

# Spatially Resolved Transcriptomes—Next Generation Tools for Tissue Exploration

Michaela Asp, Joseph Bergenstråhle, and Joakim Lundeberg\*

Recent advances in spatially resolved transcriptomics have greatly expanded the knowledge of complex multicellular biological systems. The field has quickly expanded in recent years, and several new technologies have been developed that all aim to combine gene expression data with spatial information. The vast array of methodologies displays fundamental differences in their approach to obtain this information, and thus, demonstrate method-specific advantages and shortcomings. While the field is moving forward at a rapid pace, there are still multiple challenges presented to be addressed, including sensitivity, labor extensiveness, tissue-type dependence, and limited capacity to obtain detailed single-cell information. No single method can currently address all these key parameters. In this review, available spatial transcriptomics methods are described and their applications as well as their strengths and weaknesses are discussed. Future developments are explored and where the field is heading to is deliberated upon.

## 1. Introduction

The word “spatial,” which originates from the Latin word *spatium*, meaning “space,” can be used when describing how objects relate to each other with regard to their relative positions. In biology, the spatial concept has a significant importance as it allows us to describe interactive biological networks, where each element is influenced by its surrounding environment. For instance, understanding molecular properties of individual cells within multicellular organisms can only be completely comprehended once we know their physical locations.<sup>[1]</sup> The reason is that cells within distinct tissue microenvironments express specific sets of genes since they are both influenced by,

and influencing, the cells around them. This phenomenon governs, for example, the formation of gene expression gradients along the main embryonic body axes during different stages of development. These gradients then direct the activation of the correct developmental gene programs, needed for the construction of specific organs.<sup>[2,3]</sup> Another example is tumor microenvironments, where several sub-populations of cancer cells constituting the tumor can differ from each other completely in terms of both structural features and gene expression.<sup>[4]</sup> If we zoom down even further into the microenvironment within the cell wall of an individual cell, the spatial concept becomes equally important, as intra-cellular functions and signaling require precise positional arguments within the cell. Along this line, the sub-cellular position of the RNA molecule can

infer important information about the transcriptional snapshot and its actual biological meaning. The realization of the importance of the spatial organization and the exact position of molecular features, historically unobtained in bulk and single cell experiments, have driven the technological advancements in spatially resolved transcriptomics. Here, we summarize the landscape of available spatial techniques and divide the different methods into five sub-categories; i) technologies based on microdissected gene expression, ii) in situ hybridization (ISH) technologies, iii) in situ sequencing (ISS) technologies, iv) in situ capturing technologies, and v) in silico reconstruction of spatial data. A brief historical timeline of the methods mentioned in this review is shown in **Figure 1**, while their characteristics are presented in **Table 1**.

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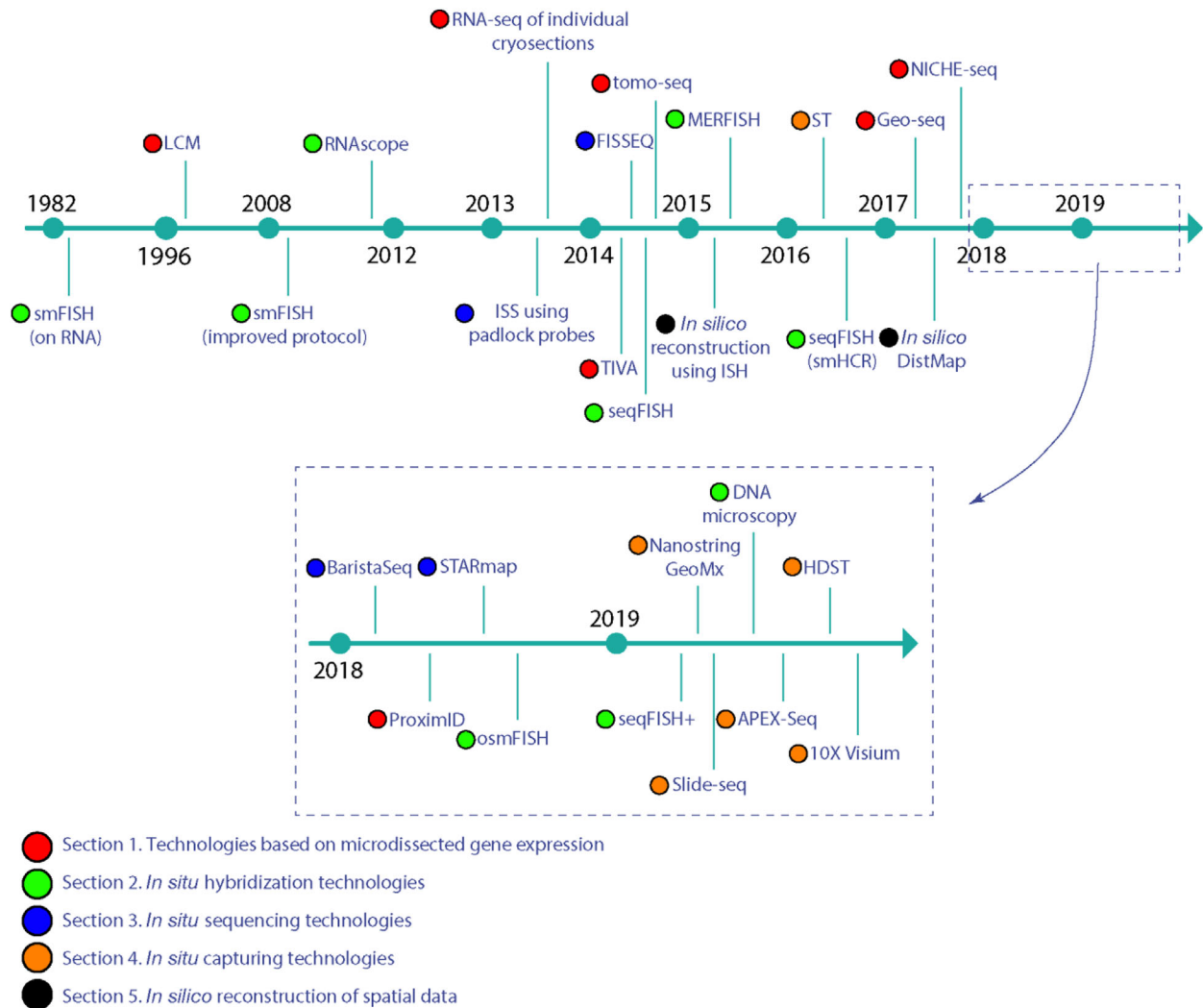
 The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/bies.201900221>

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DOI: 10.1002/bies.201900221

### 1.1. Technologies Based on Microdissected Gene Expression

The brute-force approach of capturing spatial gene expression information is simply by isolating regions of interest from within a sample. These regions can then be individually placed in test tubes for RNA extraction and subsequent gene expression profiling. While dissecting individual areas for subsequent sequencing readout allows for a complete extraction of different forms of RNA species, enabling analysis such as isoform presence, it is generally cumbersome to cover larger areas, and getting the holistic picture across an entire tissue is often not feasible. An overview of the technologies mentioned here is shown in **Figure 2**.



**Figure 1.** A brief historical timeline of the published methods mentioned in this review. Each method is categorized according to the fundamental approach they are based on.

## 1.2. Laser Capture Microdissection

Laser capture microdissection (LCM) is a technique in which a laser beam is used to cut out tissue regions identified under a microscope.<sup>[5,6]</sup> In 2017, an extended version of the LCM protocol, named geographical position sequencing (Geo-seq),<sup>[7]</sup> combined LCM with single-cell RNA-sequencing (scRNA-seq) in order to profile the transcriptomes of tissue regions as small as ten single cells. Although LCM is a robust technology, it is very labor intensive, which limits the throughput of samples to be analyzed.

## 1.3. Analyzing Individual Cryosections

Another way of producing regionalized gene expression data is to slice tissue samples into thin cryosections and prepare individual sequencing libraries from each piece of tissue. In 2013 this transcriptome-wide approach was applied to *Drosophila* embryos, where cryosectioning and sequencing was performed along the

anterior-posterior body axis, revealing both known and novel spatial gene expression patterns.<sup>[8]</sup> High quantities of input RNA were needed for the preparation of sequencing libraries, a problem which was solved by adding large amounts of carrier RNA.

## 1.4. RNA Tomography

A cryosectioning approach described in 2014, called RNA tomography (tomo-seq),<sup>[9]</sup> avoided the need of carrier RNA by linearly amplifying<sup>[10]</sup> the cDNA of individual tissue sections. Here, the authors systematically cryosectioned three identical zebrafish embryos, each along one of the three main body axes: anterior-posterior, ventral-dorsal, and left-right. A 3D transcriptional profile of the embryo was then reconstructed computationally by overlapping RNA-seq information from all cryosections. This method is superior for both RNA quantification and spatial resolution compared to the earlier cryosectioning protocol.<sup>[8]</sup> However, transcriptome-wide maps using tomo-seq can only be

**Table 1.** Comparison between spatially resolved transcriptomics technologies.

Method	Sample type	Spatial resolution	Approach	Detection efficiency	Advantages/Drawbacks	Demonstrated outside of originators lab
scRNA-seq	Fresh cells	NA	Transcriptome-wide	5–40% <sup>[56]</sup> *	No spatial resolution	Yes
LCM	Fresh-frozen or FFPE	Cellular	Targeted or transcriptome-wide	NA	+ Robust – Low throughput	Yes
Geo-seq	Fresh-frozen	10 cells	Transcriptome-wide	NA	+ More sensitive than LCM – Low throughput	No
tomo-seq	Fresh-frozen	Anatomical features	Transcriptome-wide	NA	+ Construction of 3D profiles – Needs multiple identical biological samples and therefore cannot be applied to human samples	Yes, in collaboration with inventors
TIVA	Live cells	Cellular	Transcriptome-wide	NA	+ Can be performed on live cells – Low throughput and limited analysis of human samples	No
NICHE-seq	Live cells	Cellular	Transcriptome-wide	As scRNA-seq	+ High throughput – Lost spatial information within the region of interest Method dependent on genetically engineered model organisms and therefore cannot be applied to human samples	No
ProximID	Live cells	Cellular	Transcriptome-wide	As scRNA-seq	+ Considers the actual physical interaction between cells – Low throughput	No
smFISH	Fresh-frozen or FFPE <sup>[57]</sup>	Subcellular	Targeted	Nearly 100% <sup>[58]</sup> *	+ High sensitivity – Low target throughput	Yes
RNAscope	Fresh-frozen or FFPE	Subcellular	Targeted	Not specified, very lowly abundant genes can be detected according to supplier	+ High sensitivity and robustly tested at different labs – Low target throughput	Yes
seqFISH	Fresh-frozen	Subcellular	Targeted	84% <sup>[19]</sup>	+ Highly multiplex – Need specialized equipment Cost becomes significant for increased number of targets Limited field of view	Yes, in collaboration with inventors
MERFISH	Fresh-frozen	Subcellular	Targeted	80% <sup>[21]</sup>	+ Highly multiplex – Need specialized equipment Cost becomes significant for increased number of targets Limited field of view	Yes
seqFISH+	Fresh-frozen	Subcellular	Targeted	49% <sup>[24]</sup>	+ Exceedingly high multiplex – Cost becomes significant for increased number of targets Limited field of view	No
osmFISH	Fresh-frozen	Subcellular	Targeted	Nearly 100%, but with 5.3 ± 6.9% RNA losses per cycle <sup>[25]</sup>	+ Process larger tissue areas than other multiplex smFISH techniques – Relatively low target throughput	No
DNA microscopy	Cell cultures	Cellular	Targeted	NA	+ Standard microscopy equipment – Limited demonstrated targets and samples all from the inventor's lab	No
ISS using barcoded padlock probes	Fresh-frozen and FFPE <sup>[59]</sup>	Subcellular	Targeted	30% <sup>[60]</sup>	+ Subcellular resolution Ability to detect SNVs – A priori chosen targets of limited quantity	Yes, commercialized spin-off
BaristaSeq	Cell cultures	Subcellular	Targeted	Stated higher than ISS but not explicitly demonstrated <sup>[35]</sup>	+ Apply SBS instead of SBL allow read out of up to 15 nt of actual target sequence – A priori chosen targets of limited quantity Limited field of view	No

(Continued)

**Table 1.** Continued.

Method	Sample type	Spatial resolution	Approach	Detection efficiency	Advantages/Drawbacks	Demonstrated outside of originators lab
STARmap	Fresh or fresh-frozen	Subcellular	Targeted	No less than scRNA-seq <sup>[37]</sup>	+ Increased sensitivity due to probe-complex, absence of a RT step and tissue transparency – A priori chosen targets of limited quantity Limited field of view	No
FISSEQ	Fresh-frozen or FFPE <sup>[60]</sup>	Subcellular	Transcriptome-wide	<0.005% <sup>[61]</sup>	+ Non-targeted – Low sensitivity Limited field of view	No
ST/10X Visium	Fresh-frozen	Anatomical features of 100 µm/55 µm	Transcriptome-wide	ST: 6.9% <sup>[41]</sup> 10X: higher than ST	+ Whole-mRNA analysis – Barcoded regions contain multiple cells	Yes
Slide-seq	Fresh-frozen	Anatomical features of 10 µm	Transcriptome-wide	0.3% <sup>[43,62]</sup>	+ High resolution – Does not include histology on the same tissue section and has low sensitivity	No
HDST	Fresh-frozen	Anatomical features of 2 µm	Transcriptome-wide	1.3% <sup>[44]</sup>	+ High resolution – Sparse data requires binning. Low sensitivity	No
APEX-seq	Live cells	Subcellular	Transcriptome-wide	70% <sup>[48]</sup>	+ Can be performed on live cells – Cannot be applied to human tissue	No
Nanostring GeoM	Fresh-frozen and FFPE	Custom down to 10 µm	Targeted	Not specified	+ FFPE compatible Choose between protein/RNA profiling High level of automation – Low sensitivity when using smaller ROIs Require manual choice of regions	Yes

\*External reference. Single-cell RNA-seq is not a spatial technique but is shown here for reference. The column “Demonstrated outside of the originators lab” is based on found peer-reviewed articles that present data in any context of the paper and obtain such data by using the technology outside of the inventor’s lab.

constructed using identical biological samples and thus, cannot be applied to clinical samples.

### 1.5. Transcriptome In Vivo Analysis

One technology that enables spatially resolved transcriptomics of living cells is transcriptome in vivo analysis (TIVA),<sup>[11]</sup> published in 2014. In the TIVA protocol, intact live tissue sections are first exposed to TIVA tags (each tag is a photoactivatable mRNA capture molecule). These multifunctional tags are attached to a cell-penetrating peptide, allowing them to enter the cell cytosol. Next, cells of interest are selected by using laser photoactivation, upon where TIVA tags gets activated and hybridize to mRNAs within the cell. TIVA tag-mRNA hybrids are then purified from the selected cells and the captured mRNAs can be further analyzed by RNA-seq. Although it can be applied to living tissue, its main limitation is its low throughput, as only a few single cells can be analyzed at a time. Furthermore, the application to live tissue restricts its use for the analysis of model systems.

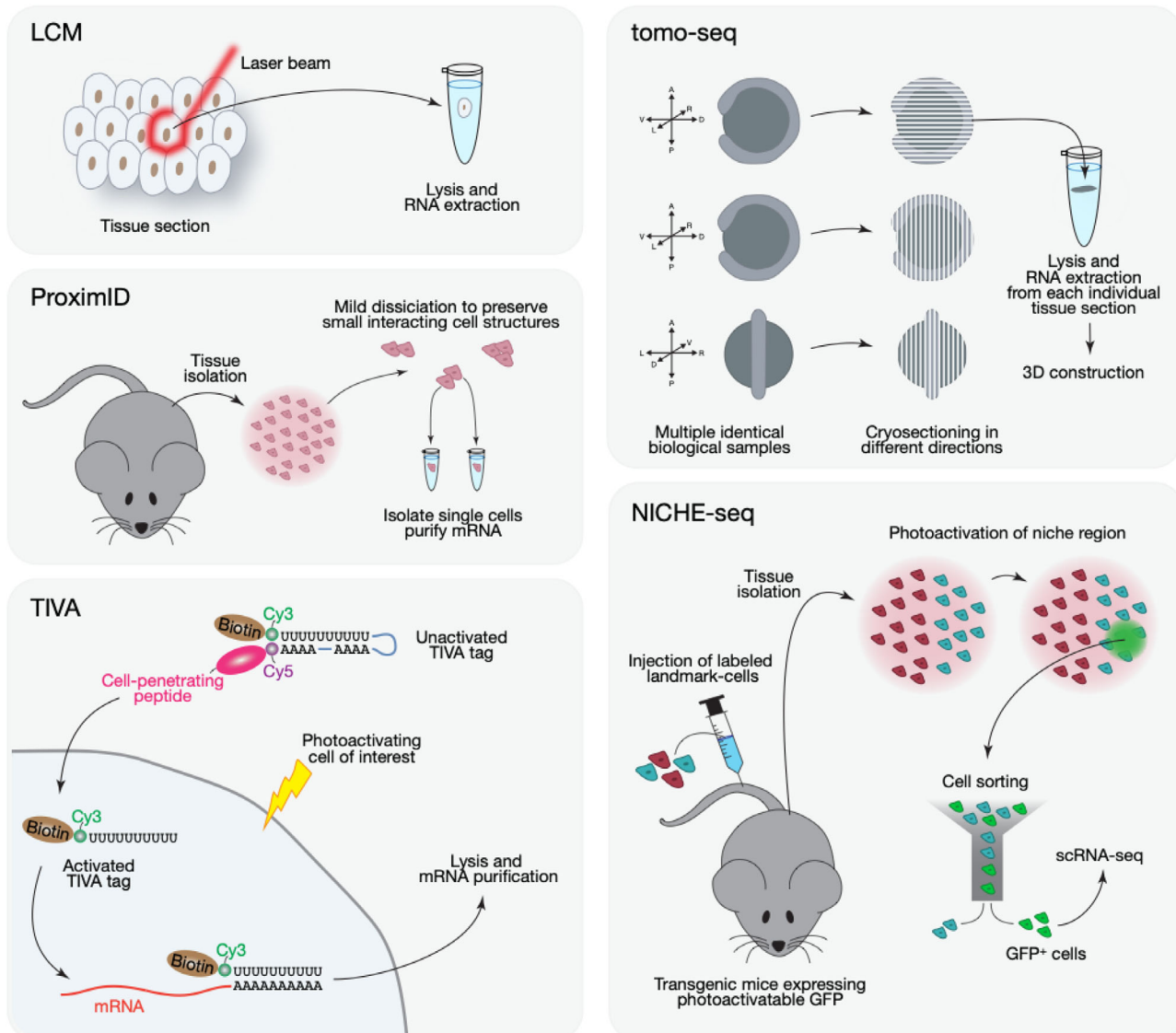
### 1.6. NICHE-Seq

An alternative technology published in 2017, also using photoactivation, is NICHE-seq.<sup>[12]</sup> As the name implies, transcriptomes of individual cells within a specific niche (an area within a tissue

that holds a specific microenvironment) are profiled. First, specific niches are visualized by intravenously transferring labeled landmark-cells (e.g., T and B cells) into transgenic mice expressing photoactivatable green fluorescent protein (GFP). Niches of interest are then photoactivated, followed by tissue dissociation, cell sorting of GFP<sup>+</sup> activated cells, and scRNA-seq. This technology is very high throughput and can analyze thousands of cells within a niche, but their exact positions within the photoactivated area are not known. As the current protocol depends on genetically engineered model organisms, it cannot be applied to human samples.

### 1.7. ProximID

Another method that can also be used for profiling niches in cellular microenvironments is ProximID, a technique published in 2018.<sup>[13]</sup> As most spatial transcriptomics technologies are focusing on whether cells are in close proximity or not, ProximID takes it one step further by looking at the actual physical interaction between cells. This is done by first dissociating tissue in a mild condition, so that small interacting structures containing two to three cells are retained. Interacting cellular structures are then manually microdissected and individual cells as subject to scRNA-seq. Nevertheless, manual microdissection makes the protocol somewhat labor intensive and is therefore limited in terms of throughput.



**Figure 2.** Overview of technologies based on microdissected gene expression. *LCM*: A laser beam is used to cut out and analyze a specific tissue region of interest. Analyzing individual cryosections omits the laser capture. *Tomo-seq*: Three identical biological samples are systematically cryosectioned in three different directions and a 3D transcriptional construction of the samples is done by overlapping data. *ProximID*: Interacting cell structures are manually dissociated into single cells and analyzed individually. *TIVA*: A photoactivatable-biotinylated-mRNA capture tag enters live cells and upon selective activation by laser hybridize to mRNAs. TIVA tagged mRNAs can thereafter be purified and analyzed. The Cy3-Cy5 groups allow for observing both cellular uptake and uncaging. *NICHE-seq*: Labeled landmark-cells are injected into transgenic mice expressing photoactivatable GFP to allow visualization of specific niches. Niche-regions are then photoactivated and the GFP expression cells can be sorted and analyzed separately.

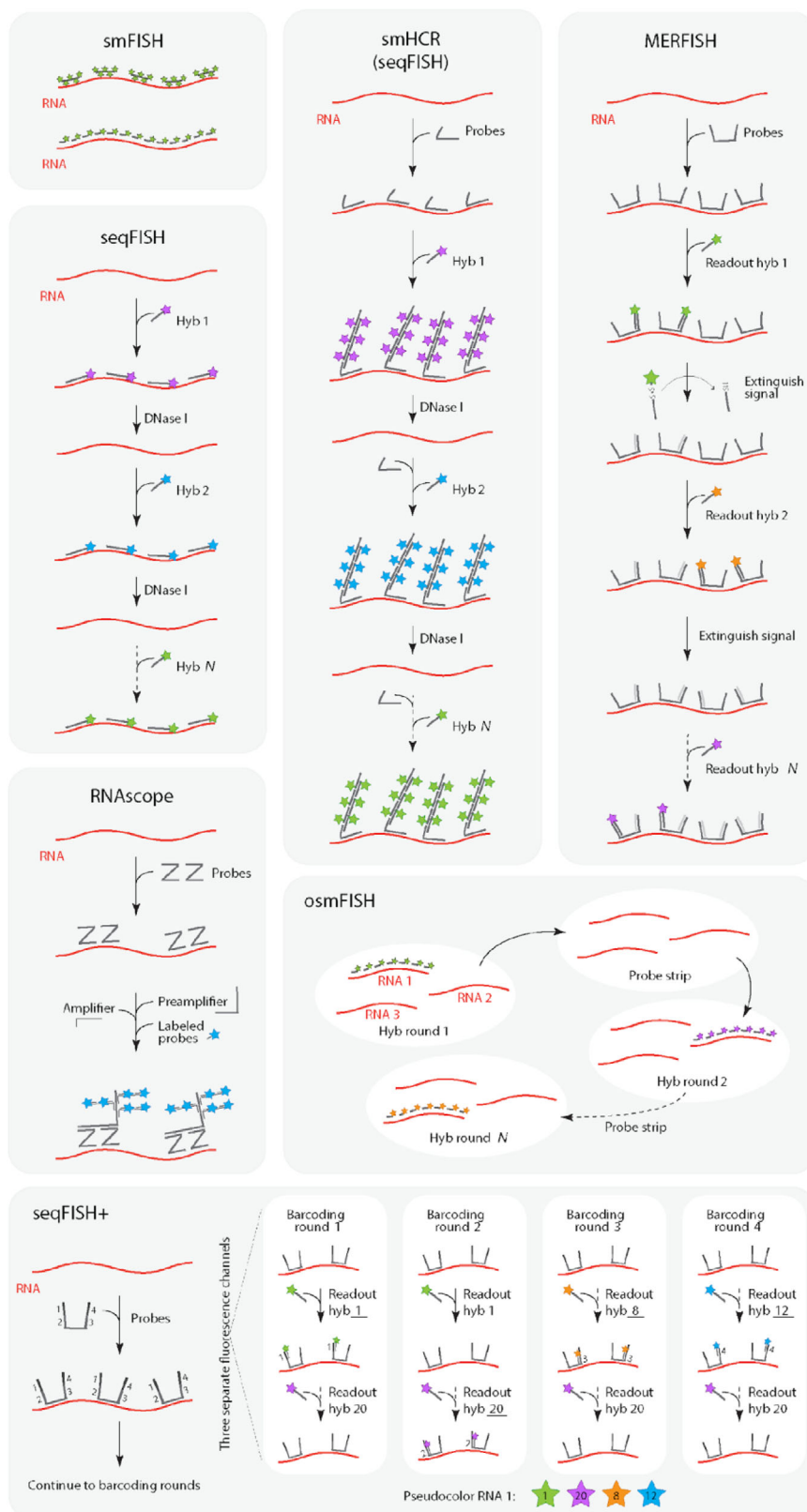
## 2. In Situ Hybridization Technologies

Instead of extracting RNA molecules from individual parts (or cells) within a tissue, one can visualize them directly in their original environment. This can be achieved by hybridizing a labeled probe complementary to the target of interest. When simultaneously detecting a larger set of different transcripts, spectral overlapping has historically been a key limitation, but recent advancements in tissue expansion methods and clever decoding strategies have enabled close to whole transcriptome readout capacity. However, at the same time this implies using even larger sets of specific probes that needs to be synthesized, and identi-

fication still rely on a priori knowledge of targets. Furthermore, increasing multiplexity implies large volumes of image processing, which is computationally demanding and introduces a limit to the field of view that can be feasibly obtained. An overview of ISH technologies mentioned here is shown in **Figure 3**.

### 2.1. Single-Molecule RNA Fluorescence In Situ Hybridization

The ISH technique has existed since the 1960s<sup>[14]</sup> and has been used for visualizing gene expression from the early 1980s





onward.<sup>[15]</sup> Fluorescently labeled probes are individually hybridized to predefined RNA targets in order to visualize gene expression in fixed tissue. Instead of using a single probe for each target, a further development of the fluorescence in situ hybridization (FISH) protocol instead utilized multiple shorter probes, targeting different regions of the same transcript. This approach, named single-molecule RNA fluorescence in situ hybridization (smFISH), gives a higher and more robust signal which enables quantitative measurements of transcripts. Initially, a set of five probes (50 bp) each coupled to five fluorophores, was used to target each transcript.<sup>[16]</sup> However, labeling probes with a large set of fluorophores is challenging due to altered hybridization characteristics and self-quenching, as well as complicates synthesis and purification. An alternative smFISH method was developed in 2008, using instead a set of  $\approx 40$  probes (20 bp) each coupled to a single fluorophore.<sup>[17]</sup> Although smFISH has high sensitivity and subcellular spatial resolution, it can only target a few genes at a time due to the inherent limitation of spectral overlaps in standard microscopy.

## 2.2. Sequential Hybridization

A multiplex smFISH approach that uses sequential hybridizations (seqFISH)<sup>[18,19]</sup> was developed in 2014. Individual transcripts are detected several times by serial rounds of hybridization, imaging and probe stripping. In every hybridization round, a pre-defined colored set of 24 encoding probes is used for each target. However, an increased number of hybridization rounds requires an increased number of smFISH probes, which makes seqFISH both expensive and time consuming. A common problem of FISH based methods is high levels of autofluorescence background from tissue samples which are thick and opaque. To deal with this issue, amplified versions have been developed where multiple readout probes are attached onto the target, for example as demonstrated by single-molecule hybridization chain reaction (smHCR).<sup>[20]</sup>

## 2.3. Multiplexed Error-Robust FISH

In an effort to side-step the extensive time of seqFISH, a multiplexed error-robust FISH (MERFISH) was published in 2015.<sup>[21]</sup> Here, non-readout probes carrying two flanking regions are first hybridized to the target transcript, and fluorescent readout-probes are then hybridized to these in several rounds of hybridization, imaging, and signal extinguishing. Computational

error-correction is performed after readout to account for imperfect hybridizations. By using a single smFISH hybridization ( $\approx 10$  h), and instead multiple readout hybridizations ( $\approx 15$  min each), the experimental time is reduced significantly. In 2018, the MERFISH technique was combined with expansion microscopy,<sup>[22]</sup> which physically enlarges tissues.<sup>[23]</sup> By widening the distance between RNA targets, the number of molecules that can be detected without spectral overlap increases.

## 2.4. Sequential Fluorescence In Situ Hybridization

In 2019, the research group behind seqFISH also adopted a somewhat similar approach as MERFISH, by using a one-step targeting with primary-probes containing flanking regions, and subsequent multiple read-out probe cycles.<sup>[24]</sup> Before imaging in seqFISH+, cross-linking between primary-probes and targets is performed using a specific hydrogel embedding protocol. Next, a series of barcoding rounds are performed, each consisting of four serial hybridizations that uses three readout probes conjugated to different colors. Each serial hybridization can be collapsed to a set of  $3 \times 4 = 12$  pseudocolors. By using multiple barcoding cycles, a high number of unique gene barcodes can be achieved. The approach allows for target multiplexing of 10 000 genes using standard confocal microscopy without the need of super-resolution equipment. However, this strategy is still based on deciding targets a priori, and implies utilizing a large set of synthesized probes.

## 2.5. Ouroboros smFISH

Another approach to avoid optical crowding was developed and optimized in 2018, resulting in a method termed ouroboros smFISH (osmFISH).<sup>[25]</sup> This is a non-barcoding technique, meaning that the number of targets are less than the previously mentioned multiplex smFISH approaches. The number of targets for osmFISH is set to the number of hybridization rounds, where each round of hybridization includes a probe set for a certain number of genes (the authors performed 13 hybridization rounds targeting 3 genes per round). After each hybridization round, an image is obtained followed by probe removal and the next run of hybridization. This procedure is rather time consuming, but the method has been semiautomated meaning hands-on time is shortened. While osmFISH has lower multiplex capacity compared to other smFISH techniques, it excels in its ability handle larger tissue areas.

**Figure 3.** Overview of ISS technologies (DNA microscopy not included). The star colors represent probes of different sequences. *smFISH*: In the original smFISH approach, a set of five probes labeled with five fluorophores each are hybridized to individual targets. Alternatively, a set of  $\approx 40$  probes coupled to a single fluorophore is used. *seqFISH*: smFISH probes are hybridized consecutively to the target and stripped off by DNase I in between each round. The same probe sequences are used in different rounds of hybridization, but probes are connected to different fluorophores. *smHCR*: seqFISH in combination with single-molecule hybridization chain reaction (smHCR) to amplify the signal. *MERFISH*: A probe set with unique flanking read out regions is used for individual targets. Readout probes are hybridized sequentially in between rounds of signal extinguishing. *RNAscope*: Two adjacent “Z-probes” hybridize to individual targets, creating a 28 bases tandem-site of the two probe tails, on which a preamplifier can bind into. For signal amplification, the preamplifier contains 20 binding sites for a second amplifier probe, which contains 20 sites for the labeled probes. *osmFISH*: smFISH probes are hybridized in a number of rounds, along probe removal in between rounds. A few targets are detected per round. *seqFISH+*: A set of 24 primary probes are hybridized per target, each probe containing four unique flanking read out regions corresponding to four barcoding rounds. For each barcoding round, readout probes are hybridized consecutively in between rounds of probe stripping. Separate fluorescent channels are used to increase the number of targets. Each target will be detected as a pseudocolor, a combination of four colors in a distinct order.

## 2.6. RNAScope

In 2011, Advanced Cell Diagnostics launched the commercial RNAScope Assay.<sup>[26]</sup> The core of the assay is the probe design, where two adjacent “Z-probes” binds to the target RNA transcript to form the required binding site for the subsequent hybridization of amplifier molecule. The hybridization cascade required for signal amplification follows the concept of stepwise binding commonly adopted in molecular assays to increase the signal to noise ratio. In July 2019, the maximum number of simultaneously detected RNA targets was increased to twelve. Low multiplex RNAScope has also been combined with imaging mass cytometry (IMC), where the DNA tree is hybridized with metal tags instead of fluorophores. Subsequently, metal-conjugated antibodies are added to the same tissue section and mass-cytometric measurements of metal abundances are conducted, allowing for simultaneous RNA and protein assessment.<sup>[27,28]</sup> This approach has so far been demonstrated for a lower number of simultaneous mRNA and protein targets, and the presented chemistry of the protocols warrants further assessments of eventual limits in epitope detection.

## 2.7. DNA Microscopy

In 2019, an optics-free mapping of nucleotides was presented dubbed “DNA Microscopy,”<sup>[29]</sup> that does not rely on physical capture from known locations, but instead on thermodynamic entropy. With standard equipment, the approach to positional reconstruction is adopted from sensor localization theory, where UMI tagged RNA targets “broadcast” their position. Outward diffusing UMI constructs are linked together in a subsequent round of random nucleotide insertion, resulting in “Unique Event Identifiers” (UEIs). The core of the concept is that the physical distance between the original sources of the targets will reflect the probability of UEI formation. After DNA sequencing of the concatemers, the prevalence will be used to infer the proximity between different targets, and thus an image can be created, together with single nucleotide information about the target. The principle for DNA microscopy implies a rather curious reverse feature compared to traditional imaging methods; sparsity of signal becomes a challenge, while high signal density implies easier reconstruction. The technique has so far only been demonstrated to a small subset of transcripts on cultured cells.

## 3. In Situ Sequencing Technologies

RNA sequencing can be performed directly on the RNA content of a cell while it remains in its tissue context. The location of the transcript can be resolved with subcellular resolution, but in order to reach sufficient signal for imaging, micrometer- or nanometer-sized DNA balls are used to amplify the signal. Due to the inherent spatial limits of the cell, there is a limit to the number of different transcripts that can be discriminated simultaneously, and, for example, different strategies to circumvent this are implemented by some of the techniques presented. An overview of ISS technologies mentioned here is shown in **Figure 4**.

### 3.1. In Situ Sequencing Using Padlock Probes

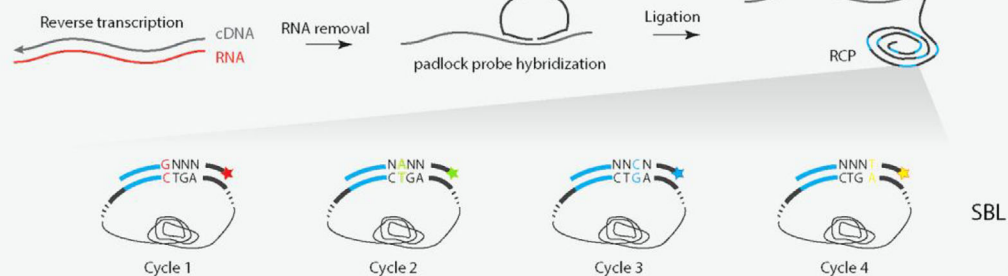
In 2013, the first ISS approach was published that used padlock probes to target known genes.<sup>[30]</sup> Within intact tissue sections, mRNAs are first reverse transcribed to cDNAs onto which a padlock probe can bind into. A padlock probe is a single-stranded DNA molecule containing regions complementary to the target cDNA. There exist two alternatives of the method, one of which the padlock probe binds to the cDNA target with a gap between the ends, and another where the ends are adjacent to each other. In the first case, the gap is filled by DNA polymerization and then, in both cases, the ends are ligated to create a circle of DNA. Target amplification is performed by a process called rolling-circle amplification (RCA), resulting in micrometer-sized RCA products (RCPs), containing numerous repeats of the padlock probe sequence. RCPs are then subjected to sequencing-by-ligation (SBL), where either the gap-filled sequence, or a four-base-long barcode within the probe with adjacent ends, is decoded. While the no-gap approach displays higher sensitivity, the gap-filling approach, where the gap could be of variable length up to 4bp, allows for reading out the actual RNA sequence of the transcript. By applying ISS to a breast cancer tissue section,<sup>[30]</sup> 31 targets were spatially localized, nevertheless, a higher number of targets has been demonstrated.<sup>[31]</sup> Compared to hybridization-based techniques, this approach has the ability to detect SNVs. Although ISS with padlock probes facilitates subcellular resolution, the number of targets is limited ( $\approx 100$ ) due to the short sequencing readout-length and the size of the RCPs. In 2017 the company CARTANA was founded to commercialize the padlock-based ISS technology by offering a sample preparation kit and service.<sup>[32]</sup> A couple of years later, in 2019, the ISS assay was automated on a microfluidic platform, significantly reducing hands-on time as well as the total protocol runtime.<sup>[33]</sup> A further development dubbed “HybISS” replaces SBL of RCPs with sequencing-by-hybridization (SBH). This method demonstrates significantly higher signal-to-noise ratio than the SBL counterpart and is thought to allow for larger gene panels. The method is demonstrated on notorious difficult human brain samples in a recent preprint.<sup>[34]</sup>

### 3.2. Barcode In Situ Targeted Sequencing

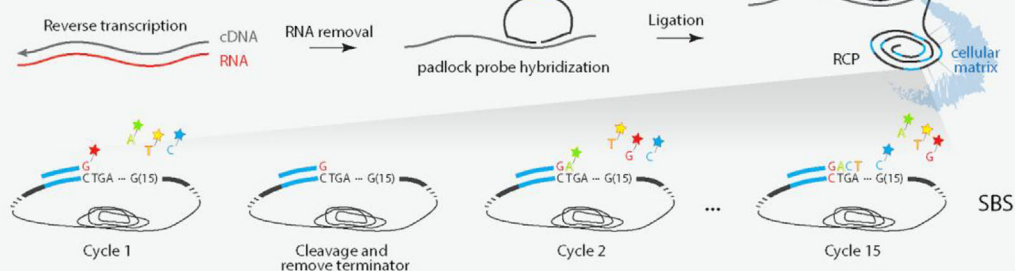
A recently developed method called Barcode in situ targeted sequencing (BaristaSeq)<sup>[35]</sup> is another gap-filling padlock based approach with increased read-length of 15 bases. To achieve such a read-length without losing efficiently as noted with the previous gap-filling ISS approach, the authors changed the polymerase enzyme typically used to one without strand displacement activity, and also note that protein engineering could potentially increase the efficiency further. The method uses sequencing-by-synthesis (SBS), which has a higher signal-to-noise ratio than SBL, and to allow for this longer sequencing, the amplicons are stabilized by cross-linking to reduce signal loss during heat cycles. The cross-linking procedure is the same as used in FISSEQ<sup>[36]</sup> (described below). In the original article, BaristaSeq demonstrate a five-fold increase in quantification of RCP formation when compared to traditional gap-fill padlock ISS, and even more compared to



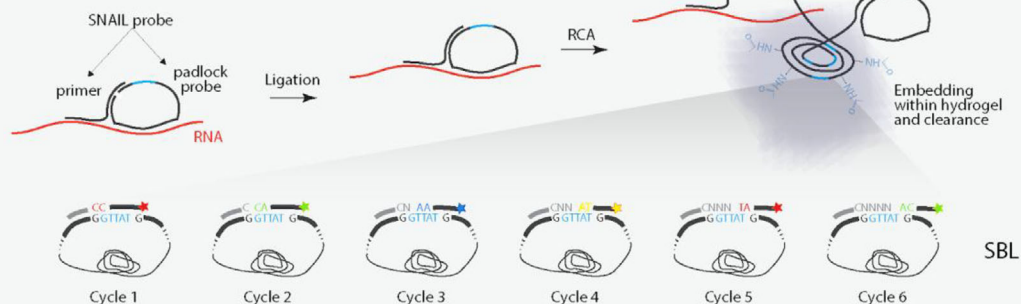
### ISS with padlock probes



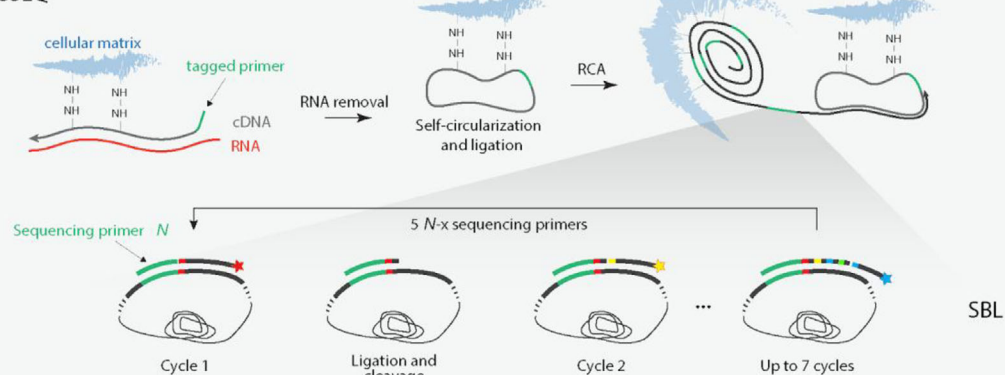
### BaristaSeq



### STARmap



### FISSEQ



FISSEQ. But it should be noted that the method is only demonstrated on cultured cells.

### 3.3. Spatially Resolved Transcript Amplicon Readout Mapping

Another recently developed approach to ISS is Spatially resolved Transcript Amplicon Readout Mapping (STARmap).<sup>[37]</sup> STARmap uses barcoded padlock probes that hybridize to the targets but avoid the reverse transcription (RT) step by adding a second primer, targeting the site next to the padlock probe. This approach avoids the efficiency hurdle of cDNA conversion and reduces noise by the addition of the second hybridization step. The construct is then amplified by RCA to generate nanometer-sized single-stranded DNA products called nanoballs. Amine-modified bases are incorporated during the RCA and used for embedding the nanoballs in a 3D scaffold by applying a hydrogel-tissue chemistry. The tissue-hydrogel complex is subsequently cleared of unbound proteins and lipids, enhancing the transparency of the tissue. Next, a modified SBL approach is applied to decode the five-base-long barcode. By using the hydrogel-tissue chemistry in combination with the modified sequencing protocol, more than 1000 genes were targeted in mouse brain tissue sections. The most eye-catching feature presented in the article introducing the method is the ability to analyze intact tissue samples in 3D, preserving the orientation of the cells in not just a mono 2D layer. However, it should be noted that this capacity is only demonstrated for 100–150 µm thick sections and a smaller number of targets.

### 3.4. Fluorescent In Situ RNA Sequencing

So far, the methods mentioned has been based on knowledge of the targets a priori, but in 2014, an untargeted method, that is, capturing all species of RNA, named Fluorescent in situ RNA Sequencing (FISSEQ) was published.<sup>[36]</sup> First, cDNA synthesis is performed using a mix of regular and modified amine-bases, together with tagged random-hexamer RT primers. Via the amine-bases, cDNA is cross-linked to its cellular environment and thereafter circularized by ligation. Subsequent RCA creates single-stranded DNA nanoballs, whose positions are maintained via cross-linking to the cellular protein matrix. Last, sequencing is performed using SBL with a read-length of 30 bases. However, in order to discriminate a large number of different targets, a partition sequencing strategy is applied by using extended sequencing primers, where nanoballs are randomly sampled so that only a subset is sequenced. Although the authors claim that the protocol is suited for most tissue types, including FFPE material, it has only been demonstrated in cultured fibroblasts, where more than 8000 genes could be detected with subcellular resolution. In 2016, the company ReadCoor was formed to commercialize the

technology after entering a licensing agreement.<sup>[38]</sup> In 2020, the company announced their first product line, including a fully integrated system which is said to be able to simultaneously detect RNA, DNA, and proteins, on the same tissue section at subcellular resolution.<sup>[39]</sup> The system will be sold together with reagent kits for custom panels and is said to work on both fresh frozen and FFPE tissue. However, details about the chemistry are not disclosed and to date no data has been published that support the claims. In 2019, the research group behind FISSEQ presented in situ Transcriptome Accessibility Sequencing (INSTA-Seq).<sup>[40]</sup> Here, sequencing in situ is used to map a regional barcode, while sequencing ex situ is done after extraction, to obtain full transcript information. Decoding the barcode is achieved via a bidirectional SBL chemistry, which sequence short reads (5 bp + 6 bp) from both ends of the cDNA fragment. In this manner, the limit of short read information from typical ISS approaches is circumvented.

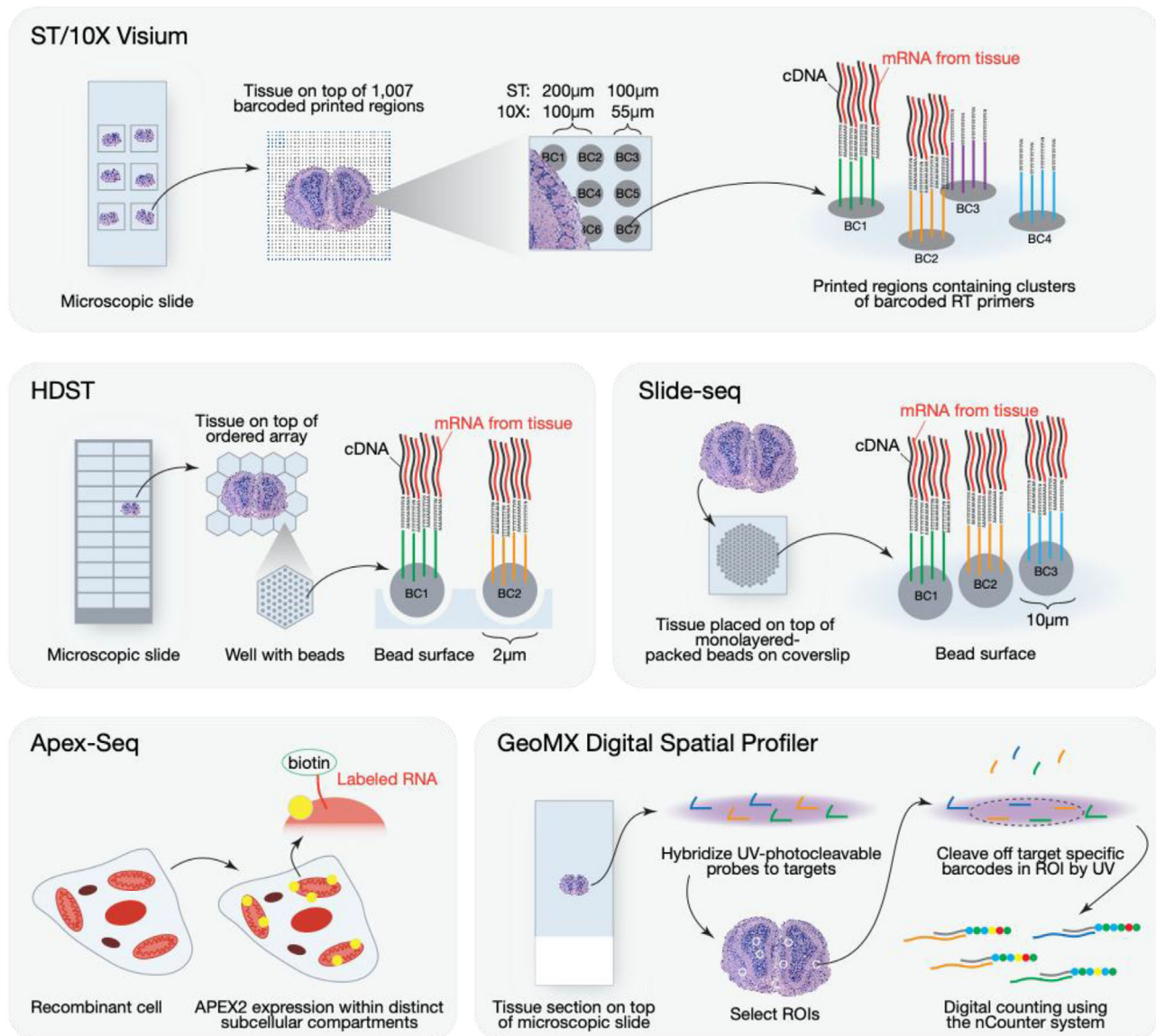
## 4. In Situ Capturing Technologies

The spatial techniques described so far have been based on either isolation of already-known tissue regions of interest, or in situ visualization of RNA molecules by hybridization or sequencing. Another approach is to capture transcripts in situ, then perform sequencing ex situ. The concept is attractive since it avoids the typical limitations of direct visualization and allows for an unbiased analysis of the complete transcriptome. However, the main hurdle for these methods is restricted RNA capture efficiency, which becomes increasingly more challenging with higher resolution (i.e., smaller capture/barcoded areas). An overview of in situ capturing technologies mentioned here is shown in Figure 5.

### 4.1. Spatial Transcriptomics

The first technique employing this approach was the Spatial Transcriptomics (ST) technology, published in 2016.<sup>[41]</sup> Thin tissue sections are placed onto glass slides which are printed with barcoded RT primer, specifying the *x* and *y* coordinates of the array. The tissue is fixed, stained, imaged, and permeabilized. During the permeabilization process, mRNA molecules diffuse vertically down to the solid surface and hybridize locally to the RT primers. RT is performed in situ, after which the tissue is removed, and the cDNA-mRNA complexes are extracted for library preparation and next generation sequencing (NGS) readout. The barcoded reads are superimposed back onto the tissue image. Although the method gives you spatially resolved whole-transcriptome information, the current barcoded regions of 100 µm in diameter, limits the resolution to ≈10–40 cells. At the end of 2018, the ST technology was acquired and

**Figure 4.** Overview of ISS technologies. ISS with padlock probes: Padlock probe-based ISS using RCA and sequencing-by-ligation (only the no-gap approach is shown here). The blue stretch indicates target barcode. BaristaSeq: Utilizes the padlock probe-approach but the RCP is cross-linked to the cellular matrix. Sequencing is performed using SBS. STARmap: A SNAIL (Specific Amplification of Nucleic Acids via Intramolecular Ligation) probe complex binds directly to RNA. RCA is performed and the RCP is embedded within a hydrogel, which is thereafter cleared from unbound proteins and lipids. A modified version of SBL is performed on the RCP. FISSEQ: cDNA is cross-linked to its cellular environment. RCA is performed on circulated cDNA and the RCP is again cross-linked to the cellular matrix. Sequencing is performed using SBL.



**Figure 5.** Overview of in situ capturing technologies. ST/10X Visium: Microscopic glass slides containing marked  $6.5 \times 6.5$  mm areas are used where thin tissue sections are placed and imaged. Each area contains 1007 (ST) or 5000 (10X Visium) printed regions of barcoded mRNA capture probes with the dimensions of 100 µm (ST) or 55 µm (10X Visium) in diameter and a center-to-center distance of 200 µm (ST) or 100 µm (10X Visium). Tissue is permeabilized and mRNAs are hybridized to the barcoded capture probes directly underneath. cDNA synthesis connects the spatial barcode and the captured mRNA, and sequencing reads are later overlaid with the tissue image. HDST: Similar approach as ST/10X Visium but instead uses an ordered bead array onto which 2 µm-sized beads are randomly deposited. Each bead contains barcoded mRNA capture primers and their x and y coordinates are decoded by rounds of hybridizations. Slide-seq: Similar approach as ST/10X Visium and HDST, but without a tissue imaging step. Here, a glass coverslip containing randomly deposited 10 µm-sized barcoded beads is used and bead positions are decoded by SBL. Apex-Seq: Recombinant cell lines expressing APEX2 in pre-known subcellular regions are used. When APEX2 gets activated by a biotin-phenol/hydrogen peroxide complex, it will tag all RNAs in its surrounding with biotin. Tagged RNAs are purified using streptavidin. GeoMX Digital Spatial Profiler: A tissue section is first exposed to a pre-known panel of barcoded hybridization probes, after which ROIs are manually selected. ROIs are subjected to UV light and target specific barcodes are cleaved off. At the time of this writing, DBIT-seq has not been published and an illustration is not included in this figure.

further developed by the company 10X Genomics, under the name “10X Visium.”<sup>[42]</sup> The 10X Visium assay displays improvements in both resolution (55 µm in diameter and smaller distance between barcoded regions) as well as time to run the protocol. At the moment, both the ST and 10X Visium assays are only offering 3' cDNA count data.

#### 4.2. Slide-Seq

Instead of printing regional barcoded RT primers onto a glass slide, one could instead attach them onto beads in solution and then dispense them on a glass surface. This approach was taken by the research team behind the Slide-seq technology in

2019,<sup>[43]</sup> where barcoded 10  $\mu\text{m}$  in diameter beads are randomly monolayered-packed onto a glass coverslip. Since the positions of the beads are not known beforehand, the beads barcode needs to be decoded in situ by using SBL prior to the sample preparation procedure. The experimental procedure is conceptually similar to the ST method, where permeabilization of the tissue section lets the mRNA within diffuse vertically downward to the barcoded beads on the surface. The method holds a resolution analogous to the size of a single cell, but in order to properly map spatially localized cell types, the sensitivity limitation of the current version implies that support by scRNA-seq data is needed. Of note is that, compared to the other in situ methods, the tissue image is obtained from an adjacent section and not from the same as the RNA data is acquired from. The impact of this will depend in large on the sample type and the biological question at hand. While tissue with highly conserved morphological structure (e.g., regions of the mouse brain) will display very high similarity across adjacent sections, samples with less structure and high degree of heterogeneity (e.g., within a tumor) will aggravate the interpretation.

### 4.3. High-Definition Spatial Transcriptomics

Shortly after the Slide-seq approach was published, another method using even smaller barcoded beads was announced, named high-definition spatial transcriptomics (HDST).<sup>[44]</sup> Here, they randomly deposit 2  $\mu\text{m}$ -sized beads containing barcoded RT primers onto an ordered bead array. As well as with Slide-seq, the beads location on the array needs to be decoded before the sample preparation procedure begins, which is done by several rounds of hybridizations. Since the spatially resolved HDST data is currently very sparse, neighboring beads covering circular areas with a diameter of  $\approx 13 \mu\text{m}$  is binned in order to enhance the read depth per region. Like for Slide-Seq, the lack of capture sensitivity demands scRNA-seq data to assist mapping of spatially localized cell types.

### 4.4. Nanostring GeoMx Digital Spatial Profiler

In March of 2019, NanoString announced the commercial launch of the GeoMx Digital Spatial Profiling instrument.<sup>[45]</sup> GeoMx differentiate itself by its ability to work on notoriously difficult FFPE samples. The instrument also has the ability to spatially profile proteins, although not on the same tissue section. It works in an iterative manner, where the user manually selects regions of interest (ROIs) via microscopy of varying sizes (10–600  $\mu\text{m}$  in diameter). These regions are then excited with UV light, triggering the release of either RNA target probe (mRNA assay) or antibody (protein assay) coupled barcoded tags. In the current commercial form, the tags are collected and quantified with the NanoString nCounter instrument, putting a limit to multiplex capacity. However, NGS readout is demonstrated in a preprint,<sup>[46]</sup> in which the authors state that this type of readout has the potential for an unlimited multiplex capacity. While in theory the chemistry and the combinatorial approach might allow for such a statement, this capacity has not yet been demonstrated. The workflow of selecting ROIs, although largely automated, makes it infeasible to an-

alyze whole tissue sections and therefore unbiased regional analysis could become difficult. While the shape and size of the ROI can be adjusted, the smallest available size of 10  $\mu\text{m}$  (approximately the size of a single-cell), suffers from low protein detection efficiency,<sup>[47]</sup> and the same resolution on RNA level has not yet been demonstrated.

### 4.5. APEX-Seq

In situ capturing technologies described so far target the spatial landscape of anatomical regions within intact tissue sections. However, there are also approaches for profiling subcellular localizations of endogenous RNAs within individual living cells. Two recent publications showing how the specific locations of RNAs within living cells is bound to their cellular functions, used the APEX-Seq method.<sup>[48,49]</sup> In APEX-Seq, one uses cell lines recombinantly expressing the enzyme APEX2 in specific subcellular regions of interest. By incubating the live cells in biotin-phenol and hydrogen peroxide, the APEX2 enzyme will tag nearby RNAs with biotin, which can then be isolated using streptavidin beads and subjected to RNA-seq. Whole-transcriptome profiles within individual cellular domains can therefore be assessed from living cells. As APEX-Seq requires recombinant technology, the methods not applicable to normal tissues.

### 4.6. Microfluidic Barcoding in Tissue Sections

A spatial method that attempts to combine spatially resolved mRNA and protein expression on the same tissue section, utilizing microfluidics and imaging, was recently presented in a preprint.<sup>[50]</sup> This method, named microfluidic Deterministic Barcoding in Tissue for spatial omics sequencing (DBiT-seq), is performed by first placing a microfluidics chip containing multiple parallel channels on top of a tissue section. For each channel, oligo-dT-tagged barcodes and/or antibody-tagged barcodes are streamed over the tissue. Next, the first chip is removed, and a second microfluidic chip is put on top of the tissue, but this time it is turned in a 90° angle to the first flow direction. New barcodes are now streamed through the parallel channels, resulting in a mosaic of 10  $\mu\text{m}$  sided rectangles of the tissue, containing combined barcodes from the first and the second stream.

## 5. In Silico Reconstruction of Spatial Data

As well as the experimental approaches described earlier, there are also computational ways of constructing spatially resolved gene expression datasets. These technologies start from dissociated single cells and aim to computationally assign them to a spatial location based on the gene expression profiles. The mapping of cells to a spatial position can either be achieved based on a reference map (where a set of informative gene signatures are used to guide the appropriate placement of cells), or, can be sought de novo based on assumptions about expression characteristics across the physical space.



### 5.1. Using a Reference Map

In 2015, two similar computational approaches using preexisting ISH reference databases were published. The first one integrated scRNA-seq data into the spatial context of a zebrafish embryo,<sup>[51]</sup> while the other focused on the brain of the marine annelid *Platynereis dumerilii*.<sup>[52]</sup> Reference maps were created by computationally dividing the tissue into smaller regions, and single cells were mapped onto the tissue based on an ISH reference of a smaller set of informative genes (<100). Another algorithm published in 2017, also mapping spatial localisation of single cells by means of an ISH reference, is DistMap.<sup>[53]</sup> Here, the research group used a larger scRNA-seq data set of dissociated *Drosophila* embryos to reconstruct a 6000-cellular virtual embryo. These approaches are suitable for well-defined biological specimen and tissue structures, but quickly gets more complicated to perform on more complex structures. Using existing ISH databases to construct spatial reference maps limits analyses to those tissue types for which ISH atlases already exist. Accordingly, this approach is not applicable to clinical samples, where paired data would be needed to create a reference. Hence, by combining scRNA-seq and spatially barcoded transcriptome data on the same biopsy, one could refine spatial maps of theoretically any type of tissue. This approach, where half of a tumor sample was subjected to scRNA-seq and the other half to ST, was recently demonstrated on pancreatic tumor samples.<sup>[54]</sup> Marker genes found in the scRNA-seq data were used to deconvolute cell type compositions of different tissue regions containing multiple cell types.

### 5.2. Not Using as Reference Map

The availability, as well as the quality of the reference, will constitute a key consideration when using the above-mentioned approaches for mapping. As such, being able to construct the spatial positioning *de novo*, without the reliance on a reference, is conceptually attractive. Once such approach, dubbed NovoSpaRc, demonstrated how this can be achieved.<sup>[55]</sup> Without any reference, the mapping is based upon assumptions about how gene expression varies across a tissue section. Namely, the core of the concept is that cells in proximity will display a higher similarity in their overall transcriptional profile than cells farther apart, and that this behavior is monotonic in its nature within a compact neighborhood of each cell. This can be formulated as an optimization problem, where the probabilistic solution prefers output that preserve pairwise distances both in gene and physical space. The concept is demonstrated on clearly defined tissue examples like the *drosophila* and zebrafish embryos, and further work would be needed to achieve detailed expression maps on more complex tissues. Furthermore, the author points out that including a set of marker genes with known expression patterns *a priori* significantly improves the result.

## 6. Integration of Data Across Different Methods

The methods described in Sections 1–5 obtain spatial transcriptome information using fundamentally different strategies. How-

ever, the outcome of these strategies are progressively converging; the targeted methods with limited multiplex capacity but with excellent resolution are steadily increasing their multiplex capacity, while, on the other end of the spectrum, whole-transcriptome capture based methods are steadily improving the resolution that they historically have been lacking. Yet, there is today no single method that has demonstrated the ultimate goal of spatially resolved transcriptomics: unbiased, subcellular, whole-transcriptome profiling with minimal technical dropouts. To approach this goal with existing methods, it is necessary to combine them and leverage their respective strengths. However, since practically all methods consume the tissue, or at least leave it in a state unsuitable for subsequent use, such efforts are hampered by the fact that they cannot be based on information from the same cells. Nevertheless, ongoing large collaborative efforts, like the Human Cell Atlas (HCA),<sup>[63]</sup> which aims to create a reference map of all human cells, are currently tackling the question of how to integrate information across diverse platforms and highlight the importance of a common coordinate framework and the ability to computationally map data from different modalities to a reference.<sup>[64]</sup> Likely, computational methods for integrating spatial with non-spatial scRNA-seq data will be fundamental in the creation of such atlases. Furthermore, these integrations could circumvent some of the current drawbacks of the spatial techniques. For example, scRNA-seq can be used to deconvolute data from spatial *in situ* capturing methods, which lack single-cell resolution, allowing the researcher to obtain proportion estimates of cell types within the capture areas.<sup>[65]</sup> Several other methods have been developed to fuse scRNA-seq with hybridization or ISS data<sup>[31,51,52,66]</sup> and lately also to fuse histological imaging data with *in situ* capturing data.<sup>[67]</sup>

## 7. Common Overlooked Drawbacks and Considerations

We would like to highlight a few easily overlooked caveats when it comes to using the spatial methods presented here, which otherwise would be missed if one only considers typical performance measurements, like resolution and multiplex capacity: i) The field of view (FOV), that is, the total size of the tissue that can be analyzed in a single experiment, will largely dictate the feasibility of the study. The FISH based methods with impressive throughput have a limited FOV and rely on a substantial number of high-resolution images, which quickly could become a bottleneck for smaller labs if larger tissues are to be profiled. ii) Software availability: Many of the methods have not yet reached the critical mass of users that results in community driven software development. While commercial suppliers are developing their own software, and there are efforts from the academic side to create unified computational pipelines, a researcher that is looking into the field will most likely be faced with the prospect of creating in-house software to address their particular questions. iii) cDNA conversion: The readout from the methods that include a RT step could be affected by enzymatic bias. iv) Sample type: FFPE tissue preservation remains common practice, but most of the spatial transcriptomics methods only work satisfactorily on fresh frozen tissue. v) Finally, an inherent limitation of current techniques is that they only provide a snapshot in time of a dynamically moving



landscape. Thus, examination of spatiotemporal transcriptomics has instead been conducted by using multiple samples from different time-points,<sup>[68,69]</sup> or using non-spatial single cell studies to mathematically infer temporal information based on assumptions about splicing.<sup>[64]</sup>

## 8. Conclusions and Future Perspectives

The importance of spatial context to infer deeper biological meaning is unquestionable, but technological constraints have historically limited broad adoption for various applications. Although spatially resolved omics is routinely used in the clinic today, for example to conduct immunohistochemistry or FISH based HER2 assessment, the practice of utilizing more comprehensive spatially resolved transcriptomic profiles to analyze tissues is today largely a basic research effort. Nonetheless, this could in the future become an important tool for personalized medicine, in particular for heterogeneous tissue types where locally acting immune responses and clonal niches could be of importance for treatment decisions. However, future clinical adoption requires both a greater understanding of which information that is clinically relevant as well as the availability of cheaper and more simplified workflows. Currently, many of the techniques presented here are lacking the necessary degree of robustness to allow for broad adoption among clinicians. For example, data from a majority of the mentioned methods have only been published from the creators' own labs. While this is to be expected given the short time since the methods were originally published, it calls into question the robustness of their performance and the amount of specialized knowledge required to apply them. Nevertheless, several commercial endeavors have been created as spin-offs from academia to pursue the goal of broader adoption.

Beyond spatially resolved transcriptomics lies the prospect to enable analysis of multiple omes, preferably on the same tissue sample. As of today, the most feasible strategy to obtain a more comprehensive multi-omic profile is to process consecutive tissue sections, where each section is subject to interrogation of different omes. Although such an analysis will not allow for multi-omic profiling of the same cells, spatial areas of interest often span multiple tissue slices and would allow for certain integrative types of analyses. Even though the prospect of multi-omics is attractive, there is still much more to learn from the transcriptome alone, and efforts that allow for direct RNA targeting, omitting the RT step, constitute an interesting new research development. Direct RNA sequencing via geometric sensitive current disruptions in nanopore based methods, where the tissue is placed directly on top of the pores, could constitute such a future method which may reveal information about RNA modifications.<sup>[70]</sup> Furthermore, strategies to computationally enhance the data has the potential to reduce the number of required experiments and decrease costs by, for example, allowing for the omission of highly correlated information and imputing results in silico from sparse data sets. We foresee that the field of computational enhancement will rapidly expand in conjunction with increased access to publicly available data sources.

A driver behind the technological development is several ongoing, large collaborative efforts to create comprehensive and publicly available atlases. In tandem, new analysis tools and standard-

ized procedures need to be put in place to allow for integration across all these different technologies, each with its own unique characteristics, data formats, biological strengths and caveats. The field of spatially resolved transcriptomics is accelerating in technological advancement, and the many biological analyses made possible by this advancement are yet to be fully explored.

## Author Contributions

M.A. and J.B. wrote the primary manuscript with significant contribution from J.L. M.A. designed and prepared the figures.

## Conflict of Interest

J.L. is a scientific advisor at 10x Genomics Inc, which holds IP rights to the ST technology.

## Keywords

gene expression, RNA, RNA-sequencing, single cells, spatial omics, spatial transcriptomics, spatially resolved transcriptomics, tissue heterogeneity

Received: November 14, 2019

Revised: March 28, 2020

Published online: May 4, 2020

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