate the hits from their pooled screening study. Finally, they used T cells engineered to express the NY-ESO-1 TCR and a chimeric receptor (TGF-βR2-41BB) combination to demonstrate improved solid tumor clearance in a xenograft model. Various already discussed sorting, cell engineering and multiomics technologies were used in combination for these studies. This highlights that the next logical extension of these technologies is the integration and automation of a few or all the required tasks within a single platform that can expedite screening and validation processes in *ex vivo* cell therapy development pipelines (Figure 4a). On the other hand, the development of precision cell engineering options, in combination with spatial multiomics profiling, can provide a different paradigm for the evolution of the next generation of *in vivo* cell therapy (Figure 4b). However, the path toward fully integrated workflows still has several challenges, as we discuss next.

Integration Challenges. Several technological and scientific hurdles still need to be overcome to make integrated cell therapy workflows using micro and nano technologies a reality. One critical challenge is scaling up some of the microfluidic processes for biomanufacturing and clinical applications. For example, many microfluidic cell sorting and cell engineering systems have limited throughputs that are not ideal for processing primary cells or patient samples where handling millions of cells within minutes is required. In this context, hybrid systems leveraging the advantages of different techniques are likely to provide optimal solutions. We have previously discussed how combinations of immunomagnetic, and hydrodynamic separation has allowed for cell sorting with high efficiency, purity and throughput. Similarly, combining electroporation and mechanoporation has the potential for efficient cell engineering of hard-to-transfect cells at high throughputs. On the single cell analysis side, cell hashing, combinatorial indexing, improved library preparation, and reduced sequencing costs will likely improve their scalability for clinical use. A second significant challenge is the actual integration of the different elements of sample preparation, followed by the isolation, engineering, culture and omics analysis of cells on a single platform in a modular fashion. This presents a massive engineering undertaking with several interdisciplinary considerations. The materials must be chosen carefully to ensure biocompatibility, enable long-term cell culture, and monitor biofouling or analyte losses during processing. A high degree of automation is also necessary to execute such complex workflows with reduced human intervention to cut down on handling steps, maintain sterility, minimize process variability, and improve reliability and robustness. Additionally various steps may need to be executed in parallel to optimize operation time and process costs. All this is crucial for manufacturing processes involving sensitive cells that have limited shelf life *ex vivo* and may exhibit degraded potency, viability and sub optimal therapeutic response over several cycles of culture. Compatibility between the various processing steps in terms of throughput, design, architecture, and seamless sample transfer from one module to another is also critical. In this context, the common microfluidic architectures of μFACS, flow through cell engineering and droplet-based cell encapsulation for omics library preparation steps make them amenable to integration but may not be ideal for all applications. On the other hand, combining different platforms requires further innovations and development. Finally, the data generated from these workflows will also be massive and developing the methodologies for the accurate association of the different data modalities, their analyses, correct interpretation, efficient storage and maintaining security is crucial. Other pertinent challenges for integration include the development of microfluidic platforms for handling complex *in vitro* models like organoids, antibody free proteomics, integration and interpretation of multimodal omics data, and omics scale analysis of less explored but clinically relevant molecular subtypes like lipids and metabolites. Finally, there is also a need for developing higher sensitivity assays for the reliable profiling of low abundance molecules from single cells such as transcription factors which play a critical role in many biological processes and diseases. There will obviously not be one universal integrated solution to all cell therapy workflow challenges. However, what can be expected in the coming years is the development of targeted platforms that implement partially integrated workflows for specific applications. Some of the developments toward such technologies are discussed in the following section.

Emerging Solutions and Opportunities. With extensive developments in the various fields discussed previously, integrated systems for genetic discovery and therapeutic development are becoming a reality. For instance, Inscripta's Onyx is an integrated platform allowing large-scale CRISPR-based genome engineering experiments.[235] The platform allows the users to design their CRISPR experiment and plasmid library. Benchtop equipment with several modular microfluidic units performs all the engineering steps such as cell culture, electroporation of the custom libraries into the cells, and processing successfully edited cells for downstream sequencing and phenotyping. All the intermediate steps such as media exchange and sample transfer on the benchtop equipment are automated and monitored, minimizing user input. The Onyx platform also provides assay kits to prepare NGS ready libraries from the edited cells and software for the analysis of sequencing results. Another example is Berkeley Light's Lightning platform, which employs a microfluidic chip for optofluidic manipulation of lymphocytes in compartments called NanoPens. This platform allows for on-chip cytokine secretion, cytotoxicity, cell−cell interaction assays, and target cell recovery for downstream sequencing.[236] Using these kinds of platforms, researchers have barely scratched the surface. Thus, there are tremendous opportunities for utilizing these technologies and developing similar integrated platforms tailored toward different cell therapy workflows. For now, most of these platforms and cell therapy workflows, in general, have been targeted toward immune cells and HSCs. However, other applications such as engineering and transplantation of

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stem cells to treat neurological, muscular, skeletal and visual disorders have shown promise but are not as mature.[237] Consequently, integrated platforms addressing these workflows also require development to accelerate these fields. Thus, it is entirely conceivable that such integrated platforms executing complex workflows for the discovery and design of cell-based therapeutics will become popular in the upcoming years. Their designs will be governed by their specific applications but will likely have some common features such as (1) high throughput, scalable, modular design to incorporate different related workflows and the ability to handle different starting sample types and sizes without significant losses; (2) high recovery and purity for target cell isolation steps; (3) cargo agnostic cell engineering at high efficiency; (4) multimodal single cell molecular information at the omics scale; (5) reduced operation time to prevent sample degradation and lower cost to expand experimental design; and (6) finally, to bring it all together, high quality and reliable data generation to improve confidence on inferences. In addition, the experimental and data processing pipelines should also be highly automated to reduce operator burden and make these systems attractive propositions for adoption as compared to traditional manual workflows.

OUTLOOK

Cellular therapy products must be safe, pure, potent, stable, and effective for clinical use. Currently, over 28 cell therapy products are globally approved for clinical use, and over 1,700 active clinical trials employ cells for therapeutic purposes. Approved cell therapy products predominantly include T cells, followed by stem cells and dendritic cells.[238] However, it took several decades of work, failures, and massive investments to get to this stage. With the advent of micro/nano technology-based platforms that can accelerate cell therapy development work, we are likely to see rapid progress toward a better understanding of disease landscapes and investigation of cell-based therapeutic solutions. At present, the use of T cells for cancer therapies has garnered the most attention. Several CAR-T cell-based therapies for oncology are already in the market and CAR-T cells for the treatment of cancers such as multiple myeloma and lymphoma are under clinical trial.[239] On the other hand, various other diseases and therapeutic cell types are relatively less studied. For example, mesenchymal stem cells (MSCs) are strong candidates for therapeutics due to their multipotent, immune evasive and anti-inflammatory nature. Hence, MSCs are being extensively investigated for the treatment of cardiovascular disorders namely ischemic heart disease and central nervous system (CNS) disorders such as multiple sclerosis.[240] Likewise, other promising examples include the use of induced pluripotent stem cells (iPSCs) for treating acute macular degeneration and neurodegenerative disorders such as Parkinson's disease, dendritic cells for uveal melanoma and liver transplantation, B-cells for autoimmune disorders like type 1 diabetes, and genome edited HSCs for monogenic disorders such as sickle cell disease and β-thalassemia.[241] Thus, we are just beginning to unlock the potential of therapeutic cells and the use of micro/nano technologies will only aid in the next generation of therapeutic advancements. For a comprehensive discussion on promising cell therapies and challenges, the readers are also guided to other excellent reviews on this topic.[237,238,240−242]

Irrespective of the type of cell therapy, their safety and clinical efficacy depend on factors such as cell sourcing, efficient engineering, targeted delivery, maintenance of cell functionality *in vivo*, scalability of cell therapy products, and comprehensive analysis of cell properties. Often, promising preclinical studies have failed in the clinic due to inconsistent performances arising from variability in cell source, isolation, culture, engineering, and poor-quality control of the product in general. Thus, there are still major biological, manufacturing, and regulatory challenges associated with the clinical translation of cell therapies. The future of cell therapy depends on the development of solutions to address these critical translational challenges. For autologous therapies (using patient's own cells), one challenge is that the quality and quantity of cells retrieved depends on the patient's condition, with the possibility of the therapeutic cell reserves being depleted or incapacitated due to illness. With a small starting sample there is always a risk of poor survival, loss of stability and risk of the culture being overtaken by undesirable cell types when the purity is low. Here, micro/nano isolation and engineering methods may be advantageous as they provide superior recovery, purity, and modification efficiency, which can lead to a higher yield of the therapeutic product from a limited starting resource. For allogenic therapies (using a donor's cells), abundant and potentially scalable cell sources are available, but immunogenicity is a significant safety risk and barrier to translation. Current allogenic cell therapies are often combined with immunosuppression regimens to control host immune response to the foreign material. Genome engineering solutions that help cells escape immune recognition but at the same time do not pose tumorigenicity risk is likely to be useful in these scenarios. Here, the use of the high-throughput omics testbeds for screening CRISPR-based genome and epigenome edits can be employed to reduce the turnaround times. Additionally, these screening methods can be used to deeply study common issues with cell therapies such as potential off target effects leading to toxicity, moderate activity, restricted trafficking, and limited stability within challenging environments *in vivo*. In addition to cell sourcing, scalability of biomanufacturing is one of the major bottlenecks to clinical translation. Scalable biomanufacturing processes for cell culture growth and genetic modification can harness the advantages of flow-based micro/nano systems that can process millions to billions of cells within several minutes and are likely to make immediate impacts in improving this aspect of cell therapy development as mentioned previously. On the efficient cell engineering and modification front, reproducibility, and the long-term durability of the therapeutic response across different batches is an unresolved problem. This is a result of complex interplay between several factors such as the variability in source material, therapeutic potency, engineering modification efficiency, cell viability, time and location of administration and interaction with the host's system among others. Micro/nano substrate-based *in vitro* cell engineering platforms that provide tighter control over cargo dosage, greater modification efficiency and improved viability are likely to address some of these issues and improve efficacy. Emerging nanoparticle-based delivery or *in vivo* cell engineering solutions on the other hand can control the distribution, specificity, and timing of therapeutic release while minimizing interaction with the host's immune system. Thus, they are also likely to help ameliorate the poor reliability of outcomes. Another grand challenge that holds true for cell therapies just like other therapeutics is the time it takes to get through several cycles of testing and validation, which adds to the already high

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manufacturing costs. To generate clinically and commercially viable cell therapies, it is imperative to adopt testing strategies that incorporate combinatorial genome editing schemes to improve specificity, therapeutic potency, and minimize off target effects. Moreover, synthetic biology approaches to generate constructs that can predictably control input−output relations within gene regulatory networks, and high-throughput multiomic analyses to quickly screen and validate results are also necessary. Such strategies can potentially reduce cycle times and lead to robust and stable therapeutic solutions at reasonable costs. Overall, independent development of various micro/nano-based platforms for cell isolation, engineering, and analysis have already had or are likely to have a positive impact on the next generation of cell therapy pipelines in the near term. In the long term, we expect interdisciplinary efforts and synergistic developments across the different fields to improve basic understanding, yield integrated solutions to streamline cell therapy pipelines, and aid in the development of "universal" cell therapy products.[241]

The emergence of micro/nano technologies and the evolution of cellular engineering and analysis methods pivoting on these technologies have transformed the landscape of biological inquiry. It is incredible to think that within a few decades of the advent of Sanger sequencing, technology has advanced to a point where we can precisely engineer thousands of individual cells and harness the power of NGS to examine their genome, epigenome, transcriptome, and proteome in parallel. In this review, we have focused on how micro and nano technologies have enabled single cell manipulation and analysis, emphasizing technologies that are being translated into commercial products and thus democratizing their use among researchers to pursue advanced lines of inquiry. Our discussion provides evidence of the continued contribution of micro and nano technologies toward the advancement of cell engineering and analysis tasks involved in cell therapy development workflows both *in vivo* and *ex vivo*. *In vivo* studies are likely to benefit from techniques that improve cell engineering precision, reduce off-target effects, and provide time-resolved single cell analysis of multiple molecular subtypes without losing spatial information. *Ex vivo* studies, on the other hand, are likely to benefit from fully automated platforms that handle all steps of the experiments with minimal manual intervention. This would help researchers focus on the experimental design and data interpretation without being involved in the complex execution steps. With further development of experimental and data analysis methods, it will not be long before such solutions are developed. We are already witnessing great strides being made toward their resolution, with micro and nano technologies at the heart of it.

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Notes

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VOCABULARY

Cell Therapy:It is a process where viable cells from a source are injected or implanted into a patient to fight diseases or to repair and regenerate damaged tissues. Often the cells are engineered prior to implantation to elicit the intended medicinal response. In autologous cell therapy the therapeutic cells are obtained from the patients themselves, while in allogenic cell therapy the donors are individuals different from the patients.

Cell Engineering:It involves modifying the genetic sequences of cells to alter their gene expression and biological function. Engineering cells has a wide range of applications such as introduction of therapeutic phenotypes, elimination of disease-causing phenotypes, and production of biologics.

Intracellular Delivery:The process of introducing foreign material into a cell without destroying it. A variety of materials can be delivered into a cell including small molecules, DNA, RNA, proteins, and engineered nanoprobes. Intracellular delivery is usually a critical step in cell engineering.

Multi-Omics:It involves quantifying and characterizing large sets of biological molecules from multiple classes simultaneously. Multiomics can involve the study of DNA (genomics), DNA modifications (epigenomics), RNA (transcriptomics), proteins (proteomics), and metabolites (metabolomics) together in various combinations.

Spatial-Omics:This encompasses a class of technologies that map the information obtained from various "omics" assays

directly onto tissues to provide spatial context to the molecular quantification.

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