PERSPECTIVES

SINGLE-CELL OMICS — INNOVATION

# Spatially resolved transcriptomics and beyond

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Abstract | Considerable progress in sequencing technologies makes it now possible to study the genomic and transcriptomic landscape of single cells. However, to better understand the complexity of multicellular organisms, we must devise ways to perform high-throughput measurements while preserving spatial information about the tissue context or subcellular localization of analysed nucleic acids. In this Innovation article, we summarize pioneering technologies that enable spatially resolved transcriptomics and discuss how these methods have the potential to extend beyond transcriptomics to encompass spatially resolved genomics, proteomics and possibly other omic disciplines.

More than a century ago, the father of modern pathology, Rudolph Virchow, inaugurated the cell era by stating that all diseases originate in cells[1]. Paradoxically, in spite of the unanimous recognition that cells are the fundamental unit of multicellular organisms, modern cellular and molecular biology knowledge is largely derived from bulk assays applied to cell populations, whereas single-cell methods have been lagging behind. However, in the past few years, several sequencing-based approaches that can interrogate the genome and transcriptome of individual cells have been developed[2–7], and the young fields of single-cell genomics and transcriptomics have been booming ever since[8–11]. The fundamental advantage of single-cell methods over bulk assays is that retaining single-cell information can reveal rare cellular properties and biologically meaningful cell-to-cell variability. However, unlike circulating blood cells, solid tissues and organs must be mechanically or enzymatically disaggregated in order for single-cell analyses to be practicable. This procedure unavoidably causes loss of positional information, making it impossible to assess how single-cell genomic and transcriptomic landscapes are spatially organized throughout a tissue.

A comprehensive review of single-cell DNA and RNA sequencing technologies and their applications is beyond the scope of this Innovation article, and has been presented elsewhere[12–14]. Here, we summarize pioneering technologies to perform RNA measurements *in situ* or by means that preserve spatial information, and discuss how these methods provide a foundation that, in our opinion, will ignite a new field of spatially resolved omics, which encompasses integrative acquisition of genomic, transcriptomic, proteomic and possibly other omic data, while retaining positional information. A systematic comparison of the characteristics of each of these methods is shown in TABLE 1.

## The importance of *in situ* methods

The development of *in situ* methods in the past decades has had a major role in advancing our ability to integrate functional and spatial information in biological studies[15–23]. These methods have enabled traditionally function-oriented fields such as molecular genetics and biochemistry to progressively converge with more structure-focused fields such as embryology[24] and histology[25], thus 'setting the stage' for spatially resolved molecular analyses of biological processes. For example, combination of genetic tools with *in situ* hybridization techniques has been instrumental in revealing how gradients of gene expression are formed along the main embryonic axes at various developmental stages, and how these gradients dictate the proper execution of developmental programmes and organ formation[26–28]. In parallel, immunocytological and immunohistological techniques have greatly expanded our understanding of the structural organization of normal and pathological tissues, enabling a level of morphological classification that would be otherwise impossible to achieve, particularly for distinguishing between different cancer types[29]. Moreover, the ability to visualize specific DNA, RNA and protein molecules *in situ* has markedly expanded our knowledge of how molecular processes are organized at the subcellular level. For example, DNA fluorescence *in situ* hybridization (DNA FISH) has been widely used to study the spatial organization of chromosomes within the nucleus, revealing how chromosomes tend to occupy distinct nuclear regions — known as chromosomal territories — that are positioned with respect to each other in a nonrandom manner[30]. Similarly, *in situ* visualization of proteins using immunofluorescence, fluorescent fusion proteins[17] or proximity ligation assays (PLAs)[19] has been instrumental in studying highly spatially organized cellular processes such as vesicular trafficking[31], nuclear import and export signalling[32], and assembly of endogenous complexes[33]. Finally, combination of MS2 tagging[34], the ParB–INT system[35] or the CRISPR–Cas system[36] with microscopy has recently opened up the possibility to observe and quantify fine-scale cellular events, such as RNA splicing at single-molecule resolution[37,38], repair of DNA breaks[35] or movements of specific DNA loci directly in living cells[39,40].

## The need for spatially resolved omics

In spite of the enormous impact that *in situ* methods have had in multiple fields in biology and medicine, a common feature of many *in situ* methods is that only a handful of markers can be simultaneously assessed at a given time in a biological sample.

Table 1 | **Current methods and technologies for spatially resolved transcriptomics and other omic measurements**

| | smFISH | Padlock probes and RCA | Branched FISH | LCM | Microtomy sequencing | TIVA | ISS | FISSEQ | Imaging mass cytometry |
|---|---|---|---|---|---|---|---|---|---|
| **Sample** | Fixed cells and tissues; purified RNA | Fixed cells and tissues; purified DNA or RNA | Fixed cells and tissues; possibly purified DNA or RNA | Fixed tissues | Fixed and fresh tissues | Live cells | Fixed cells and tissues | Fixed cells and tissues | Fixed tissues |
| **Target** | RNA | DNA; RNA | RNA | RNA; DNA; proteins | RNA; possibly DNA and proteins | RNA | RNA | RNA | Proteins |
| **Type** | Targeted | Targeted | Targeted | Targeted or non-targeted | Non-targeted | Non-targeted | Targeted | Non-targeted | Targeted |
| **Variable measured** | Abundance; SNVs; fusion transcripts; splice variants; subcellular localization | Abundance; SNVs; fusion transcripts; splice variants; subcellular localization | Abundance; subcellular localization | Abundance; possibly SNVs, fusion transcripts and splice variants | Abundance; possibly SNVs, fusion transcripts and splice variants | Abundance; possibly SNVs, fusion transcripts and splice variants | Abundance; possibly SNVs, fusion transcripts and splice variants | Abundance; possibly SNVs, fusion transcripts and splice variants | Abundance; protein modifications |
| **Single-cell?** | Yes | Yes | Yes | Yes | No | Yes | Yes | Yes | Yes |
| **Spatial resolution** | Subcellular | Subcellular | Subcellular (except the nucleus) | Anatomical or cellular | Anatomical | Cellular | Cellular | Cellular | Subcellular |
| **Morphology assessment** | Yes | Yes | Yes | Yes | No | Yes | Yes | Yes | Yes |
| **Throughput (number of cells)** | Low to medium | Low to medium | Low to medium | Medium | High | Low | Low to medium | Low to medium | Very high |
| **Throughput (number of genes or proteins)** | Low to medium | Low to medium | Medium | High | High | High | Low | High | Low |
| **Estimated efficiency** | ~90% | ~30% | NA | NA | ~5–10% | NA | NA | NA | NA |
| **Readout** | Microscopy; flow cytometry | Microscopy; flow cytometry | Microscopy; flow cytometry | Microarray; RNA-seq; MS | RNA-seq; possibly MS | RNA-seq | Microscopy | Microscopy | MS |
| **Technical difficulty** | Easy | Easy | Easy | Moderately easy | Moderately easy | Moderately difficult | Difficult | Difficult | Difficult |
| **Refs** | 23,53–58, 60–63 | 64–68 | 70,71 | 72,74,76–78 | 79–81 | 82 | 83 | 85 | 87,88 |

FISH, fluorescence *in situ* hybridization; FISSEQ, fluorescent *in situ* RNA sequencing; ISS, *in situ* sequencing; LCM, laser capture microdissection; MS, mass spectrometry; NA, not available; RCA, rolling circle amplification; RNA-seq, RNA sequencing; smFISH, single-molecule RNA fluorescence *in situ* hybridization; SNV, single-nucleotide variant; TIVA, transcriptome *in vivo* analysis.

Moreover, these assays typically measure only one molecule type (DNA, RNA or protein), thus limiting the study of complex and spatially organized regulatory networks within cells and tissues. With the advent of next-generation sequencing technologies in the past decade[41], it is now possible to perform unbiased measurements of genomic[42], epigenomic[43] and transcriptomic[44] landscapes in cells and tissues, revealing systems-level design principles and regulatory networks that could not have been discovered by traditional gene-oriented approaches. In principle, performing multiple spatially resolved omic measurements in the same biological sample, and possibly reiterating the procedure for multiple samples collected at various time points from the same source, followed by high-dimensional data analysis could reveal completely new spatiotemporal interdependencies that would advance our understanding of how complex biological systems operate.

An immediate application of this approach would be to measure chromatin states, gene expression, and protein abundance and activity for thousands of genes in multiple regions of the developing embryo at various developmental stages. Another exciting application of spatially resolved omics would be to measure how gene expression profiles and epigenetic states are spatiotemporally co-regulated in the brain during different mental activities. The acquisition of such high-dimensional data from various anatomical regions of the brain at different time points would bring us closer to understanding how molecular and cellular processes collectively cooperate

to produce high-order mental functions such as perception and consciousness. Finally, spatially resolved omic methods would greatly enable the assessment of the molecular basis and clinical impact of intratumour heterogeneity[45–51]. There is a strong expectation that measuring genetic and phenotypic diversity in tumours will help to predict responsiveness to molecular targeted drugs and to facilitate patient selection in clinical trials[45,46]. Indeed, more than 10 ongoing trials are currently evaluating the clinical impact of tumour heterogeneity[46], which indicates a rapidly growing interest in the topic and the need for developing quantitative methods that can capture multiple features of intratumour heterogeneity.

## Imaging-based methods

***smFISH.*** For more than three decades, *in situ* hybridization of RNA molecules with sequence-specific probes, followed by target visualization using a chromogenic reaction, has been instrumental for revealing embryo patterning and studying developmental processes[52]. A quantitative development of this approach came from the introduction of single-molecule RNA fluorescence *in situ* hybridization (smFISH) techniques[23,53,54]. In smFISH, DNA oligonucleotides that are complementary to the target RNA are labelled with fluorescent dyes, which enable visualization of individual transcripts as diffraction-limited spots (FIG. 1a,b). Using this approach, a panel of 15 cell type-specific marker transcripts were charted *in situ* in the mouse intestinal crypt, which unravelled the design principles of stem cell progenitor hierarchy formation in the crypt during embryonic development[55]. Other promising applications of smFISH include splice variant[56] and fusion transcript[57,58] detection, particularly in complex tissues and tumour sections to study spatial heterogeneity. With the emergence of single-cell transcriptomic assays, smFISH also represents a powerful means to validate individual RNA expression values from single-cell transcriptome-wide measurements, as recently shown for a variety of transcripts in mouse embryonic stem cells[59]. However, although this approach enables robust transcript quantification in morphologically intact tissues, the number of transcripts that can be visualized simultaneously in the same sample is small (usually 1–3) owing to limited availability of fluorophores with non-overlapping spectra, which prevents highly multiplex measurements.

To increase multiplexing, spatial or spectral mRNA barcoding was combined with super-resolution microscopy[60] (FIG. 1c). In this approach, rather than comprising pools of oligonucleotides, smFISH probes consist of pools of oligonucleotide pairs conjugated to photo-switchable dyes. One of the two oligonucleotides carries an emitter dye, while the other is coupled to an activator dye, and both oligonucleotides hybridize to the same target molecule adjacent to each other. Hence, a signal is generated only upon specific hybridization of both oligonucleotides to the same target mRNA. In spatial barcoding, sets of four oligonucleotide pairs with identical dye pairs are used to visualize distinct portions of a transcript, and each set has a different dye pair in different transcript regions. After imaging, the identity of targeted transcripts is deduced from the order by which distinct fluorescence signals are arrayed in two dimensions. In order to induce linearization of RNA molecules and to keep the sequence of colours along each mRNA molecule confined to two-dimensional (2D) rather than 3D space — which would render reconstruction of the colour order more challenging — cells have to be mechanically compressed and flattened. This is hardly applicable to tissues and must be avoided when retaining information about RNA subcellular localization is important. Instead, spectral barcoding can be applied in such cases by designing oligonucleotide pairs that are coupled to the same fluorophore pair scattered all along the same mRNA target and that are intermingled with other oligonucleotide pairs conjugated to different fluorophore pairs (FIG. 1c). In this case, the molecular identity is encoded only by the combination of colours — but not by the order — of each RNA molecule. Using this approach, 32 mRNAs involved in the cellular response to calcium stress were visualized and quantified simultaneously in hundreds of single yeast cells, demonstrating the existence of two clusters of responsive genes with different regulatory architecture[60].

In a recent improvement of the method, spatial barcoding and spectral barcoding were substituted by the use of sequential barcoding[61] (FIG. 1c). In this approach, each transcript is identified through multiple cycles of hybridization, imaging and probe removal. In each cycle, a given transcript is detected using a specific smFISH probe labelled with one fluorophore, and the probe is removed after imaging using DNase I. In the next cycle, the same transcript is detected with the same probe but labelled with a different fluorophore. The temporal pattern of colours, which change at every hybridization cycle, defines a transcript. In this manner, the number of transcripts that can be visualized in a single cell scales as $F^N$, where $F$ is the number of dyes and $N$ is the number of rounds of hybridization. Thus, as few as 4 dyes and 8 hybridization rounds are, in principle, sufficient to detect all of the transcripts present in one cell.

Another application of smFISH is the detection of single-nucleotide variants (SNVs) and point mutations in RNA molecules *in situ*[62,63]. Using this approach, the oncogene *BRAF* V600E mutation was detected with 56% sensitivity and a false discovery rate of 7% in a melanoma cell line[63]. A clear advantage of this method over *in situ* DNA genotyping[64] is the high statistical power afforded by the fact that the number of RNA molecules per cell is typically larger than the corresponding genomic locus copy number.

***Padlock probes.*** An alternative method to visualize RNA molecules *in situ* uses padlock probes and rolling circle amplification (RCA)[65–68]. Each padlock probe consists of one single-stranded DNA molecule, approximately 100-nucleotide in length, with the 5′ and 3′ portions (~20-nucleotide-long homology arms) complementary to the same target. Upon hybridization, the homology arms become adjacent to one another and form a circle-like structure with a nick in between the two ends, while the internal portion of the probe (the linker) forms a loop. The nick is then ligated *in situ*, transforming the linear padlock probe into a circle, which can now be amplified using an isothermal DNA polymerase. This process leads to the formation of an RCA product (RCP) and accumulation of thousands of identical copies of the padlock probe sequence. Using fluorescently labelled detection oligonucleotides complementary to the linker, RCPs can be visualized *in situ* as bright fluorescence spots (FIG. 1d). As ligation of the homology arms depends on perfect complementarity of the 5′ and 3′ ends to the target, the presence of a point mutation in this region will prevent the circularization and generation of the signal. Hence, by designing two types of padlock probes — one complementary to the wild-type sequence and one to the mutant sequence — it is possible to discriminate between the two alleles using different linker sequences. Using this approach, the closely related α- and β-isoforms of mouse actin could be distinguished in tissue sections, and an oncogenic *KRAS* mutation frequently found in human cancers was

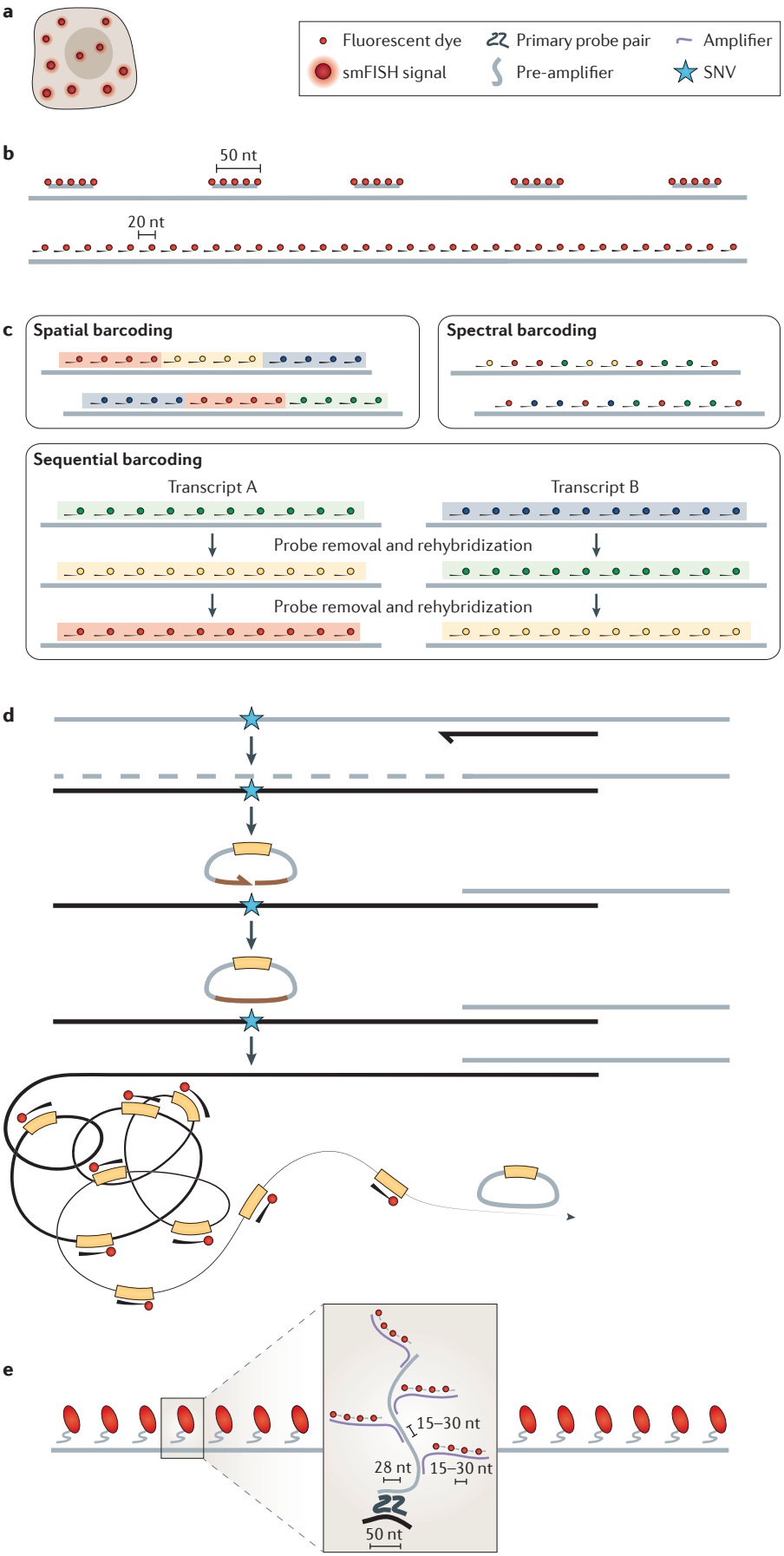


Figure 1 | **Imaging-based methods for spatially resolved transcriptomics.** **a** | In single-molecule RNA fluorescence *in situ* hybridization (smFISH), following hybridization, individual mRNA molecules are visualized using fluorescence microscopy and appear as diffraction-limited fluorescence spots within cells. The number and position of these spots can be accurately measured using automated image processing software tools. **b** | smFISH probes consist of DNA oligonucleotide sets that are complementary to a specific RNA target (grey lines) and that are conjugated with desired fluorophores (red). In the original approach[23], oligonucleotides (40–50 nucleotides (nt) in length) containing 5 fluorophores are used (upper panel). An alternative method[53] uses 20-nt-long oligonucleotides (typically 30–50 of them), each labelled with a single fluorophore molecule (lower panel). **c** | Multiplex smFISH can be achieved by spatial, spectral and sequential barcoding[60,61]. In spatial barcoding, each transcript is encoded by the linear sequence of colours with which individual sets of four oligonucleotides are labelled. In spectral barcoding, it is the specific mixture of colours that identifies a transcript. In sequential barcoding, a given transcript is detected multiple times through hybridization, imaging and probe removal cycles, and appears in a different colour during each cycle. **d** | RNA detection and genotyping can also be performed using padlock probes and rolling circle amplification (RCA)[69]. Locked nucleic acid (LNA)-modified oligonucleotides (black single-headed arrow) prime reverse transcription upstream of the site of a single-nucleotide variant (SNV). After *in situ* cDNA preparation, a padlock probe consisting of two homology arms (brown) separated by a linker sequence (yellow) hybridizes to the target cDNA, and is circularized by ligation only on molecules containing the corresponding SNV. The padlock probe is then amplified using an isothermal DNA polymerase, producing a local accumulation of hundreds to thousands of linker copies. Fluorescently labelled oligonucleotides (red spots) are finally used to visualize RCA products. **e** | In branched DNA fluorescence *in situ* hybridization (bDNA FISH)[71], primary probe pairs are designed to bind in close vicinity to a short sequence (usually 50 nt) in the target mRNA. A tree-like structure is then formed onto the primary probe pair by adding a long pre-amplifier DNA filament (the 'trunk') to which multiple amplifier filaments (the 'branches') are hybridized. Finally, amplifier filaments are hybridized with short oligonucleotides, each of which is labelled with a single fluorophore. Primary probes ensure high specificity, while the branched structure acts as signal multiplier. Parts **d** and **e** are adapted from REF. 69 and REF. 71, respectively, Nature Publishing Group.

successfully detected in a mixture of wild-type and mutant cells[69]. As target recognition requires two simultaneous binding events and a successful ligation, this method is highly specific.

***Branched DNA probes.*** A third method for *in situ* detection of RNA molecules is based on branched DNA probes[70]. In this approach, numerous primary probe pairs are designed to hybridize to a transcript of interest. Upon hybridization, each primary probe pair serves as a scaffolding site from which a branched structure of additional probes is built up through multiple hybridization steps, ultimately allowing the recruitment of a large number of short fluorescently labelled oligonucleotides for signal amplification (FIG. 1e). To achieve high-throughput RNA measurements *in situ*, branched DNA probes were combined with automated liquid handling and high-content imaging, which enabled the expression of 928 selected genes to be measured in parallel in hundreds of single HeLa cells[71]. In addition, by co-staining for various localization markers, several metrics reflecting subcellular mRNA distribution were computed, enabling classification of the 928 genes into 5 clusters with distinctive subcellular patterning[71]. This imaging-based, high-throughput approach was highly reproducible and sensitive, and it yielded mean expression values comparable to those obtained with high-throughput RNA sequencing (RNA-seq). However, one limitation is that high throughput is achieved by parallelizing hundreds of specific hybridization reactions in separate wells of multiwell plates. This means that the location and mRNA level of only one gene are measured for all cells in a given well, preventing multiplexing. Another limitation — similarly affecting all of the smFISH applications described above — is that if a large number of targets is to be analysed, then the cost of probe synthesis quickly grows above a threshold that is incompatible with the budget of most research laboratories.

## Sequencing-based methods

***LCM.*** Laser capture microdissection (LCM) is a powerful technology that enables the isolation of cells or small tissue regions from defined anatomical locations[72,73]. Data sets derived from these samples can then be annotated with the known original location, thus preserving spatial information. Nucleic acids can be extracted from LCM-captured cells and used in a variety of downstream applications, including gene expression microarrays and RNA-seq[74–76]. Importantly, a variety of genome-wide nucleic acid amplification methods are now available that can be applied to amplify LCM-extracted DNA and RNA, especially when the size of laser-ablated regions is very small[4,14]. In two landmark papers, microarray analyses of RNA extracted from hundreds of discrete regions in the prenatal[77] and adult[78] human brain revealed widespread anatomical variability in gene networks, which is reflective of developmental processes and of the distribution of major cell types. These data are now part of the Allen Brain Atlas, a comprehensive resource integrating gene expression and neuroanatomical information for humans and mice. In the future, similar application of spatially resolved proteomic and possibly other omic assays to different developmental stages and various disease processes will further expand this formidable data set, casting new light on the astonishing complexity of the brain.

***Microtomy sequencing.*** A second approach for spatially resolved transcriptomics based on RNA extraction from discrete tissue regions is serial microtomy sequencing. In this method, RNA is extracted from individual thin tissue cryosections and subjected to sequencing. In a proof-of-principle application of serial microtomy sequencing of *Drosophila melanogaster* embryos, known and novel spatial patterns of gene expression were identified[79], indicating that this approach can be used to obtain spatially resolved transcriptional maps in complex structures when single-cell resolution is not required. However, a caveat is that carrier RNA must be added to retrieve a sufficient amount of RNA from each cryosection for preparation of a sequencing library, which limits the number of usable sequence reads obtained from each section. Moreover, separate libraries must be obtained from each section, limiting the number of sections that can be sequenced and therefore the achievable spatial resolution.

To improve spatial resolution and quantification, a modified approach named tomo-seq was recently developed[80] based on an earlier method for single-cell transcriptomics[7]. In tomo-seq, RNA is extracted from individual thin cryosections using the TRIzol reagent, and oligodeoxythymidine primers carrying section-specific barcodes are used to initiate reverse transcription, allowing sample pooling before library preparation[80]. In addition to barcodes, primers also contain the T7 phage transcriptional promoter sequence, which enables linear cDNA amplification by *in vitro* transcription, thus reducing amplification biases in comparison to other RNA amplification methods[7] (FIG. 2a). When identical samples are available (such as in the case of genetically identical model organisms), they can be sectioned separately along each Cartesian coordinate in order to obtain transcriptional maps in one, two or three dimensions. Two and three identical samples are required to generate 2D and 3D maps, respectively[80,81]. By applying tomo-seq to genetically identical zebrafish (*Danio rerio*) embryos collected at different developmental stages, and using cluster analysis to identify similar spatial patterns of gene expression, an atlas of developmental transcriptional dynamics in two and three dimensions was generated, which revealed previously unknown spatial patterns for many genes[80]. Despite lacking single-cell resolution, this approach is straightforward and can be readily applied to other developmental systems, as well as to adult organs or large anatomical structures, to provide an unprecedented picture of gene expression in space. However, as already noted, 2D and 3D maps cannot be generated when multiple identical samples are not available, as in the case of human clinical specimens.

***TIVA.*** Another recently developed approach for spatially resolved transcriptomics based on RNA extraction and sequencing is transcriptome *in vivo* analysis (TIVA)[82]. In TIVA, photoactivatable tags — which consist of multiple functional groups and a trapped poly(U) oligonucleotide coupled to biotin — are first introduced into live cells using a disulphide-linked cell-penetrating peptide attached to the tags. In selected cells or tissue regions of interest, the poly(U) sequence is then unblocked by photoactivation, while a Cy3–Cy5 pair is used to signal both the uptake and the uncaging of tags based on fluorescence resonance energy transfer (FRET). Finally, poly(A)-tailed mRNA molecules recognized by freed TIVA tags are extracted using streptavidin capture of the biotin group and are processed for RNA-seq (FIG. 2b). TIVA transcriptome profiling of selected neurons within mouse hippocampal slices and human biopsy samples of the frontal cortex accurately recapitulated tissue-specific gene expression profiles[82]. In single neurons labelled in their tissue context, but not in cultured neurons, TIVA revealed bimodally expressed transcripts, which is consistent with gene expression noise being driven predominantly by extrinsic factors in tissue microenvironments[82].

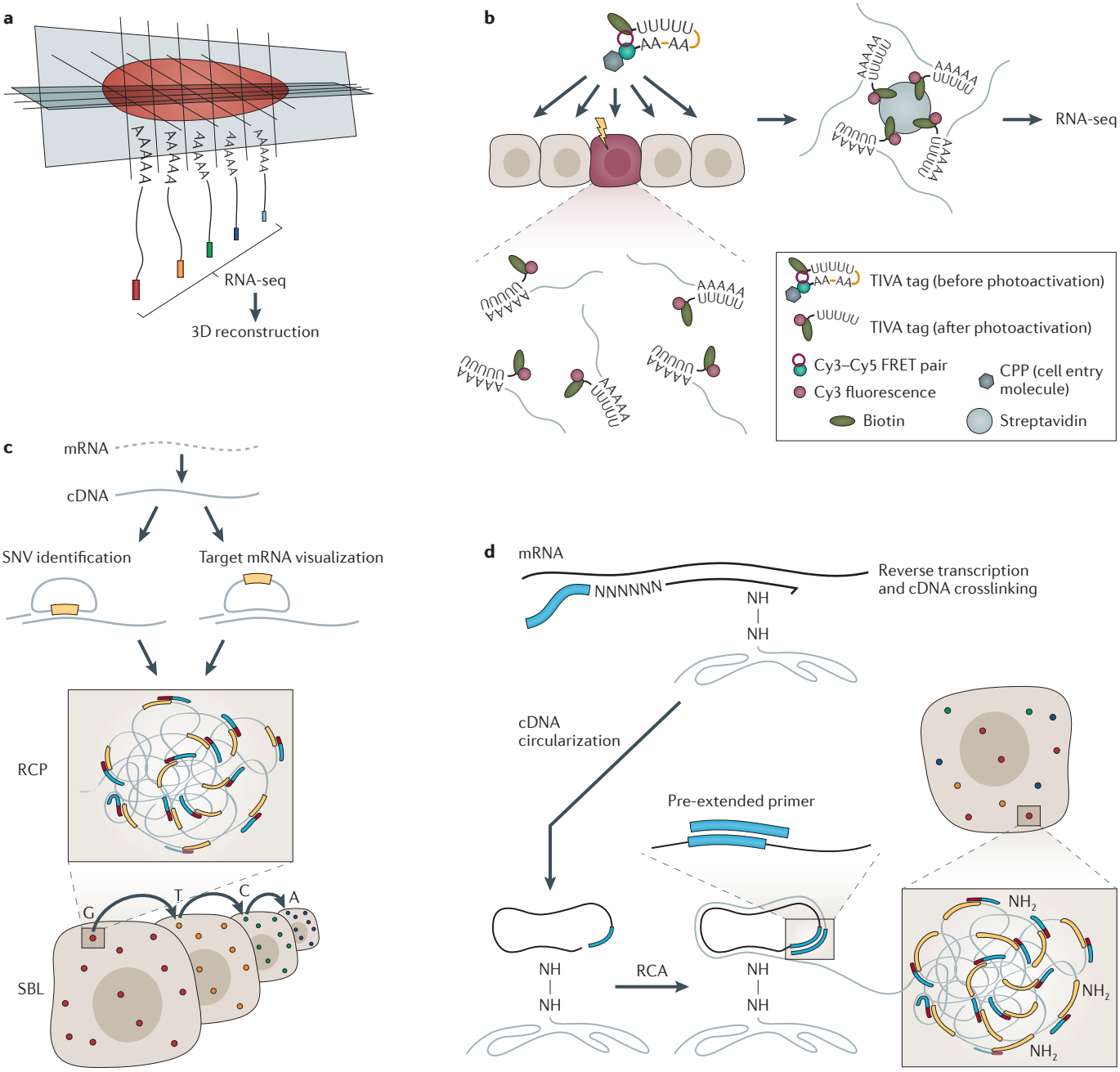


Figure 2 | **Sequencing-based methods for spatially resolved transcriptomics.** **a** | In tomo-seq, fixed embryos (or organs or tissue fragments) are serially dissected into small sections along Cartesian axes, and RNA is extracted from each section[80]. Barcoding (coloured rectangles) enables pooling of RNA from multiple sections before RNA sequencing (RNA-seq). Algorithms such as those used in computer tomography enable the generation of three-dimensional (3D) transcriptional maps. **b** | In transcriptome *in vivo* analysis (TIVA)[82], a photoactivatable biotinylated tag is introduced into living cells by a cell-penetrating peptide (CPP). The tag contains a poly(U) sequence masked by two poly(A) stretches joined by photocleavable linkers (brown). Upon photoactivation of selected cells (magenta), the poly(A) stretches are released so that mRNA molecules can be bound by the freed poly(U) TIVA tags, captured by streptavidin and processed for RNA-seq. A Cy3–Cy5 pair is used to signal tag uptake and uncaging based on fluorescence resonance energy transfer (FRET). **c** | In *in situ* RNA-seq[83], RNA is converted to cDNA, followed by hybridization of target-specific padlock probes, probe circularization, rolling circle amplification (RCA) and formation of a RCA product (RCP). Sequencing-by-ligation (SBL) primers (cyan) are used to decode the sequence (yellow) of either a 4-base stretch encompassing a single-nucleotide variant (SNV; left) or a target-specific 4-base sequence barcode incorporated in the padlock probe (right). At each sequencing step, RCPs appear as bright fluorescence spots within cells, and the sequence of bases is deduced from the temporal colour order of each spot. **d** | In fluorescent *in situ* RNA sequencing (FISSEQ)[85], RNA is reverse transcribed in fixed cells using random hexamers with a 5′ tag (cyan), and cDNA is circularized and crosslinked to the cellular protein matrix. A pre-extended oligonucleotide complementary to the tag (cyan) primes RCA of circularized cDNA, and the resulting RCP is crosslinked to the cellular protein matrix. SOLiD SBL is used to sequence 30 bases downstream of the tag. Part **c** is adapted from REF. 83, Nature Publishing Group. Part **d** is adapted from Lee, J. H. *et al.* Highly multiplexed subcellular RNA sequencing *in situ*. *Science* **343**, 1360–1363 (2014). Reprinted with permission from AAAS.

**In situ *sequencing.*** A second group of sequencing-based technologies for spatially resolved transcriptomics uses direct *in situ* sequencing without involving RNA extraction and amplification steps. By combining padlock probes, RCA and sequencing-by-ligation (SBL), a pioneering method was devised for *in situ* detection of either 4-base sequence barcodes inserted in padlock probes targeting selected transcripts, or 4-base sequences encompassing an expressed SNV[83]. In both cases, RNA is first converted to cDNA, followed by hybridization of specific padlock probes to cDNA, probe circularization, RCA and finally

## Glossary

**Cluster analysis**
A set of statistical methods used in many scientific disciplines to group objects within a data set based on similarity.

**CRISPR–Cas system**
(Clustered regularly interspaced short palindromic repeat–CRISPR-associated protein system). A genome editing technique based on the bacterial immune system. The Cas9 protein and an RNA guide are used to specifically edit or visualize a given sequence of DNA.

**DNA fluorescence *in situ* hybridization**
(DNA FISH). An *in situ* hybridization technique in which probes consisting of short (<500 nucleotides) fluorescently labelled DNA fragments are hybridized to the complementary target sequences in the nucleus.

**High-content imaging**
A subfield of microscopy using fast and automated imaging systems that can acquire thousands of cell micrographs from different regions on a microscope slide or separate wells of multiwell plates. It is frequently applied to detect morphological cellular changes in drug and small interfering RNA (siRNA) library screens.

**High-dimensional data analysis**
A collection of statistical methods and visualization tools used to analyse and represent data with dozens to thousands of dimensions. In spatially resolved omics, the intrinsic high dimensionality of omic data is multiplied by the number of spatial locations analysed, producing mega- or giga-dimensional data sets that need to be correlated among each other in order to identify spatially organized expression patterns and regulatory networks. Efficient handling of these mega-dimensional omic data sets requires extraordinary computational efforts and novel statistical tools that are being developed for the analysis of 'big data' in many areas of science and business.

**Immunocytological and immunohistological techniques**
A group of methods that visualize specific proteins or other antigens directly in cells (immunocytology) or tissues (immunohistology) using antibodies coupled to fluorophores or enzymes.

***In situ* hybridization techniques**
A set of methods that use DNA or RNA probes which bind selectively to specific DNA or RNA target sequences by Watson–Crick or Hoogsteen base pairing. Probes can be conjugated to fluorescent dyes (such as in fluorescence *in situ* hybridization (FISH)) or to enzymes that catalyse a chromogenic reaction (such as the horseradish peroxidase in chromogenic *in situ* hybridization (CISH)).

***In situ* methods**
A vast collection of methods used for the detection of nucleic acids or proteins directly in cells or tissues while preserving spatial information.

**Laser capture microdissection**
(LCM). A technique in which a laser is used to ablate small groups of cells or even single cells up to relatively large regions within a tissue section mounted on a special support matrix, and subsequently to transfer the captured material into test tubes for downstream processing.

**Locked nucleic acid**
(LNA). A synthetic RNA nucleotide in which the ribose group is modified with an extra bridge connecting the 2′ oxygen and the 4′ carbon. Incorporating LNAs into DNA or RNA oligonucleotides increases the specificity and sensitivity of detection in a number of assays.

**Mass cytometry**
A technology that combines flow cytometry with mass spectrometry, which enables simultaneous detection of dozens of antigens in thousands of single cells using antibodies tagged with isotopically pure metal reporters. Based on current isotope availability, up to ~100 proteins or protein modifications can be detected simultaneously in thousands of single cells using mass cytometry.

**MS2 tagging**
A technique that uses the MS2 coat protein of the MS2 bacteriophage to detect RNA molecules owing to the high binding affinity of MS2 for a specific RNA stem–loop structure. Upon insertion of the MS2 recognition sequence in the RNA of interest, the intracellular localization of the RNA can be monitored in live cells using GFP-tagged MS2.

**Padlock probes**
Oligonucleotide probes consisting of target-complementary homology arms connected by linker sequences. Upon recognition of the target, the extremities of the probe are ligated, and the circle created is amplified through rolling circle amplification (RCA). The resulting product is detected using fluorescence *in situ* hybridization (FISH) probes. Padlock probes and RCA combine high specificity of detection with high signal amplification, and they have been used in numerous applications *in vitro* and *in situ*.

**ParB–INT system**
A technique used to investigate the position and dynamics of DNA in living cells based on the interaction between the bacterial ParB protein and the *parS* sequence. Insertion of a cluster of *parS* sequences in a genomic region of interest allows its visualization using fluorescently tagged ParB.

**Proximity ligation assays**
(PLAs). A method that can detect proteins and protein–protein interactions with high specificity. The protein of interest is recognized by two primary antibodies raised in different species, followed by binding of species-specific secondary antibodies conjugated to a short DNA oligonucleotide. Upon specific binding of both primary antibodies to the target (two epitopes either on the same protein or on two distinct interacting proteins), the two oligonucleotides are brought together, which aids the formation of a circle from an extra pair of oligonucleotides added to the mixture by the use of *in situ* ligation. Fluorescent detection is then achieved using rolling circle amplification.

**Rolling circle amplification**
(RCA). A process by which circularized padlock probes are amplified through the continuous action of an isothermal DNA polymerase. The result is a single-stranded DNA concatemer containing many copies of the original padlock probe sequence in tandem.

**Sequence barcodes**
Short unique DNA sequences, usually inserted in synthetic oligonucleotides, that are used to keep track of sample identity during nucleic acid amplification procedures prior to sequencing. A particular type of sequence barcode are Unique Molecular Identifiers used for counting absolute numbers of DNA or RNA molecules that are originally present in a sample before nucleic acid amplification by methods such as PCR.

**Sequencing-by-ligation**
(SBL). A next-generation sequencing method based on sequential ligation of fluorescently labelled probes starting from an adaptor primer bound to a template DNA fragment. Multiple rounds of ligation, detection and fluorophore cleavage are performed using a pool of differently labelled oligonucleotides. In each cycle, only the oligonucleotide that is complementary to the query sequence is ligated and its specific fluorescence detected. SBL constitutes the core of the SOLiD and Complete Genomics technology.

**Single-molecule RNA fluorescence *in situ* hybridization**
(smFISH). An *in situ* hybridization method by which individual RNA molecules are specifically visualized in cells or tissues using pools of 20–50 complementary short DNA oligonucleotides, each of which is coupled to the same fluorophore. Single RNA molecules appear as diffraction-limited fluorescence spots, which can be accurately counted using unbiased automated procedures.

**Spatially resolved omics**
A new technology-driven field in which high-throughput genomic, epigenomic, transcriptomic, proteomic and possibly other omic data are collected from cells or tissues by means that preserve positional information and analysed using high-dimensional data analysis techniques. So far, most of pioneering efforts in this newly developed field have focused on RNA measurements, but we predict that measurements at the DNA and protein levels will soon ensue.

**Super-resolution microscopy**
A form of light microscopy that allows the acquisition of images with a resolution not limited by the diffraction limit of light (that is, in the range of dozens of nanometres or less). Super-resolution microscopy methods include STED (stimulated emission depletion), SSIM (saturated structured illumination microscopy), STORM (stochastic optical reconstruction microscopy) and PALM (photoactivated localization microscopy).

*in situ* SBL (FIG. 2c). In the first approach, 4-base barcodes are inserted into the non-hybridizing region of padlock probes and serve as identification tags for the transcripts targeted by the unique homology arms of the probes. Using this approach, the expression levels of 39 transcripts were successfully profiled in breast cancer tissue sections, including the 21 transcripts of OncoType DX, which is a proprietary gene expression signature used to guide patient selection for adjuvant chemotherapy in breast cancer[84]. In the second approach, padlock probes are designed such that the homology arms hybridize 4 bases apart from each other, with an SNV-containing gap in between. During probe circularization, the gap is filled in using the sequence containing the SNV site as a template. Using this strategy, a 4-base sequence encompassing codon 12 of the human *KRAS* oncogene was interrogated, and one mutated cell could be found in a background of 1,000 wild-type cells, thus demonstrating that the method has good sensitivity in finding rare cells in morphologically preserved tissues.

Using a combination of molecular tools similar to the ones described above, a recent improvement moved from targeted to untargeted *in situ* sequencing and markedly expanded the number of sequenced transcripts[85], which represents an important step forward. In this next-generation fluorescent *in situ* RNA sequencing (FISSEQ) approach, RNA is first reverse transcribed in fixed cells using tagged random hexamers, the low priming efficiency of which allows reduced representation of the cellular transcriptome. cDNA is then circularized, and clonally amplified RCPs are finally sequenced using SOLiD SBL with a 30-base read. To further reduce the crowding of transcripts that are to be sequenced (which would result in overlapping, unresolvable fluorescence spots), one could pre-extend the primer complementary to the tag that is attached to the hexamers used for reverse transcription, so that only a subset of transcripts is primed. Moreover, both the product of reverse transcription and clonally amplified RCPs are crosslinked to the cellular protein matrix, creating a 3D *in situ* RNA-seq library within the cell (FIG. 2d). Using a robust image-processing algorithm based on read alignment to reference sequences rather than signal intensity, a good correlation (Pearson's correlation coefficient >0.5) was obtained between transcript levels measured by FISSEQ and levels detected by RNA-seq of extracted RNA for moderately expressed genes. Importantly, FISSEQ was able to detect differential RNA subcellular localization and successfully captured known gene expression changes in a wound-healing assay. Even though this method represents a next-generation version of the original FISSEQ protocol, it remains to be seen how robustly it can perform in complex samples, such as in multidimensional tissue sections.

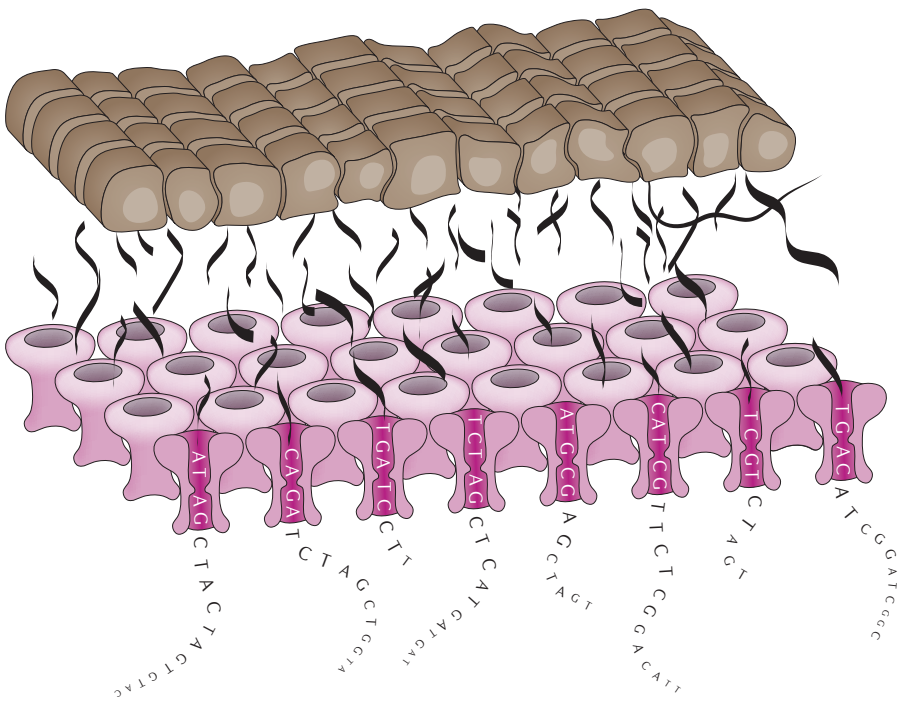


Figure 3 | **A future technology for spatially resolved omics: tissue-to-nanopore *in situ* sequencing.** DNA and RNA are forced out of a tissue section using an electrophoretic field. The tissue section is overlaid onto a nanopore sequencing device, so that DNA and RNA molecules are immediately sequenced as they flow out of the tissue section.

## Emerging spatially resolved omics

In addition to methods that enable RNA measurements with spatial definition, two landmark methods based on mass cytometry[86] have recently been developed to support spatially resolved measurements of multiple proteins and post-translational modifications in tissue sections[87,88]. To preserve spatial information, a scanning laser was used to ablate a mass-cytometry-labelled tissue section laid onto a solid support following a zigzag path[87,88]. During laser ablation, the tissue material was sent in real time through high-temperature plasma, followed by analysis using mass spectrometry. By synchronizing the speed of laser ablation with the time needed for the sample to travel to the mass spectrometer, 100 (REF. 88) and 32 (REF. 87) proteins and protein modifications were measured simultaneously with high spatial resolution in formalin-fixed, paraffin-embedded breast cancer tissue sections, delineating cell subpopulations and highlighting intratumour heterogeneity. These technologies pave the way to spatially resolved proteomic measurements, and they also have the potential to spark next-generation methods of immunohistochemistry that will revolutionize our understanding of disease processes and how pathology diagnostics is performed[89].

## Conclusions and perspectives

An extraordinary convergence of imaging and high-throughput sequencing technologies with methods that preserve positional information enables, for the first time, the measurement of up to thousands of different transcripts in cells and tissues while preserving spatial information. We anticipate that systematic application of these spatially resolved transcriptomic approaches in the coming years would have a profound impact in many fields, revealing the spatial architecture of complex transcriptional networks in normal and diseased tissues and organs, as well as contributing to the discovery of subcellular RNA localization patterns and the investigation of their functional implications. In parallel, technologies for spatially resolved proteomics are also emerging, and we anticipate that this paradigm will soon

be extended to other omic disciplines such as genomics and epigenomics, sparking the field of spatially resolved omics. The ultimate goal of all of these efforts will be to produce a comprehensive systems-level portrait of thousands of different molecular states — including DNA sequence variation and copy number, DNA and chromatin modifications, 3D genome organization, RNA and protein abundance and modifications — at various locations within cells and tissues, bringing us closer to a systems-level understanding of how complex multicellular organisms function by the coordinated action of millions of molecules simultaneously.

Despite great excitement driven by the broad range of potential applications, the path towards spatially resolved omics is still long and awaits bold new technologies to emerge. We envision that development of methods and technologies for spatially resolved transcriptomics and, more generally, spatially resolved omics would follow two complementary avenues. In the first avenue, new methods will be developed to detect large numbers of DNA and RNA molecules directly *in situ*, using either imaging or sequencing devices. Development of devices that support automated and fast cycles of smFISH hybridization, imaging and probe stripping (such as the sequential barcoding strategy (FIG. 1c)) will enable the expression of hundreds of genes to be measured in single cells from large tissue areas in a relatively short time and at affordable cost. Robust smFISH signals can be detected after as little as 5 minutes under special hybridization conditions[90]. Simultaneous DNA and RNA quantification in single cells is also feasible[21], and these 'cycleFISH' approaches are likely to be implemented in the near future. Additionally, RCA — which can amplify *in situ* hybridization signals by thousands of times — could enable the multiplexing of DNA and RNA measurements *in situ* in large tissue areas using low-magnification objective lenses. In parallel, it is very likely that a large amount of effort will be dedicated to adapt existing DNA and RNA sequencing approaches such that they can be applied directly to cells and tissue sections. We envision that third-generation sequencing devices, such as Oxford Nanopores, might be integrated with electrophoretic systems to force DNA and RNA molecules to move directly from a tissue section into the nanopores, where sequencing takes place (FIG. 3). Using high-density nanopores, this approach would enable *in situ* DNA and RNA sequencing at single-cell or even subcellular resolution.

A second avenue in the development of new methods and technologies for spatially resolved omics will be the integration of nucleic acid barcoding and genome-wide amplification methods with sequencing technologies. Nucleic acids or intact chromatin could be isolated from entire tissue sections as in microtomy sequencing, or from small tissue regions using LCM. Barcoding and nucleic acid amplification from each section or region, followed by sample pooling and construction of a sequencing library, would enable affordable sequencing of DNA and RNA simultaneously from multiple tissue sections or areas. Spatially resolved epigenomic assays — for example, chromatin immunoprecipitation followed by sequencing (ChIP–seq) and DNA methylation profiling[91] — might also be applicable based on the use of nucleic acid amplification methods such as linear DNA amplification (LinDA)[92]. A major task ahead will be to develop methods that enable simultaneous whole-genome and whole-transcriptome barcoding and amplification with spatial resolution, while leaving cells and tissues intact for subsequent targeted analyses, for example, using FISH and immunofluorescence methods. The ability to perform spatially resolved, multi-assay omic measurements in low-input samples would markedly advance our ability to carry out high-throughput correlative studies in precious clinical specimens and model organisms. Importantly, these efforts will strongly benefit from methods that increase the transparency and penetrability of tissues — such as CLARITY[93,94], whole-body clearing[95] and CUBIC[96] methods — which enable tissue labelling and deep imaging in the context of intact organs. We are confident that, with this growing panoply of methods and technologies, spatially resolved omics will soon become a mature field and will substantially advance our knowledge of complex multicellular organisms.

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**Competing interests statement**

The authors declare no competing interests.

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