scTrends: A Living Review of Commercial Single Cell and Spatial ‘Omic Technologies

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Original Appendix to Cell Genomics review

Appendix 1: Commercial single cell omic technologies

Microfluidics-based methods

10x Genomics / Chromium platform

10x Genomics, Inc., founded by Serge Saxonov, Ben Hindson and Kevin Ness in 2012 was the first company aiming to commercialize a high-throughput single cell RNA-seq method based on microfluidic devices. Their technology relies on a paper published in 2017[1] where they describe the cell encapsulation in Gel bead in EMulsion (GEM), in-droplet mRNA capture, reverse transcription barcoding, bulk cDNA amplification and subsequent Illumina library preparation. 10x Genomics has largely evolved and is now proposing, in addition to all the single cell Chromium products, analysis pipelines and instrumentations, spatial transcriptomics.

When using the Chromium single cell gene expression kits, cells are co-encapsulated with a compressible barcoded bead and a lysis/reverse transcriptase mix in monodisperse water-in-oil emulsions generated using a triple-inlet plastic chip connecting to vacuum lines, allowing for the processing of up to 8 samples in parallel. Since the barcoded beads are compressible, they can be loaded accurately into ~60-70% of droplets, the cell loading follows Poisson statistics and depends on the initial loading concentration. Once the cells are encapsulated, the barcoded beads dissolves due to the thiolated properties of the reverse transcriptase mix, hereby releasing the oligo(dT) primers which will bind the polyadenylated tails of mRNA molecules, as well as A-rich stretches of the transcriptome (mainly intronic), hereby initiating reverse transcription and cDNA generation. Terminal transferase activity from the reverse transcriptase results in the 3' addition of untemplated cytosines on the cDNA, providing a binding substrate for riboguanosines found on the template-switching oligo (TSO), enabling the addition of the PCR reverse template to the 3' end of the cDNA. After incubation, the emulsions are broken to recuperate the cDNA molecules which are subsequently amplified using limited-step PCR. For the 5' gene expression kits, the TSO contains a barcode and is bead-bound, for 3' gene expression solutions, the oligo(dT) is barcoded and bead-bound. After cDNA amplification, the molecules are randomly fragmented and only transcripts containing the 10x barcode and the Illumina read 2 adapter (ligated after cDNA amplification) will be amplified during the indexing PCR. In addition to looking at gene expression, it is now possible to combine those technologies with cell-surface protein abundance measurements using barcoded antibodies based on the CITE-seq concept[2] or CRISPR perturbations by co-sequencing transcripts and sgRNAs within the same single cell. Whilst the history of perturb-seq (pooled CRISPR screens with single cell readouts) is out of scope for this review, we note that the original protocols (perturb-seq[3], CROP-seq[4], CRISP-seq[5]) have now been industrially standardized via "direct capture perturb-seq" using specialized beads.

Ultimately, as mutational information is seldom used, 10x Genomics has recently released a new single cell gene expression flex kit also compatible with simultaneous measurement of cell surface proteins. This protocol has the advantage of being more sensitive than the classical 3'/ 5' workflows, works with fixed cells and is compatible with Formalin-Fixed Paraffin Embedded (FFPE) and degraded samples. This is a probe-based approach that is not relying on cDNA synthesis (the probes are directly sequenced) and is only currently available for mouse and human genomes.

10x Genomics also has specific kits to sequence full length V(D)J sequences for paired B- or T-cell receptors, the Chromium single cell Immune profiling. Amplified full length cDNA from poly-adenylated mRNAs is used to amplify full-lenght V(D)J fragments using specific primers hybridizing to the constant region of the TCR or BCR sequences. After enzymatic fragmentation, size selection, end repair, A-tailing and Illumina read 2 sequence ligation, libraries are further amplified using i5-P5 and i7-P7 primer sequences and ready for sequencing.

Beyond transcriptomics, the epigenome can also be measured at single-cell resolution, for example, using the assay for transposase-accessible chromatin with sequencing (ATAC-seq) method, whereby one can preferentially sequence regions of DNA that are available to being transcribed. To achieve this, cells are reduced to nuclei and a tn5 transposase is used to insert Illumina Read 1 and Read 2 in regions of open chromatin in bulk. The tagmented nuclei are then loaded in the Chromium instrument and the emulsions are subjected to barcoded PCR after release from the dissolvable beads to add unique cell barcodes as indices as well as the Illumina P5 and P7 adapters. The emulsions are then released and the product can be cleaned-up and sent for sequencing. The Multiome kit allows for both single cell ATAC-seq and RNA-seq within the same nuclei, and cytoplasmic RNA is lost.

Mission Bio / Tapestri platform

Several commercial players have focused on developing single-cell DNA decoding platforms (besides the aforementioned epigenetic read-out mentioned such as ATAC-seq), mainly aiming to resolve mutational payload of targeted regions in the genome. The main player in the field, Mission Bio, founded by Adam Abate and Adam Sciambi, has put forward the Tapestri instrument which relies on a dual droplet microfluidic set-up[6,7]. Herein, 1.) first a cell encapsulation step in water-in-oil emulsions at high-throughput for cell compartmentalization followed by protease-mediated lysis, and 2.) a droplet merging system where an emulsion containing a single-cell barcode linked to the region targets and PCR master-mix are merged with the lysate from the previous step using coalescence-induced merging, enabling gene panel amplification while maintaining the single-cell compartment using PCR in emulsions, which also adds the Read1 and Read2 sequences as overhangs at each end of the amplicon[8]. The emulsions can then be opened to release the barcoded amplicons and a final PCR adds the i5-P5 and i7-P7 indices to make the fragments compatible with next-generation sequencing. Because Tapestri is largely optimized for emulsion PCR and uses a droplet merging module to introduce the barcode to the lysate, they have been largely able to operate without litigation for patent infringements from 10x Genomics or Bio-Rad.

Atrandi Biosciences / Onyx platform

Atrandi Biosciences, formerly known as Droplet Genomics, is a company established in 2016, dedicated to developing microfluidics systems. Their unique speciality lies in developing droplet instrumentation such as the Onyx to conduct high-throughput analysis of tens of thousands of cells, facilitated by a syringe pump system, which can be used for standard single-cell RNA-seq or for encapsulating single cells within semi-permeable capsules[9,10] (SPCs) using UV light treatment. These SPCs are notably stable under a variety of reaction conditions. Such stability enables the introduction of washes between process steps, setting up the necessary conditions for a range of downstream reactions, including but not limited to, ligation, reverse transcription, and PCR. The semi-permeable nature of the capsules broadens their applicability beyond RNA-based transcriptomics to encompass targeted sequencing. Atrandi Biosciences currently advocates for the use of a combinatorial split-and-pool barcoding method, a technique also adopted by Parse Biosciences. However, their product offering is presently limited to kits for droplet generation. This implies that the process of cell barcoding necessitates an in-house, tailored approach.

Plate- & Microwell-based methods

Takara Bio / SMART platform

SMART-seq is a sequencing technique that was developed to enhance read coverage across transcripts and was developed on top of the Illumina sequencing platform[11]. It utilizes a template-switching mechanism at the 5’ end of the transcript; developed by Clontech and eventually acquired by Takara Bio, who made several enhancements to increase efficiency.

The original SMART-seq protocol operated by lysing each cell in a hypotonic solution, and then converting polyadenylated RNA to full-length cDNA using oligo(dT) priming. SMART template-switching technology was used to append a PCR handle to the cDNA, which was then amplified via PCR. The amplified cDNA was converted into a library using Covaris shearing, followed by ligation of adapters or Tn5-mediated tagmentation via Nextera technology.

In 2013, Picelli *et al.* introduced SMART-seq2[12], featuring several improvements over the original protocol. These included the use of Locked Nucleic Acids as a substitute for the ribose bases in the Template switch oligonucleotide, a higher MgCl2 concentration, the inclusion of betaine, and the elimination of the purification step to enhance yield.

A more sensitive short-read sequencing method than SMART-seq3 was introduced by Hagemann-Jensen *et al.* in 2020[13]. It utilized 5' UMIs in the template switch oligonucleotide for precise quantification and a Tn5 modification within the primer site to differentiate 5' UMI-tagged reads from internal reads. Other enhancements involved replacing KCL with either NaCl or CsCl during reverse transcription, and the inclusion of 5% PEG in reverse transcription, leading to improved sensitivity and yield, likely due to the reduction in RNA secondary structures. They also added Guanosine-5'-triphosphate (GTP) or deoxycytidine triphosphate (dCTPs) to promote and stabilise the template switching[14].

Takara Bio's commercial version of SMART-seq, SMART-seq v4, incorporates several features of the academic protocols. More recently, the SMART-seq series has been upgraded to increase throughput and miniaturize the approach for more efficient library generation

automation[15]. This was realized by further evolving SMART-seq2 using Superscript IV as the reverse transcriptase instead of Superscript II, reducing the reaction time, increasing the quantity of dCTP, and substituting a troublesome component in the oligonucleotide with riboguanosine.

Honeycomb Biotechnologies / HIVE platform

The Seq-Well method was first published in 2017 by the Shalek and Love labs at MIT[16], combining barcoded poly(A) RT capture-oligo functionalized GEM beads with micro-fabricated picowell PDMS arrays. Cells are applied across the picowell plates and efficiently drop by gravity into the picowells that each contain a poly(A) RT capture-oligo functionalized GEM bead which uniquely barcodes cellular mRNA upon cell lysis. Seq-Well provides a cost-effective and simplified solution for cell/bead co-encapsulation that works well with low sample inputs[17] and challenging sample preparation environments such as remote locations and BSL restricted facilities that exclude application of droplet-based scRNA-seq platforms. The open-source nature of the Seq-Well has served to increase the democratization and wide-spread adoption of scRNA-seq globally and the protocol has been widely validated in resource-limiting environments[18].

The Seq-Well assay has been heavily optimized and improved using a randomer based on-bead amplification to improve gene capture, and is now approaching comparable data quality to other SOTA methods. The assay is under active development and has so far been extended to co-detect additional cellular modalities such as mutations[19], TCR-receptors[20], and cell-hashing.

Established in 2018, Honeycomb Biotechnologies has expanded from the foundational Seq-Well academic solution developed at MIT, focusing on crafting a comprehensive solution for single-cell sequencing. Their key product, "HIVE", represents a single-use, disposable device intended to capture, store, and analyze biological samples at single-cell resolution, circumventing the need for specialized equipment. Notably, their microwell technology can potentially preserve sensitive cells such as granulocytes more effectively than some microfluidic techniques. The HIVE v1 offers a significant storage capacity, being able to maintain samples for up to nine months. The company has strategically targeted the clinical trial market, leveraging the convenience in sample collection and storage that their product offers. In addition, Honeycomb Biotechnologies has secured a commercial agreement with Revvity (formerly known as PerkinElmer) to market reagent kits.

BD Biosciences / Rhapsody platform

Originating from the acquisition of Cellular Research[21,22], BD Biosciences (itself a subsidiary of Becton Dickinson) developed the Rhapsody platform as a comprehensive single-cell multiomics system designed to capture and analyze multimodal data from thousands of individual cells concurrently. The system is constructed around three key components: the BD Rhapsody Express System, the BD Rhapsody Scanner, and BD's proprietary Molecular Indexing technology.

The BD Rhapsody Express System is an efficient benchtop instrument that utilises microwell-based single-cell partitioning technology. It allows the capture of individual cells from a

wide array of sample types. Post-capture, these cells are barcoded and prepared for subsequent analysis of various molecular markers, including both RNA and proteins.

Complementing the Express System is the BD Rhapsody Scanner. It's an advanced imaging tool designed to visualize the single cells isolated by the Express System. Using brightfield and dual band fluorescence imaging, it provides precise measurements of cell capture and multiplet rates. These measures can be used to gauge the quality of the single-cell capture workflow. The system also incorporates BD's proprietary Molecular Indexing technology, oligo-conjugated antibodies to measure protein expression by sequencing, which enables simultaneous detection of protein and mRNA expression in a single cell. The antibody specific oligonucleotides are captured, amplified and sequenced alongside mRNA in a single workflow using BD Rhapsody, a massively parallel single cell analysis system, a system that is analogous to the approach of CITE-seq.

While not unique to the field, the Rhapsody platform offers a selection of features that may be of interest to immunology researchers. Specifically, the system has the capacity for 24 antibody-based fluorophore measurements, and full-length V(D)J analysis.

Singleron Biotechnologies / SCOPE platform

Singleron Biotechnologies (“Singleron”), founded in 2018 in Nanjin China, offers the single-cell SCOPE series of products of which the GEXSCOPE single-cell RNA library was first released in 2019, and appears to be a successor to the CEL-seq[23] and CEL-seq2[24] protocols. The company has an international presence, boasting research laboratories and office space in Germany, the USA and its home country of China. Singleton leverages its proprietary Matrix platform, which is a microwell based method, to deliver a fully automated process capable of capturing between 500 to 30,000 cells per run.

In addition to its core technology, Singleron has developed an extensive suite of consumables to support advanced single-cell analyses. These include the GEXSCOPE RNA library preparation kit, which simplifies the process of generating a single-cell RNA library and the sCircle kit, a full-length V(D)J sequencing kit similar to the BD rhapsody platform.

One of the main strengths of the SCOPE platform is the ability to customize the capture sequences on the beads (FocuSCOPE), allowing researchers to more easily enrich regions of interest using a standardized service. The bead system employed by Singleron is also labeled with multiple capture sequences, providing the potential for simultaneous single-cell poly(A) and ATAC sequencing. Unique services provided by SCOPE platform are the DyneSCOPE kit, a kit that allows researchers to measure nascent RNA over time and at a single-cell resolution, and ProMoSCOPE, which allows for the detection of cell surface N-Acetyllactosamine at a single cell resolution.

Combinatorial indexing methods

Parse Biosciences / Evercode platform

Parse Biosciences (“Parse”) is a relative newcomer to the scRNA-seq field, with the company founded upon the SPLiT-seq technology first published as a preprint in 2017[25]. First authors of the paper Alex Rosenberg and Charlie Rocco along with George Seelig established the spin out

Parse Biosciences in 2020 to develop commercially available kits initially known as “Parse Evercode WT". The combinatorial indexing method implemented in the Parse Evercode protocol can scale to 1M cells in a single barcoding experiment, meaning that on a cost-per-cell basis this protocol can achieve impressive economies of scale. The Parse system has been heavily optimized with the *Evercode-v2* system reaching a stage of maturity where the data quality is comparable with SOTA scRNA-seq technologies on several benchmark samples such as HEK/3T3 and PBMC[26].

Parse kits currently use 4x rounds of barcoding, using a reverse transcription step to append the first barcode and sample-specific information, two further ligation reactions to append a 2nd and 3rd well-specific by in-cell ligation along with a UMI sequence. Cells are then pooled and split one final time before they are lysed and a final barcode is introduced by PCR during library construction. Finally, after sequencing each cell transcriptome is demultiplexed by combining those reads which contain an identical four-barcode combination.

The initial RT step is based on both poly(A) and random hexamer priming, which allows Parse to capture more unprocessed transcripts and non-coding RNAs than 3’ poly(A) capture systems alone, making it particularly suited to nuclei, as well as intact cells.

Parse has carried out additional development on the Evercode platform, offering the ability to perform parallel TCR/BCR-seq, perform targeted gene capture, as well as co-detection of sgRNA used in single-cell CRISPR screening where the advantages of massively increased scale synergise well in the context of large pooled CRISPR screening (CROP-seq) panels. In addition, Parse provides a complete computational pipeline and analysis platform to analyze the generated sequencing library data.

Scale Biosciences / ScaleBio platform

Scale Bioscience (“Scale”) uses an instrument-free combinatorial indexing-based single-cell workflow that was developed based on the sci-RNA-seq protocol first published in 2017[1]. The protocol has been further developed and heavily optimized by the Shendure and Trapnell labs at the University of Washington[27], now termed sci-RNA-seq3. Scale Biosciences scientific co-founders are Jay Shendure, Cole Trapnell along with Frank Steemers and Gary Nolan who have collectively made significant advances in the development of split-pool barcoding technology[28,29]. Cells in the Scale workflow can be fixed, adapting the fixative protocol from sci-RNA-seq3 and frozen allowing for improved experimental flexibility and minimization of potential batch effect.

Scale Biosciences’ 3’ scRNA-seq kit currently uses 3x level indexing strategy, which allows sequencing of up to 125k cells per experiment multiplexing up to 96 samples. In this workflow cells a first barcode is incorporated during first strand cDNA synthesis during reverse transcription, the second barcode is introduced by hairpin ligation and finally the third barcode is introduced through a tagmentation step and final libraries generated through index PCR to generate sequencer platform compatible libraries.

The core combinatorial indexing process of the Scale assay has been extended to other modalities and Scale also offers CRISPR screening compatible modifications the 3’ scRNA-seq kit and a similar format assay for scATAC-seq and scMethylation-seq[30]. In addition, Scale provides a complete computational pipeline and analysis platform to analyze the generated sequencing library data.

Miscellaneous methods

Scipio Bioscience / Asteria platform

Paris-based Scipio Bioscience offers an instrument-free scRNA-seq methodology based on the RevGel-seq protocol[31]. Their first product, the Asteria kit for scRNA-seq, has been launched in Q2 2022. The selling points of the kit and Scipio's methodology are no starting/equipment costs and a benchtop solution to prepare poly(A), cytoplasmic RNA libraries from single cells for Illumina sequencing (and assumed compatible with other sequencing platforms).

In brief, single cells in a cell suspension are captured onto single DNA-barcoded, and oligo(dT)-tailed, beads according to a Poisson distribution. To encourage colocation of cells with beads, cells are labeled with bifunctional chemical linkers: a poly(A) oligonucleotide attached to hydrophobic moiety. In a tube, in dilute conditions, bead-cell pairs are immobilized by gelification. Cell lysis takes place in the gel and released RNA is hybridized to barcoded beads. The key concept here is that RNA hybridization onto beads is much faster than diffusion of transcripts through the gel, and no absolute separation such as plate wells or droplets are needed to obtain single-cell measurements. After bead-RNA recovery from the gel, reverse transcription and library amplification are performed.

The molecular biology of the technology is compatible with multimodal measurements of RNA and for example guide RNA or epitope-barcoded proteins but have not yet been advertised by Scipio Biosciences. Naturally, the current version of the technology is ineffectual when profiling nuclei as the linker is not designed for this purpose. However, one unique opportunity for technology development is to consider cells partially attached to an extracellular matrix (ECM) and uniquely barcode and sequence cytoplasmic mRNA from individual cells – avoiding the need for isolated cells in suspension.

Scipio Bioscience provides their Cytonaut software, a cloud-based pipeline that covers the entire single-cell analysis process and is compatible with various open-source analysis packages.

Fluent BioSciences / PIPseq platform

Fluent BioSciences launched in 2018 with the PIPseq platform, with underlying technology development originating at co-founder Adam Abate's laboratory at UCSF. The current iteration of the so-called "PIP-seq" methodology was made public in October 2022 via bioRxiv and subsequently published in Nature Biotechnology in March 2023[32], with the hyphen dropped in the resulting commercial solution.

PIPseq compartmentalizes single cells with barcoded polyacrylamide templates and lysis reagents into water-in-oil droplets by a simple vortexing step and thus can be considered a virtually instrument-free approach. Cells are lysed by increasing the temperature releasing cellular mRNA that is captured on polyacrylamide beads with immobilized barcoded poly(T) sequences. After breaking the emulsion and individual droplets, barcoded RNA molecules are reverse-transcribed and amplified for sequencing. Usage of monodispersed polyacrylamide beads assists particle-template emulsification resulting in monodisperse droplets similar to those generated by microfluidic devices; a principle previously introduced in 2018[33]. In microfluidic emulsification, droplets are created sequentially and therefore the number of droplets scales

with instrument run time; in contrast, templated emulsification monodispersed droplets are generated in parallel within a test tube and therefore the number of droplets scales only with the test tube volume. PIPseq is compatible with multimodal measurements of RNA, single guide RNA (for pooled CRISPR screens) and oligonucleotide-linked epitopes for surface protein quantification.

Fluent BioSciences provide their PIPseeker Software for primary analysis of the PIPseq sequencing libraries. From FASTQ files as input summary metrics, diagnostic plots, clustering and differential gene expression tables can be obtained. Generated feature-barcode count matrices are compatible with the more frequently used open-source analysis packages.

CS Genetics

CS Genetics has developed a novel instrument-free single cell RNAseq assay that uses new solution-phase chemistry rather than physical partitioning between cells to enforce single cell indexing. The company has invented a new combined cell pairing and indexing reagent ("CPair") comprised of paramagnetic beads each coated on their surface with unique oligonucleotides from a library of indexing oligonucleotides along with molecules that allow the beads to bind non-specifically and with high affinity to cells. Cells are mixed with the CPair reagent such that 1:1 cell–bead binding events are strongly favoured. Cells paired with CPair beads are dispersed in a viscous buffer and subjected to a short high-temperature incubation followed by slow cooling in a standard thermocycler. This causes cell lysis and releases the indexing oligonucleotides from the CPair surface. The indexing oligonucleotides hybridize to mRNA molecules released from lysed cells.

Single cell indexing is maintained during the hybridization process by the high viscosity of the buffer. This "kinetic confinement" constrains diffusion of mRNA molecules and indexing oligonucleotides such that it strongly favors indexing of mRNA from a given cell by an oligonucleotide released from the CPair bead to which that cell was bound. CS Genetics are commercialising this approach as their SimpleCell® product and claims that it has advantages in workflow simplicity, scalability (to many hundreds to thousands of individual samples) and costs

Appendix 2: Commercial spatial omic technologies

Sequencing-based methods

10x Genomics / Visium platform

10x Genomics commercialized the Visium Spatial Gene Expression assay in 2019, a commercially scaled and enhanced version of the earlier Spatial Transcriptomics technology[34]. Using Visium, a fresh frozen tissue section is placed upon a 6.5x6.5mm capture area of a glass slide. The capture area is lined with polyadenylated spatially-barcoded probes arranged in spots of 55 µm diameter and spaced 100 µm apart (center to center distance). The tissue is fixed with

cold methanol, stained with hematoxylin and eosin (H&E), imaged and finally permeabilized to release the RNA. The mRNA is captured by the surface probes and reverse-transcribed, elongating the probes with the cDNA sequence. A second strand complementary to the primed cDNA is then synthesized and transferred to tubes, where it is used as a template for library preparation compatible with Illumina sequencers. An advantage of Visium is its compatibility with the standard H&E staining, which allows the integration of the spatial transcriptome information within the common workflow of morphological/pathological analysis.

Analogous to other methods based on poly(A) capture, Visium has the advantage of not being limited to the species for which custom probes are available. However, due to the dimension of the spots and the distance between them, it does not have single-cell resolution, but a range from 1 to 10 cells is captured by each spot. Adaptations of the technology include protein co-detection with immunofluorescence or with DNA-tagged antibodies, adapted protocols for formalin-fixed paraffin-embedded (FFPE) tissue, and the ability to spike-in probes for custom gene analysis. Of note, the FFPE adaptation is based on the incubation of the tissue with probes that target almost all the murine or human transcriptome; such probes are not yet available for other species, but 10x Genomics offers guidelines on how a user may design them.

Recent advancements include the commercialization of the Visium CytAssist, an instrument which enables the analysis of tissuesections already placed on non-Visium slides. In this workflow, the tissue section with the probes which are then blotted towards a receiver Visium slide with the aid of the CytAssist. Of note, the CytAssist Visium slides are offered in two formats: one with two standard 6.5x6.5mm capture areas, and one with two larger ones of 11x11mm. Another notable advancement is the commercialization of Visium HD, which enables the analysis of FFPE tissue sections at 2 μm resolution.

10x Genomics developed the Space Ranger pipeline for automatic image alignment and FASTQ processing, and the Loupe Browser software for semi-automatic image alignment and basic analysis and visualization. Several packages have been developed for deconvolution of Visium spatial transcriptomics data with single cell transcriptomic data[35].

Curio Bioscience / Curio Seeker platform

Recently formed Curio Bioscience is a privately held company based in Palo Alto, California, capitalizing on the research of academic co-founders Samuel Rodriques, Evan Macosko and Fei Chen, who developed the Slide-seq technology in 2019[36]. This has since been developed into the Curio Seeker platform.

In Curio Seeker, a fresh frozen tissue section is placed onto a 3x3mm or 10x10mm capture areas covered by a monolayer of tightly packed 10 μm spatially indexed beads, wherein barcodes and positions are identified by sequencing by oligonucleotide ligation and detection (SOLiD). The library preparation follows the same strategy used in Visium, where the RNA is captured by tissue permeabilization and reverse transcribed into a spatially barcoded cDNA. With a resolution of 10 μm it is possible to capture the transcriptome from ~1-2 cells upon each bead.

Thus far, only fresh-frozen samples are compatible with Curio Seeker; no adaptations are currently available for H&E staining or protein co-detection. The newest product from the company is the Curio Trekker, which enables the spatial analysis of single nuclei with the approach that was previously published as Slide-tags.

The Seeker Bioinformatics pipeline is used to process the FASTQ files into a spatial transcriptomic map of the tissue section.

NanoString Technologies / GeoMx platform

NanoString Technologies ("NanoString") is a publicly-held biotech company founded in 2003. Its products are based on a digital molecular barcoding technology invented at the Institute for Systems Biology (ISB) in Seattle under the direction of Leroy Hood. NanoString's spatial products include the GeoMx Digital Spatial Profiler (GeoMX DSP), launched in 2019, and the CosMx Spatial Molecular Imager (CosMx SMI).

The NanoString GeoMx DSP is a platform capable of analyzing the RNA or protein content of different regions of interest (ROIs) from a whole FF or FFPE tissue section[37]. In GeoMx DSP, the tissue is incubated with probes tagged with UV photo-cleavable oligonucleotides. Slides are then stained with fluorescent antibodies or other stainings and subsequently scanned. The captured fluorescent images are used to select ROIs with standard geometric shapes (rectangle or circle) or custom polygons; ROIs may be further segmented into discrete compartments or areas of illumination (AOIs) using the NanoString segmentation algorithm or a custom one. The GeoMx DSP instrument collects UV-cleaved oligonucleotides from the AOIs into the wells of a collection plate. Quantification occurs using the nCounter for panels of less than 300 gene targets or processed into an Illumina-compatible library and counted through NGS for larger gene panels.

The system is probe based; probesets offered by NanoString include the Cancer Transcriptome Atlas (CTA) with over 1,800 gene targets, the immune pathways panel with 84 gene targets, as well as the human and mouse whole-transcriptome panels with over 18,000 gene targets. For protein analysis, the minimal ROI area must be at least 2,000 μm2 (equivalent to ~20 cells); for RNA analysis, it must be at least 30,000 μm2 (equivalent to ~100-200 cells). A maximum of 380 ROIs, each of maximum 660x785 μm, can be run in a single scan.

NanoString offers the AtoMx Spatial Informatics Platform, a cloud-based software with analyses and collaboration tools. The GeoMx NGS Pipeline is used to convert FASTQ sequencing files produced with GeoMx DSP into digital count conversion (DCC) files, and runs on NanoString's standalone software or Illumina's DRAGEN. GeoMX data produced with nCounter can be processed on an nCounter Pro or MAX/FLEX AnalysisSystem or SPRINT Profiler to generate reporter count conversion (RCC) files. Both DCC and RCC can be uploaded to GeoMx DSP in the Data Analysis Suite, which performs quality control checks and generates analysis plots.

MGI / STOmics platform

Established in 2020, STOmics provides 3' spatially-resolved transcriptomics through its proprietary SpaTial Enhanced REsolution Omics-Sequencing (Stereo-seq) technology. In Stereo-seq, frozen tissue sections are loaded onto DNA nanoball (DNB) lithographically etched chips (patterned arrays)[38]. Subsequently, unique molecular identifiers (UMI) and poly(T) sequence-containing oligonucleotides are ligated onto each spot through hybridization with an oligonucleotide sequence containing the CID. The tissue is then fixed and permeabilized, the mRNA is captured onto the nanoballs and reverse-transcribed, and the cDNA is primed with the

CID of the nanoball that is captured by. The cDNA is then amplified, used as template for library preparation, and sequenced together with the CID. Stereo-seq Transcriptomics Set library appears to require sequencing on DNBSEQ-G400RS, MGISEQ-2000RS and DNBSEQ-T7RS platforms only.

An adaptation of the protocol is available for PFA fixed and FFPE samples. H&E staining is not recommended by the standard protocol, but an adaptation with immunofluorescence is provided. Protein profiling is enabled also through the StereoCITE protocol, which provides the multiplexing of more than 100 DNA tagged antibodies. DNB patterned arrays are prepared depositing DNB templates containing random barcodes on the patterned array, which are then incubated with primers and sequenced to retrieve the coordinate identity (CID) of every DNB. The standard STOmics-GeneExpression-S1 Chip has a 1x1 cm RNA capture unit, with around 400 million nanoballs of 220 nm diameter and 500 nm center to center distance. However the biggest Stereo-seq sequencing chip is 13x13cm, and it has more than 100 billion pixel resolution.

STOmics provides the Image Studio software for image processing, the SAW pipeline for reads processing and quantification, and StereoMap for visualization and additional analysis.

Imaging-based methods

10x Genomics / Xenium platform

Xenium platform launched by 10X Genomics in 2022 builds on the In-situ-sequencing technologies developed by Swedish company Cartana and Boston based company ReadCoor. Cartana was spun out of the Mats Nilsson lab at Stockholm University and ReadCoor came out of work from George Church's lab at Harvard Medical School and the Wyss Institute. In 2020 10X Genomics acquired both companies to gain intellectual properties and key technology advances in the *in situ* field.

Xenium *in situ* analysis uses padlock probes and rolling circle amplification chemistry[39]. Padlock probes are linear oligonucleotides consisting of backbone sequence and two arms which are complementary and hybridize to their target mRNA molecules[40]. Once the probe arms are stably hybridized to their target, a ligase is added to seal the junction between the probe regions that have hybridized to RNA. Ligation of the probe ends on the targeted RNA generates a circular probe. Only ligated probes are enzymatically amplified by rolling circle amplification (RCA) resulting in rolling circle product (RCP). RCP contains hundreds of copies of the padlock probe with the gene specific barcode creating robust and strong signals. The signal is detected with fluorescent dyes in a subset of imaging cycles.

Xenium currently supports fresh frozen and FFPE samples and can multiplex 5000 genes with the latest Xenium prime 5k chemistry. The tissue has to be placed on a 10X Genomics proprietary Xenium slide with an imageable area 12mm X 24mm. To detect the gene expression Xenium uses either targeted pre-designed gene panels or customized genes. The pre-designed gene panels were designed to cover major cell types for specific tissues and consist of genes selected from single cell atlas data and literature. Pre-designed gene panels can accommodate up to 100 additional add-on custom genes.

Genes which are highly expressed can generate a lot of fluorescent signals which are too close to each other and will cause an effect called optical crowding. To avoid optical crowding Xenium uses a sparse codebook where each gene is detected in a subset of cycles and channels. Another way to reduce optical crowding is to reduce the number of probesets per gene. By default, Xenium uses up to eight probesets per gene. Single cell data is used to model the expected optical density and based on this the number the probesets can be tuned to reduce the optical crowding. The goal of the panel design is to select genes which are highly cell type-specific with biologically-informative expression patterns. Most of the tissues suffer from autofluorescence, which is a challenge of imaging-based methods. Xenium protocol diminishes unwanted autofluorescence by autofluorescence quenching which enhances signal-to-noise ratio.

The acquired data are processed in parallel with imaging. Xenium onboard analysis includes decoding of the transcripts, cell segmentation and clustering. For data visualization 10X Genomics developed a visualization software called Xenium Explorer. Xenium Explorer can be also used to compare gene expression in different manually selected tissue microenvironments and to overlay IF/IHC, H&E and DAPI images from the same section to explore the pathology. 10X Genomics also provides the option to reanalyze the Xenium data using Xenium Ranger analysis pipeline. Xenium Ranger can be used to relabel or reassign the transcripts or to resegment the cells.

NanoString Technologies / CosMx platform

NanoString Technologies (“NanoString”) was spun out of the Institute for Systems Biology in 2003 and offers discovery and translational research solutions.
CosMx SMI platform is an integrated system that is based on cyclic *in situ* hybridization (ISH) and multiplexed barcoded antibody staining with non-enzymatic signal amplification. CosMx is compatible with FFPE, FF, TMAs and organoid samples on standard pathology-lab standard glass slides and is able to image user defined regions of interest, ranging from 0.25 - 300mm2 per slide, across 4 slides in a single experimental run.

CosMx currently offers probe panels to measure expression of over 6000 RNA targets, with an option of supplementing up to 200 additional custom RNA targets and up to 120 proteins, offering the highest plexity of RNA and protein targets on the market at the moment. Presently, the predesigned gene panels extend only to Human and Mouse. The ISH probes have an encoding length of 35-50 nt and 5 probes per gene are typically designed and are readout across 16 rounds of imaging and decoding[41]. Throughput wise, NanoString advertises that depending on the size of the target assay panel, the on-instrument time for CosMx SMI for processing four samples for 16mm2 area is approximately 1 day and will take an estimated ~0.5 slide per day for 100mm2 samples.

An advantage of CosMx over other imaging platforms is that it is compatible with both RNA and protein panels, making it a one stop platform for the study of RNA and Protein expression. Moreover, the platform detects fluorescent signals in all 3 xy and z dimensions, allowing the detection and decoding of signals over a 3D volume, increasing detection efficiency.

A distinction between the GeoMx platform and the CosMx platform is that GeoMx allows for whole transcriptome study, while the CosMx platform offers the advantage of single cell subcellular resolution studies.

Similar to GeoMx, the AtoMx SIP also supports CosMx data for visualization as well as the possibility to build custom analysis modules and pipelines for further analysis.

Vizgen / MERSCOPE platform

Vizgen's MERSCOPE platform is the commercial version of MERFISH (multiplexed error-robust FISH) developed in the Zhuang lab at Harvard[42–44]. Vizgen Inc. was founded in 2019 to commercialize the technology and has strong ties to the original inventors. The platform was launched in 2021 with wider availability of the instruments in 2022, several months earlier than competing technologies.

MERSCOPE utilizes DNA probes that are hybridized to up to 500 target RNA species. Each targeting probe contains unique sequences that allow for hybridization of shorter readout probes attached to a fluorophore. When imaged, all fluorophores associated with the probes on an individual RNA molecule produce a single molecule fluorescent signal in one or multiple readout channels. After imaging, fluorophores are chemically removed by reducing the disulfide bond attaching them to the readout probes[43]. This is a rapid process that reduces the runtime to less than two days, but also means readout probes are permanently associated with their binding site. Multiple rounds of hybridization with different combinations of readout probes, imaging and fluorophores removal generate a unique barcode that is used to identify the position of targeted RNAs. This information is output as transcript coordinates together with DAPI and poly(T) images of the sample and three optional membrane stains.

MERSCOPE can also detect up to 6 target RNA species through sequential smFISH, for example to detect high-expressors that otherwise induce optical crowding. Alternatively, up to 6 user-provided primary antibodies can be detected with Vizgin-provided secondary antibodies.

The original MERFISH approach has been adapted to increase target numbers up to 1000[45,46], to enable epigenomic readouts[47] and assess chromatin conformation[48]. These show the potential of future MERSCOPE iterations.

A typical MERSCOPE run takes two days including imaging, decoding and data export and generates data for up to 140, 300,500 or 960 custom RNA targets across a 100 mm² field of view (300 mm² in the upcoming MERSCOPE Ultra) for frozen or FFPE sections. This makes it a very flexible technology and has allowed it to be adapted to many tissues and organisms from mapping the mouse brain[49], human cortical development[50] to complex tumors[51] and even plant pathology[52]. However, lead times for custom panels are long and few predesigned panels are available. FFPE-compatibility that has so far only been shown for samples with relatively high RNA quality (MERSCOPE Data Release Program), potentially limits its efficacy with typical clinical samples.

MERFISH is one of the most well-documented spatial transcriptomics technologies and MERSCOPE instruments have now been tested in the field in significant numbers for two years. This makes this technology poised to be one of the first to transition to larger scale projects as seen in initiatives by the Allen Institute to map the mouse brain[49] and create an atlas of Alzheimer's disease). What is currently still lacking is standardization with a lot of parameters in

the core MERSCOPE protocol and sample prep needing to be dialed in for specific tissues and use-cases.

MERSCOPE experiments generate proprietary VZG files that are compatible with Vizgen's Visualizer software for viewing transcripts, cell boundaries, images, selecting regions of interest and importing and exporting information for cell clustering. Data is also output in open formats like CSV or TIFF for transcript coordinates, cell segmentation polygons and DAPI, poly(T) as well as cell boundary images for all captured z-planes. These can be used for cell segmentation with the Vizgen Post-processing Tool (VPT) with watershed as well as Cellpose[53] options available. Since output files are in widely used formats, fully custom data analysis with non-Vizgen tools is an option for advanced users.

Resolve Biosciences / Molecular Cartography platform

Resolve Biosciences ("Resolve"), headquartered in Monheim am Rhein, Germany, is a spin-out of QIAGEN where the foundation of their Molecular Cartography (MC) technology was developed.

The chemistry behind MC was never fully disclosed and only basic information on the number of imaging rounds, optical performance, probe design and signal calling is publicly available[54,55]. MC is based on combinatorial smFISH, similar to MERFISH. Multiple short DNA oligonucleotides are hybridized to each of the up to 100 target RNA species in a tissue section. These oligonucleotides can be reversibly labeled with one of two fluorophores using a proprietary approach and will generate one fluorescent signal for each individual RNA molecule when imaged. The identity of RNA molecules is determined by combinatorial imaging over 8 rounds of labelling and removal of fluorophores. The resulting fluorescent code identifies targeted RNA molecules with a claimed high sensitivity matching traditional smFISH, resolution (~ 300 nm) and specificity (typically <0.5 % false positives). It has been speculated on the probe chemistry used by Resolve before[56] and the company holds IP (US Patents: 20220205028, 20220235402, 20230227907) that might be connected to its basic principles.

One of the advantages of MC appears to be the flexibility of the technology with publications or preprints in different mammalian systems covering liver atlasing[55] and toxicology[57], breast atlasing[58], neurobiology[59], oncology[60], cardiology or developmental biology and organoids[61,62], but also zebrafish[54], xenopus[63], drosophila[64] or plant biology[65].

In spite of only targeting 100 RNAs in a given experiment, MC yields good results for cell-typing supporting the claims of high sensitivity and specific made by the manufacturer.

The main constraint of MC is the small area that can be processed per experiment. Every slide comes with 8 placement areas for samples that share a total imageable area of 40 mm² between them – an average of about 5 mm² per sample. Since the fluidics of the instrument always process all 8 placement areas and no smaller reagent kits are available, running fewer samples is not feasible and a total processing time of 80 hours per slide limits throughput. Resolve has a roadmap for the addition or protein detection and compatibility with FFPE samples, but no data is publicly available at the time of writing. A second generation MC-instrument that is meant to address many of the above was announced in 2022 (GenomeWeb) and is expected in 2024. Until its release, the utility of MC for clinical or high-throughput applications is limited, but the technology could excel in basic and early-stage clinical research where specificity and sensitivity often outweigh other factors.

An advantage for MC is a robust probe design interface for the generation of fully custom probe panels. The transcriptomes of at least 17 species from mammalian, other vertebrate, insect and plant species can be targeted at the time of writing. Most of these are not available as standard for other technologies that focus on human and mouse. For human projects, relevant MC targets can be selected with integrated scRNAseq expression data of 25 tissues from the Human Protein Atlas. Each MC run creates <40 GB of data: transcript coordinates in csv files as well as single-plane brightfield and maximum projection DAPI images of all fields of view. Data can be viewed in an ImageJ plugin provided by Resolve or through the web-based ReCognize tool for registered customers (https://my.resolvebiosciences.com/). No integrated solution for cell segmentation is available, but user-generated imageJ-style cell boundaries can be readily imported into ReCognize for clustering and visualization.

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