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Single cell- and spatial 'Omics revolutionize physiology

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Abstract

Single cell multi- 'Omics and Spatial Transcriptomics are prominent technological highlights of recent years, and both fields still witness a ceaseless firework of novel approaches for high resolution profiling of additional omics layers. As all life processes in organs and organisms are based on the functions of their fundamental building blocks, the individual cells and their interactions, these methods are of utmost worth for the study of physiology in health and disease. Recent discoveries on embryonic development, tumor immunology, the detailed cellular composition and function of complex tissues like for example the kidney or the brain, different roles of the same cell type in different organs, the oncogenic program of individual tumor entities, or the architecture of immunopathology in infected tissue are based on single cell and spatial transcriptomics experiments. In this review, we will give a broad overview of technological concepts for single cell and spatial analysis, showing both advantages and limitations, and illustrate their impact with some particularly impressive case studies.

KEYWORDS

multi-omics, single cell technologies, spatial transcriptomics

1 | INTRODUCTION TO SINGLE CELL TECHNOLOGIES

From the onset of Next Generation Sequencing technologies, many research groups and then companies started to develop protocols for amplification of minute amounts of nucleic acids to accommodate the needs of research projects with limited input material. The detection sensitivity of these assays quickly arrived at the single cell level, which enabled researchers for the first time to resolve the genetic and transcriptional heterogeneity of cell types and cellular states in complex tissues with sequencing-based methods.[1–3] The tremendous benefits for research areas from cancer genomics to developmental biology became obvious when these approaches developed from ultra-low-input protocols for few single cells to highly scalable assays that enabled the investigation of the (patho-)physiology of complex organ systems.[4–6] A recent review article on single cell technologies applied to the human kidney[7] highlights the enormous impact on research in the field of physiology. Here, single cell technology-based studies are reviewed that dissect the genetic programs, pathways, and mechanisms of cellular crosstalk underlying physiological kidney development and function, as well as the changes that occur in different pathological conditions. Driven by international efforts and concerted initiatives,[8] numerous innovations have contributed to a vast increase in throughput, sensitivity and scope, and in the meantime, a vast range of genomic, transcriptomic and epigenomic read-outs have become accessible at the single cell level (Figure 1).

Below, we will highlight some of the major technological concepts at the foundation of what is now known as the *single cell multi- 'Omics* field.

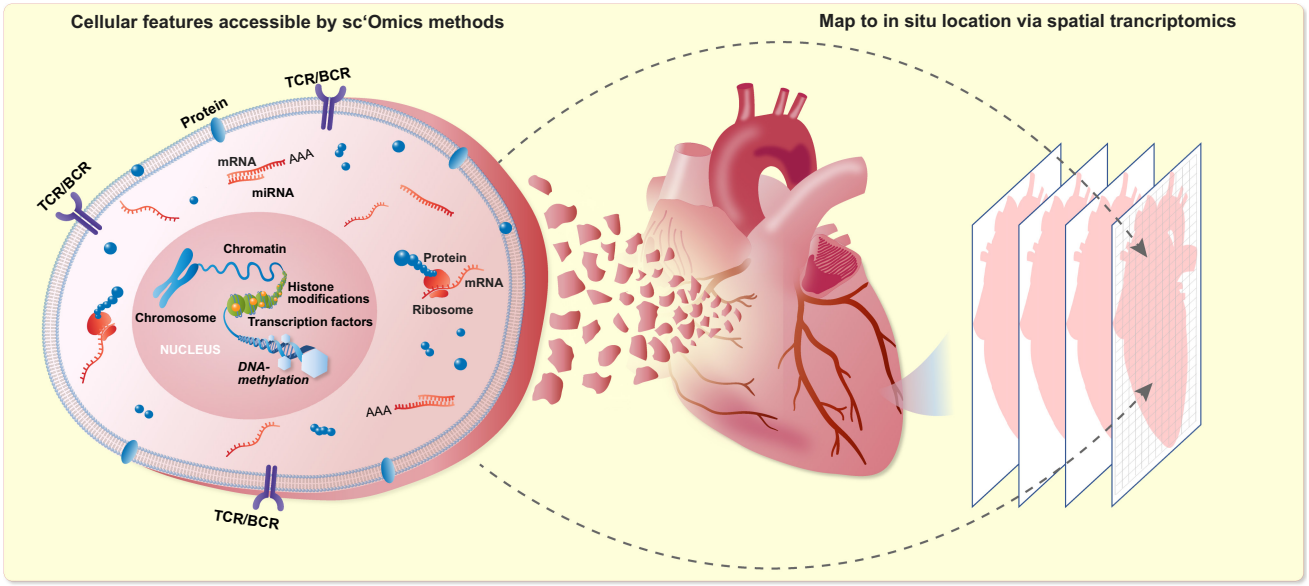

FIGURE 1 Cellular features accessible by single cell and spatial 'Omics methods.

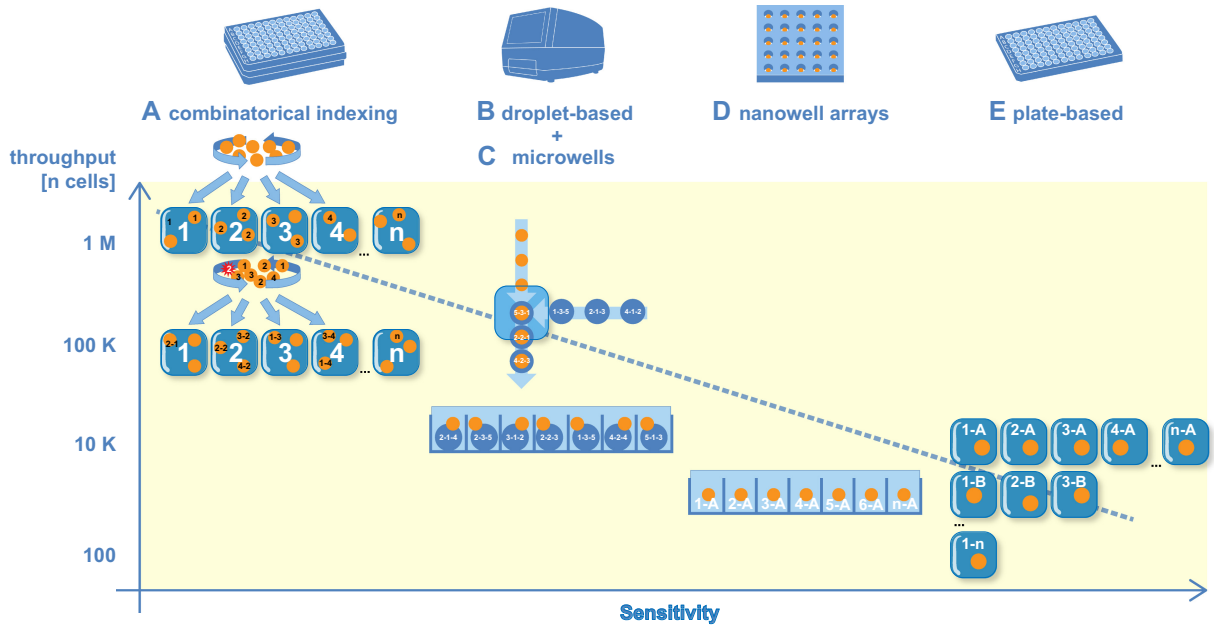

FIGURE 2 schematic view on single cell technological concepts and the associated throughput and resolution/sensitivity (cells are depicted in orange and beads in blue). **(A)** the split&pool approach of the combinatorial indexing concept assembles, one after one, index combinations that are unique for individual cells circumventing the need for compartmentalization. (**B)** droplet-based barcoding partitions single cells together with a barcoded gel bead in oil droplets where the cDNA synthesis takes place. (**C)** microwell-arrays partition cells together with uniquely barcoded microbeads that capture cellular mRNA for reverse transcription ad subsequent cDNA amplification. (**D and E):** The concept of assay miniaturization in nanowell arrays or plates down-scales library preparation protocols to finally generate one individual sequencing library per cell.

Common to all concepts is the labeling of nucleotide molecules from an individual cell with short artificial DNA sequences, representing the "cellular barcode", followed by deep sequencing. However, the different concepts show a reverse correlation of throughput and sensitivity[9] (Figure 2) which also shapes the applications they are mainly used for (Table 1). Tools exist that can help with decision making on study design concerning sample size and number of cells (e.g., How Many Cells | Satija Lab). During subsequent bioinformatic analysis, cells are clustered based on the similarity of their molecular profiles to identify cell types, states and trajectories.

TABLE 1 single cell technologies in a nutshell

	Assay miniaturization	Pico/nano/micro-well-plates and arrays	Droplet-based	Combinatorial indexing
Ease of use	++	++	+++	+(++[a])
Prominent advantage	High sensitivity	Gentle to cells	Robustness, high recovery rate (~60%)	Scalability
Limitation	High costs	Few multi-omics combinations	Limited cell size	Rather sparse data, low recovery rate
Major application	Deep analysis (e.g., isoforms) in systems of lower complexity	Sensitive suspensions that do not tolerate cell sorting or microfluidics	High throughput analysis of complex systems with limited source material	High throughput analysis of complex systems with unlimited source material

[a]Ease of use for combinatorial assays may increase with first commercial solutions.

While some excellent reviews provide detailed insights into the wealth of available bioinformatic tools and analysis strategies,[10] we will here focus on the molecular techniques.

2 | CONCEPT OF ASSAY MINIATURIZATION IN PLATES

The oldest approaches for sequencing of RNA or DNA from individual cells simply resemble miniaturized versions of bulk library preparation protocols combined with different techniques of pre-amplification. Here, cells are first isolated via microdissection, pipetting, or more commonly cell sorting into 96- or 384-well plates, with the advantage that cell lysates can be stored at −80°C and kept safely for months until the project is ready to proceed. The released nucleic acids then undergo PCR- or IVT-based amplification and barcoding reactions, with adjusted enzyme mixes and reduced reaction volumes, which decreases NGS library preparation costs per cell to less than one-tenths compared to bulk protocols. Since one individual NGS library is generated per cell, plate-based procedures are quite labor intensive compared to later methods, and the number of available indices provided by the adapter system limits the throughput or at least the number of pooled single-cell-libraries that can be sequenced together and thus the potential scale of projects. However, despite the tremendous increase in cell numbers in later single cell omics approaches, plate-based methods are far from obsolete and new variations still appear on a regular basis. Their major advantage is an unmatched sensitivity (unique molecules detectable per cell, e.g., for mRNA ~100 k/cell, Figure 2), high flexibility, and the possibility of further biophysical fractionation for multi-omics approaches.[11–14] Some of the (unwanted) complexity of the cell suspension can be easily reduced by flow cytometry, and many assays like the profiling of genomic copy number variations or genome-wide DNA methylation require comparably deep sequencing data (in general several Gb/cell), which makes larger cell numbers cost-prohibitive anyways. No special equipment (other than a flow cytometry device) or major upfront investment in consumables is presupposed but the usage of a reliable liquid handler is helpful, as dispensing of volumes of few µl or even below 1 µl is unadvisable to do manually, especially for 384-well plates. Todays "gold standard" of plate-based transcriptome sequencing are the protocols from the SMART-Seq ("switching mechanism at 5' end of RNA template") family,[15,16] with Smart-seq 3[17] and its modifications[18–20] being the newest family members that also introduce a unique molecular identifier to eliminate PCR duplicates

and can achieve a detection sensitivity of more than 10 k genes per cell. While the more scalable concepts described below usually detect the 3′end of polyadenylated RNA, SMARTseq offers full-length coverage of transcripts, making isoform detection possible. As an example, full length SMARTseq has been used in a project by Booeshaghi et al to identify specific isoform markers for different cell types in the mouse cortex. Used in conjunction with spatial RNA capture and gene-tagging methods, this enabled the inference of spatially resolved isoform expression. In a comprehensive analysis of 6160 mouse primary motor cortex cells, the authors provide examples of isoform specificity and isoform shifts that would have been masked in sc 3′mRNA analysis. Additionally, they showed that isoform specificity helps to further resolve cell identities, and that a multi-platform analysis of single-cell transcriptomic data leveraging multiple assay types provides a comprehensive atlas of transcription in the mouse primary motor cortex that improves on the possibilities offered by any single technology alone.[21]

As mentioned above, the plate-based concept also enables multi-omics via physical fractionation. Splitting cytoplasm and nucleus, or capturing poly-A transcripts from whole cell lysate, enables parallel transcriptome and genome or methylome sequencing from the same cell to obtain a functional read-out of (epi)allelic variation.[22] Cytoplasm can also be split for parallel profiling of miRNA and mRNA in the same cells to study post-transcriptional regulation.[23] Single cell ChipSeq[24,25] has been demonstrated as well, and numerous variations of plate base-assays exist that have been described in detail elsewhere.[26] Multiomics assays are summarized for all major concepts in Table 2.

3 | CONCEPT OF PICO/NANO/MICROWELL-PLATES AND ARRAYS

Several commercial solutions have been introduced that use specialized equipment and dedicated kits for further miniaturization of single cell RNA-seq reactions, enabling parallel processing of thousands of cells. ICell 8 (Takara) uses a nanowell-chip with predispensed barcoded adapters in which scRNA or scATAC-Seq library preparation is conducted, while Seq-Well[39] (commercialized by Honeycomb), GEXSCOPE chemistry (Singleron) and BD Rhapsodie (BD Biosciences) use arrays of microwells that can each accommodate a magnetic bead with covalently attached barcoded DNA primers for reverse transcription of mRNA. Since the cell barcodes are introduced already at the RT step, they are independent from the final library index, which facilitates sample multiplexing during sequencing. While cell sorting can be used to preselect viable cells, or to enrich for rare cell types, a cell sorting device is not a requirement as the procedures start from cellular suspensions of a defined concentration. The number of cells per well then follows a Poisson distribution, which means that cell counting needs to be as exact as possible and remaining duplicates need to be controlled by visual inspection under a microscope and/or with bioinformatic correction. While these commercial solutions achieve a considerable increase in cellular throughput, in some cases matching the output of droplet-based barcoding approaches (see below), they offer less flexibility in terms of multi-omic approaches and sometimes require an initial investment in laboratory equipment (e.g., BD Rhapsodie or ICell 8 systems) which is not the case for plate-based and combinatorial indexing approaches. However, most

TABLE 2 Road map for single cell multi 'Omics

Concept	Combination of read-outs	Protocol examples
Combinatorial indexing	scRNA + scATAC	sci-CAR,[27] SNAREseq2,[28] CoTECH,[29] SHAREseq,[30] Paired-seq[31]
	scRNA + scChIPseq	Paired-Tag[32]
	scRNA + scGenome	sci-L3-RNA/DNA[33]
	scGenome + scHiC	s3GCC[34]
Droplet-based	scRNA + scATAC	Multiome (10x genomics), SNAREseq[35]
	scRNA + scATAC + epitopes	TEAseq,[36] DOGMAseq[37]
	scRNA + epitopes	CITEseq[38]
	scATAC + epitopes	ASAPseq[37]
Plate-based	scRNA + scATAC + scMethylome	scNMTseq[11]
	scRNA + scGenome	G&Tseq[13]
	scRNA + sc-miRNA	SMALLseq[23]

assays can be combined with protein detection from the same cells via barcoded antibodies, and some specialized applications such as detection of metabolically labeled RNA are commercially only available in this format.

4 | CONCEPT OF DROPLET-BASED BARCODING

In 2015, a major technological advance enabled scaling of scRNA-seq reactions to thousands of cells. Key to the concept was to encapsulate cells in microdroplet emulsions together with enzymes and beads that carry barcoded adapters for reverse transcription.[40] Here, each cell is captured inside a reaction volume in the sub-nanoliter range, in which the nucleic acid amplification and barcoding is performed, representing an extreme version of the miniaturization approaches described above, although the higher scalability of droplet-based approaches comes with slightly reduced detection sensitivity (Figure 2). The barcoded beads can either be magnetic with adapaters covalently linked to the bead surface for mRNA capture, or dissolvable to release RT primers into the nanoliter reaction volume of the droplet, enabling more efficient reverse transcription.

The microfluidics used for droplet generation are sensitive to clogging and put upper limits on the maximum cell size, but with the growing use of isolated nuclei also cells naturally larger than 40 µm in diameter can be processed without this risk. Some cell types however, like for example neutrophils, are known to be sensitive to the microfluidics procedure and subsequently underrepresented in the data. Both variable sensitivity as well as cellular sizes are known to cause biases in the observed proportions of cell types which is probably the major disadvantages of microfluidic systems. While open-source platforms are available,[35,36,40–42] droplet-based sequencing is usually carried out with the use of commercial devices and dedicated kits. The number of devices placed in individual labs is dramatically increasing, making the droplet-based concept the most widely used and the most accessible. Cell sorting is not mandatory, but often used to remove debris and dead cells, or to pre-enrich rare cell populations of interest. Since the cellular barcode is introduced during RT as part of the oligo(D)T capture oligo, only the 3´ends of transcripts are read out during short read sequencing. However, several groups have performed long-read sequencing of cDNA from droplet-based approaches and provided bioinformatic solutions that enable isoform detection from this data.[43,44]

Main Vendors for commercial solutions are dolomite bio (Nadia instrument), Illumina/BIO-RAD (ddSEQ), mission bio (tapestri, only for a targeted genomic approach), and 10x Genomics[45] (Chromium). The latter already includes a wide portfolio of additional omics layers and multi- O'mics solutions (T&B-cell receptor repertoire, CRISPR screens, CITEseq,[38] scRNA+ scATACseq,[46] Table 2). Disregarding the infrastructure investment, the cost for the droplet-based approach is less than one tenth compared to the plate-wise approach in the range of few cents per cell. One excellent example how droplet-based RNAseq can be used for the generation of broad gene expression atlases is the work of Büchler et al. describing fibroblast lineages in health and disease. In this study, fibroblast atlases were constructed by integrating single-cell transcriptomic data from about 230 000 fibroblasts across 17 tissues and several disease states. Two universal fibroblast transcriptional subtypes were identified across tissues. The analysis suggested that these cells can serve as a reservoir that can yield specialized fibroblasts across a broad range of steady-state tissues and activated fibroblasts associated with pathogenicity in cancer, fibrosis, arthritis and inflammation.[47]

5 | CONCEPT OF COMBINATORIAL INDEXING

Single cell combinatorial indexing (sci) was first published in 2015 for a chromatin accessibility approach,[48] circumventing the need for compartmentalization of individual cells and therefore paving the way for almost unlimited throughput. Here, nucleic acids are tagged with cellular barcodes inside permeabilized cells in a multiwell plate, each well providing a different barcode. Cells from all wells are then pooled and redistributed across the next multiwell plate for tagging with a second barcode (Figure 2). This procedure is performed several times to label the cells with individual barcode combinations. The last barcode contains the PCR handle for library amplification as in the plate- or droplet-based approaches.[49,50] The final combination of barcodes has a high probability to be unique for a single cell. After a (significant) initial investment in manufactured oligos, the processing costs per cell are comparably low (<1 cent/cell). This concept has been applied to many modalities like chromatin accessibility,[48,51] chromatin conformation,[52,53] genome sequencing,[54] transcriptomes,[50,55–57] methylomes,[58] scChiPseq[27] and also several multi 'O'mics approaches[27–33] (Table 2). Since the procedure involves a series of enzymatic reactions, each of them not 100% efficient, initial iterations of the concept provided only shallow information that was limited to several hundred UMIs per cell (Figure 2). In addition, much of the sequencing effort is wasted on aggregates of nuclei and incomplete cellular barcodes.

Of all concepts presented here, combinatorial indexing is also the one with the lowest cell recovery rate (<10%), which makes it only useful for projects with non-limited cell numbers. The absence of a "barcode-white-list" and many incomplete and therefore not attributable barcodes make this concept bioinformatically particularly challenging. However, especially for developmental studies on whole organisms the enormous throughput excels the lower resolution. Most published studies are based on homebrew protocols, but first commercial products are already entering the market (Parse Biosciences; SCALE Biosciences) and promise a substantial increase in sensitivity.

As an impressive example, Cao et all. Investigated the transcriptional dynamics of mouse organogenesis at single-cell resolution. Using the concept of combinatorial indexing, they profiled the transcriptomes of around 2 million cells derived from 61 embryos staged between 9.5 and 13.5 days of gestation. The resulting 'mouse organogenesis cell atlas' provides a global view of developmental processes during that time frame. Hundreds of cell types and 56 trajectories could be identified, many of them only detectable because of the depth of cellular coverage, and collectively defining thousands of corresponding marker genes.[59]

6 | INTRODUCTION TO SPATIAL TECHNOLOGIES

While single cell omics approaches can provide an overview over the cell types and states that reside in a tissue, the function of complex tissues strongly depends on the correct positioning of their cells in space. Cells in local tissue microenvironments display distinct molecular properties and gene expression programs that enable them to exert their specific physiological functions, and at the same time shape their local niche via juxtacrine and paracrine signaling and intercellular interactions. The importance of this complex spatial organization becomes apparent when tissue architecture is disrupted in disease contexts like infections, inflammatory processes or cancer. Accordingly, changes in tissue architecture have long been used as diagnostic read out in histopathology, supported by molecular detection methods like in situ hybridization or immunohistochemistry. However, these methods only provide snapshots of a low number of transcripts or proteins per experiment. In the last decade, a host of new methods have been introduced that promise more comprehensive read outs of spatial gene expression patterns, paving the way for a molecular understanding of 3D tissue homoeostasis and of the pathogenic mechanisms that disturb cellular organization in disease settings.

Spatial omics technologies fall into two major categories: 1) NGS-based methods like laser capture microdissection (LCM) and spatial barcoding (SB), and 2) imaging-based methods like in situ hybridization (ISH) and in situ sequencing (ISS) (Figure 3). Each concept has its distinct advantages and disadvantages with respect to sensitivity, coverage, spatial resolution, labor intensity, dependency on specialized equipment, and data integration, and no single method currently excels at each of these aspects. The right choice of method therefore depends on the sample type and biological question at hand (Table 3).

7 | CONCEPT OF LASER CAPTURE MICRODISSECTION (LCM)

In LCM, a UV or IR laser beam is used to cut out and capture areas from mounted tissue sections on special glass slides for further processing. The first successful attempts to obtain untargeted transcriptomic read outs from LCM samples date back to the late 1990s, when several groups integrated laser capture microdissection (LCM) with IVT and cDNA microarrays.[60,61] LCM has since been used in hundreds of studies and numerous biological systems and disease contexts and still remains the most widely used spatial technique,[62] likely due to the availability of commercial LCM systems in core facilities. While most of LCM-based studies assay hundreds to thousands of cells per selected tissue area, LCM-RNAseq has recently achieved single cell resolution.[63–68] LCM has also been employed to profile additional modalities like DNA methylation, revealing for example the epigenetic basis of liver zonation[69]. Sequencing data from serial whole tissue sections has been used to reconstruct 3-dimensional gene expression maps in Zebrafish embryos and Drosophila.[70–72] Despite its widespread usage, LCM remains labor intensive and requires specialized equipment for sample collection. The potential throughput of LCM is also hindered by the difficulty to automate the necessary selection of cells or tissues areas for analysis. In addition, isolation of intact RNA can be challenging after the required tissue fixation, sectioning and dehydration steps.

Very recently, a variation of optical microdissection has been introduced with the Nanostring GeoMX spatial profiler, in which unique barcodes are released from hybridization probes or conjugated antibodies in selected tissue areas by UV irradiation and then read out by NGS for multiplexed protein and RNA profiling.[73] The detection sensitivity is still lower compared to LCM, so that distinct tissue areas or groups of at least 20 or 200 cells have to be selected for protein or RNA analysis, respectively. Integration with other data types can help to overcome this limitation. Jerby-Arnon et al. for

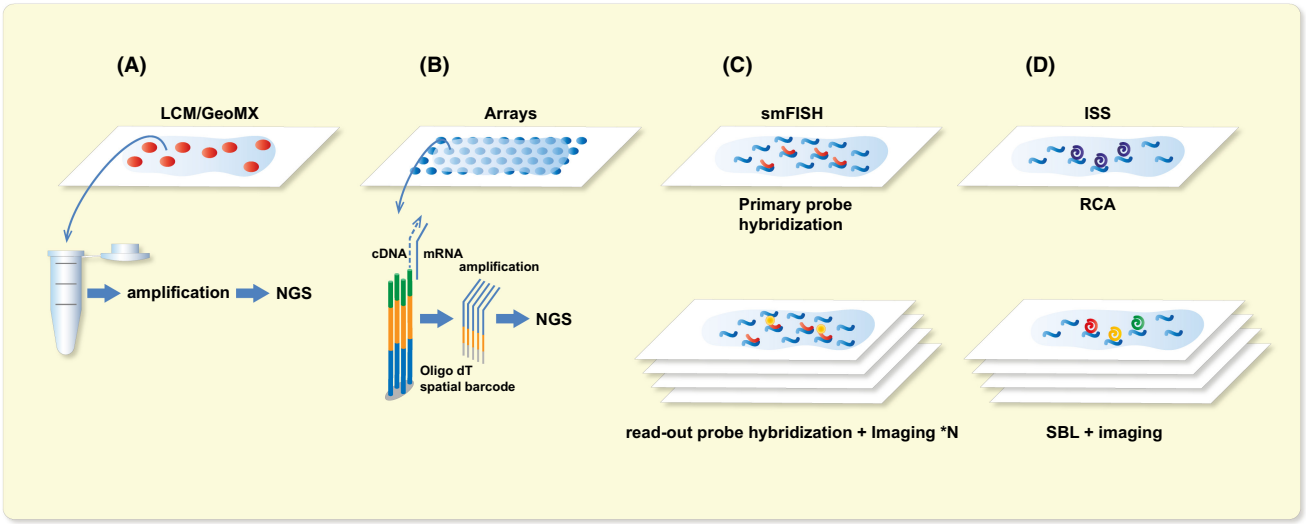

FIGURE 3 schematic view on the major spatial technology concepts. **(A)** In laser capture microdissection, selected tissue areas are cut out using a UV or infrared laser and transferred to a collection tube for nucleic acid amplification and library preparation. (**B**) In array-based technologies, tissue sections are mounted on a glass slide with pre-printed capture arrays of spatially barcoded oligo(d)T oligos. Upon tissue lysis, polyadenylated RNAs are captured by the oligo(d)T sequence and reverse transcribed on the slide. cDNA is then collected and amplified for library preparation and deep sequencing. Sequence reads can eventually be mapped back to their initial spatial coordinates via the spatial barcode. **(C)** In smFISH, fixed tissue sections are incubated with panels of gene specific hybridization probes, followed by serial hybridization and imaging of fluorescent readout probes. (**D**) In ISS, gene specific padlock probes are hybridized to RNA or cDNA in situ, closed by ligation and amplified with rolling circle amplification. The DNA circles are then detected with secondary fluorescent hybridization probes or decoded by SBL.

TABLE 3 Main spatial technology concepts

	LCM	Arrays	Single molecule FISH	In situ sequencing
Sensitivity	++ (+)[a]	+	+++	+ (++)[d]
Resolution	Subcellular (100 cells)[b]	50 µM (5 µM)[c]	Subcellular	Subcellular
Panel size	Whole transcriptome	Whole transcriptome	Thousands	Hundreds
Automatization	Yes	No (in development)	Yes	Yes

Note: [a+b]GeoMX; [c]Visium HD; [d]Xenium.

instance combined two different scRNAseq approaches with ChiP-Seq and spatial gene expression profiling via the GeoMx platform, to characterize the genetic and immune mechanisms that shape the oncogenic programs in synovial sarcoma, which enabled them to uncover a malignant subpopulation of sarcoma cells in immunodeprived niches.[74] The compatibility with FFPE material, large field of view, multiplexed protein and transcriptome profiling capability, and easy integration of the NGS read out with pre-existing omics data have the potential for more widespread application of this technology to clinical samples, especially for analyses that do not require true single cell resolution. It should be noted, however, that also here distinct tissue areas need to be selected for analysis during the instrument run, necessitating a clear understanding of the tissue histology, and the availability of fluorescent labeled antibodies that specifically highlight the tissue regions or cells of interest.

8 | CONCEPT OF ARRAY-BASED METHODS / SPATIAL BARCODING

NGS-based acquisition of spatially resolved gene expression information across whole tissue sections, without the need to manually preselect areas of interest, was first reported in 2016 as Spatial Transcriptomics.[75] Here, tissue sections are placed on a glass slide carrying an array of spatially barcoded oligo(d)T-capture probes. After methanol fixation and H&E staining, the tissue is permeabilized, and the released polyadenylated transcripts are captured

and reverse transcribed on the slide surface. The cDNA is then amplified and converted into NGS libraries. Since the spatial address of each barcode is known, the location of each transcript can be reconstructed from the sequencing data. Commercially available capture slides (10X Genomics Visium) currently have a spot diameter of 55 μM with 100 μM distance, meaning that one spot usually accommodates multiple cells. However, a version with 5 μm spots is under development and expected to be released in 2022. Nevertheless, even smaller spots only provide local transcript counts that potentially overlap with more than one cell as the thickness of sections as a third dimension as well as lateral diffusion of transcripts after permeabilization needs to be considered. To partially overcome this limitation in resolution, a growing number of bioinformatic solutions exist for integration with single cell RNAseq data from the same tissue, matched sample, or ideally adjacent section, which enables the mapping of cell-types and states to distinct spot locations and greatly leverages the power of array-based assays.[76–81]

Since its inception, several research groups have reported variations of the concept, including the use of microbead monolayers instead of spotted barcode arrays,[82,83] and the repurposing of next generation sequencers to generate such arrays.[84,85] The resulting capture arrays have unmatched spatial resolution of just 0.5 – 1 μM, approaching subcellular resolution. A capture area of >40 cm^2 has been generated, although practical application to large sample areas such as whole human brain tissue sections remains to be demonstrated. Besides high cost, the biggest limitation of commercially array-based solutions is still the robustness of the library preparation itself. In current workflows, tissue sections have to be mounted directly on the capture arrays, and the tissue quality and sectioning greatly impact on the success of the experiment. In addition, lysis conditions have to be optimized individually for each tissue type. Recently, commercial capture slides for FFPE samples have been introduced to provide access to the vast trove of archived clinical samples, and an automated device for the transfer of pre-existing FFPE sections to capture arrays has been released, enabling prior selection of optimal sections based on H&E or antibody staining, which might help to overcome some of these limitations. An impressive example for array-based methods was published by Boyd et al., who used a droplet-based assay for scRNAseq-Seq in combination with spatial transcriptomics to investigate the immunopathology of acute respiratory distress syndrome in a mouse model of acute influenza infection. They were able to identify a population of hyperactivated fibroblasts in the lower respiratory tract that secrete matrix metalloproteases for remodeling of the local microenvironment upon viral infection. Spatial transcriptomics enabled them to locate that population in areas of interstitial inflammation in the distal airways, where fibroblast induced tissue remodeling and cytokine release lead to robust immune cell infiltration at the expense of lung function. Strikingly, the authors also highlighted the significance of these findings in a clinical setting, where the observed levels of Adamts4 in the lower respiratory tract of human intensive care patients were strong predictors of prolonged multiple organ dysfunction syndrome, prolonged acute hypoxic respiratory failure, and fewer ventilator-free days.[86]

9 | CONCEPT OF IMAGING-BASED TECHNOLOGIES

Imaging-based approaches provide the reverse trade-off between sensitivity and gene throughput compared to capture arrays, offering subcellular and single molecule resolution with high detection sensitivity, but mostly restricted to targeted gene panels. In situ hybridization of fluorescently labeled complementary probes has been used in the last 40 years to visualize gene expression in tissue sections,[87] and the sensitivity of the approach was greatly enhanced with the advent of single molecule FISH,[88,89] in which multiple fluorescent probes are hybridized to the same target to enable the quantitative measurement of transcript counts. Adaption of the method to histological samples paved the way for an understanding of spatial tissue organization and homeostasis at unprecedented resolution, which the authors first showcased in a detailed analysis of stem cell dynamics in the mouse small intestine[90,91] The fluorescent signal can be further enhanced by amplifier probes that form tree-like structures on a single target-specific hybridization probe,[92,93] an approach that was commercialized in 2012 as RNAscope.[94] RNAscope allows the parallel detection of small numbers of genes in FFPE sections, and automatization of the labeling and imaging procedure enabled profiling of ~50 genes in the mouse somatosensory cortex from adjacent tissue sections.[95] Alternatively, probes can be stripped after imaging to perform additional rounds of hybridization.[96]

In the last decade, multiplexed versions of single molecule FISH have been developed that enable simultaneous profiling of hundreds and even thousands of genes in parallel. The key innovation was the use of combinatorial labelling strategies where individual transcripts are repeatedly probed in different colors to increase the number of transcripts that can be read out with a limited number of available fluorophores. Initially, this entailed the simultaneous hybridization of probes with different fluorophores along individual transcripts, which in combination with super resolution microscopy enabled the parallel profiling of 32 genes in

yeast.[89] Shortly afterwards, the same group introduced seqFISH, in which probes are stripped off by DNAse treatment after imaging, and additional rounds of hybridization greatly increase the number of possible color combinations.[97] Subsequent iterations of seqFISH improved the sensitivity and specificity of the assay, which enabled multiplexed detection of hundreds of genes in the same tissue section.[98,99]

A major step towards imaging-based profiling of entire transcriptomes was the design of gene-specific probes with custom barcode sequences, which are recognized by secondary fluorescent probes in subsequent hybridization rounds. This approach was first introduced by MERFISH and enabled the generation of theoretically unlimited barcode combinations, irrespective of the length or sequence of the target RNA.[100] In the first implementation of MERFISH, a series of 14 hybridization rounds enabled the multiplexed detection of 1000 genes cells with ~80% detection efficiency.[100] MERFISH was later combined with expansion microscopy to reduce the fluorescent signal density, which enabled profiling of 10 000 genes in tissue culture cells.[101,102] In the meanwhile, the signal to noise ratio of MERFISH has been further improved by additional tissue clearing[103] and branched DNA amplification of read out probes, providing close to 100% detection efficiency.[104]

A similar approach is used by SeqFISH+ which has been used to assay 10 000 genes in the mouse cortex, subventricular zone, and olfactory bulb with 47% detection efficiency.[105]

The work of Zhang et al impressively demonstrates how even a medium sized panel of 258 genes can be used in MERFISH to obtain highly resolved spatial maps of complex tissues, in this case for the mouse primary motor cortex. Identification of 95 neuronal and non-neuronal cell clusters across 300 000 cells, enabled them to resolve the laminar fine structure of excitatory and inhibitory neurons within cortical layers. The authors finally demonstrate how the integration of MERFISH measurements with retrograde fluorescent labelling can be used to trace the projection patters of neurons and resolve the complex network of interactions between neuronal clusters and their target regions.[106]

While single molecule FISH and especially RNAscope are widely used as validation tools, highly multiplexed FISH methods have still not spread far beyond the inventor's laboratories. However, with the advent of commercial automated platforms that are currently close to market release or in early access programs, these technologies will soon become available to a larger audience of researchers and core facilities. While not all technical specifications have been released by the time of writing, all automated platforms will enable parallel profiling of hundreds of genes with the option for multiplexed protein detection. Considerable efforts have been made by the developers to improve data processing and cell segmentation, and all solutions deliver single cell gene expression matrices that can readily be integrated with NGS-based data sets.[107,108] Important differences may lie in the capacity for parallel slide processing, and in the time needed for slide read-out, which can take several days and thus substantially limit the potential throughput. It is also important to note that all automated imaging-based systems still require extensive manual sample processing for probe hybridization and antibody staining prior to probe read-out in the instrument. Successful FISH experiments are highly dependent on a large number of technical factors,[109] and accordingly great efforts are being made by commercial suppliers to provide optimized protocols and probe designs. Benchmarking the robustness of these approaches for a diverse range of primary tissue samples that may have gone through different fixation and storage conditions will be an important task during the implementation of the first generation of highly multiplexed FISH-based instruments.

10 | CONCEPT OF IN SITU SEQUENCING

An alternative imaging-based approach is in situ sequencing of transcripts directly in the tissue. This was first demonstrated in 2013, when it was used to profile the expression of 31 genes in breast cancer tissue.[110] In the first iteration of the approach, transcripts were reverse transcribed in situ and gene-specific padlock probes were then hybridized to the target cDNAs. The nick in the padlock probes is closed by ligation, or by a DNA polymerase, and the circularized probes are amplified to DNA nanoballs by rolling circle amplification. Sequencing by ligation is then used to read out a 4 nt barcode on the padlock probes, or the 4 nt gap sequence. Signal amplification reduces the number of required padlock-probes per gene to ~5, which means that smaller genes or isoforms can be probed. At the same time, while the padlock probe design confers high specificity, the sensitivity of in situ sequencing is much lower compared to single molecule FISH, and the number of genes that can be detected simultaneously is limited to several hundred due to the size and diffusion of the DNA nanoballs. However, like other spatial technologies, in situ sequencing-based methods are still rapidly evolving. Several studies presented variations of the workflow, such as direct hybridization of padlock probes to mRNAs,[111,112] stabilization of the DNA nanoballs by crosslinking,[113,114] additional tissue clearing and the use of a hybridization-based barcode read-out[115,116] all with the aim to increase sensitivity and throughput.

The tremendous power of combined scRNAseq and spatial profiling over time has recently been showcased by La Manno et al[117] By sampling the embryonic mouse brain each day between embryonic day E7 and E18, the authors identified more than 800 cellular states that together describe a developmental program for the major functional elements of the brain. The authors then mapped the spatial expression of key developmental genes via ISS to reveal how neuronal progenitors are spatially organized during patterning of the nervous system.

All of the above-described imaging-based methods rely on predesigned panels of gene-specific detection probes. However, already in 2014 fluorescent in situ RNA sequencing (FISSEQ) was introduced with the aim of untargeted transcriptome profiling.[118] Here, transcripts are reverse transcribed in situ with random hexamer containing primers, and cDNAs are then circularized and directly sequenced by SBL. Although the original version of FISSEQ was very inefficient and time consuming, the concept has recently been combined with expansion microscopy and additional sequencing of the amplicons ex situ, which greatly improved sensitivity and accuracy of the approaches.[118].

So far, only ISS has been applied in a larger number of publications outside of the inventor's laboratory. However, the first commercial platform will be introduced in 2022 under the name Xenium, using direct binding of padlock probes followed by limited rolling circle amplification and hybridization-based barcode read out from the resulting DNA nanoballs. Initial panels are expected to target up to 500 genes, but the numbers are projected to increase in the future. As for the highly multiplexed FISH-based methods, the initial sample processing and primary probe hybridization are still manual, and thorough benchmarking will be required to compare the robustness of the workflow for different source materials.

11 | OUTLOOK

Single cell and spatial omics technologies are still evolving at a rapid pace, and additional commercial platforms are continuously entering the market. Provided that the low sensitivity of the initial combinatorial indexing workflows can be overcome by the recently launched commercial solutions, the number of addressable cells can be drastically increased due to their virtually unlimited scaling capacity. At the same time, higher throughput versions of droplet-based approaches have been demonstrated,[50] and the number of addressable cells can also be scaled up by increasing the surface area of microwell chips, eventually shifting the practical limitation of single cell 'Omics approaches to the subsequent sequencing cost.

At the same time, spatial methods will likely witness a similar increase in addressable modalities as has been seen for single cell approaches. While the first commercial platforms enable simultaneous RNA and protein profiling, additional modalities such as open chromatin detection has recently been demonstrated and can be expected to become accessible to a wider range of potential users in the future.[119] In addition, recent proof of concept studies have utilized combinatorial indexing approaches for spatial profiling, which might mature into commercially available automated solutions in the future.[120]

A major challenge that we have not covered in this review concerns the steps that precede and follow the actual measurements: the availability of high-quality input material might be the biggest hurdle when aiming for informative single cell or spatial data. The way how input material is obtained, stored, and processed has a major impact on the quality and composition of cell suspensions or tissue sections, and will influence the observed transcriptional states, e.g., because of prior cellular stress. Especially valuable will be workflows for fixation and storage of tissues or cell suspensions that are compatible with downstream protocols, which would facilitate the collection and simultaneous processing of large sample cohorts. Ideally, also the upstream processing of tissue sections for spatial analysis prior to the final read-out step may become automized in the future, which would greatly facilitate access to users outside specialized labs and core facilities.

Single cell- and Spatial O'mics analysis brings clinicians closer to the bench, as the quality requirements for input material are high and the precise planning and scheduling of experiments is therefore of utmost importance.

Bioinformatic analysis needs to account for biases and background noise and, especially, the bioinformatic integration of multiple samples or omics layers requires expertise far beyond standard next generation sequencing analysis. For deep analysis and interpretation, a team of experts from different fields is therefore needed.

One additional challenge or limitation we face are the still enormous costs of single cell experiments – much more projects would be feasible if these experiments would be more affordable and a possible translation to diagnostics seems to be prohibitive with current costs. How can library preparation costs be reduced? Similar to the increase in throughput from plates to micro/nano/pico wells to droplets to combinatorial indexing concepts (Figure 2), the volume, in which the biochemical reactions take place, shrinks up to the cellular volume itself. Low reaction volume goes along with low consumable costs and if combinatorial indexing strategies improve in sensitivity and flexibility, these will probably gain in importance.

The rapid pace of single cell- and Spatial 'Omics, and especially their combination and integration, will tremendously enhance our understanding of life processes in cells, organs and organisms in the years to come.

Maybe that's what he had in mind:

> "Progress in science depends on new techniques, new discoveries and new ideas, probably in that order." — *Sydney Brenner, Nobel Prize laureate 2002 for medicine and physiology*.

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CONFLICT OF INTEREST

The authors declare to have no conflict of interest.

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REFERENCES

1. Tang F, Barbacioru C, Wang Y, et al. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods*. 2009;6(5):377-382.
2. Kurimoto K, Yabuta Y, Ohinata Y, Saitou M. Global single-cell cDNA amplification to provide a template for representative high-density oligonucleotide microarray analysis. *Nat Protoc*. 2007;2(3):739-752.
3. Ramsköld D, Luo S, Wang Y-C, et al. Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. *Nat Biotechnol*. 2012;30(8):777-782.
4. Method of the Year 2013. *Nat Methods*. 2014;11(1):1.
5. Marx V. Method of the year: spatially resolved transcriptomics. *Nat Methods*. 2021;18(1):9-14.
6. Method of the Year 2019: Single-cell multimodal omics. *Nat Methods*. 2020;17(1):1.
7. Schreibing F, Kramann R. Mapping the human kidney using single-cell genomics. *Nat Rev Nephrol*. 2022;18:347-360. doi:10.1038/s41581-022-00553-4
8. Rajewsky N, Almouzni G, Gorski SA, et al. LifeTime and improving European healthcare through cell-based interceptive medicine. *Nature*. 2020;587(7834):377-386.
9. Ding J, Adiconis X, Simmons SK, et al. Systematic comparison of single-cell and single-nucleus RNA-sequencing methods. *Nat Biotechnol*. 2020;38(6):737-746.
10. Zappia L, Theis FJ. Over 1000 tools reveal trends in the single-cell RNA-seq analysis landscape. *Genome Biol*. 2021;22(1):301.
11. Clark SJ, Argelaguet R, Kapourani C-A, et al. scNMT-seq enables joint profiling of chromatin accessibility DNA methylation and transcription in single cells. *Nat Commun*. 2018;9(1):781.
12. Macaulay IC, Haerty W, Kumar P, et al. G&T-seq: parallel sequencing of single-cell genomes and transcriptomes. *Nat Methods*. 2015;12(6):519-522.
13. Macaulay IC, Teng MJ, Haerty W, Kumar P, Ponting CP, Voet T. Separation and parallel sequencing of the genomes and transcriptomes of single cells using G&T-seq. *Nat Protoc*. 2016;11(11):2081-2103.
14. Clark SJ, Smallwood SA, Lee HJ, Krueger F, Reik W, Kelsey G. Genome-wide base-resolution mapping of DNA methylation in single cells using single-cell bisulfite sequencing (scBS-seq). *Nat Protoc*. 2017;12(3):534-547.
15. Picelli S, Björklund ÅK, Faridani OR, Sagasser S, Winberg G, Sandberg R. Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat Methods*. 2013;10(11):1096-1098.
16. Picelli S, Faridani OR, Björklund ÅK, Winberg G, Sagasser S, Sandberg R. Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc*. 2014;9(1):171-181.
17. Hagemann-Jensen M, Ziegenhain C, Chen P, et al. Single-cell RNA counting at allele and isoform resolution using Smart-seq3. *Nat Biotechnol*. 2020;38(6):708-714.
18. Hagemann-Jensen M, Ziegenhain C, Sandberg R. Scalable full-transcript coverage single cell RNA sequencing with Smart-seq3xpress. *bioRxiv*. 2021, Preprint.
19. Hahaut V, Pavlinic D, Cowan C, Picelli S. Lightning Fast and Highly Sensitive Full-Length Single-cell sequencing using FLASH-Seq. *bioRxiv*. 2021, Preprint.
20. Isakova A, Neff N, Quake SR. Single-cell quantification of a broad RNA spectrum reveals unique noncoding patterns associated with cell types and states. *Proc Natl Acad Sci U S A*. 2021;118(51):e2113568118. doi:10.1073/pnas.2113568118
21. Booeshaghi AS, Yao Z, van Velthoven C, et al. Isoform cell-type specificity in the mouse primary motor cortex. *Nature*. 2021;598(7879):195-199.
22. Angermueller C, Clark SJ, Lee HJ, et al. Parallel single-cell sequencing links transcriptional and epigenetic heterogeneity. *Nat Methods*. 2016;13(3):229-232.
23. Hagemann-Jensen M, Abdullayev I, Sandberg R, Faridani OR. Small-seq for single-cell small-RNA sequencing. *Nat Protoc*. 2018;13(10):2407-2424.
24. Ai S, Xiong H, Li CC, et al. Profiling chromatin states using single-cell itChIP-seq. *Nat Cell Biol*. 2019;21(9):1164-1172.
25. Kaya-Okur HS, Wu SJ, Codomo CA, et al. CUT&Tag for efficient epigenomic profiling of small samples and single cells. *Nat Commun*. 2019;10(1):1930.
26. Chen Y, Song J, Ruan Q, et al. Single-cell sequencing methodologies: from transcriptome to multi-dimensional measurement. *Small Methods*. 2021;5(6):2100111.
27. Cao J, Cusanovich DA, Ramani V, et al. Joint profiling of chromatin accessibility and gene expression in thousands of single cells. *Science*. 2018;361(6409):1380-1385.
28. Plongthongkum N, Diep D, Chen S, Lake BB, Zhang K. Scalable dual-omics profiling with single-nucleus chromatin accessibility and mRNA expression sequencing 2 (SNARE-seq2). *Nat Protoc*. 2021;16(11):4992-5029.
29. Xiong H, Luo Y, Wang Q, Yu X, He A. Single-cell joint detection of chromatin occupancy and transcriptome enables higher-dimensional epigenomic reconstructions. *Nat Methods*. 2021;18(6):652-660.
30. Ma S, Zhang B, LaFave LM, et al. Chromatin Potential Identified by Shared Single-Cell Profiling of RNA and Chromatin. *Cell*. 2020;183(4):1103-1116.e20.
31. Zhu C, Yu M, Huang H, et al. An ultra high-throughput method for single-cell joint analysis of open chromatin and transcriptome. *Nat Struct Mol Biol*. 2019;26(11):1063-1070.
32. Zhu C, Zhang Y, Li YE, Lucero J, Behrens MM, Ren B. Joint profiling of histone modifications and transcriptome in single cells from mouse brain. *Nat Methods*. 2021;18(3):283-292.

33. Yin Y, Jiang Y, Lam K-WG, et al. High-Throughput Single-Cell Sequencing with Linear Amplification. *Mol Cell*. 2019;76(4):676-690.e10.
34. Mulqueen RM, Pokholok D, O'Connell BL, et al. High-content single-cell combinatorial indexing. *Nat Biotechnol*. 2021;39(12):1574-1580.
35. Chen S, Lake BB, Zhang K. High-throughput sequencing of the transcriptome and chromatin accessibility in the same cell. *Nat Biotechnol*. 2019;37(12):1452-1457.
36. Swanson E, Lord C, Reading J, et al. Simultaneous trimodal single-cell measurement of transcripts, epitopes, and chromatin accessibility using TEA-seq. *elife*. 2021;10:e63632. doi:10.7554/eLife.63632
37. Mimitou EP, Lareau CA, Chen KY, et al. Scalable, multimodal profiling of chromatin accessibility, gene expression and protein levels in single cells. *Nat Biotechnol*. 2021;39(10):1246-1258.
38. Stoeckius M, Hafemeister C, Stephenson W, et al. Simultaneous epitope and transcriptome measurement in single cells. *Nat Methods*. 2017;14(9):865-868.
39. Gierahn TM, Wadsworth MH, Hughes TK, et al. Seq-Well: portable, low-cost RNA sequencing of single cells at high throughput. *Nat Methods*. 2017;14(4):395-398.
40. Macosko EZ, Basu A, Satija R, et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell*. 2015;161(5):1202-1214.
41. Grosselin K, Durand A, Marsolier J, et al. High-throughput single-cell ChIP-seq identifies heterogeneity of chromatin states in breast cancer. *Nat Genet*. 2019;51(6):1060-1066.
42. Klein AM, Mazutis L, Akartuna I, et al. Droplet Barcoding for Single-Cell Transcriptomics Applied to Embryonic Stem Cells. *Cell*. 2015;161(5):1187-1201.
43. Tian L, Jabbari JS, Thijssen R, et al. Comprehensive characterization of single-cell full-length isoforms in human and mouse with long-read sequencing. *Genome Biol*. 2021;22(1):310.
44. Wang Q, Boenigk S, Boehm V, Gehring NH, Altmueller J, Dieterich C. Single cell transcriptome sequencing on the Nanopore platform with ScNapBar. *RNA*. 2021;27:763-770. doi:10.1261/rna.078154.120
45. Zheng GXY, Terry JM, Belgrader P, et al. Massively parallel digital transcriptional profiling of single cells. *Nat Commun*. 2017;8(1):14049.
46. Satpathy AT, Granja JM, Yost KE, et al. Massively parallel single-cell chromatin landscapes of human immune cell development and intratumoral T cell exhaustion. *Nat Biotechnol*. 2019;37(8):925-936.
47. Buechler MB, Pradhan RN, Krishnamurty AT, et al. Cross-tissue organization of the fibroblast lineage. *Nature*. 2021;593(7860):575-579.
48. Cusanovich DA, Daza R, Adey A, et al. Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science*. 2015;348(6237):910-914.
49. Lareau CA, Duarte FM, Chew JG, et al. Droplet-based combinatorial indexing for massive-scale single-cell chromatin accessibility. *Nat Biotechnol*. 2019;37(8):916-924.
50. Datlinger P, Rendeiro AF, Boenke T, et al. Ultra-high-throughput single-cell RNA sequencing and perturbation screening with combinatorial fluidic indexing. *Nat Methods*. 2021;18(6):635-642.
51. Domcke S, Hill AJ, Daza RM, et al. A human cell atlas of fetal chromatin accessibility. *Science*. 2020;370(6518):eaba7612. doi:10.1126/science.aba7612
52. Ramani V, Deng X, Qiu R, et al. Massively multiplex single-cell Hi-C. *Nat Methods*. 2017;14(3):263-266.
53. Ramani V, Deng X, Qiu R, et al. Sci-Hi-C: A single-cell Hi-C method for mapping 3D genome organization in large number of single cells. *Methods*. 2020;170:61-68.
54. Vitak SA, Torkenczy KA, Rosenkrantz JL, et al. Sequencing thousands of single-cell genomes with combinatorial indexing. *Nat Methods*. 2017;14(3):302-308.
55. Cao J, Zhou W, Steemers F, Trapnell C, Shendure J. Sci-fate characterizes the dynamics of gene expression in single cells. *Nat Biotechnol*. 2020;38(8):980-988.
56. Cao J, Packer JS, Ramani V, et al. Comprehensive single-cell transcriptional profiling of a multicellular organism. *Science*. 2017;357(6352):661-667.
57. Rosenberg AB, Roco CM, Muscat RA, et al. Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding. *Science*. 2018;360(6385):176-182.
58. Mulqueen RM, Pokholok D, Norberg SJ, et al. Highly scalable generation of DNA methylation profiles in single cells. *Nat Biotechnol*. 2018;36(5):428-431.
59. Cao J, Spielmann M, Qiu X, et al. The single-cell transcriptional landscape of mammalian organogenesis. *Nature*. 2019;566(7745):496-502.
60. Luo L, Salunga RC, Guo H, et al. Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat Med*. 1999;5(1):117-122.
61. Sgroi DC, Teng S, Robinson G, LeVangie R, Hudson JR, Elkahloun AG. In vivo gene expression profile analysis of human breast cancer progression. *Cancer Res*. 1999;59(22):5656-5661.
62. Moses L, Pachter L. Museum of spatial transcriptomics. *bioRxiv*. 2021, Preprint. doi:10.1101/2021.05.11.443152
63. Peng G, Suo S, Cui G, et al. Molecular architecture of lineage allocation and tissue organization in early mouse embryo. *Nature*. 2019;572(7770):528-532.
64. Tirosh I, Izar B, Prakadan SM, et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science*. 2016;352(6282):189-196.
65. Chen J, Suo S, Tam PP, Han J-DJ, Peng G, Jing N. Spatial transcriptomic analysis of cryosectioned tissue samples with Geo-seq. *Nat Protoc*. 2017;12(3):566-580.
66. Moor AE, Harnik Y, Ben-Moshe S, et al. Spatial reconstruction of single enterocytes uncovers broad zonation along the intestinal villus axis. *Cell*. 2018;175(4):1156-1167.e15.
67. Nichterwitz S, Benitez JA, Hoogstraaten R, Deng Q, Hedlund E. LCM-Seq: a method for spatial transcriptomic profiling using laser capture microdissection coupled with PolyA-based RNA sequencing. *Methods Mol Biol*. 2018;1649:95-110.
68. Nichterwitz S, Chen G, Aguila Benitez J, et al. Laser capture microscopy coupled with Smart-seq2 for precise spatial transcriptomic profiling. *Nat Commun*. 2016;7(1):1-11.
69. Brosch M, Kattler K, Herrmann A, et al. Epigenomic map of human liver reveals principles of zonated morphogenic and metabolic control. *Nat Commun*. 2018;9(1):1-11.
70. Junker JP, Noël ES, Guryev V, et al. Genome-wide RNA Tomography in the Zebrafish Embryo. *Cell*. 2014;159(3):662-675.
71. Holler K, Neuschulz A, Drewe-Boß P, et al. Spatio-temporal mRNA tracking in the early zebrafish embryo. *Nat Commun*. 2021;12(1):1-13.
72. Combs PA, Fraser HB. Spatially varying cis-regulatory divergence in Drosophila embryos elucidates cis-regulatory logic. *PLoS Genet*. 2018;14(11):e1007631.

73. Merritt CR, Ong GT, Church SE, et al. Multiplex digital spatial profiling of proteins and RNA in fixed tissue. *Nat Biotechnol*. 2020;38(5):586-599.
74. Jerby-Arnon L, Neftel C, Shore ME, et al. Opposing immune and genetic mechanisms shape oncogenic programs in synovial sarcoma. *Nat Med*. 2021;27(2):289-300.
75. Ståhl PL, Salmén F, Vickovic S, et al. Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. *Science*. 2016;353(6294):78-82.
76. Dong R, Yuan GC. SpatialDWLS: accurate deconvolution of spatial transcriptomic data. *Genome Biol*. 2021;22(1):1-10.
77. Biancalani T, Scalia G, Buffoni L, et al. Deep learning and alignment of spatially resolved single-cell transcriptomes with Tangram. *Nat Methods*. 2021;18(11):1352-1362.
78. Lohoff T, Ghazanfar S, Missarova A, et al. Integration of spatial and single-cell transcriptomic data elucidates mouse organogenesis. *Nat Biotechnol*. 2021;2021:1-12.
79. Cable DM, Murray E, Zou LS, et al. Robust decomposition of cell type mixtures in spatial transcriptomics. *Nat Biotechnol*. 2021;2021:1-10.
80. Andersson A, Bergenstråhle J, Asp M, et al. Single-cell and spatial transcriptomics enables probabilistic inference of cell type topography. *Commun Biol*. 2020;3(1):1-8.
81. Elosua-Bayes M, Nieto P, Mereu E, Gut I, Heyn H. SPOTlight: seeded NMF regression to deconvolute spatial transcriptomics spots with single-cell transcriptomes. *Nucleic Acids Res*. 2021;49(9):e50.
82. Vickovic S, Eraslan G, Salmén F, et al. High-definition spatial transcriptomics for in situ tissue profiling. *Nat Methods*. 2019;16(10):987-990.
83. Rodriques SG, Stickels RR, Goeva A, et al. Slide-seq: A scalable technology for measuring genome-wide expression at high spatial resolution. *Science*. 2019;363(6434):1463-1467.
84. Chen A, Liao S, Cheng M, et al. Large field of view-spatially resolved transcriptomics at nanoscale resolution. *bioRxiv*. 2021, Preprint. doi:10.1101/2021.01.17.427004
85. Cho CS, Xi J, Si Y, et al. Microscopic examination of spatial transcriptome using Seq-Scope. *Cell*. 2021;184(13):3559-3572.e22.
86. Boyd DF, Allen EK, Randolph AG, et al. Exuberant fibroblast activity compromises lung function via ADAMTS4. *Nature*. 2020;587(7834):466-471.
87. Singer RH, Ward DC. Actin gene expression visualized in chicken muscle tissue culture by using in situ hybridization with a biotinated nucleotide analog. *Proc Natl Acad Sci*. 1982;79(23):7331-7335.
88. Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. Imaging individual mRNA molecules using multiple singly labeled probes. *Nat Methods*. 2008;5(10):877-879.
89. Lubeck E, Cai L. Single-cell systems biology by super-resolution imaging and combinatorial labeling. *Nat Methods*. 2012;9(7):743-748.
90. Lyubimova A, Itzkovitz S, Junker JP, Fan ZP, Wu X, van Oudenaarden A. Single-molecule mRNA detection and counting in mammalian tissue. *Nat Protoc*. 2013;8(9):1743-1758.
91. Itzkovitz S, Lyubimova A, Blat IC, et al. Single-molecule transcript counting of stem-cell markers in the mouse intestine. *Nat Cell Biol*. 2011;14(1):106-114.
92. Player AN, Shen LP, Kenny D, Antao VP, Kolberg JA. Single-copy gene detection using branched DNA (bDNA) in situ hybridization. *J Histochem Cytochem*. 2001;49(5):603-611.
93. Urdea MS. Synthesis and Characterization of Branched DNA (bDNA) for the Direct and Quantitative Detection of CMV, HBV, HCV, and HIV. *Clin Chem*. 1993;39(4):725-726.
94. Wang F, Flanagan J, Su N, et al. RNAscope: A Novel in Situ RNA Analysis Platform for Formalin-Fixed, Paraffin-Embedded Tissues. *J Mol Diagn*. 2012;14(1):22-29.
95. Bayraktar OA, Bartels T, Holmqvist S, et al. Astrocyte layers in the mammalian cerebral cortex revealed by a single-cell in situ transcriptomic map. *Nat Neurosci*. 2020;23(4):500-509.
96. Codeluppi S, Borm LE, Zeisel A, et al. Spatial organization of the somatosensory cortex revealed by osmFISH. *Nat Methods*. 2018;15(11):932-935.
97. Lubeck E, Coskun AF, Zhiyentayev T, Ahmad M, Cai L. Single-cell in situ RNA profiling by sequential hybridization. *Nat Methods*. 2014;11(4):360-361.
98. Goh JJL, Chou N, Seow WY, et al. Highly specific multiplexed RNA imaging in tissues with split-FISH. *Nat Methods*. 2020;17(7):689-693.
99. Shah S, Lubeck E, Zhou W, Cai L. In situ transcription profiling of single cells reveals spatial organization of cells in the mouse hippocampus. *Neuron*. 2016;92(2):342-357.
100. Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science*. 2015;348(6233):aaa6090. doi:10.1126/SCIENCE.AAA6090/SUPPL_FILE/PAP.PDF
101. Wang G, Moffitt JR, Zhuang X. Multiplexed imaging of high-density libraries of RNAs with MERFISH and expansion microscopy. *Sci Rep*. 2018;8(1):1-13.
102. Xia C, Fan J, Emanuel G, Hao J, Zhuang X. Spatial transcriptome profiling by MERFISH reveals subcellular RNA compartmentalization and cell cycle-dependent gene expression. *Proc Natl Acad Sci U S A*. 2019;116(39):19490-19499.
103. Moffitt JR, Hao J, Bambah-Mukku D, Lu T, Dulac C, Zhuang X. High-performance multiplexed fluorescence in situ hybridization in culture and tissue with matrix imprinting and clearing. *Proc Natl Acad Sci U S A*. 2016;113(50):14456-14461.
104. Xia C, Babcock HP, Moffitt JR, Zhuang X. Multiplexed detection of RNA using MERFISH and branched DNA amplification. *Sci Rep*. 2019;9(1):1-13.
105. Eng CHL, Lawson M, Zhu Q, et al. Transcriptome-scale super-resolved imaging in tissues by RNA seqFISH+. *Nature*. 2019;568(7751):235-239.
106. Zhang M, Eichhorn SW, Zingg B, et al. Spatially resolved cell atlas of the mouse primary motor cortex by MERFISH. *Nature*. 2021;598(7879):137-143.
107. He S, Bhatt R, Birditt B, et al. High-plex multiomic analysis in ffpe tissue at single-cellular and subcellular resolution by spatial molecular imaging. *bioRxiv*. 2021, Preprint. doi:10.1101/2021.11.03.467020
108. Petukhov V, Xu RJ, Soldatov RA, et al. Cell segmentation in imaging-based spatial transcriptomics. *Nat Biotechnol*. 2021;2021:1-10.
109. Young AP, Jackson DJ, Wyeth RC. A technical review and guide to RNA fluorescence in situ hybridization. *PeerJ*. 2020;8:e8806.
110. Ke R, Mignardi M, Pacureanu A, et al. In situ sequencing for RNA analysis in preserved tissue and cells. *Nat Methods*. 2013;10(9):857-860.
111. Sountoulidis A, Liontos A, Nguyen HP, et al. SCRINSHOT enables spatial mapping of cell states in tissue sections with single-cell resolution. *PLoS Biol*. 2020;18(11):e3000675.

112. Lee H, Salas SM, Gyllborg D, Nilsson M. Direct RNA targeted transcriptomic profiling in tissue using Hybridization-based RNA In Situ Sequencing (HybRISS). *bioRxiv*. 2020, Preprint. doi:10.1101/2020.12.02.408781
113. Chen X, Sun YC, Church GM, Lee JH, Zador AM. Efficient in situ barcode sequencing using padlock probe-based BaristaSeq. *Nucleic Acids Res*. 2018;46(4):e22.
114. Chen X, Sun YC, Zhan H, et al. High-Throughput Mapping of Long-Range Neuronal Projection Using In Situ Sequencing. *Cell*. 2019;179(3):772-786.e19.
115. Wang X, Allen WE, Wright MA, et al. Three-dimensional intact-tissue sequencing of single-cell transcriptional states. *Science*. 2018;361(6400):eaat5691.
116. Gyllborg D, Langseth CM, Qian X, et al. Hybridization-based in situ sequencing (HybISS) for spatially resolved transcriptomics in human and mouse brain tissue. *Nucleic Acids Res*. 2020;48(19):e112.
117. la Manno G, Siletti K, Furlan A, et al. Molecular architecture of the developing mouse brain. *Nature*. 2021;596(7870):92-96.
118. Alon S, Goodwin DR, Sinha A, et al. Expansion sequencing: Spatially precise in situ transcriptomics in intact biological systems. *Science*. 2021;371(6528):eaax2656.
119. Deng Y, Bartosovic M, Kukanja P, et al. Spatial-CUT&Tag: Spatially resolved chromatin modification profiling at the cellular level. *Science*. 2022;375(6581):681-686.
120. Su G, Qin X, Enninful A, et al. Spatial multi-omics sequencing for fixed tissue via DBiT-seq. *STAR Protoc*. 2021;2(2):100532.

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