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Use of Single Cell -omic Technologies to Study the Gastrointestinal Tract and Diseases, From Single Cell Identities to Patient Features

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Abstract

Single cells are the building blocks of tissue systems that determine organ phenotypes, behaviors, and function. Understanding the differences between cell types and their activities might provide us with insights into normal tissue functions, development of disease, and new therapeutic strategies. Although -omic level single cell technologies are a relatively recent development that been used only in laboratory studies, these approaches might eventually be used in the clinic. We review the prospects of applying single cell genome, transcriptome, epigenome, proteome, and metabolome analyses to gastroenterology and hepatology research. Combining data from multi-omic platforms and rapid technological developments could lead to new diagnostic, prognostic, and therapeutic approaches.

Keywords

heterogeneity; transcriptomics; proteomics; metabolomics; epigenomics

During the last decade, significant technological advances occurred in the biomedical sciences due to rapid development of next-generation sequencing and mass spectrometry (MS) technologies. Today, we can easily generate comprehensive data on the genomic, epigenome, metabolome/lipidome, and proteome from patient specimens.[1-3] This information, generated at sample level, enables studies of pathogenesis and identification of

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disease subtypes.[1, 2, 4] Although bulk approaches have enabled discoveries based on inter-patient or inter-sample variation, intra-sample heterogeneities carried by individual cells over time are lost.[5] This information is critical in understanding disease mechanisms; different tissue functions are carried out by specific cell types, and disease progression, such as in cancer relapse or metastasis, are usually mediate by small populations of cells.[6, 7]

To address cellular level heterogeneities, researchers developed new methods and techniques to generate high-throughput and multi-dimensional data at the single cell resolution (Fig. 1). The emerging single cell biology field is revolutionizing basic sciences by addressing sophisticated biological questions in developmental biology.[8] In the clinic, single cell level metrics such as the Immunoscore[9] can affect decisions about management of patients with cancer. Furthermore, in patients with gastrointestinal malignancies, tumors with increased cellular heterogeneity tend to respond poorly to treatment.[10, 11] Increasing our understanding of these phenomena and obtaining higher resolution single cell data, especially from patients, might improve care. We review single cell technologies and how they can be used to study digestive diseases. However, we emphasize that single cell -omics level technologies have only been applied in a research setting, including translational research, and cannot yet be used to make actionable clinical decisions.

Candidate-based Approaches

Analyses of molecular features of individual cells are not new to clinical research—pathologists have been using microscopy, immunohistochemistry, and in situ hybridization for decades to identify cells, such as infiltrating lymphocytes, in human tissues. Flow cytometry is used to perform immunophenotype analyses of blood cells in patients with immune-mediated and other diseases. These techniques are known as candidate-based approaches, because they require probes to detect specific molecules.

Advances have mainly focused on exceeding the limitations of spectral overlap in fluorescence to enable highly multiplexed (such as multi-analyte) analyses at the single cell level, such that machines can potentially identify cells from multivariate measurements in lieu of a human expert.[12] There are 2 main ways by which multiplexing is achieved. First, elemental isotope labeling of antibodies enable mass spectrometric deconvolution of probes with minimal spill over. Metal antibody labeling results in flow cytometry (mass cytometry, also known as CyTOF)[13] and microscopy modalities (imaging mass cytometry, multiplex ion beam imaging)[14, 15] capable of analyzing 30–40 analytes at single cell resolution.

Mass cytometry has been optimized to study signaling within the intestinal epithelium[16], and immune cells in the lamina propria.[17] For example, Chuang et al studied immune cells infiltration in lamina propria samples from patients with Crohn's disease using CyTOF; they found monocytes that carried a frameshift mutation in *CSF2RB* had reduced responses to granulocyte-macrophage colony stimulating factor.[18] Martin et al also used CyTOF to identify unique fibroblasts and immune cells in ileal tissues from patients with Crohn's disease that contribute to resistance to tumor necrosis factor antagonists.[19]

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Iterative schemes can be used with microscopy, such that different probes are applied in different imaging rounds even though the same detection labels are used. With advanced registration approaches[20], multi-channel images, analogous to standard 3- or 4- channel fluorescence images, are produced. Iterative schemes can be used to detect protein[21-25] or transcripts[12, 26], with differences between these schemes being the detection method (for example, direct conjugate vs tagged nucleic acids) affecting iteration times. Gerdes et al stained 61 protein antigens in tissue microarrays comprising 747 samples from patients with colorectal cancer (CRC); they found extensive heterogeneity among tumors that corresponds to specific signaling pathways.[27] Using multiplex protein imaging, Ligorio et al found that cancer-associated fibroblasts contribute to heterogeneity within pancreatic tumors.[28] Algorithms to analyze multi-dimensional images can be segmentation-based to produce single cell resolution data similar to mass cytometry[29, 30], or pixel based[31]. The unifying theme behind these studies revolves around the discovery of new, unexpected cell populations that associate with disease processes. These efforts are made possible by labeling tissues with multiple probes that can detect a wide variety of cell types, and for combinatorial labeling to define unexpected cell types or states. The experiment therefore acts a screen for unexpected cell populations that correlate to phenotype, which fulfills a fundamentally different goal than single-target approaches, such as immunohistochemistry, which a single marker to query a single cell population.

Candidate-based approaches are most ready to be adopted for human research due to their congruence with traditional pathology analyses, and machine learning on large sets of histological images has already been explored.[32, 33] However, candidate-based approaches require accurate probes—it is a challenge to determine specificity and sensitivity of detection for probes (such as antibodies) in human tissues, due to lack of control samples. Furthermore, measurements of preselected markers provide only a narrow scope of new insights into factors that contribute to disease development.

Single-Cell Analysis of Transcriptomes from Cell Suspensions

Untargeted sequencing does not depend on candidate probe quality, because sequencing data are mostly categorical in nature (4 nucleotides- ACGT). Single-cell RNA sequencing (scRNA-seq) is one of the most mature single cell genomics tools for studying cell heterogeneity.[34] Since 2016, there has been a large increase in studies using scRNA-seq analysis, due to the advent of droplet-based techniques that enable high-throughput analyses of thousands of cells instead of hundreds.[35-37] Different protocols provide different aspects of transcriptomic information (Supplemental Table 1), such as isoform information from full-length analysis[38] and differential gene expression based on 3' poly(A) capture transcript counting.[39] For a review on scRNA-seq, see refs[34, 40]. We focus on aspects most relevant to human research.

Single-cell RNA sequencing involves isolation, containment, and processing of single cells into nucleic acid libraries for sequencing. An important pre-analytical variable lies in tissue collection and handling. Ischemic conditions and dissociation of single cells can introduce significant artefacts to cell transcriptomes. Fresh specimens collected from accessible surgical or biopsy samples, immediately delivered for processing, yield the highest quality

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data. Single cells are dissociated by exposure to enzymes such as trypsin or collagenase, followed by mechanical trituration. Optimization in fresh tissue processing, such as cold protease dissociation, enables minimized perturbation during single cell isolation.[41] For tissue types where dissociation is challenging or situations in which times of ischemia cannot be controlled, tissues can be flash frozen, so quality of nucleic acids can be preserved and nuclei can be isolated for single nuclei (sn) RNA-seq.[42, 43] snRNA-seq has the advantage of being amendable to retrospectively collected frozen archive specimens, but it should be noted that nuclei possess less material compared with what is in an entire cell, with marked differences in representation in transcripts.[44] Regardless of protocols used, relative abundances of particular cell types might be artificially changed due to selective filtering and retrieval during tissue preparation steps.

A variety of methods, from plates to microdroplets[35, 36], exist to contain single cells such that cDNA libraries from individual cells can be prepared (Table 1). The most technically amenable methods are droplet based[35-37] or nanowell based.[45-47] These are high-throughput approaches that require less manual handling due to barcoding (Fig. 1), miniaturization, and automation, and commercial counterparts exist for turnkey applications. Although there are nuances to library preparation downstream that results in different coverage[48-51], all scRNA-seq libraries require amplification, due to the low amount of starting material. From small amounts of RNA, technical noise arises due to Poisson sampling, especially for less abundant transcripts.[39] Researchers should balance the cost benefit of using exponential amplification (such as PCR) and linear amplification (such as in vitro transcription).[51-53] Exponential amplification is easy and requires less technical skills and awareness, but results in amplification bias of highly abundant transcripts that requires more sequencing (and therefore, higher costs) to overcome. Linear amplification allows low abundance transcripts to be represented but is a multi-day process requiring multiple steps in handling RNA. Ziegenhain et al and Wang et al performed comparative analyses of different scRNAseq methods that could be informative for choosing particular methods for particular applications.[54, 55]

There are several alternatives for downstream processing and analysis of sequencing data (see ref[56]). Mapping of FASTQ data can be achieved via a variety of mapping algorithms including TopHat, STAR, and Kallisto Bustools[57]. Popular data analysis packages such as Seurat[58] or Scanpy[59] can be used for downstream analyses. These software tools already incorporate dimension reduction algorithms for visualization of multi-dimensional data in lower dimensional space, such as t-distributed stochastic neighbor embedding (t-SNE)[60], and uniform manifold approximation and projection (UMAP).[61]

These tools allow data points, and therefore cells with similar expression profiles, to be grouped, enabling identification and analysis of cell types and states. One significant challenge, however, is the quality of single cell level data that results from incomplete sampling of the transcriptome. These noisy data require additional attention paid to quality control regarding the filtering of low quality cells [62], as well as non-reliant and irrelevant gene features[63]. Of note for scRNA-seq applications in human studies is the eventual acquisition and analysis of datasets from large cohort of patients (Fig. 2). Researchers have

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developed tools[64, 65] that can compare entire single cell landscapes that can be used for subtyping samples and patients.

Slide-based Spatial Transcriptome Analyses

Transcriptomic analysis is routinely performed on single cell suspensions where spatial context is lost. The location of cells determines their interactions with other cells, via short- and long-range signaling mechanisms[66], and their identities and behaviors as a function of environment (such as hypoxia or normoxia).[67] Furthermore, cells in tissues, especially in patients with diseases, are spatially heterogeneous. For instance, Crohn's disease intestinal tissues contain pathological skip lesions that are interspersed amongst uninflamed areas.[68] Suspension-based scRNA-seq in this case produces data on admixtures of healthy and affected tissues, blending out disease-specific signals. In situ analysis does not require dissociation nor single cell retrieval, thus should maintain accurate representation of cell types of the native tissue. To produce spatially resolved analyses, researchers rely on candidate-based approaches for targeted analyses. To scale these approaches to the genome level, seqFISH+[12] and MERFISH[26] use temporal fluorophore barcoding with hamming distance correction to extend the number of mRNA measured to the scale of 10,000. For temporal barcoding to work, individual molecules of mRNA must be resolved, which limits the volume of tissue that can be profiled. These approaches are therefore best for high-, sub-cellular resolution imaging with high-end microscopes that are not always accessible outside of research settings. As such, recent studies have combined seqFISH+ with expansion microscopy to visualize gene expression at deeper, organelle levels.[69]

At the other end of the spectrum, genome-scale data can be obtained when single cell resolution is not needed. For instance, laser capture microdissection can be used in a low-throughput manner, even with fixed specimens, to profile transcriptomes of a few to 10s of cells.[70, 71] For lower-resolution tissue profiling, multiple groups have developed techniques that involve spatial barcoding in lieu of single cell barcoding.[72, 73] In general, these approaches immobilize barcoded oligonucleotides[73] or oligonucleotide-conjugated beads onto slides[72]; tissue sections are then cut and permeabilized, enabling mRNA capture by the underlying oligos. The oligos are then released, pooled, and prepared as a library in a similar manner to scRNA-seq. The spatial barcode that was incorporated into the cDNA during capture could be mapped back to the original position in the tissue section.

These methods couple spatial information for analysis of tissue architecture and global transcriptome profiling; they have been used to characterize infiltration of immune cells into tumors during immunotherapy.[73] The resolution of these approaches is on the order of 50–100 microns, such that each barcoded pixel contains information about dozens of cells. Improved protocols with fewer than 10 micron resolution are being developed, although data quality, in terms of number of transcripts detected, is compromised.[74] Nevertheless, slide-based digital pathology and scRNA-seq have become more common, so these combined approaches could be rapidly advanced for analysis of tissues from patients.

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Application of Single-cell Transcriptome Analyses to Gastroenterology

scRNA-seq can be used to evaluate tissue heterogeneity, and the cell types responsible for a particular function or disease in the gastrointestinal tract[75-77] and liver[78, 79] is a direct application of scRNA-seq. In a basic science setting, scRNA-seq has been used for finding new populations of endocrine[80, 81] and tuft cells[75, 82] in the gut epithelium, as well as immune[83] and stromal cells.[84] Because scRNA-seq is an endpoint assay where insights are derived from a snapshot in time when tissue is collected, it can applied to range of specimens from model organisms to human patients.[85] scRNA-seq forms the basis of the next stepping stone towards precision medicine, akin to prior efforts such as TGCA and CPTAC. Large national and international consortia are being formed, utilizing scRNA-seq to characterize large cohorts of healthy and diseased tissues. Here, we will highlight studies utilizing scRNA-seq on human patient specimens.

A major application of scRNAseq is in cancer biology where intra- and inter-tumor heterogeneity is a major challenge in rational therapy design.[1, 11, 86] Our descriptions of cancer phenotypes and subtypes rely mostly on bulk sequencing or proteomic assays that mask the potentially important cell populations, such as those that are metastasis-capable, whereas scRNA-seq can be used to deconvolve cellular heterogeneity. Furthermore, a comprehensive characterization of tumor landscape by scRNA-seq, including immune and fibroblast infiltration within the microenvironment, can possibly be applied to describe different disease subtypes and predict patient prognosis .[28] Some recent studies have already shown successful application of scRNA-seq to pre-cancers and cancers of the digestive system, where cancer cell genotypes, phenotypes, and the microenvironment are systematically dissected. Zhang et al discovered a dynamic relationship of T cells in CRC and identified subgroup of CRC that are likely going to show favorable responses to immune-checkpoint blockade.[87] Bian et al dissected 10 CRC using multi-omics approaches that provide fundamental insights for understanding the molecular alterations that occur during primary CRC progression and metastasis.[88] Another study examined the transcriptional dynamics of different gastric cancer subtypes using scRNA-seq where they identified OR51E1 as a marker for unique endocrine cells in the early-malignant lesion in gastric cancer.[89] Owen et al studied Barrett's esophagus, that progress to esophageal cancer, using scRNA-seq and found transcriptional similarity between Barrett's and normal esophagus.[90] A recent liver cell atlas identified the cellular composition of liver and unraveled the phenotypic changes that occur in hepatocellular carcinoma.[78] Using scRNAseq, Zheng et al identified infiltrating T cells with distinct functions in liver cancer that provided valuable insights to understand the immune landscape and prognostic potential in this cancer.[91] Immunotherapy is gaining traction in providing sustained responses in many cancer types. However, only a fraction of patients will respond to immunotherapy, as partly determined by the degree of immune cell infiltration into the intra-tumoral space of the naive tumor.[92, 93] scRNA-seq can contribute to immunotherapy by deeply characterizing the immune microenvironment as to predict patient response.

Inflammatory bowel disease are characterized by the chronic inflammation of the intestines.[94] Complex conditions such as IBD where shifts in cellular ecosystem is correlated to disease phenotype can be dissected by scRNA-seq. Parikh et al performed a comparative

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study between IBD and normal colon to identify specific cell type that drives IBD[77] They found a pH sensing and proton channel OTOP2-expressing colonic absorptive cells at the top of the crypt that are often dysregulated in IBD as well as cancer. Using scRNAseq, they also dissect cell type-specific responses to colonic inflammation and find that WFDC2 secretion is required for mucus barrier integrity. scRNA-seq has also been used to characterize a network of IgG plasma cells, inflammatory mononuclear phagocytes, activated T cells, and stromal cells in patients with Crohn's disease resistant to tumor necrosis factor antagonists.[19] Smillie et al generated a single cell atlas of ulcerative colitis to map the GWAS risk variants precisely to cell types and pathways where they characterized specific cell subsets responsible for inflammation and therapy resistance.[95] Kinchen et al performed single cell profiling of colonic mesenchymal cells that revealed unexpected heterogeneity and mesenchymal remodeling associated with inflammation and barrier dysfunction in IBD.[96] Single-cell analyses can be used to study microenvironment communication mechanisms and increase our understanding of complex gastrointestinal disorders.

Although clinical decisions cannot yet be made based on scRNA-seq data, they can be used to determine patient prognoses and disease subtypes.[97, 98] scRNA-seq data might also be used as a reference to deconvolve cell compositions from existing bulk RNA-seq data, with algorithms such as MuSiC, xCell, and CIBERSORTx.[99-101] The promise here is for new patient categories to be defined with finer resolution, such that possible outcomes of new and formerly diagnosed patients can be predicted from a retrospective resource. Despite the promise and wide accessibility of single cell transcriptome analysis methods, there are limitations that preclude their use in the clinic. There are still many places in transcriptome analysis protocols where artefacts can be introduced, reducing reproducibility and interpretation—the technical and computational expertise required is quite high. The cost and turn-around required for interpretable results might be unacceptably high for clinical decision making. Lastly, the utmost need for accessible fresh tissue to generate the best quality scRNA-seq data is a challenge for many clinics. Combination of all these factors results in a high barrier of adoption, but many groups are moving toward solving these problems.

Single-cell Epigenetic Analyses

Layers of epigenetic information connect the genome to the transcriptome. [102, 103] Microfluidic paradigms used to determine single cell transcriptomes have been expanded for studies of single cell epigenomes. These technologies have led to methods for investigating chromatin accessibility, protein–DNA interactions, chromosome conformation, and DNA methylation in single cells.

Single-cell assays for transposase-accessible chromatin using sequencing (scATAC-seq) target accessible genomic regions by exploiting the kinetic favorability of Tn5-mediated transposition reactions with DNA unincorporated by nucleosomes. These captured genome sequences can be cis-acting DNA elements poised for transcription or regulation by transcription factors. Borrowing the microfluidic platforms of single cell transcriptomics, several scATAC-seq methods have been established (see studies by Cusanovich et al, Buenrostro et al, and Lareau et al[104-106]). These methods isolate individual cells using plated

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micro-wells, integrated fluidic circuits, and encapsulation into nanoliter droplets, respectively. Each nucleus therefore produces a single barcoded library of genomic fragments enriched for regions of accessible genomic loci.

Single-cell chromatin conformation capture methods can be used to identify cis-acting DNA elements and determine their physical proximities to potential regulators. Topologically associated domains and long-range chromatin interactions mediated by loop structures can be probed by 3C methods; more recently, at single cell resolution. Hi-C, and its single cell variants like sc-Hi-C and sci-Hi-C, developed by Nagano et al and Ramani et al, combine chromatin crosslinking, restriction digestion, and proximity-based ligation to create libraries that capture spatially proximal DNA fragments.[107, 108] sci-Hi-C, in particular, isolates nuclei in microwells and incorporates combinatorial indexing. These methods result in a single library per cell, containing fragments that represent pairs of proximally adjacent genomic loci. Although it is not exactly a single cell chromatin conformation capture method, a modified form of ATAC-seq, called ATAC-See, developed by Chen et al, permits covalent tagging of accessible chromatin with visualizable fluorophores. This allows for visualization by microscopy and subsequent high-throughput sequencing.[109]

Single-cell chromatin immunoprecipitation methods target protein–DNA interactions within single, isolated cells. These methods retain the same strategy as their bulk approaches, relying on specific antibody-protein interactions. Drop-ChIP, a method developed by Rotem et al, takes protein-associated genomic fragments generated from droplet-isolated single cells and tags them with unique DNA barcodes.[110] These nanoliter droplets, which contain the contents of a single cell, are broken and aggregated for immunoprecipitation and library generation. This information can also be obtained using CUT&Tag, described by Kaya-Okur et al. This method uses protein-A-tethered Tn5 transposons to localize these elements to protein-bound antibodies.[111] Target-localized transposons fragment the genome in a way that enriches target protein-associated loci. These reactions are amenable to nanowell-based single cell isolation systems, because antibody binding and transposon introduction can be performed at a bulk level before isolation. Both of these methods produce sequencing libraries that contain cis-acting regulatory elements associated with the antibody-targeted protein.

Single-cell methylation and hydroxymethylation (sc-5mc and sc-5hmc) assays measure covalent modifications on genomic cytosine residues. Often, these modifications are enriched in CpG islands—high concentrations can result in silencing or reversible downregulation of gene expression.[112] These methods are classified by their sodium bisulfite dependence, where dependent methods convert cytosine residues into sequencing-detectable uracil. Single-cell genome-wide and reduced-representation sequencing methods, which depend on bisulfite conversion, have been developed to capture varying breadths of the methylome.[113, 114] In contrast, single cell CpG island methylation sequencing combines methylation-sensitive restriction digestions with multiple displacement amplification to generate a sequencing library enriched for loci associated with methylated CpG islands, while avoiding destructive bisulfite conversions.[115] Other new methods include scAba-seq, which targets 5hmc and retains strand-specific information through bisulfite-independent, but glucosylation-dependent enzymatic reactions.[116]

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Like single cell transcriptomes, epigenetic data from single cells can be used in human research, possibly to determine patient prognoses or select therapy. Bormann et al examined the CpG island methylator phenotype along with cell of origin signatures in colorectal tumor tissues and identified epigenetically defined subtypes of tumors that correlated with patient survival.[117] Other tumor types have epigenetic heterogeneity along with functional heterogeneity. Litzenburger et al used scATAC-seq to demonstrate that differences in chromatin accessibility associated with sensitivity of cancer cell lines to drugs.[118]

Despite promise of single cell epigenomics, there is no integrated epigenomic method that could capture all levels of epigenetic modifications simultaneously. Nevertheless, as approaches for epigenomic analyses of single cells develop, we expect applications for gastrointestinal tissues and diseases.

Single-cell Metabolomics

Metabolites are small molecules (typically fewer than 2000 Da) including amino acids, sugars, lipids, small peptides, and break down products of drugs. These molecules can regulate cell structure, fuel, signaling, enzyme regulation, and pathogenesis. [119] It is a challenge to analyze metabolites because they are highly dynamic, [120] with vast structural diversity and many isomers.[121] Single-cell analysis introduces additional demands because metabolites cannot be amplified, necessitating ultrasensitive analytical assays.

MS is the most widely used technology for analysis of single cell metabolomes.[122] MS is label-free, untargeted (it can detect thousands of molecules concurrently), sensitive (fM limits of detection), and specific (it can discriminate molecules that differ by less than 0.001 Da).[123] Single-cell MS analyses of metabolomes are typically performed as either profiling experiments, in which a single spectral signature is collected from each cell type, or as an imaging MS experiment to visualize metabolite distributions within a cell or across a tissue microenvironment (Figure 3A). In all cases, special care needs to be taken to ensure the metabolic profile of the cell is not perturbed during sample preparation because of the relatively high rate of turnover of some metabolites.[124]

Most metabolomic profiling experiments of single cell types are performed on cell cultures or isolates from tissue. Targeted cell populations are separated by manual manipulation,[125] fluorescence-activated cell sorting, microarray cell printing,[126] or using microfluidic devices[127] prior to MS analysis. There have been significant advances in development of technologies for rapid MS analysis of single cells in suspensions and on surfaces. For example, a single-probe MS sampling technology was developed to analyze live single cancer stem cells.[128] The single-probe MS system uses a dual-bore capillary to deliver the lysis and extraction solvent to the cell surface and carry the molecular extract to the mass spectrometer for analysis by electrospray ionization. By bringing the single-probe tip directly in contact with individual cells, dispersed onto a glass slide, researchers were able to perform single cell metabolomic analyses of living cells with no sample preparation.

In a significant advancement in throughput, Neumann et al were able to perform MS lipid analysis on 30,000 individual cells using matrix-assisted laser desorption/ionization

(MALDI).[125] They achieved this by dissociating cells onto a slide and using a multimodal imaging approach to identify cells for MS analysis. Fluorescence microscopy was used to determine locations of intact cells for MALDI data acquisition. Using this technology, researchers detected more than 500 lipid features and were able to determine 101 significantly different cell clusters, using a combination of t-SNE and Louvain-Jaccard clustering. Others have been working to extend single cell metabolome profiling to more complex cell systems and tissues. For example, Portero et al coupled microsampling with capillary electrophoresis and electrospray ionization MS to enable in situ metabolite quantification in live cells and tissues.[129]

Recent advances in MS instrumentation and data processing have enabled imaging MS while maintaining spatial resolution.[130, 131] The imaging MS experiment is typically performed on tissue sections, which were thawed and mounted onto MS compatible glass slides. Ions are produced at each position (pixel) within a designated region of the tissue resulting in the generation of a mass spectrum for each pixel. In a single imaging experiment, hundreds of metabolites are detected and are visualized as images by plotting the intensities for each molecule over array of pixels.

Although all imaging MS technologies are conceptually similar, in that they generate spatial molecular data, MALDI[132] and secondary ion MS (SIMS)[133, 134] are most commonly used for high spatial resolution of metabolomes. MALDI imaging is a laser-based surface analysis method in which the sample is coated with a light-absorbing matrix that assists in desorption and ionization of endogenous molecules. Spatial resolution is defined by the diameter of the focused laser beam at the sample surface. MALDI imaging is routinely performed at higher than 5 μm resolution; specialized setups can achieve 1 μm pixel sizes with good molecular coverage. SIMS imaging is similarly performed except that no matrix is used, and a tightly focused ion beam is used for surface sampling, allowing for pixel sizes down to 100 nm. However, SIMS typically induces molecular fragmentation during the sampling process, significantly complicating data interpretation.

Prentice et al used MALDI imaging MS to identify specific phospholipid and glycolipid isoforms in pancreatic islets.[135] They performed serial imaging MS and immunofluorescence microscopy to correlate metabolite signals with specific cell types. Multimodal imaging studies have also been performed to combine MALDI and SIMS imaging for spatial metabolomics.[136] This workflow was used to generate complimentary high spatial resolution SIMS and high molecular content MALDI images of human colon cancer tissue. The combination of SIMS and MALDI data can be used to determine localizations of lipids and cholesterol esters in regions of tumor cells, stroma, and necrosis.

Imaging and single cell metabolomic profiling are developing fields with rapidly advancing technologies focused on improving spatial resolution and molecular specificity. These innovations are leading to finer spatial fidelity, more complete molecular coverage, and ultimately a deeper understanding of the molecular characteristics and interactions between cells in tissue microenvironments.

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Single-cell Proteomics

Cell structure and function are largely determined by the proteome—the complement of proteins expressed within the cell at a given time.[137] Yet, a corresponding technology for unbiased and broad profiling of the protein expression at the single cell level has been lacking. Single cells contain only picogram total amounts of protein, and as no amplification strategy is available, effectively analyzing these trace samples poses an enormous challenge. Consistent improvements in instrumentation (such as liquid chromatography, electrospray ionization, and MS) have extended protein detection limits to the single cell level[138], but conventional sample processing is incompatible with single cells due to physical challenges, such as nonspecific adsorption of proteins to the surfaces of well plates and inefficient digestion kinetics.

Recent innovations have helped us overcome this bottleneck, such than in-depth proteome profiling of single cells is now feasible. For example, a microfluidic approach to sample processing, called nanodroplet processing in 1 pot for trace samples (nanoPOTS),[139] has reduced sample preparation volumes from 10s or 100s of microliters to approximately 200 nanoliters, greatly reducing surface exposure and corresponding protein losses and increasing protein concentrations for efficient digestion kinetics. This is accomplished using a robotic nanopipetting system for liquid handling, which is capable of subnanoliter dispensing accuracy and submicrometer positioning control. The nanopipettor interfaces with a microfabricated glass nanowell chip, which replaces the conventional well plate and reduces surface exposure in each well to approximately 1 mm^2 Coupled with evaporation controlling mechanisms, highly sensitive and miniaturized nanoflow liquid chromatography, and latest-generation MS instruments, the nanoPOTS platform enables several hundred to more than 1000 protein groups to be identified in label-free, single cell analyses.

In addition to miniaturizing sample preparation and increasing the sensitivity of analytical instrumentation, researchers have analyzed single cells using tandem mass tag (TMT) barcoding, which allows multiple single cell proteomes to be determined in 1 liquid chromatography-MS analysis. This approach can increase measurement throughput and proteome coverage. Budnik et al first applied TMT labeling to the analysis of single mammalian cells with the SCoPE-MS workflow,[140] in which isobarically labeled single cells were analyzed in the presence of a larger carrier sample comprising hundreds of cells. The peptide signals from the combined carrier and single cell samples facilitated identification, and peptides from each cell were differentiated based on their corresponding reporter ion intensities following MS/MS fragmentation. Importantly, TMT workflows have recently been combined with miniaturized sample processing to take advantage of improved sample processing efficiency and multiplexing (Fig. 3B). These advances have been applied to studies of single cells[140, 141] and trace samples[139, 142], including those relevant to the digestive system. [139, 142] For example, liver tissues were profiled with 50 μm resolution[142], and 10-μm sections of individual pancreatic islets were studied from patients with and without type 1 diabetes [139]. Researchers were able to compare the 2 proteomes at the single-islet level. Of the nearly 3000 proteins profiled from the islets, approximately 300 were found to differ significantly in level between the two groups.

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Compared with single cell immunoblotting[143], which is an antibody-based assay designed to analyze a small number of proteins (approximately 10) per cell, MS-based single cell proteomics can quantify nearly 1000 proteins/cell in an unbiased, label-free analysis. However, every cell essentially occupies its own liquid chromatography-MS run, so single cell proteomic analysis requires more time, coupled to automation for analysis. This is one of the barriers to MS-based methods, and keeps them from being widely adopted.

Analyses of metabolomes and proteomes of single cells can identify differences in functions among seemingly identical cells; this information might be used in development of therapeutic agents. However, technologies for broad proteome and metabolome profiling at and near the single cell level are in their infancy; improvements in coverage, measurement throughput, and automation are needed before they can used routinely in human research.

Integration of Multi-omic Technologies

For a comprehensive picture of a cell, researchers require information on the genome, transcriptome, epigenome, and metabolome to be integrated (Fig. 4). Thus, comprehensive multi-omic profiling of single cells is the next frontier of development. Examples of current technologies include simultaneous profiling the transcriptome and chromatin-accessibility landscape[144, 145], and the transcriptome plus a set of candidate protein markers.[146] On the computational side, in silico multi-modal analytical frameworks permit exploration of phenomena indescribable by a single type of data alone and can often operate outside of the restrictions of multi-omic experimental methods. Frameworks such as Seurat v3 use distributions of cells collected by separate scRNA-seq and scATAC-seq experiments to co-embed cells into the same low-dimensional space. Further examples borrow other principles of multi-modal analysis, based on, but are not limited to, Bayesian modeling, graph theory, or deep learning. Respective examples include BREM-SC[147], iOmics-PASS[148], and SAUCIE[149]. Combinatorial multi- omics approaches could reveal different layers of heterogeneities that govern biology and disease phenotypes, but their prospect of being implemented in human research remains in question.

Future Directions

Although none of technologies for -omic analysis of single cells are ready for routine use in the clinic, many are used in human research and provide information about disease development and treatment. The degree by which each technology is adopted into clinical studies depends on its ease of use, precision, and reproducibility in generating data from minute amounts of material (Fig. 5). Some technologies, such as single cell proteomics, require sophisticated instruments and will probably be limited to specialized use in near term. Others, such as droplet-based scRNA-seq, is already available widely. A recent case study reported use of data from scRNA-seq analysis to guide treatment of drug-induced hypersensitivity syndrome.[150] The next few years will be an exciting time as various atlasing efforts will test the limit of these technologies in generating data that can illuminate basic scientific knowledge and inform biomedical decisions.

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Supplementary Material

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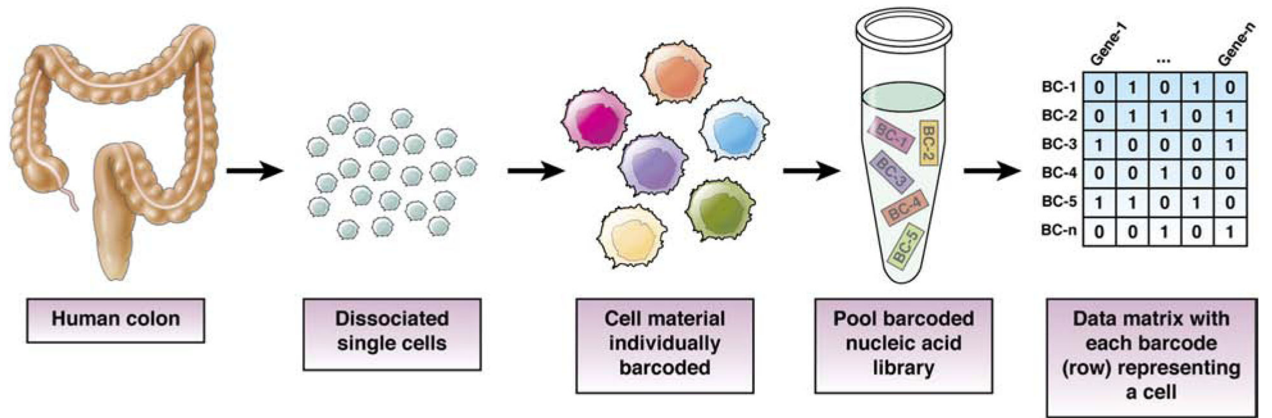

Figure 1.

Barcoding Scheme for High-throughput Genomics Analysis of Single Cells

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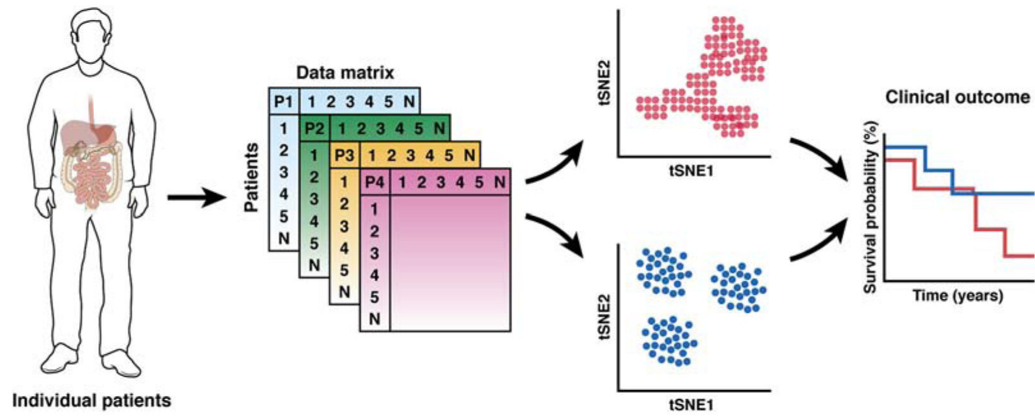

Figure 2. Potential Clinical Application of Single-cell -omic Technologies

Single-cell -omics profiles of digestive organs from cohort of patients may reveal single cell landscapes that predict clinical outcome.

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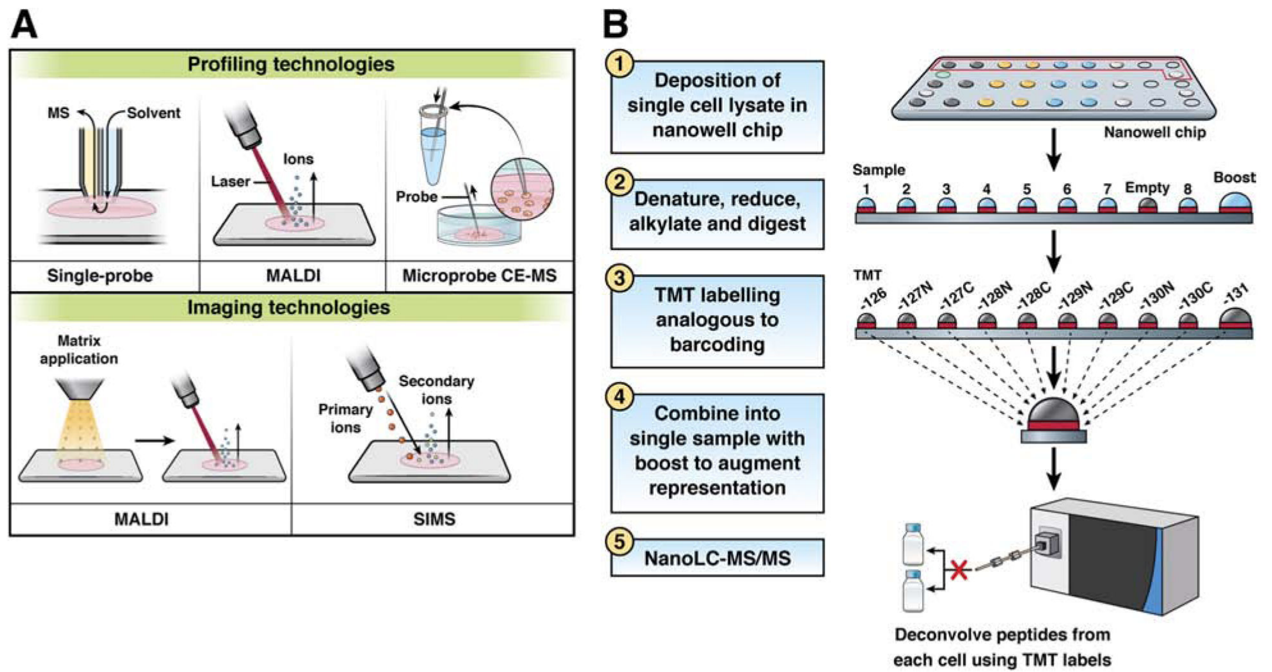

Figure 3. MS-based Technologies for Analysis of Single Cells

(A) Schematics of common single cell metabolomics profiling and imaging technologies.

(B) Workflow of the nanoPOTS-TMT-based single cell proteomics platform.

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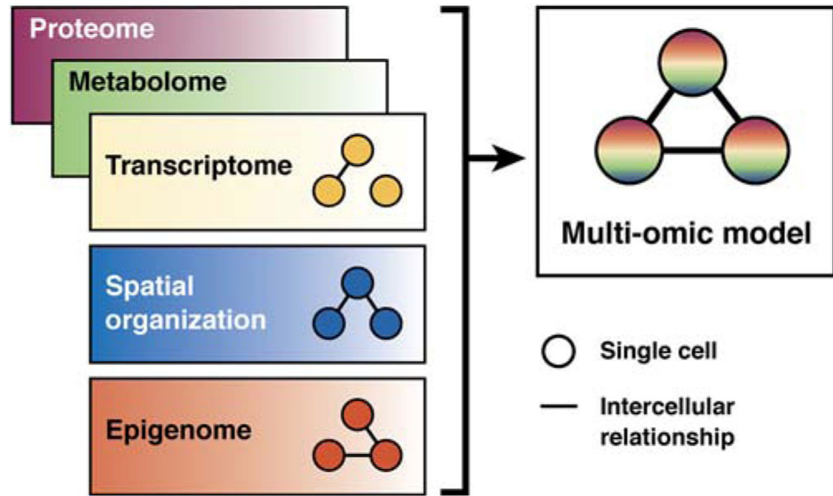

Figure 4.
Integration of Multi-omic Data

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Technical maturity

scRNAseq	scEpigenomics	Spatial transcriptomics	scMetabolomics	scProteomics
Tang et al., 2009	sc-Hi-C	ST	Neumann et al., 2019	NanoPots
STRT-seq	sc-ATAC-seq	Slide-seq	Sun et al., 2019	Scope-MS
SMART-seq	sci-ATAC-seq	HDST	Portero et al., 2019	
CEL-seq	Drop-ChIP			
Quartz-seq	sc-RRBS-seq			
Dp-seq	ATAC-see			
MARS-seq	sc-Aba-seq			
SCRB-seq	sc-WGBS-seq			
Cyto-seq	sc-CGI-seq			
SC3-seq	sci-Hi-C			
inDrop-seq	CUT&Tag			
Drop-seq	dsc-ATAC-seq			
10X Chromium				
Seq-Well				
DroNc-seq				
sciRNA-seq				
SPLiT-seq				

Figure 5. Technical Maturity of Single-cell -omic Technologies

Technical maturity is the time it takes to run the samples, the equipment requirements for these techniques, and the personnel expertise necessary for successful application. Abbreviations and citations: STRT-seq (Single-cell Tagged Reverse Transcription, Islam et al (2011)); SMART-seq (Switching mechanism at 5′ end of the RNA transcript, Ramskold et al (2012)); CEL-seq (Cell expression by linear amplification and sequencing, Hashimshony et al (2012)); Quartz-seq (Sasagawa et al (2013)); Dp-seq (Designed Primer-based RNA-sequencing strategy, Bhargava et al (2013)); MARS-seq (Massively parallel RNA single cell sequencing, Jaitin et al (2014)); SCRB-seq (single cell RNA barcoding and sequencing, Soumillon et al (2014)); Cyto-seq (Gene expression cytometry, Fan et al (2015)); SC3-seq (single cell mRNA 3-prime end sequencing, Nakamura et al (2015)); inDrop-seq (Indexing Droplets sequencing, Klein et al (2015)); Drop-seq (Individual cell in Droplets, Macosko et al (2015)); 10X Chromium (Zheng et al (2017)); Seq-well (Sequencing in nanowell, Gierahn et al (2017)); DroNc-seq (Droplet based single-nuclear sequencing, Habib et al(2017)); sciRNA-seq (single cell combinatorial indexing RNA sequencing, Cao et al (2017)); SpLiT-seq (split-pool ligation-based transcriptome sequencing, Rosenberg et al (2017)); sc-Hi-C (Single-cell Hi-C, Nagano et al (2013)); sc-ATAC-seq (Single-cell assay for transposase-accessible chromatin using sequencing, Buenrostro et al (2015)); sci-ATAC-seq (single cell indexing ATAC-seq, Cusanovich et al (2015)); Drop-ChIP (Droplet based single cell ChIP sequencing, Rotem et al (2015)); sc-RRBS-seq (Single-cell reduced-representation bisulfite sequencing, Guo et al (2015)); ATAC-see (Assay for transposase accessible chromatin with visualization, Chen et al (2016)); sc-Aba-seq (Single-cell 5hmC sequencing by AbaSI nuclease, Mooijman et al (2016)); sc-WGBS-seq (Single-cell whole genome bisulfide

sequencing, Gravina et al (2016)); sc-CGI-seq (CpG island (CGI) methylation sequencing for single cells, Han et al (2017)); sci-Hi-C (single cell combinatorial indexed Hi-C, Ramani et al (2019)); CUT&Tag (Cleavage Under Targets and Tagmentation, Kaya-Okur et al (2019)); dsc-ATAC-seq (droplet single cell assay for transposase-accessible chromatin using sequencing, Lareau et al (2019)); ST (Spatial Transcriptomics, Stahl et al (2016)); Slide-seq (Slide based spatial sequencing, Rodriques et al (2019)); HDST (High-Density Spatial Transcriptomics, Vickovic et al (2019)); NanoPOTS (nanodroplet processing in one pot for trace samples, Zhu et al (2018)); Scope-MS (Single Cell ProtEomics by Mass Spectrometry, Budnik et al (2018)).

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Table 1:

Cell Capture and Barcoding Methods for Genomic Analyses of Single Cells

	Micropipetting	Laser Capture Microdissection	Flow Sorting	Droplet	Nanowell	Combinatorial Indexing
Input Material	tissue in situ	tissue in situ	suspension	suspension	suspension	suspension
Input Cell Requirement	No lower bound	No lower bound	~10^5	~10^4	~10^2	~10^6
Cell Output	< 10^2	< 10^2	~10^3	~10^3	~10^3	~10^5
Cell Capture	cell by cell picking	cell by cell picking	automated	automated	deposition by gravity	combinatorial scheme
Downstream Steps	well by well	well by well	well by well	pooled	pooled	pooled
Ease of Use	hard	hard	moderate	easy	moderate	moderate