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Spatial Single Cell Technologies for Exploring Gastrointestinal Tissue Transcriptome

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Abstract

In the gastrointestinal (GI) system, like in other organ systems, the histological structure is a key determinant of physiological function. Tissues form multiple layers in the GI tract to perform their specialized functions in secretion, absorption, and motility. Even at the single layer, the heterogeneous cell population performs a diverse range of digestive or regulatory functions. Although many details of such functions at the histological and cell biological levels were revealed by traditional methods such as cell sorting, isolation, and culture, as well as histological methods such as immunostaining and RNA *in situ* hybridization, recent advances in spatial single cell technologies could further contribute to our understanding of the molecular makeup of GI histological structures by providing a genome-wide overview of how different genes are expressed across individual cells and tissue layers. The current minireview summarizes recent advances in the spatial transcriptomics field and discusses how such technologies can promote our understanding of GI physiology.

I. Introduction

Histology is the study of the microscopic anatomy of biological tissues, determined through various microscopic imaging methods (1). The structure of each tissue is directly related to its physiological function. The structure is often altered during disease contexts, leading to the formation of characteristic histopathological structures, which could be detected through microscopic analysis and used for the diagnosis of specific diseases.

The histological structure of the gastrointestinal (GI) wall has been well defined and described (1). The GI wall, from the mid-esophagus to the anus, exhibits the conserved structure composed of major layers, including the mucosa, submucosa, and external muscle layers. The mucosa is subdivided into the epithelium, lamina propria, and muscularis mucosa. The epithelium is composed of highly heterogeneous cell populations, which

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Declaration of Interests

J.H.L is an inventor on pending patent applications related to Seq-Scope. H.M.K. is presently an employee of Regeneron Pharmaceuticals, in which he owns stock and stock options.

perform specialized functions in digestion, such as secretion and absorption. For instance, in the small intestine, absorptive function is mainly mediated by enterocytes, while goblet cells secrete mucus and enteroendocrine cells secrete diverse hormones. These cell types were produced by the division and differentiation of stem cells, which are located at a special histological niche, such as the intestinal crypt. The diversity of cell types has been previously explored through microscopic cell morphology or expression of tissue-specific marker genes and proteins. For the latter, examination of spatial gene expression in the tissue is critical for identifying specific cell types. Recent advances in single cell RNA-seq (scRNA-seq) dramatically improved our understanding of different cell types and their specific molecular markers in the GI organs. To put this cell diversity information into a context of spatial biology, histological techniques to detect gene expression, such as immunohistochemistry (detecting protein expression) or RNA *in situ* hybridization (detecting mRNA expression), have therefore been essential for the study of single cell type location and function in the GI organs (2).

Although these techniques are powerful for confidently detecting gene expression and can provide microscopic spatial information that can reveal single cell and subcellular structures, they are limited by their inherent low-throughput characterization since only 1-4 molecular species can be profiled in a single histology session. These limitations could be overcome by transcriptomic profiling using RNA-seq (3). Bulk RNA-seq, however, does not have a single cell or histological resolution; therefore, even though we can have information about the RNA expression level, we do not know how RNA expression is distributed throughout different cells and tissue areas.

scRNA-seq addresses this challenge by dissociating tissue into a single cell suspension, and by profiling each single cell transcriptome separately (4). This can reveal the full heterogeneity of the single cell transcriptomes comprehensively for liquid biosies; however, for solid tissues, it still erases all the spatial information of where the cells are originally located in the tissue. Recent developments of various spatial techniques (Figure 1; Table 1) can address this issue by providing spatial resolution to the transcriptomic studies (5). This review will provide a brief overview of currently available spatial transcriptomics solutions and discuss how such technologies can promote our understanding of GI physiology and pathology by examining several recent studies utilizing the technology.

II. Various Spatial Transcriptomics Technologies and Their Applications to GI Organs.

II-1) scRNA-seq Techniques & In-Silico Reconstruction of Spatial Information

Most currently available non-spatial scRNA-seq methods involve three experimental steps: tissue dissociation and preparation of a single cell suspension, molecular barcoding for the single cell transcriptome, and preparation of the library containing both barcoded single cell information and gene information. The scRNA-seq methods can be classified according to how the single cell transcriptome is barcoded for single cell multiplexing and de-multiplexing. Early scRNA-seq technologies prepared separate libraries for individual cells (6); therefore, single cell molecular barcoding was not necessary or had to be done

manually. However, this method has a clear limitation in scalability as it can only profile a small number of cells. Utilization of nanoliter droplets as a method for encapsulating single cells with a barcoded oligonucleotide source that can be attached to cDNA enabled massively parallel profiling of the single cell transcriptomes (4), and it is widely adopted in commercial platforms (e.g. Chromium from 10X Genomics (7)), as well as in-house platforms (e.g. Drop-seq (8), inDrop (9)). Single cell barcode sorting using nanowells is another method enabling massively parallel scRNA-seq analysis, and it is also implemented in customized in-house platforms (e.g. CytoSeq (10) and Seq-Well (11)) or commercial platforms (e.g. BD Rhapsody (12)). Finally, combinatorial barcoding directly performed on fixed cells can also enable tracing of transcriptomic information into single cells (e.g. Split-Seq (13) and sciRNA-seq (14)). In some configurations, two or more of these methods could be combined to further increase the throughput of scRNA-seq (e.g. scifi-RNA-seq (15)). Massive improvements in throughput and the quality of scRNA-seq profoundly revolutionized the field and enabled us to approach the transcriptomic heterogeneity in single cells found in the tissue.

However, there are some inherent limitations in the scRNA-seq approach due to the tissue dissociation step that enables single cell isolation. During tissue dissociation, some sensitive cell types may be lost, damaged, or altered in their transcriptomic contents. There are cell types, such as mature adipocytes and elongated myofibers, which are particularly challenging for single cell isolation. Also, for most solid tissues, many cells die or lyse during dissociation and sorting; these caveats introduce inherent bias in the estimation of cell type composition from the tissue. To overcome these limitations, single nucleus RNA-seq (snRNA-seq) was devised and performed, as the size of the nuclei is more homogeneous than the size of single cells, and the nuclei can be conveniently isolated from difficult tissues and cell types (16). In addition, snRNA-seq could be performed with preserved frozen tissues, while frozen solid tissues are not typically compatible with scRNA-seq (17). Still, the transcriptome information recovered from snRNA-seq is more sparse than the one from scRNA-seq as it only captures transcripts in the nuclei. A more serious problem of scRNA-seq and snRNA-seq with solid tissues is that the cell dissociation or nuclei isolation procedure inherently erases all the spatial information, which is important for interpreting the results in the histological context.

However, for tissue types that have a defined histological zonation of transcriptome phenotypes, this problem can be alleviated by performing computational spatial reconstruction using spatial RNA expression patterns obtained through independent methodologies (18, 19). Since GI organs have well-characterized zonation structures, scRNA-seq has been combined with other techniques so that each single cell transcriptome can be sorted through histological zonation patterns.

For instance, in the liver, it has been very well characterized that hepatocytes show clear zonation patterns across the hepatic lobule, spanning from the portal triad (artery, portal vein, and bile duct) to the central vein (20). Hepatocytes located close to the portal triad are exposed to an environment rich with nutrients and oxygen, while hepatocytes located close to the central vein are exposed to hypoxic and nutrient-sparse conditions. Using landmark genes that show characteristic gene expression patterns across the portal-central axis,

hepatocellular scRNA-seq data were sorted to reveal spatial transcriptome patterns (21). The results suggested that over 50% of all genes expressed in the liver show spatial zone-specific expression patterns. Many genes were expected to show spatially non-monotonous expression patterns, which may indicate a layer-specific metabolic function in the liver (21). A similar strategy was used to compare the single cell transcriptomes of normal and fatty liver hepatocytes and revealed zone-specific hepatocellular responses to nutritional overload (22). Zonation-based spatial reconstruction can also be used to infer the global pattern of spatial gene expression changes in response to circadian inputs (23).

The liver zonation pattern was also examined in non-hepatocytes such as hepatic stellate cells (HSCs), which play an important role in liver physiology and fibrosis (24). Similar to the hepatocellular patterns, scRNA-seq of HSCs revealed portal vein- and central vein-associated HSCs, and the latter was found to become active myofibroblasts, which produce collagen during liver fibrosis (24). Liver sinusoidal endothelial cells (LSECs) were also similarly profiled to show zonation patterns across the portal-to-central axis based on several landmark gene expression patterns (25, 26). LSEC zonation patterns were also assessed through paired sequencing with the hepatocytes attached to the LSEC (27). Identification of cell surface markers showing zonation patterns also allowed for the spatial sorting of liver cells, which enabled the comprehensive molecular profiling of cells residing in different zones (28, 29). scRNA-seq analysis of the human liver identified specific HSCs, macrophages, and LSEC clusters found in the cirrhotic liver, and their cluster type markers were highly expressed in the fibrotic lesion (30), indicating that, in addition to zonation structure, spatial information is critical for understanding the pathological structure of liver inflammation and fibrosis.

Nevertheless, many of the spatial features may be difficult to reconstruct precisely *in silico*, without relying on global axes such as zonation or circadian time. Moreover, the inference of global axes such as zonation could be locally confounded by the pathological alterations of transcriptomes. Computational reconstruction is also prone to overfitting and may misrepresent the actual biological information. Therefore, the information from scRNA-seq becomes more useful if integrated with spatial transcriptomic technologies. For example, methods such as robust cell type decomposition (RCTD (31)) and conditional autoregressive-based deconvolution (CARD (32)) leverages cell types identified from scRNA-seq data as reference to deconvolute cell types from spatially-resolved transcriptomics data, obtained through several methods that will be described below.

II-2) In situ Imaging-based Techniques

Molecular detection techniques, such as immunohistochemistry or RNA *in situ*, when combined with microscopical techniques such as fluorescence and confocal microscopy, can provide high-resolution images that capture where different biomolecules are found across the histological space (2).

Even though a single session of each imaging technique can only monitor 1-4 molecular species due to the limitations of available spectra for fluorophores, the tissue section can be erased and stained with a completely new set of probes, so that the imaging can be repeated,

and the data can be multiplexed for an unlimited time in principle. Developments in tissue embedding and clearing methods enabled such multiple staining/erasing cycles from a single tissue section. Furthermore, the combinatorial barcoding (Seq-FISH (33)) or error-correcting binary code (Mer-FISH (34)) could be used to reduce the necessary staining/erasing cycles required to monitor many different genes, with elevated confidence and a decreased error rate. One of the potential limitations of these methods is that in a cytosolic structure with high concentrations of RNAs in a limited space, spatial decoding of FISH image data could be challenging due to the proximity of different transcripts. These problems can be partially addressed with the usage of expansion microscopy (35). Still, to profile the whole transcriptome, many rounds of staining/erasing cycles need to be performed, and many images should be obtained and analyzed. Confident decoding of such a huge image dataset can be computationally challenging and requires a large amount of computational resources. Some efforts have been made towards streamlining these procedures through automation, and several companies, such as Vizgen (Mer-FISH), Spatial Genomics (Seq-FISH), and Nanostring (CosMx Spatial Molecular Imager (36)), are working on providing commercial solutions for these methods.

Another technology that is based on *in situ* imaging is *in situ* sequencing. In this technique, sequencing reactions such as sequencing by ligation (e.g. ISS (37), FISSEQ (38) and STARmap (39)), sequencing by synthesis (e.g. BaristaSeq (40)), and sequencing by hybridization (HybISS (41)) are directly performed in the tissue, and fluorescence signals are used to decode the sequence. To enable sequencing, the specific sequence signatures are amplified *in situ* through rolling circle amplification, which will generate DNA nanoballs that can be used for sequencing by ligation or sequencing by synthesis. Like the *in situ* hybridization techniques, *in situ* sequencing techniques are also limited by the cellular space where nearby signals cannot be easily distinguished and separately decoded. Again, tissue expansion microscopy techniques can be used to produce a better resolution and more confident sequencing process (e.g. Ex-Seq (42)).

The limitation of *in situ* imaging techniques, including both *in situ* hybridization and sequencing techniques, is that they require extensive high-resolution imaging and analysis, which can be technically demanding and necessitates a large-scale investment of time and resources, like the prolonged use of expensive equipment. Computational imaging analysis could be also challenging, as it would need to cover a wide tissue area. In most cases, they are focused on a limited number of genes, making unbiased transcriptome analysis difficult. Nevertheless, by providing an extremely fine microscopic spatial resolution, these methods could confidently identify small spatial features, such as single cell types and shapes, as well as subcellular structures.

Single molecule-detecting FISH (smFISH) has been used to either identify landmark transcription that can guide spatial reconstruction of scRNA-seq data or confirm spatial features inferred by scRNA-seq data analysis (21). smFISH was also used to identify transcriptional burst effects in single cells (43). Multiplexed *in situ* hybridization was also performed for analyzing the spatial transcriptome of the developing mouse liver (through Mer-FISH (44)) and gut (through Seq-FISH (45)) and revealed embryo-specific structures in

these organs (44, 45). Seq-FISH is also currently used to spatially profile the human tissue transcriptome, including the liver and intestine, as a part of the HuBMAP consortium (46).

II-3) Histological segmentation methods

Various methodologies were also developed to capture region(s) of interest (ROI) from the histology area, and comprehensively characterize its gene expression content. Laser capture microdissection (LCM), which uses a laser beam to isolate ROI directly from the tissue (47), has been applied for diverse analysis for more than a decade. LCM could be combined with scRNA-seq to perform single cell analysis with spatial information (LCM-Seq (48) and GEO-Seq (49)).

More recently, a solution for regional spatial profiling, NanoString GeoMx Digital Spatial Profiler (DSP), has been commercially released (50). The solution first targets various probes with specific barcode sequences to their corresponding biomolecules. Then, those probes are released into the solution through light-mediated cleavage. By limiting light activation in specific regions of the tissue, GeoMx DSP can analyze multiple ROIs with transcriptome- or proteome-level coverage (50).

LCM-based experiments were utilized for investigating liver zonation (51, 52) and zonation of intestinal epithelial cells (53). These studies provided essential information regarding tissue zonation that were extremely helpful for guiding scRNA-seq analyses by identifying zonation landmark genes as described in the earlier section (21). Combined with a modified scRNA-seq strategy, such as paired cell sequencing (27) and cell clump sequencing (54), these landmark genes were used to characterize the spatial heterogeneity of single cells across diverse cell types. More recent NanoString DSP technologies were also used for characterizing the gene expression patterns in developing intestinal tissues in mouse embryos (55).

Unlike *in situ* imaging techniques, which are basically a targeted approach for detecting defined molecular features, these histological segmentation methods enable unbiased molecular quantitation covering the whole transcriptome. Nevertheless, these methods are primarily based on ROI selection, so even though they are less molecularly biased, they could be histologically biased by ROI selection; unexpected molecular features in certain biologically relevant (but unexpected) spatial patterns may not be detected. These limitations could be addressed by the spatial barcoding technologies described in the next section.

II-4) Spatial Barcoding technologies.

Spatial barcoding technologies are based on an array of oligonucleotides that have a barcode sequence that encodes the coordinate of a given position. The first method utilizing spatial barcoding was Spatial Transcriptomics (ST), published in 2016 (56). ST initially utilized an oligonucleotide microarray, whose spot-to-spot distance is 200 μm (56). In this method, the tissue is overlaid onto the array and imaged, and tissue RNA is released so that it can be captured by oligo-dT-tagged spatial barcode-containing oligonucleotides in the microarray (57). Then, the reverse transcription is conducted, and the library, whose components are chimeric molecules containing both spatial barcode and cDNA sequences, is constructed and sequenced. From the sequence results, spatial images can be reconstructed based on

the known coordinates of each barcode sequence, and each spot can be analyzed through standard statistical techniques to identify different cell types and gene expression features.

This is a revolutionary method because it can profile the spatial gene expression for the whole transcriptome in an unbiased and scalable manner. The procedure is intuitive because it is simply an RNA-seq with spatial information. However, the low resolution of the original ST has been a major hurdle for its widespread adoption; even after extensive optimization and commercial product development by 10X Genomics, the most recent version of ST (branded as Visium) provides 100 μm center-to-center resolution (58). Considering that the naked human eye has a resolution of around 40 μm, many tissue features, including histological layers and heterogeneous single cells, could be obscured with this analysis. So, many different techniques have been developed to improve the resolution of ST.

For instance, Slide-Seq and HDST utilize barcoded microbeads with a diameter of around 10 μm (Slide-Seq (59)) or 2 μm (HDST (60)), to encode the spatial barcode sequence. Accordingly, the center-to-center spatial resolution is 15-20 μm (Slide-Seq) or ~4 μm (HDST), exhibiting around an order of magnitude improvement over the first-generation ST. In these methods, barcode sequences in the bead are generated through the split-pool synthesis method; therefore, the barcode in each bead will be randomly synthesized. Such barcode sequences can be decoded through sequencing by ligation (Slide-Seq) or sequential FISH (HDST) methods; therefore, the spatial coordinates for each barcode sequence can be determined. Although these methods can produce a high resolution, they are limited with low capture sensitivity; read depth per region is very low (e.g. <1 UMI/μm^2 in HDST), so high-resolution cell type mapping analysis is limited. However, recent improvements in library preparation chemistry may improve the RNA capture efficiency so that they can be more widely adopted (Slide-SeqV2 (61)).

Another method, named Deterministic Barcoding in Tissue for spatial omics sequencing (DBiT-seq), uses a microfluidic device for spatial barcoding (62). Unlike other methods that capture RNA by solid-supported oligonucleotides, this method diffuses the spatial barcode into the tissue. Accordingly, it has shown a substantially higher RNA capture efficiency over previously available methods (~12 UMI/μm^2), albeit providing a small feature size whose center-to-center resolution can go down to 20 μm. Barcode diffusion also makes it easy to capture molecular signatures other than transcriptome (62–65), as further discussed below in future perspectives.

More recently, the resolution of spatial barcoding technology has been brought down to submicrometer ranges through the repurposing of next-generation sequencing technologies. Beijing Genome Institute (BGI) announced that it developed a technology named Stereo-Seq, which repurposes their DNA nanoball sequencing platform into spatial molecular barcoding, providing nominal resolution down to the 0.5-0.7 μm range (66). However, as observed with the bead-based technologies described above, Stereo-Seq initially suffered from a low RNA capture efficiency, forcing the investigators to aggregate transcriptome information into 36 μm-sided squares, lowering the effective resolution. The reported transcriptome capture efficiency was initially ~1.7 UMI/μm^2, which was lower than that of ST/Visium (~3.6 UMI/μm^2). This rate was improved in recent studies (67), but most

analyses were still done with 36 μm-sided squares, which are larger in size than most single cells.

At a similar time to the announcement of Stereo-Seq, another spatial barcoding technology based on the Illumina sequencing platform, named Seq-Scope (68, 69), was independently reported. Like Stereo-Seq, Seq-Scope has an ultra-high center-to-center resolution of spatial barcodes, 0.5-0.8 μm range (~0.6 μm on average). An encouraging finding from Seq-Scope is that it also exhibits high transcriptome capture efficiency, up to ~23.4 UMI/μm^2, which is among the highest of all available reported values from spatial barcoding technologies at the time (69). Based on the ultra-high resolution and efficient transcriptome capture output, Seq-Scope can visualize unprecedented details of spatial molecular features, including histological, cellular, and subcellular features. Importantly, Seq-Scope relieves investigators from the computational burden of image processing as the Illumina sequencing platform automatically translates the raw images into spatially coordinated barcode sequences. Shortly after the report of Stereo-Seq and Seq-Scope, another spatial barcoding method based on a custom-built sequencing-by-synthesis platform, named PIXEL-seq, was reported (70). PIXEL-seq also has a micrometer center-to-center resolution (~1 μm) and an efficient transcriptome capture efficiency (~10 UMI/μm^2). In summary, the developments of these ultra-high-resolution technologies suggest that spatial barcoding technologies now have the potential to examine the spatial transcriptome with a high resolution and in an unbiased way.

Another important recent development is that these spatial barcoding technologies can be combined with scRNA-seq, enabling spatial single cell analysis. For example, XYZeq segments the histological section into microwells that have a 500 μm center-to-center resolution and performs the spatial barcoding reaction in the microwell before each segment is combined and subjected to scRNA-seq (71). In contrast, sci-Space uses a grid of spatially barcoded oligonucleotides on a slide, which are transferred to the nuclei of overlaid tissue so that it can be used to track their originated spatial location in a subsequent snRNA-seq (72). The center-to-center resolution of sci-Space is reported to be ~220 μm. Even though the spatial resolution of these spatial single cell or single nucleus methods is relatively low, it is possible for these techniques to be applied with higher-resolution spatial barcoding methods to produce spatially resolved transcriptomic data in both single-cell and sub-micrometer resolution. In addition, the development of ultra-high-resolution spatial barcoding methods may also enable simple histology-based single cell segmentation, which may enable spatial single cell analysis even without tissue dissociation. Indeed, Seq-Scope was able to perform such spatial single cell analysis through histology- or transcriptome-based cell segmentation methods (69).

Compared to other technologies, spatial barcoding technologies are scalable; therefore, it could be easily applicable to various problems in the GI biology. For instance, the first-generation spatial barcoding methods, specifically ST and 10X Visium, have become commercially successful and very popular in the field, and various groups have used these methods for investigating the GI histological transcriptome. For instance, the original ST was used for investigating the spatial transcriptomes of adult mouse liver (73), human embryonic liver (74) and intestines (75, 76), inflamed intestinal tissues in Crohn's disease (76), and injured mouse colon (77). Thorough studies were also performed on various

neoplastic tissues in GI system, such as gastric (78), pancreatic (79, 80) and colon cancers (81). However, as discussed above, the limitation in resolution seriously impeded getting pathophysiologically important spatial information from these datasets because a single spatial data point of ST/Visium can contain a large number of cells. Therefore, instead of serving as a stand-alone spatial solution, ST/Visium was used to provide rough spatial information to already existing scRNA-seq datasets, which provided single cell-resolution information.

High-resolution spatial barcoding methods were also experimentally applied to understand the GI spatial transcriptome. In its initial proof-of-concept study, Slide-Seq was applied to mouse liver tissue and identified periportal and pericentral hepatocellular populations from the histological space (59). Seq-Scope was applied to both normal and injured mouse liver and revealed continuous zonation of normal hepatocytes and increased infiltration of non-parenchymal cells during active liver injury (69). In the injured liver, Seq-Scope was able to detect different macrophage and HSC subpopulations mediating homeostatic and inflammatory roles, exhibiting distinct spatial localization patterns (69). Seq-Scope was also applied to colon tissue and revealed the major histological layers of the colonic wall, as well as the different cell types present in epithelial and non-epithelial cells in each layer (69). Importantly, this cell type mapping information is congruent with underlying findings from traditional histology; nonetheless, the Seq-Scope data was much more informative than traditional histology in identifying specific cell types and revealed the histological structure of transcriptomic features.

III. Conclusion and Future Perspectives

Many GI tissues are characterized by small, repeating structural units mediating the parenchymal functions of the organ, which are composed of diverse cell types performing specialized functions. Such fine structures are subjected to diverse pathological alterations during the disease process. For instance, tissue inflammation and dysfunction often involve damage in the parenchymal tissue, malfunction in tissue homeostasis, and massive infiltration of non-parenchymal inflammatory cells. To understand the physiological and pathological processes of the GI tissues, high-resolution spatial profiling is essential.

For this, we have reviewed the major spatial transcriptome methodologies and discussed how these methods were applied for studying the GI system (Figure 1). Various scRNA-seq technologies can give information on the true single cell resolution and reveal the cellular heterogeneity of an organ (Figure 1A); however, the spatial information should be guessed through existing landmark information determined through histological segmentation analysis or *in situ* imaging methods. Histological segmentation is a powerful tool to perform differential expression between different areas of the tissue (Figure 1B); however, selection of ROI can limit the spatial output and discoverable novel spatial features. *In situ* imaging techniques can provide microscopic spatial resolution and reveal structural details of diverse gene expression (Figure 1C); however, demanding requirements of imaging and data processing capabilities, as well as technical issues, could limit the information output, such as number of genes, for detection. Finally, spatial barcoding techniques could comprehensively profile the spatial transcriptome (Figure 1D); however,

the original versions have limited spatial resolution, and the newer high-resolution single cell-level technologies are yet to be made available to broader society. In addition, the capture rate of rarely expressed genes is not great in spatial barcoding technologies when compared to imaging-based technologies. These limitations might be addressed through further optimization and/or additional technical breakthroughs. Various startup and established companies work on streamlining in situ imaging techniques to make it more accessible to scientific community, while there are also efforts of making high-resolution ST commercially available. Further improvements in either in situ imaging or molecular barcoding technologies may enable near-saturated spatial transcriptome characterization in microscopic resolution, which might offer one-size-fits-all solutions for tissue molecular characterization. However, until that time arrives, these different methodologies are currently complementary to each other, and should be chosen carefully depending on the nature of the tissue and scientific questions to be addressed.

These technologies have the potential to reveal information beyond transcriptomic information, such as proteomic and epigenomic-level information. scRNA-seq has been already combined with proteomic and epigenomic solutions to perform single cell multi-Omics (82–84). Histological segmentation methodologies were originally developed as a multi-omics platform (47, 50), and *in situ* imaging technique is specifically amenable for the multiplexed detection of proteins, which has been recently implemented through the Co-Detection by Indexing (CODEX) platform (85), iterative indirect immunofluorescence imaging (4i) platform (86, 87), or Nanostring CosMx platform (36). Spatial barcoding techniques were also recently expanded to perform spatial profiling of protein expression (62, 63, 88, 89), chromatin accessibility (64, 90), and epigenomic signatures (65). These advances enable spatial multi-omics analysis of GI tissues that can enable better understanding of the molecular basis underlying diverse pathophysiology.

The advances in technology should be also followed up by new discoveries enabled by the new techniques. Identification of specific biological questions and question types that can be better solved by these techniques would also be essential, as it will demonstrate the utility of these new techniques. Specifically, bioinformatic and statistical tools for comprehending the information from large-scale datasets would be critical, as the raw data produced from spatial transcriptomics experiments can hardly be analyzed without automated computation. Especially, the ultra-high resolution spatial transcriptomics technologies pose us new challenges in computational scalability in the software tools as the number of spatial barcodes per unit region dramatically increases (e.g. ~120/mm^2 for Visium and >1M/mm^2 for Seq-Scope). At the same time, as there will be a limited number of mRNAs that can be captured per unit region, statistical methods that can robustly infer fine-scale spatial information from a limited number of reads per spatial barcode will become increasingly more important. The vast amount of new information revealed through these technologies may enable many new discoveries that cannot be done with former methodologies, such as the unbiased diagnosis of human pathologies and development of new therapeutic rationale specific for certain disease subtypes.

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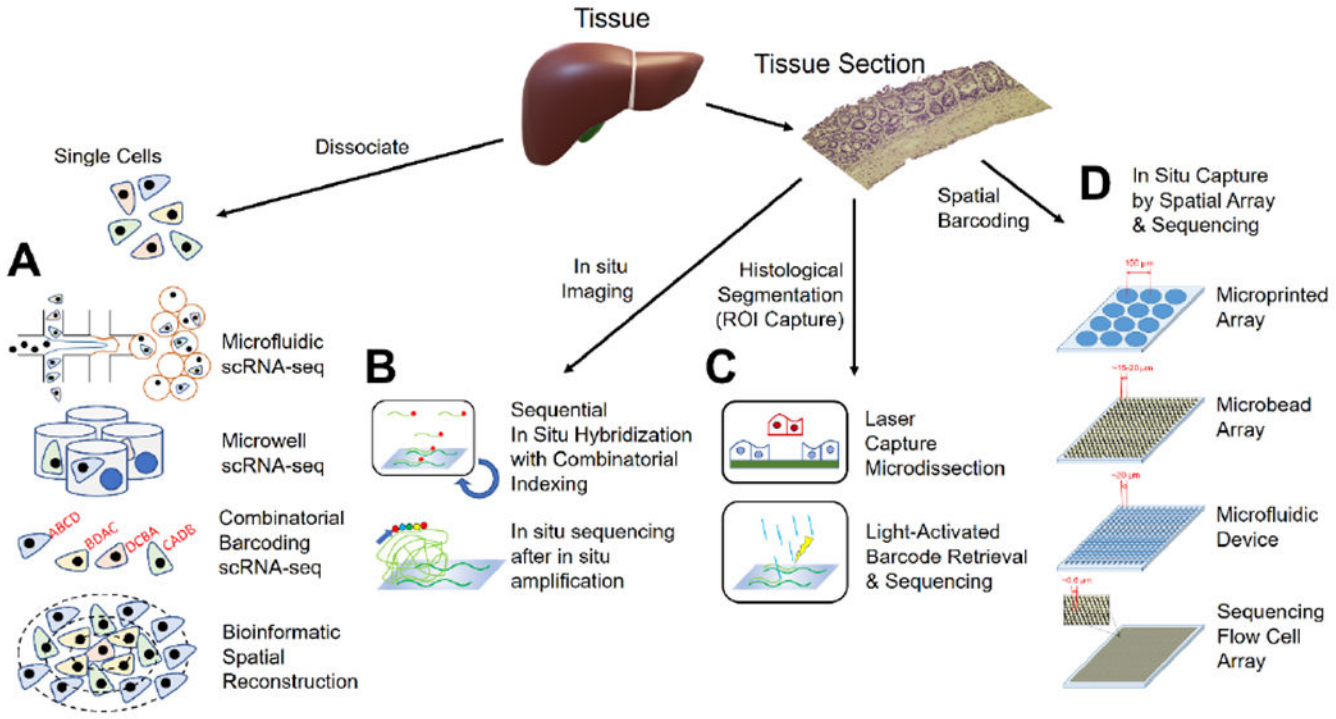

Figure 1.

Overview of Various Spatial Single Cell Transcriptomics Methodologies including (A) Single Cell RNA-seq (scRNA-seq) methods, (B) *In situ* Imaging methods including sequential *in situ* hybridization or *in situ* sequencing methods, (C) Histological Segmentation methods, and (D) Spatial Barcoding and *in situ* capture methods.

Table 1.

Characteristics of major technologies available for spatial transcriptome analysis

Technology		Pros	Cons
scRNA-Seq (e.g. 10X Chromium (7))		▪ Well-established commercial solutions available ▪ Cell dissociation and sorting minimizes cross-cell contamination ▪ High transcriptome capture rate per cell	▪ Destroys spatial information during dissociation ▪ May induce stress responses during dissociation ▪ May introduce bias by eliminating vulnerable cell population during dissociation
In situ Imaging (e.g. Mer-FISH (32))		▪ Ultra-high microscopic resolution ▪ High detection rate for rare transcripts	▪ Technically and computationally demanding ▪ Microscopic optical challenges in decoding abundantly expressed genes ▪ More appropriate for targeted analysis over unbiased screen
Histological Segmentation (e.g. GeoMx DSP (50))		▪ Well-established commercial solutions available ▪ Enables unbiased whole transcriptome analysis	▪ Limited and potentially biased spatial information due to ROI selection
Spatial Barcoding	Overall	▪ Enables unbiased whole transcriptome analysis ▪ Enables unbiased spatial analysis ▪ Straightforward methodology ▪ Relatively low cost for implementation (no special equipment necessary other than sequencing)	▪ Lower detection rate for rare transcripts ▪ Possibilities of transcript diffusion during capture ▪ Difficulties in cell segmentation and isolating single cell transcriptome
	Microprinted Array (e.g. 10X Visium (58))	▪ Well-established commercial solutions available	▪ Limited spatial information due to low resolution (~100 μm)
	Microbead Array (e.g. Slide-Seq (59))	▪ Relatively high resolution (4-20 μm)	▪ Difficulties in producing uniform array with spatial feature information ▪ Relatively low capture rate – continue to improve. ▪ Custom array generation and raw image analysis can be technically and computationally demanding.
	Microfluidic Device (e.g. DBiT Seq (62))	▪ Relatively high resolution (20-100 μm) ▪ High transcriptome capture rate due to barcode infusion into tissue	▪ Limited number of spatial barcodes (50 x 50 coordinates)
	Next-Generation Sequencing Array (e.g. Seq-Scope (69))	▪ Ultra-high spatial resolution (0.5-1 μm) ▪ High transcriptome capture rate due to tight arrangement of spatial features. ▪ Does not require raw image processing as spatially coordinate barcodes are automatically produced from the Illumina platform (for the case of Seq-Scope).	▪ Deep sequencing is necessary to perform high-resolution analysis ▪ Solution not commercially available yet