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Next-generation sequencing-based spatiotemporal transcriptomics: the next wave of spatial transcriptomics

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Highlights:

- Nucleic acid barcoding strategies for NGS-based spatial transcriptomics platforms are reviewed.
- The evolution from spatial to spatiotemporal transcriptomic technologies is explored.
- Metabolic RNA labeling tools for capturing high-resolution RNA dynamics are highlighted.
- The pathological applications, current bottlenecks, and future directions of NGS-based spatiotemporal transcriptomics are discussed.

Abstract: Tissues serve as the functional units of multicellular organisms with intricate spatial organizational complexity. Next-generation sequencing (NGS)-based spatially resolved transcriptomics (SRT) delineates *in situ* gene expression heterogeneities, establishes high-fidelity associations between transcripts and spatial pixels by segmenting a tissue section into spatial pixels, and elucidates the pivotal roles of cellular spatial organization in biological processes and complex pathological mechanisms. The synergistic integration of nucleic acid barcoding and high-throughput sequencing technologies has rapidly advanced the development of these spatial modalities regarding throughput, resolution, cost-effectiveness, and sensitivity. In this review, we summarize the state-of-the-art sequencing-based spatial transcriptomics, with a primary emphasis on the methodologies. We first introduce two major categories of typical nucleic acid barcoding platforms (*in situ* barcoding-based and barcoding array-based) used for spatial localization and transcriptome profiling. Then, the burgeoning trend of spatial transcriptomics towards spatiotemporal transcriptomics, which integrates SRT with the temporal dimension to provide more holistic landscapes of gene expression networks, is discussed. We also highlight spatiotemporal transcriptomics based on metabolic RNA labeling that provides unprecedented resolution to resolve transcriptome-wide dynamics in space and time. The emerging applications of these technologies in providing mechanistic insights into complex pathological mechanisms are also discussed. Finally, the perspectives on current

bottlenecks and future direction of spatiotemporal transcriptomics are provided.

Keywords: spatiotemporal transcriptomics; spatial nucleic acid barcoding; next-generation sequencing; metabolic RNA labeling

1. Introduction

In multicellular organisms, tissue function and disease progression—particularly during embryonic development and within the tumor microenvironment—are profoundly dependent on spatial cellular organization and intercellular interactions [1]. Although cellular heterogeneity can be resolved at high resolution by next-generation sequencing (NGS)-based single-cell transcriptomics, native spatial information is inherently obliterated by the requisite tissue dissociation process [2]. To circumvent this technical limitation, spatially resolved transcriptomics (SRT) has been developed [3]. By leveraging diverse physicochemical strategies and nucleic acid barcoding, precise correlations between transcripts and their *in situ* spatial coordinates are established [4]. Through this spatial mapping, high-dimensional molecular landscapes are reconstructed while spatial context is preserved, thereby providing a breakthrough paradigm for the elucidation of cellular networks and complex pathological mechanisms [5].

Early acquisition of spatial transcriptomic data predominantly relied on two main avenues, physical spatial isolation and *in situ* imaging, both of which struggle to balance throughput, resolution, and detection efficiency [6]. Physical isolation techniques represented by laser capture microdissection (LCM) [7], such as Geo-seq [8], achieve the alignment and integration of transcriptomic and spatial data by precisely excising regions of interest (ROIs) under a microscope for subsequent RNA extraction and sequencing. However, LCM relies heavily on manual or semi-automated repetitive operations, resulting in exceedingly low detection throughput. Furthermore, it struggles to achieve authentic single-cell resolution within dense tissues, rendering it inadequate for the comprehensive analysis of whole-tissue sections [9].

Distinct from macroscopic physical excision, imaging-based *in situ* hybridization (ISH) or *in situ* sequencing (ISS) technologies opt to directly detect target transcripts on tissue sections [10]. These technologies utilize DNA probes to label mRNAs and achieve the quantification and localization of mRNAs via fluorescence imaging [11]. Imaging-based SRT technologies feature single-molecule resolution, demonstrating significant advantages in delineating subcellular transcriptomic atlases and revealing precise intracellular molecular interactions. Despite achieving subcellular resolution, these approaches necessitate extensive imaging to acquire a full view of gene expression in tissues and predominantly rely on targeted probes to detect predefined gene panels, thereby suffering from prolonged experimental procedures and low throughput.

Given these constraints, the spatial transcriptomics field urgently requires an unbiased and genome-wide methodology with high throughput and high spatial resolution. An ideal way to profile spatial gene expression is to segment a tissue section into “pixels” as much as possible and perform NGS for RNAs in each pixel while preserving its spatial location. Therefore, the spatial gene expression patterns can be reconstructed with high resolution and high accuracy. However, conventional strategies relying on physical compartmentalization, such as LCM or tissue microdissection, face insurmountable bottlenecks in practical application. For these optical microdissection methods, as spatial resolution is progressively enhanced, the required number of physical partitions and consequently individual sequencing libraries

scales quadratically, increasing from a baseline of 1 to 4, 16, and beyond 100 (Figure 1). This geometric expansion not only incurs prohibitive time, labor, and reagent costs but also introduces confounding batch effects. To resolve this inherent trade-off between throughput and resolution, researchers have introduced spatial barcoding technology to enable pooled sequencing. Contrasting with physical dissection methods, current array-based capture platforms (e.g., 10x Genomics Visium, Stereo-seq) utilize this approach. Prior to pooling all targeted RNA for the construction of a single sequencing library, predefined DNA sequences are utilized to *in situ* allocate unique coordinate identifiers to each individual pixel, thereby bridging the capture of microscopic-scale information with the efficiency of macro-scale library construction.

DNA barcodes possesses exponential encoding capacity. A nucleotide sequence of length N, composed of the four bases A, T, C, and G, can yield 4^N orthogonal barcode combinations. This implies that a mere fragment of sequence is sufficient to assign a globally unique identifier to every single molecule within a tissue section spanning a vast field of view. Furthermore, capture probes with DNA barcodes for each pixel can be densely arranged within an extremely confined physical space. This molecular characteristic perfectly satisfies the stringent probe density requirements for high sensitivity and high spatial resolution in single-cell and subcellular multi-omics analyses. Most critically, the inherent programmability of DNA arrays enables the establishment of a deterministic mapping relationship between barcode sequences and physical coordinates (X_i,Y_j) across a two-dimensional plane. This ingenious design effectively translates the two-dimensional physical space of the tissue into a one-dimensional digital sequence space. Due to the high barcoding complexity and efficiency of DNA barcodes, spatial DNA barcoding has been integrated with NGS to establish NGS-based spatial transcriptomics for genome-wide profiling of spatial gene expression with high resolution, high efficiency, and low cost.

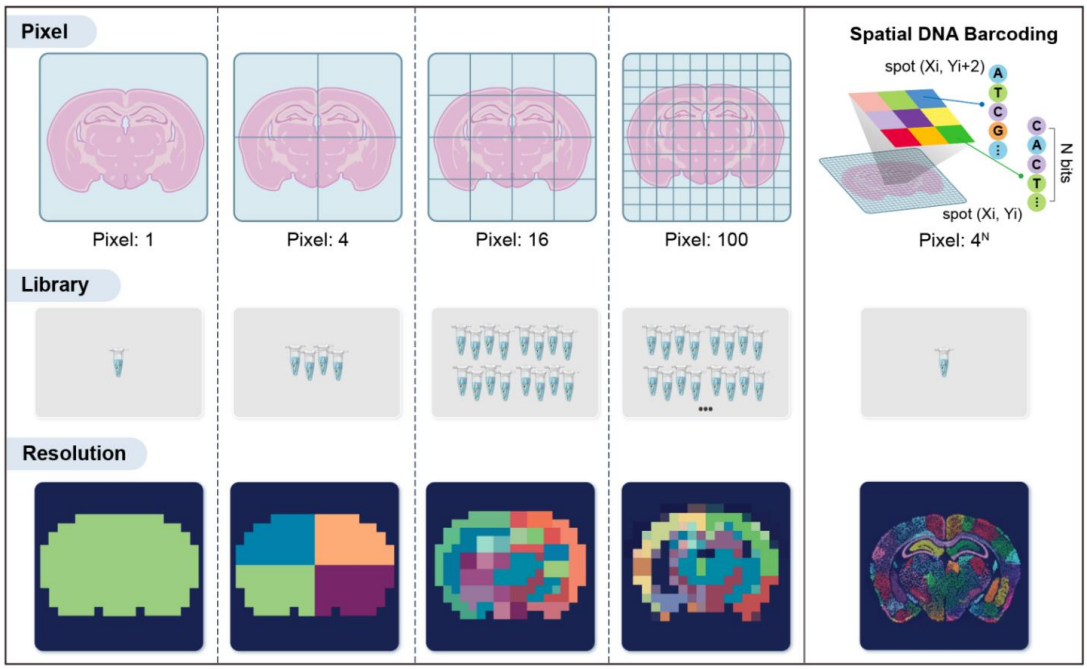

Figure 1. Schematic of spatial resolution evolution and spatial DNA barcoding.

In this review, we first summarize the recent advances of NGS-based spatial transcriptomics technologies and discuss their principles. We focus on different nucleic acid barcoding strategies that encode spatial location information into RNAs in tissues, converting physical positional information into

nucleic acid sequences readable by high-throughput NGS. We explore how these innovations progressively overcome the physical bottlenecks of conventional techniques regarding throughput, spatial resolution, and detection sensitivity, while systematically summarizing their unique technical advantages and applicable scenarios. Then, we highlight NGS-based spatiotemporal transcriptomics, a cutting-edge direction of NGS-based spatial transcriptomics that adds a temporal dimension to conventional spatial transcriptomics. We discuss different strategies (e.g., time-series snapshots, genetic barcode tracing, and metabolic RNA labeling) to profile the spatiotemporal dynamics of gene expression in biological processes. We emphasize that metabolic RNA labeling-based spatiotemporal transcriptomics enables the unbiased *in situ* capture of transcriptome-wide RNA dynamics, providing a novel tool for deciphering the spatiotemporal regulatory principles of gene expression and deepening our understanding of health and disease. Finally, the current challenges and future directions of NGS-based spatiotemporal transcriptomics are also discussed.

2. Spatial DNA barcoding strategies for NGS-based spatial transcriptomics

Based on the distinct core mechanisms of spatial barcode introduction, spatial DNA barcoding and NGS-based spatial transcriptomics can be classified into two primary categories (Figure 2). The first one is *in situ* coding technologies, which directly introduce spatial tags to transcripts within the native tissue cells prior to nucleic acid extraction and library preparation for sequencing. The second one is *in situ* capture technologies, which employ pre-fabricated microarrays with spatially barcoded capture probes to capture mRNAs released from tissues while simultaneously accomplishing positional anchoring, followed by sequencing to decode the spatial information.

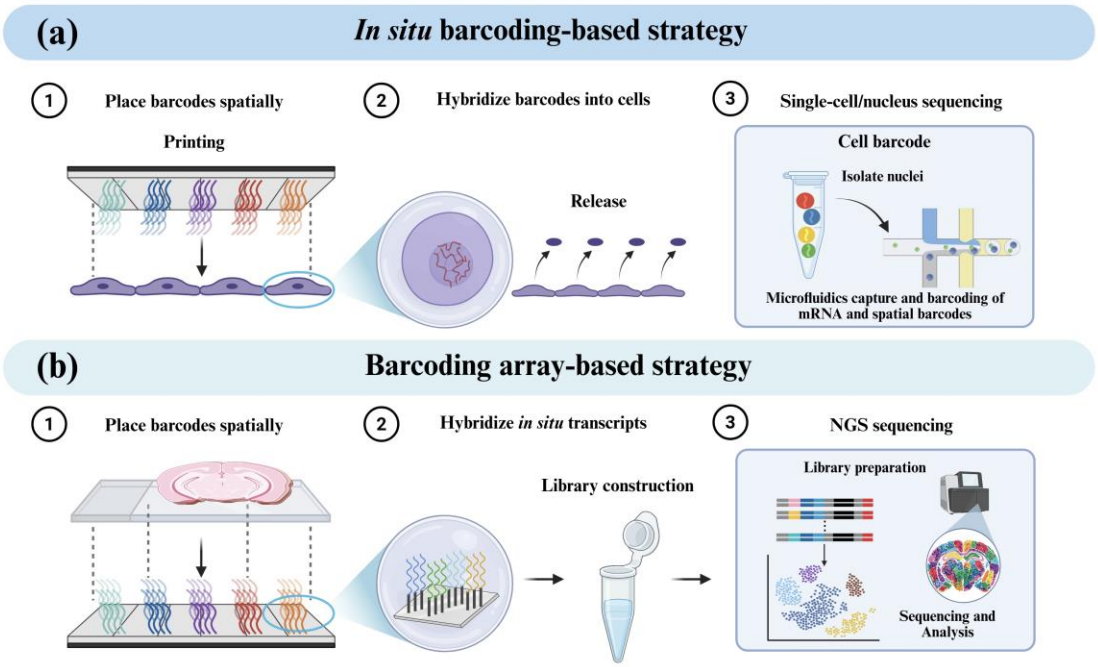

Figure 2. Overview of the nucleic acid barcoding strategies in NGS-based spatial transcriptomics. Schematic illustrating the two core paradigms: **(a)** *in situ* barcoding-based (direct barcode introduction within tissue) and **(b)** barcoding array-based (microarray-based mRNA capture with pre-fabricated spatial barcodes).

2.1. In situ barcoding-based strategies

In the context of tissue, the direct encoding of spatial information into RNA sequences is facilitated by the hybridization of engineered DNA barcode probes with target RNA or cDNA, coupled with subsequent ligation and amplification processes. Through the ultimate readout of these barcodes via sequencing, the precise spatial localization of transcripts and the comprehensive profiling of gene expression are enabled (Figure 3).

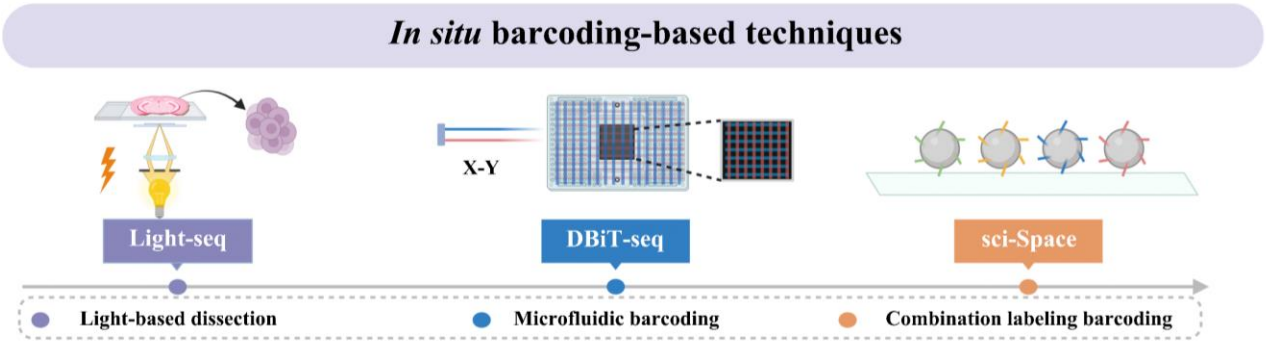

Figure 3. Summary of the typical *in situ* barcoding-based techniques.

2.1.1. Photochemistry-based *in situ* barcoding technologies

(1) Photocaging

Photocaging strategies leverage light as a high-precision spatial switch. By actively directing the cleavage of photolabile caging tags on photosensitive chemical moieties, these methods enable the precise transcriptomic labeling of specific ROIs within complex tissues.

Lovatt *et al*. designed a photoactivatable mRNA capture molecule featuring a photocaged hairpin structure for single-cell transcriptome *in vivo* analysis (TIVA) [12]. This probe utilizes an o-nitrobenzyl (ONB) photocleavable group to cage the capture sequence, keeping the poly(2′-deoxy-2′-fluorouridine) capture oligos, a poly-U analog, strictly inactive in the absence of light. Upon targeted illumination of live cells, the uncaging reaction is triggered to expose the capture oligos, allowing them to bind the polyA tails of intracellular mRNAs *in situ*. While this enabled the first minimally invasive and targeted extraction of single-cell transcriptomes directly within live tissues, TIVA remains applicable only to live samples. Furthermore, because a single experimental round labels only a limited number of cells, achieving large-scale, high-throughput spatial analysis across whole tissues is highly challenging.

To enable high-resolution spatial transcriptome analysis of fixed tissues, Honda *et al*. developed transcriptome profiling coupled with photoisolation chemistry (PIC) [13]. This technique employs photocaged oligodeoxynucleotides (caged ODNs) as primers for *in situ* reverse transcription (RT). By modifying the deoxythymidine residues with 6-nitropiperonyloxymethyl (NPOM) photoprotective groups, the resulting NPOM-dTs leverage steric hindrance to strictly suppress the read-through extension activity of the polymerase. Following the definition of ROIs via immunostaining, targeted spatial photouncaging activates the synthesis process, permitting the extraction of cDNA-mRNA hybrids. Coupled with DMDs, PIC ultimately achieves transcriptome profiling at the level of subcellular and subnuclear microstructures.

To overcome the throughput bottlenecks inherent in preceding endogenous capture or synthesis technologies, Krummel's group developed ZipSeq, a technique that utilizes light-directed printing to directly conjugate spatial barcodes onto live cell surfaces [14]. Specifically, ultraviolet (UV) illumination uncages the NPOM groups on membrane-bound anchor strands, permitting the exposed overhangs to hybridize with spatially encoded Zipcode (ZC) strands. This strategy successfully assigns DNA tags to cells within precise ROIs. During subsequent single-cell RNA sequencing (scRNA-seq), these surface-tagged ZC strands are co-sequenced alongside intracellular mRNAs to enable spatially resolved transcriptomic analysis. Through multiple rounds of illumination, ZipSeq enables multiplexed ROI labeling, offering theoretically infinite scalability for its multiplexing encoding capacity. In the tumor microenvironment, ZipSeq revealed novel gene expression patterns tied to distinct histological structures, thereby illuminating how local microenvironments regulate cellular transcriptional heterogeneity.

(2) Photocleavage

Photocleavage strategies employ UV light as a spatial optical scalpe l. By directing the targeted cleavage of specific photocleavable fluorescent tags, this approach precisely releases pre-encoded spatial information.

To achieve spatial multiplex analysis of both transcriptomes and proteins across distinct ROIs in tissue sections, Merritt *et al*. developed the digital spatial profiling (DSP) system [15]. This technology utilizes UV-photocleavable linkers, typically o-nitrobenzyl derivatives, to integrate three essential moieties into a single functional unit: a unique indexing oligo for quantification, an affinity reagent (antibody or mRNA probe) for target recognition, and a photocleavable linker. Coupled with a programmable digital mirror device (DMD) and iterative illumination, the system sequentially releases the indexing oligos within different ROIs. Following collection via microcapillary aspiration, separate sequencing libraries are constructed to read out proteins and mRNAs. While DSP is capable of single-cell resolution with an mRNA analysis throughput of over 1000 genes, its reliance on pre-designed targeted probes and the requirement for separate sequencing libraries for distinct ROIs make it incompatible with unbiased unknown gene profiling and whole-slide high-throughput spatial barcoding.

To extend photocleavage-based *in situ* barcoding from localized targeting to global single-cell resolution, Macosko *et al*. developed Slide-tags, a technique utilizing dense arrays of spatially indexed DNA-barcoded beads [16]. In this approach, intact tissue sections are mounted onto 10 μm DNA-barcoded bead substrates. Each bead is functionalized with spatial barcode oligos, which encode positional information, via photocleavable linkers. Global UV illumination triggers the photocleavage reaction, releasing the spatial barcodes to tag cellular nuclei, thereby assigning spatial coordinates to the captured mRNAs. Through its application to the mouse hippocampus, Slide-tags achieved highly sensitive transcript capture, establishing a new benchmark for spatial transcriptomic profiling at intrinsically single-cell resolution.

(3) Photocrosslinking

Diverging from the aforementioned bond-cleavage mechanisms, photocrosslinking strategies leverage photo-induced bond formation to establish *in situ* covalent conjugation.

Yin's group developed Light-Seq, utilizing light-directed DNA barcoding of cDNAs for multiplexed spatial indexing sequencing (Figure 4) [17]. This technique utilizes 3-cyanovinylcarbazole nucleoside (CNVK), an ultrafast photo-cross-linker, to anchor spatial DNA barcodes directly and

covalently *in situ* onto cDNAs within ROIs under 365 nm UV illumination. Subsequently, RNase H treatment is applied to specifically degrade the RNA within the hybrid strands, permitting the gentle extraction of barcoded cDNAs while preventing sample loss. By enabling the joint analysis of morphology, tissue context, and transcriptome within the same cells, this technology achieves a comprehensive, multidimensional measurement of cell states and intercellular interactions. Furthermore, Light-Seq enables rare cell transcriptomics analysis, identifying specific biomarkers for a very rare neuronal subtype in mouse retinal sections for the first time.

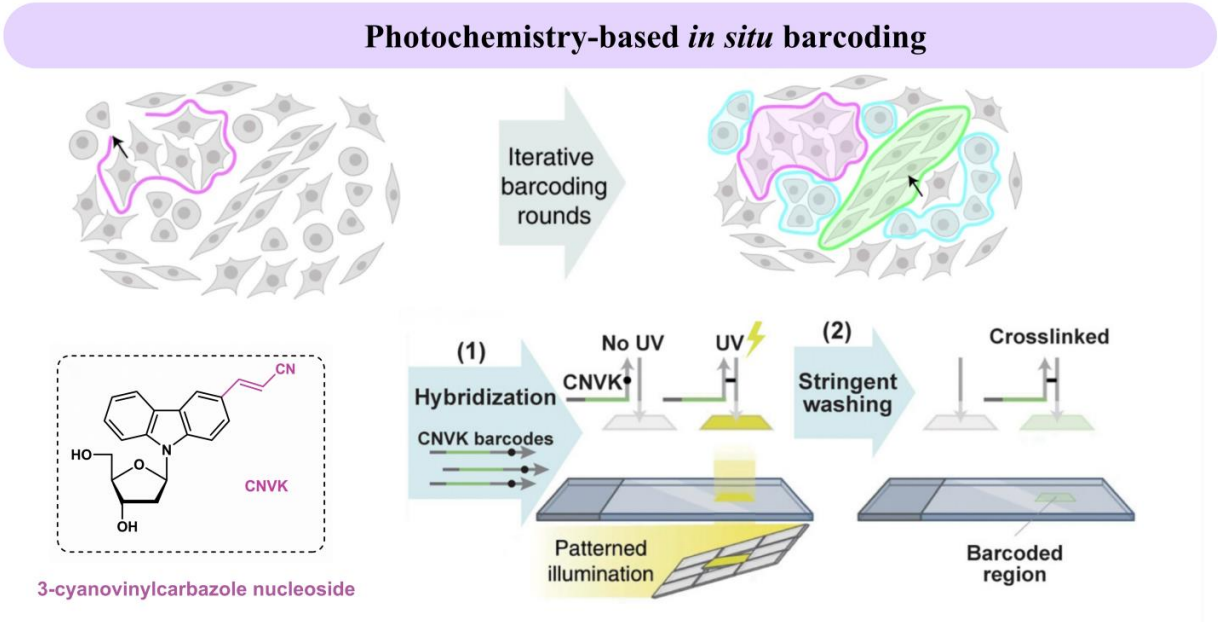

Figure 4. Photochemistry-based *in situ* barcoding technologies. These techniques use light as a high-precision spatial switch for targeted ROI labeling or global barcode release. Reprinted with permission [17]. Copyright 2022 Springer Nature.

In summary, photochemistry-based strategies offer superior localization precision and sensitivity, rendering them ideal for rare cell identification and non-destructive tissue profiling. However, the substantial reliance on iterative illumination or complex biochemical extraction protocols results in cumbersome workflows and limited spatial indexing throughput. These inherent constraints hinder the direct extension of such methodologies to the high-throughput mapping of large-scale whole tissues.

2.1.2. Microfluidics-based *in situ* barcoding technologies

Microfluidics-based *in situ* barcoding strategies capitalize on physical confinement and fluid dynamics. By perfusing oligo solutions of DNA barcode directly across the tissue via parallel microchannel networks, these approaches effectively overcome the throughput bottlenecks inherent to optical targeting.

Fan's group developed deterministic barcoding in tissue for spatial omics sequencing (DBiT-seq) [18]. This technique employs a pair of perpendicular polydimethylsiloxane (PDMS) microchannel chips to establish an orthogonal microfluidic network across tissue sections. Initially, barcodes A are delivered into the permeabilized tissue via the first set of parallel microchannels, followed by the introduction of barcodes B through the perpendicular set. At the intersections, they undergo *in situ* enzymatic ligation to

generate distinct combinatorial barcodes, forming a two-dimensional tissue pixel mosaic for position indexing. By flexibly altering the channel width, the system achieves tunable spatial resolutions ranging from 10 μm to 50 μm. Furthermore, DBiT-seq demonstrates robust compatibility with clinical formalin-fixed paraffin-embedded (FFPE) samples and enables the simultaneous spatial co-mapping of whole transcriptomes and hundreds of proteins, offering a reliable approach for the high-resolution profiling of complex tissue microenvironments.

To achieve multiplexed analysis of tissue samples, Wirth *et al*. developed the Multiplexed Deterministic Barcoding in Tissue (xDBiT) workflow, optimizing the biochemical efficiency and operational throughput of DBiT-seq (Figure 5) [19]. By designing serpentine microchannel chips, xDBiT spatially barcodes nine tissue sections in parallel, expanding the barcoded area to 1.17 cm^2. Diverging from the DBiT-seq protocol, xDBiT performs the reverse RT reaction across a whole tissue section, followed by two rounds of lower-temperature ligation reactions within the microchannels to introduce spatial barcodes. This strategy mitigates the leakage risk associated with these channels because the ligation steps require shorter incubation times and lower temperatures than reverse transcription inside the channels, and dehydrating the tissue beforehand improves chip attachment. Concurrently, it enhances overall ligation efficiency by increasing ligase concentration and performing reverse transcription across the whole tissue to maximize enzyme availability. With these chemistry optimizations, xDBiT achieves a 3.0-fold increase in both read and gene counts per spot. Consequently, xDBiT holds great potential for research projects focused on profiling 3D tissue or organ atlases and mapping spatiotemporal expression dynamics in longitudinal studies.

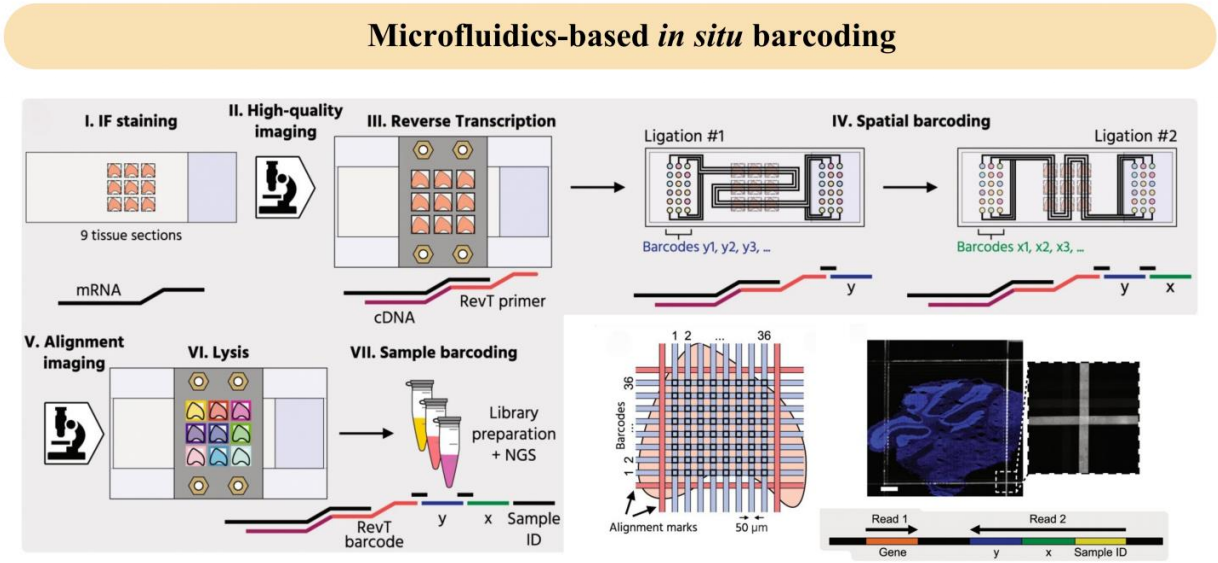

Figure 5. Microfluidics-based *in situ* barcoding technologies. Orthogonal microchannel designs that deliver barcodes via perpendicular or serpentine networks to achieve combinatorial spatial indexing at tunable resolutions. Reprinted with permission [19]. Copyright 2022 Springer Nature.

Collectively, microfluidics-based strategies provide high-throughput capabilities and operational simplicity while maintaining robust compatibility with clinical specimens. Nevertheless, the integrity of precision microchannels is frequently compromised by fluid leakage or clogging when applied to uneven tissue surfaces. Additionally, since the spatial resolution of these platforms generally reaches only near-

single-cell dimensions, the direct delineation of authentic single-cell boundaries within complex tissues remains a significant challenge.

2.1.3. Physical permeation-based *in situ* barcoding technologies

When profiling large-scale macroscopic tissues, relying exclusively on microfluidic networks often fails to sustain stable fluidic boundaries. To overcome this limitation, physical permeation strategies capitalize on macroscopic spatial confinement and concentration-gradient-driven diffusion to execute "split-pool" spatial indexing.

Lee *et al*. developed XYZeq, a technique integrating physical partitioning with permeabilization-diffusion strategies, to achieve spatially resolved scRNA-seq of tumor tissues (Figure 6). In this approach, tissue sections are physically partitioned into 500 μm microwells for a first round of *in situ* reverse RT, which assigns spatial barcodes [20]. The cells are subsequently pooled and split for a second round of polymerase chain reaction (PCR) indexing to generate combinatorial cellular barcodes. Through these two rounds of combinatorial barcoding, the RT spatial index and PCR cellular index act together to constitute a unique spatial barcode for each single cell, enabling the precise mapping of single-cell transcriptomic data back to their original tissue locations following sequencing. This method demonstrates robust signal specificity in resolving the regional heterogeneity of complex tissues, such as the tumor microenvironment.

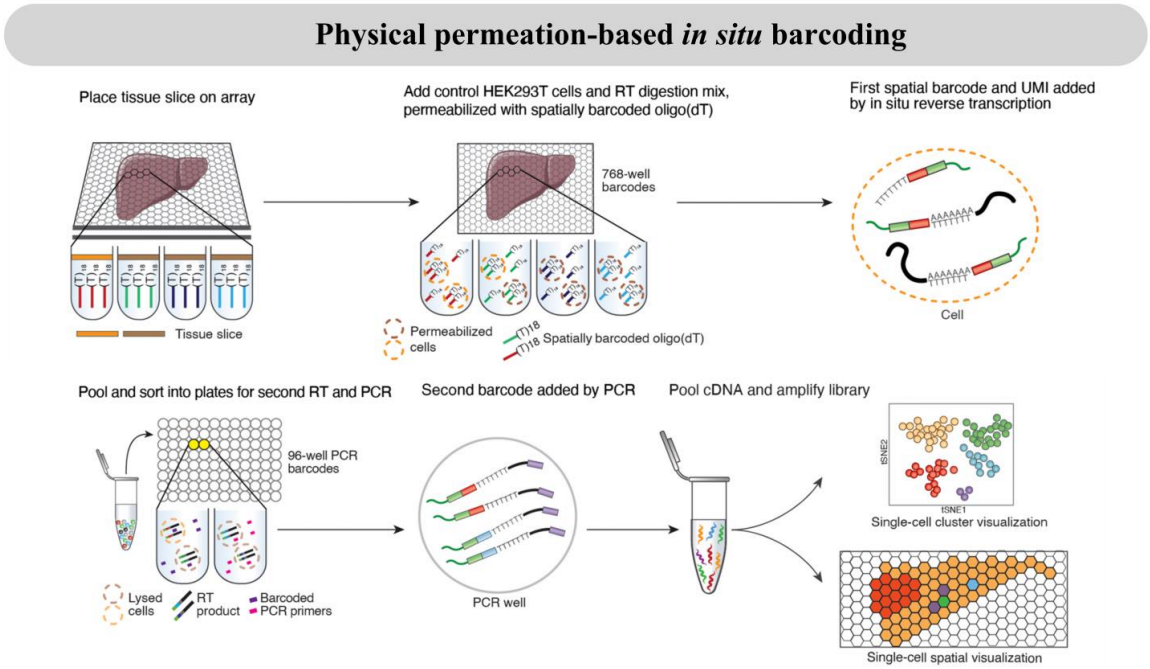

Figure 6. Physical permeation-based *in situ* coding technologies. Split-pool strategies utilizing physical confinement and diffusion to assign spatial barcodes via multi-round combinatorial indexing. Reprinted with permission [20]. Copyright 2021 The American Association for the Advancement of Science.

Trapnell's group developed sci-Space, which integrates flexible hydrogel media with a multi-round combinatorial indexing system to achieve embryo-scale, single-nucleus spatial transcriptomic mapping [21].

This technique pre-prints spatial hashing oligos onto agarose-coated slides to construct a gridded array uniquely marked by combinations of sector and spot barcodes. Leveraging the porous characteristics of the hydrogel, these barcodes are guided to vertically permeate into tissue nuclei, thereby implanting primary spatial position tags. Subsequently, the intact nuclei are extracted and subjected to multiple rounds of split-pool combinatorial indexing between 96-well plates and PCR plates, ultimately reconstructing large-scale spatial transcriptomic maps of the tissue at single-cell resolution. This evolution from diffusion-based tagging to high-throughput indexing enables the simultaneous capture of transcriptomes and spatial coordinates from hundreds of thousands of nuclei within a centimeter-scale macroscopic field of view, providing a powerful tool to decipher dynamic cellular migration patterns during embryonic development.

Collectively, physical permeation-based strategies facilitate the mapping of macroscopic tissues at an exceptionally low cost while retaining the high capture rate characteristic of single-cell sequencing. Nevertheless, their profound reliance on uncontrollable molecular Brownian motion exacerbates lateral diffusion, thereby inherently restricting their authentic resolution to the multicellular regional level.

2.2. Barcoding array-based strategies

Barcoding array-based strategies utilize primers of spatially barcoded arrays to capture RNA at corresponding tissue locations *in situ* (Figure 7). By subjecting the captured RNA to *ex situ* sequencing and subsequently employing computational algorithms for the visual analysis of the integrated spatial barcodes, a comprehensive tissue transcriptional atlas can be reconstructed.

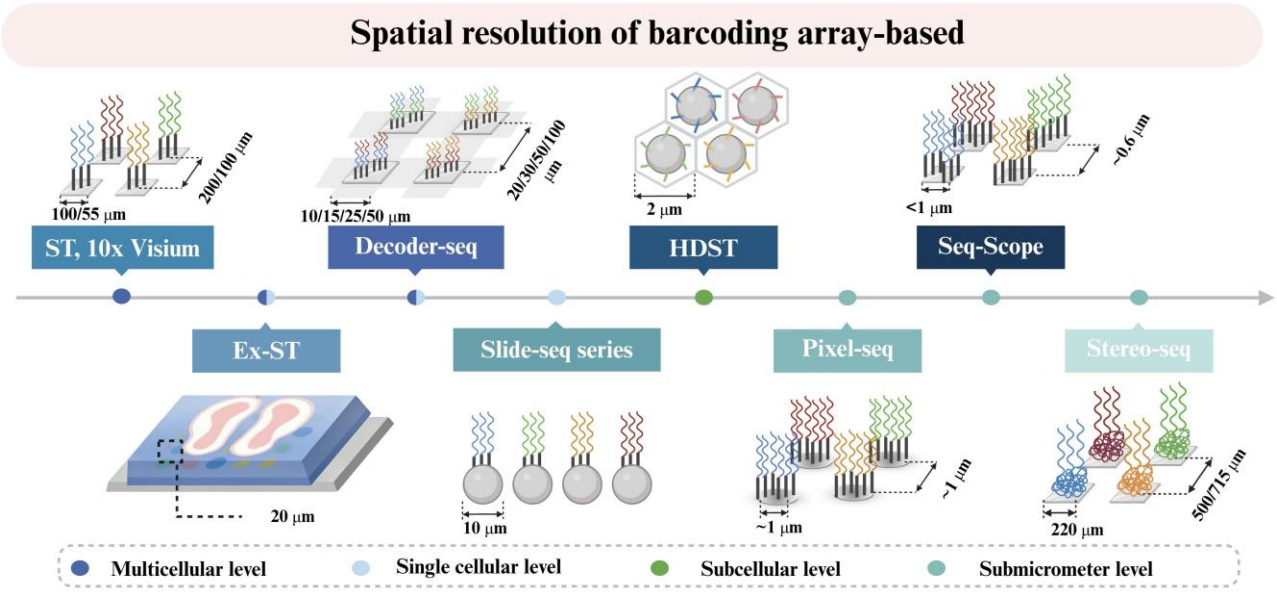

Figure 7. Summary of the spatial resolution of typical barcoding array-based techniques.

2.2.1. Barcoding arrays with multicellular resolution

Printed array-based barcoding platforms pioneered the *in situ* capture and barcoding of RNAs from tissue sections at multi-cellular resolution, establishing the technological foundation during the early phase of NGS-based spatial transcriptomics.

In 2016, Stahl *et al*. developed spatial transcriptomics (ST), which was the first spatial barcoding method [22]. As a pioneering milestone in the field, its core lies in utilizing a microarrayer spotting robot to construct a spatially barcoded array on glass slides. Featuring capture spots with a diameter of 100 μm and a center-to-center distance of 200 μm, this platform employs polyT oligos to *in situ* capture mRNA molecules within tissue sections. Because each individual spot physically encompasses 10 to 50 cells, the initial resolution of ST was primarily confined to multicellular regions or the macroscopic tissue level.

Building upon ST, 10x Genomics integrated and optimized its physical architecture and biochemical workflows to launch the widely applied Visium platform [23]. This system reduces the capture spot diameter from the original 100 μm to 55 μm and compresses the center-to-center distance to 100 μm, significantly enhancing spatial sampling density and data quality. Furthermore, comprehensive upgrades to probe chemistry and tissue permeabilization systems enable robust compatibility with diverse tissue sample types, including cryosections and FFPE tissues. Nevertheless, constrained by the physical limits of microarray spotting, the 55 μm spots still encompass several heterogeneous cells within most complex tissues; consequently, downstream analysis remains highly reliant on computational deconvolution algorithms to infer detailed cellular compositions.

2.2.2. Barcoding arrays with near single-cell resolution

To engineer spatial transcriptomic substrates with feature sizes approaching near-single-cell dimensions, researchers have pioneered three core methodologies: tissue expansion, random bead deposition, and substrate material enhancement. The implementation of these strategies effectively propels spatial profiling toward ultra-high resolution.

Constrained by factors such as droplet evaporation, merging, and instrumental accuracy, generating barcoded spots approaching a single-cell diameter via microarray spotting remains highly challenging. To overcome this limitation, rather than directly shrinking spot sizes, Wang's group developed expansion spatial transcriptomics (Ex-ST) [24]. Drawing upon the isotropic expansion principles of expansion microscopy, this technique embeds tissue specimens into a polyacrylate gel. Subsequent enzymatic digestion and physical expansion allow the system to bypass the hardware limits of conventional chips. During this protocol, endogenous RNA molecules are covalently anchored to the gel network; subsequently, water-driven swelling induces an approximately 2.5-fold linear expansion of the hydrogel, physically increasing intercellular distances. The expanded sample is then mounted onto standard Visium arrays with a 55 μm spot size for transcript capture, improving the equivalent spatial resolution to a nearly 20 μm scale. While preserving whole-transcriptome capture capabilities, this strategy significantly enhances the analytical resolution for rare transcripts and fine tissue structures, objectively compensating for the precision deficits of commercial hardware sensors through the physical deformation of the specimen itself.

Beyond tissue expansion strategies, shrinking the dimensions of spatial barcodes offers an alternative route to near-single-cell resolution. Chen's group developed Slide-seq [25], a technique utilizing split-pool synthesis to fabricate 10 μm DNA-barcoded beads, which are subsequently randomly packed onto glass slides to construct a capture array. Its upgraded iteration, Slide-seqV2 [26], systematically optimizes biochemical efficiency by refining the enzymatic system for second-strand synthesis, thereby overcoming tissue-induced inhibition during the reverse transcription reaction.

Consequently, this modification yields an average capture of approximately 550 UMIs per bead. Such sensitivity successfully supports the mapping of neuronal processes and developmental trajectories at the single-cell level, signifying that random bead-based capture protocols are gradually approaching the detection sensitivity of scRNA-seq technologies.

To overcome the capture bottlenecks of two-dimensional planar surfaces, Yang's group developed the dendrimeric DNA coordinate barcoding design for spatial RNA sequencing (Decoder-seq) [27]. This technique employs a microfluidics-based combinatorial barcoding strategy to preassemble barcoded DNA arrays onto 3D dendrimer substrates. Featuring abundant active primary amino groups, these dendrimers enable the conjugation of high-density amino-terminated oligos via a disuccinimidyl suberate cross-linker. Such a 3D architecture not only mitigates the steric hindrance inherent to traditional planar chips but also extends the effective contact distance between probes and tissue mRNAs, significantly enhancing capture accessibility. Consequently, at a near-cellular resolution of 15 μm, this technology achieves a robust capture yield of 40.1 UMIs per square micrometer (μm²), providing a powerful platform for profiling the spatial distribution of lowly expressed transcripts, including mouse olfactory receptor (Olfr) genes.

While maintaining robust detection performance, Zhao's group developed microfluidics-assisted grid chips for spatial transcriptome sequencing (MAGIC-seq) [28], providing a cost-effective platform for large-area spatial transcriptomic mapping through an innovative microfluidic chip design and chemical cross-linking system. Integrating carbodiimide chemistry with spatial combinatorial indexing, this technology employs customized microfluidic grid chips to directly deliver spatial barcodes onto the tissue surface, enabling tunable detection resolutions ranging from 10 μm to 50 μm. By overcoming the capture area limitations inherent to conventional microfluidics, MAGIC-seq efficiently profiles macroscopic tissue sections, such as the whole mouse brain. Consequently, its high-throughput and economical attributes establish a critical foundation for constructing large-scale developmental atlases and 3D tissue models.

2.2.3. Barcoding arrays with subcellular resolution

Early explorations of sub-cellular resolution were primarily achieved by downscaling spatial capture features to the micrometer scale. Lundeberg's group developed high-definition spatial transcriptomics (HDST) [29], a technique that performs spatial barcoding by randomly packing 2 μm DNA-barcoded beads into high-precision hexagonal microwell arrays. Fabricated via a split-and-pool ligation reaction, these beads are subsequently decoded through sequential hybridization. Leveraging its subcellular resolution, the platform successfully identified transcripts exhibiting preferential nuclear localization. Nevertheless, restricted by the technical difficulties associated with synthesizing and densely packing micrometer-scale DNA beads, this class of barcoding methods remains challenging in achieving submicrometer spatial resolution.

To mitigate signal loss arising from physical gaps in bead arrays and further improve capture efficiency, 10x Visium HD employs semiconductor-grade photolithography to fabricate a continuous probe grid comprising 2 μm × 2 μm pixels [30]. This seamless matrix arrangement captures tissue-released mRNAs *in situ*, substantially elevating transcript capture rates within these micrometer-scale pixels. Additionally, the platform incorporates an RNA-templated ligation mechanism that utilizes paired probe

hybridization to significantly enhance analytical fidelity for degraded samples. While the continuous grid resolves issues of signal continuity, practical analysis typically requires data binning to 8 μm or 16 μm to ensure a robust statistical signal-to-noise ratio.

2.2.4. Barcoding arrays with submicron resolution

As spatial transcriptomics advances toward sub-micrometer resolution, a wide field of view, and high throughput, random barcoding strategies utilizing DNA clusters and nanoballs have emerged as a core technological trajectory.

Seq-Scope, developed by Cho *et al*. [31], alongside open-source platforms Nova-ST [32] and Open-ST [33] that share the identical Illumina flow cell repurposing logic, achieves submicrometer resolution to enable spatial transcriptomic profiling at the single-cell and subcellular levels. These methods directly leverage solid-phase bridge amplification on flow cell surfaces to generate high-density DNA cluster arrays featuring a diameter of ~300 nm and a center-to-center distance of ~600 nm. Within each cluster, the oligonucleotides comprise a PCR/read adaptor, a unique spatial barcode, and a cleavable polyT sequence. Following spatial barcode readout via sequencing-by-synthesis for position indexing, subsequent cleavage exposes the polyT tail for *in situ* mRNA capture. By bypassing the sampling frequency constraints inherent to conventional microfluidics and capitalizing on semiconductor-grade manufacturing, this hardware repurposing strategy reduces preparation costs by nearly 80%. Consequently, it provides a high-throughput platform for resolving nanoscale transcriptomic atlases within neuronal synapses and complex tissue microenvironments.

The costly and time-consuming process of decoding spatial barcode sequences poses significant challenges for upscaling array production. To address this bottleneck, Gu's group developed polony-indexed library-sequencing (Pixel-seq) [34], utilizing polony gel stamping to achieve the repeatable and scalable replication of DNA cluster arrays (polonies). In this workflow, polonies are fabricated via bridge amplification on an elastomeric, cross-linked polyacrylamide gel to serve as templates. Through DNA polymerase-catalyzed chain extension, these templates are subsequently copied onto multiple gels. Because both primers and templates are covalently conjugated to mitigate DNA diffusion, this gel-to-gel replication reliably maintains the original resolution and sequence information. Consequently, decoding sequencing is required for only a single or a few gels within a replication series. By generating high-density polonies (~0.6–0.8 million per mm^2) with a feature diameter of ~1 μm and minimal feature-to-feature gaps, this technique—following cell segmentation—facilitates high-resolution spatial transcriptomic profiling of specific cell types, including periglomerular-type and mitral/tufted cells.

In pursuit of expanded capture areas and nanoscale precision, Chen *et al*. developed spatial enhanced resolution omics-sequencing (Stereo-seq) [35], establishing a new benchmark for subcellular resolution through rolling circle amplification (RCA) and high-precision deposition processes. By utilizing DNA nanoball (DNB) technology, this platform constructs an ultra-high-density capture surface on glass slides featuring a center-to-center distance of merely 500 nm. Within this grid, each nanoball—measuring ~0.22 μm in diameter—encodes an independent coordinate identifier. Facilitated by these regularly arrayed nanostructures, the system achieves an unprecedented 13 cm × 13 cm large-area array, empowering the systematic profiling of cross-organ developmental dynamics and complex disease atlases. Furthermore, the latest iteration, Stereo-seq V2 [36], integrates a random primer strategy; this

upgrade not only enhances transcript capture within FFPE specimens but also enables the simultaneous sequencing of both host and pathogen transcriptomes.

To address the low capture rates typical of submicrometer resolutions, Guo's group developed Salus-STS [37], pushing the boundaries of sensitivity through the dual dimensions of biochemical affinity and probe loading capacity. By optimizing the chemical environment of the chip substrate in tandem with a secondary bridge amplification step, this technique elevates the coupling density of barcoded probes to 55,000 molecules per square micrometer (μm²). When evaluated on highly heterogeneous tissues such as the mouse testis, the platform successfully captured over 14,000 valid UMIs within a 10 × 10 μm² single-cell equivalent area. This substantial enhancement in sensitivity ensures the retention of a robust statistical signal-to-noise ratio even at spatial resolutions of 2 μm or finer, thereby facilitating the precise delineation of cellular boundaries and functional tissue zones.

3. Static snapshot-based spatiotemporal transcriptomics

While NGS-based spatial transcriptomics maps tissue architecture through static snapshots, deciphering dynamic cellular trajectories and the true evolutionary history of cells from discrete sectional data remains a challenge. To resolve the temporal dimension, static snapshot-based spatiotemporal transcriptomics integrates advanced computational algorithms, multi-timepoint high-frequency continuous sampling, and genetic barcode tracing to reconstruct continuous trajectories. This approach yields instantaneous dynamic predictions and definitive physical lineage evidence, unifying cell identity, developmental history, and tissue architecture. Consequently, it refines the resolution of organogenesis and disease progression for predictive precision medicine.

3.1. Instantaneous dynamics prediction based on single-point sampling of tissue sections

Deciphering dynamic cellular trajectories from static tissue sections is fundamental to resolving the temporal dimension. By synergistically integrating augmented information density at single-point sampling with advanced computational algorithms, this paradigm facilitates instantaneous dynamic predictions.

Stickels and colleagues utilized the optimized Slide-seqV2 [26] technology to characterize the spatiotemporal development of the mouse neocortex at near-cellular resolution. The core breakthrough of this platform is its high transcript detection efficiency, achieving an RNA capture efficiency of approximately 50% compared to droplet-based single-cell RNA-seq techniques. This remarkable sensitivity enables the effective capture of splicing information, which can be integrated with the scVelo trajectory inference method to order spatial features along predicted latent time. Consequently, this approach successfully recapitulated the established radial developmental axis of the neocortex, representing neuronal migration toward the cortical plate within a single tissue section. Such high-resolution profiling not only identifies dendritically localized mRNAs in neurons but also demonstrates that improving the capture density of spatial measurements enables the identification of underlying continuous genetic programs.

3.2. Reconstruction of biological evolutionary trajectories based on continuous multi-timepoint sampling

Spatiotemporal omics facilitates the reconstruction of dynamic biological trajectories from static snapshots through the implementation of multi-timepoint, high-frequency continuous sampling. This

paradigm is instrumental in deciphering complex physiological phenomena, notably tissue development and regeneration.

Utilizing the submicron-resolution Stereo-seq platform [35], Wang *et al*. constructed a comprehensive three-dimensional spatiotemporal transcriptomic atlas spanning the entire Drosophila developmental trajectory from embryogenesis to the larval stage [38]. By systematically sampling across these developmental windows, their research precisely delineated the spatial specification and dynamic migration trajectories of cellular states within diverse organ primordia. Furthermore, integrating the Spateo computational framework enabled the investigators to identify pivotal regulatory factors orchestrating organ morphogenesis, effectively elucidating the temporal alternation of their expression profiles within specific spatial niches throughout development.

Wei *et al*. systematically profiled the axolotl brain regeneration process through dense temporal sampling spanning various developmental stages (St. 44 to adulthood) and multiple time points post-injury (2 to 60 DPI) (Figure 8) [39]. This high-resolution profiling identified a critical population of injury-induced progenitor cells (reaEGCs). Cellular dynamics analysis revealed that these progenitors undergo clear sequential state transitions during tissue repair. Upon injury activation, reaEGCs proliferate to cover the wound surface before progressing through intermediate progenitor and immature neuron stages, ultimately terminally differentiating into mature neurons to restore damaged neural circuits. Crucially, this study demonstrates for the first time at single-cell spatial resolution that the core regulatory logic of axolotl brain regeneration highly recapitulates the embryonic neurogenesis program alongside injury-specific regulatory modules. Ultimately, this work provides a foundational spatial transcriptomic atlas for investigating vertebrate brain regeneration.

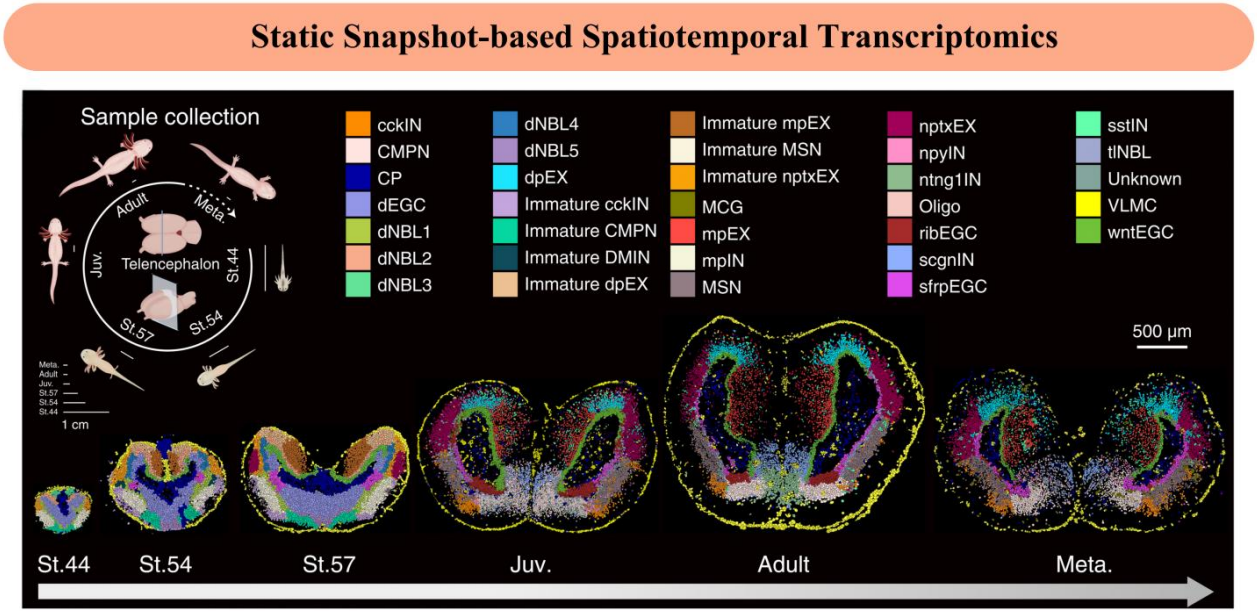

Figure 8. Static snapshot-based spatiotemporal transcriptomics. Reprinted with permission [39]. Copyright 2022 The American Association for the Advancement of Science.

3.3. Spatial clonal and lineage dynamics profiling based on genetic barcode tracing

While advanced computational dynamic models can infer developmental trajectories from static transcriptomic data, resolving the true evolutionary history of cells still requires definitive physical lineage evidence. In recent years, spatial clonal tracking technologies based on genetic barcoding have emerged as a new paradigm, providing a new solution for dissecting the spatiotemporal dynamics of complex tissues.

Ratz *et al*. developed Space-TREX by integrating spatial transcriptomics with *in vivo* barcoding to perform *in situ* gene expression analysis and clonal tracking in mouse brain sections [40]. To implement this, they utilized a TREX vector with a U6-driven 30-bp random DNA sequence, which is transcribed into a polyadenylated non-coding cloneID engineered with a 3' capture sequence. Following delivery into apical progenitors via in utero electroporation (IUE), these unique identifiers are stably inherited by cellular progeny. The research team analyzed eight consecutive 10 μm coronal sections barcoded at embryonic day 9.5 (E9.5) and harvested at postnatal day 14 (P14) to quantitatively evaluate spatial gene patterns, cell types, and clonal distributions. Mapping these shared cloneIDs to physical coordinates enables the reconstruction of dynamic migration pathways, including tangential migration or radial clustering from discrete spatial distributions, effectively unifying cell identity, lineage history, and tissue architecture within a single framework.

He *et al*. established Spatial iTracer to deeply explore the mechanisms of cell fate establishment in induced pluripotent stem cell (iPSC)-derived organoids [35]. To achieve temporal resolution, the system leverages a doxycycline-inducible CRISPR-Cas9 scarring mechanism where the administration of doxycycline triggers Cas9-mediated insertions and deletions at a synthetic target locus within the reporter transcript at designated windows to create unique molecular scars recording lineage history. By mapping these multimodal recorders to physical coordinates and integrating long-term four-dimensional (4D) light-sheet microscopy, the researchers confirmed regional clonality in the nascent neuroepithelium and evaluated the effects of mosaic TSC2 mutations on lineage alterations. This synergistic strategy effectively unifies cell identity, lineage history, and tissue architecture within a single analytical framework to bridge the gap between static spatial maps and dynamic biological processes.

By synergistically applying NGS-based spatial transcriptomics and dynamical algorithms, static snapshot-based spatiotemporal transcriptomics can resolve continuous trajectories from discrete sectional data. The integration of genetic barcoding provides definitive physical evidence for lineage origins, unifying cell identity, developmental history, and tissue architecture. This paradigm shift refines the resolution of organogenesis and disease progression, marking a pivotal advancement toward predictive precision medicine.

4. Metabolic RNA labeling-based spatiotemporal transcriptomics

Although static snapshot-based spatiotemporal transcriptomics can reconstruct complex tissue evolutionary trajectories, the rapid changes in transcription, the coordinated regulation of RNA synthesis and degradation rates, and the dynamic cellular interactions driving cell fate decisions remain incompletely understood through spatial inference alone. To address this bottleneck at the mechanistic level, metabolic RNA labeling-based spatiotemporal transcriptomics has emerged as a cutting-edge chemical strategy. By utilizing nucleoside analogs to label newly transcribed RNAs, this technology precisely distinguishes

nascent RNAs from pre-existing RNAs. This time-resolved approach unbiasedly captures RNA turnover dynamics for thousands of genes within tissues, providing an unprecedented microscopic perspective on heterogeneous and dynamic gene expression and establishing a foundational anchor for the temporal dimension in spatiotemporal transcriptomics.

4.1. Metabolic RNA labeling at the single-cell level

Metabolic RNA labeling-based transcriptomics sequencing was first achieved at the single-cell level. By introducing nucleoside analog labeling to scRNA-seq, metabolic RNA labeling-based single-cell transcriptomics enables the precise chemical differentiation between newly transcribed and pre-existing RNAs in single cells. This paradigm establishes a fundamental methodological foundation for the high-resolution elucidation of transcriptional bursting and dynamic gene regulatory mechanisms [41].

Introduced by Florian Erhard *et al*., single-cell SLAM-seq (scSLAM-seq) [42] stands as a pioneering milestone that integrates metabolic RNA labelling with scRNA-seq, predominantly relying on plate-based platforms such as SMART-seq [43]. Its core principle involves tagging nascent RNA with 4-thiouridine (4sU), followed by IAA-mediated alkylation. This biochemical reaction induces base recoding, yielding characteristic U-to-C conversions (manifesting as T-to-C mismatches in sequencing data) at the labelled sites. By processing individual cells within isolated wells, this technique delivers exceptional gene coverage and transcript capture sensitivity, rendering it highly advantageous for deeply profiling the bursting kinetics of individual genes. Nevertheless, this "one-cell-per-well" operational paradigm inherently restricts experimental throughput, typically limiting the capacity to merely hundreds or thousands of cells. Coupled with the elevated costs of single-cell library preparation, such a platform remains challenged in facilitating the large-scale atlas mapping of complex tissues.

Wu's group developed scNT-seq [44], leveraging droplet microfluidics to improve the throughput. In this workflow, labeled single cells and barcoded beads are co-encapsulated into nanoliter-scale droplets, enabling the rapid capture of tens of thousands of cells through *in situ* lysis and hybridization. Following nascent RNA tagging via an optimized IAA chemical recoding process, the microfluidic system facilitates bead retrieval and single-cell library preparation, drastically elevating analytical throughput and slashing the sequencing cost per cell. Nevertheless, constrained by the randomness of Poisson statistics, the effective co-encapsulation rate of single cells and beads within this platform remains limited. Furthermore, the emulsion environment struggles to thoroughly deplete ambient cell-free RNA, which to some extent compromises the quantification accuracy of newly synthesized transcripts.

Yang's group developed Well-TEMP-seq, a platform leveraging microwell arrays governed by size exclusion and quasi-static fluidic principles to achieve high-efficiency single-cell barcoding after metabolic RNA labeling (Figure 9a) [45]. By designing a unique dual-layer microwell structure, this technique utilizes physical geometric constraints to ensure the precise co-occupancy of a single cell and a single bead, thereby boosting pairing efficiency to approximately 80%. Operationally, Well-TEMP-seq introduces an *in situ* on-bead alkylation strategy, completing the chemical recoding directly on the bead surface post-lysis to minimize the loss of trace samples during complex biochemical reactions. Furthermore, its quasi-static fluidic system facilitates the efficient depletion of background noise, such as ambient RNA, leading to more accurate identification of nascent transcripts. This optimized workflow

provides a high-quality data foundation for the subsequent construction of high-resolution transcriptional dynamics vector fields.

4.2. Metabolic RNA labeling at the in vivo level

To capture the spatiotemporal RNA dynamics in tissue, it is necessary to label newly transcribed RNAs of cells in the tissues. *In vivo* metabolic labeling technologies advance dynamic chemical labeling strategies to the scale of intact living organisms, effectively circumventing the perturbation of authentic physiological states inherent to in vitro manipulations. This paradigm enables the precise tracking of transient transcriptional dynamics across cell subpopulations directly within their *in vivo* native microenvironments.

Miska and colleagues developed SLAM-ITseq [46], an approach integrating genetic models with chemical labeling. The core of this technique relies on a specialized transgenic mouse model that utilizes the Cre-LoxP recombination system to drive the target-cell-specific expression of Toxoplasma gondii uracil phosphoribosyltransferase (UPRT). Following the *in vivo* administration of 4-thiouracil (4-TU), the system capitalizes on the absence of an efficient endogenous 4-TU salvage pathway in mammals. Consequently, only cells expressing the exogenous UPRT can convert 4-TU into 4-thio-UMP for subsequent incorporation into nascent RNA transcripts. This strategy allows researchers to extract total RNA directly from intact tissues for IAA conversion and sequencing. By quantifying T-to-C base-conversion signatures, this method accurately resolves cell-type-specific transcription rates within the native *in vivo* microenvironment while completely bypassing the need for physical isolation techniques such as fluorescence-activated cell sorting (FACS).

To bypass the conventional reliance on transgenic models for *in vivo* labeling, Yang's group developed Dyna-vivo-seq for the systemic profiling of tissue dynamics in wild-type animals (Figure 9b) [47]. By intravenously administering highly permeable, low-toxicity 4-thiouridine (4sU) via the tail vein, this technology leverages the circulatory system to rapidly deliver the label to organs, such as the kidney, achieving real-time tagging of nascent transcripts across all cell populations. The platform further integrates the high-sensitivity Well-paired-seq2 microwell system [48], utilizing quasi-static fluidic properties to perform *in situ* on-bead chemical recoding. Such a strategy substantially enhances the identification precision of newly synthesized RNA from trace *in vivo* specimens. When applied to acute kidney injury (AKI), this approach first identified a proximal tubule (PT) subpopulation with high turnover rates and confirmed their heightened vulnerability during ischemia-reperfusion injury (IRI), providing pivotal evidence for resolving the cellular kinetic foundation of organ damage.

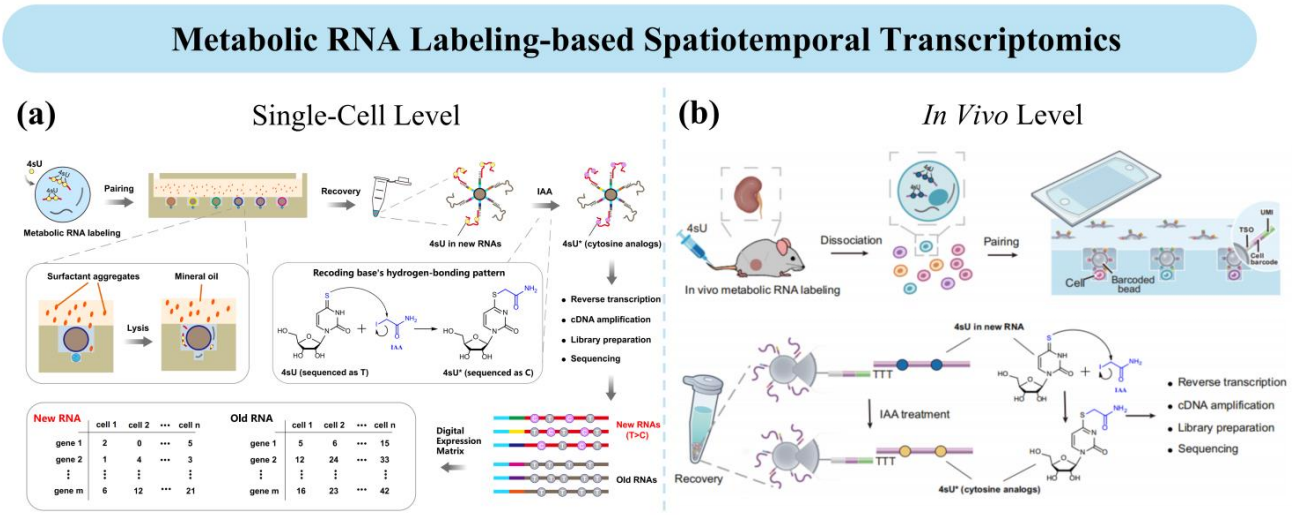

Figure 9. Metabolic RNA labeling-based spatiotemporal transcriptomics. Hierarchical integration from **(a)** single-cell level (Well-TEMP-seq) to **(b)** *in vivo* level (Dyna-vivo-seq) and spatial level (Spatial NT-seq), enabling direct quantification of RNA synthesis and degradation rates within native tissue architecture. Reprinted with permission [45,47]. Copyright 2023 Springer Nature and 2024 Springer Nature.

Following Dyna-vivo-seq, other *in vivo* metabolic RNA labeling-based single-cell transcriptomics technologies were also reported. For example, Fan *et al*. introduced scIVNL-seq to capture *in vivo* transcriptional bursts via pulse administration of 4sU through the tail vein [49]. In a Salmonella infection model, this approach confirmed that the new-to-total RNA ratio (NTR) provides higher sensitivity than total transcript abundance for pinpointing early cellular activation extremes. Furthermore, the platform revealed that intestinal epithelial cells (IECs) can rapidly express MHC-I and function as antigen-presenting cells to directly initiate adaptive immunity. Such findings effectively reconstruct the temporal logic of innate-to-adaptive transitions through the lens of molecular dynamics.

Metabolic labeling has successfully enabled the capture of cellular temporal dynamics within *in vivo* physiological contexts. However, conventional tissue dissociation inherently disrupts the original spatial organization of the sample. To facilitate the synchronous acquisition of spatiotemporal datasets while maintaining native tissue architecture, researchers have coupled metabolic labeling with spatial capture arrays, effectively establishing a new paradigm in spatiotemporal omics.

4.3. Metabolic RNA labeling at the spatial level

After establishing *in vivo* metabolic RNA labeling, it becomes possible to develop metabolic RNA labeling-based spatiotemporal transcriptomics by integrating *in vivo* metabolic RNA labeling with NGS-based spatial transcriptomics.

Wu's group developed Spatial NT-seq, a transgenic-free spatial transcriptomic technology designed to map tissue-wide RNA turnover kinetics *in vivo* [50]. This platform integrates highly permeable 4sU metabolic labeling with high-throughput spatial barcoding arrays to perform *in situ* chemical recoding

directly on tissue sections. By leveraging base-conversion signatures, this technique differentiates nascent transcripts from pre-existing transcripts at single-molecule resolution, thereby generating the first whole-transcriptome spatial turnover atlases. Furthermore, its companion analytical framework, *in vivo* Timescope, effectively decouples the regulatory contributions of transcriptional synthesis and degradation to total gene expression. When applied to the mammalian brain, this approach identified the dentate gyrus (DG) as a spatial hotspot for RNA turnover. Importantly, it unveiled a kinetics scaling mechanism wherein neurons coordinate synchronized synthesis and degradation rates to rapidly respond to external stimuli, providing a transformative dimension for investigating the spatiotemporal plasticity of brain function.

5. Conclusion and outlook

The technological evolution of spatial transcriptomics represents a persistent trade-off among resolution, throughput, and sensitivity, which is fundamentally anchored in the advancement of spatial barcoding chemistry. By transitioning from the precision of photochemical labeling and microfluidic parallelism in *in situ* coding strategies to the iteration of microarrays, bead arrays, and photolithographic grids in *in situ* capture strategies, physical limits have been pushed from multicellular resolution to submicron and even nanometer scales, thereby enabling the mapping of subcellular architectures. However, a critical bottleneck remains in balancing subcellular resolution, genome-wide coverage, and an ultra-large field of view, as these three parameters are not simultaneously satisfied by any current platform. Because static spatial atlases are no longer sufficient to decipher the intricate dynamics of development and disease, a paradigm shift from static snapshot-based spatiotemporal transcriptomics to metabolic RNA labeling-based spatiotemporal transcriptomics is necessitated. By fusing metabolic labeling with spatial capture arrays, RNA synthesis and degradation rates can be directly quantified within the native tissue context, effectively establishing true spatiotemporal resolution for the first time.

Although current spatiotemporal transcriptomics technologies persistently encounter challenges, future technological iterations are anticipated to advance along three core trajectories: balancing high resolution with high sensitivity, integrating multi-omics dimensions, and enhancing clinical accessibility. Key breakthrough areas encompass optimizing spatial resolution and transcript capture efficiency at subcellular or single-molecule levels, enabling the in-situ co-detection of multi-omics modalities, adapting single-molecule long-read sequencing for spatial applications, and improving compatibility with clinical archival samples, such as FFPE tissues. Concurrently, the universality and accessibility of these platforms will be further elevated by streamlining technical workflows and controlling costs.

At the frontier of technological evolution, the in-situ integration of *in vivo* metabolic RNA labeling with high-resolution spatial transcriptomics is regarded as one of the most groundbreaking future directions. By preserving the high-throughput and high-resolution advantages of existing spatial transcriptomics while introducing the temporal dimension of transcriptional dynamics, a technological leap from three-dimensional spatial localization to four-dimensional spatiotemporal dynamics is facilitated by this pathway. Current metabolic labeling predominantly relies on short-read sequencing, resulting in the loss of splice isoform information. However, by incorporating long-read technologies, the production rates of specific isoforms can be observed within four-dimensional space. For instance, in mammalian neural development and plasticity research, the *in situ* tracking of the minute-scale synthesis and turnover of synapse-associated splice variants is enabled, providing novel tools to decipher

the spatiotemporal regulatory mechanisms of complex brain functions, such as learning and memory or neural circuit remodeling.

The core value of spatial metabolic labeling extends beyond the resolution of RNA transcriptional dynamics. It serves as a kinetic bridge connecting multi-omics dimensions. Future applications could be built upon this framework to construct a comprehensive dynamic monitoring system encompassing spatial metabolic labeling, translatomics, and proteomics. Furthermore, through multimodal integration with spatial metabolomics data, the real-time regulation of transcriptional turnover rates in adjacent cells by the local accumulation of specific metabolites (e.g., lactate in the tumor microenvironment or succinate in the inflammatory microenvironment) can be resolved *in situ*, thereby unveiling the feedback regulatory loops between metabolites and transcription within the tissue architecture.

Regarding clinical translational applications, the massive repository of clinical FFPE samples can be leveraged alongside the compatibility of existing spatial transcriptomic platforms to develop virtual chronological models of disease progression based on spatial transcriptomic signatures, enabling the dynamic disease evolution processes to be deduced from static archival samples. In oncology research, future emphasis will be shifted from merely detecting checkpoint protein (e.g., PD-L1) expression to analyzing the activation states and transcriptional lifespans of immune cells at the tumor margin via metabolic labeling. By comparing kinetic alterations before and after therapeutic interventions, the spatial kinetic signatures of early drug-resistant clones can be identified. Concurrently, the clinical translation of this technology is restricted by the central bottleneck of the biosafety of *in vivo* metabolic labeling. The most popular nucleoside analog, 4sU, has undergone only short-term safety validation in rodents, while its long-term safety and *in vivo* metabolic characteristics in primates and humans remain insufficiently elucidated. Consequently, the development of safer and more permeable unnatural nucleoside analogs, or the utilization of nanocarriers to achieve targeted metabolic labeling, is deemed crucial for advancing this technology into clinical trials.

In the future, driven by the deep integration of AI algorithms with biophysical and transcriptional regulatory principles, spatiotemporal transcriptomics is poised to progressively become a routine diagnostic modality in precision medicine. This technological paradigm not only establishes finer spatial molecular subtyping criteria for diseases but also facilitates the dynamic prediction of cellular interaction cascades within the tissue microenvironment following therapeutic interventions. From the fundamental principles of developmental biology to the precision pharmacology of clinical oncology, our comprehension of the essence of life is being reshaped by spatiotemporal transcriptomics technologies with unprecedented depth and breadth.

Declaration of generative AI and AI-assisted technologies

During the preparation of this manuscript, the authors used generative AI tools only to improve language and readability. Specifically, the authors used Gemini for language polishing only in entire manuscript. The authors take full responsibility for the content of the manuscript.

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Authors' contribution

Conceptualization, Shichao Lin and Chaoyong Yang; writing—original draft preparation, Yiqiao Wang and Jiawei Lin; writing—review and editing, Zhi Zhu, Shichao Lin and Chaoyong Yang; supervision, Shichao Lin and Chaoyong Yang; funding acquisition, Shichao Lin and Chaoyong Yang. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

Chaoyong Yang holds the position of Editorial board member for *Life Analysis* and has not peer reviewed or made any editorial decisions for this paper.

Abbreviations

Abbreviations	Full terms
cckIN	Cck+ inhibitory neuron
CMPN	Cholinergic, monoaminergic, and peptidergic neuron
CP	Choroid plexus
dEGC	Developmental ependymoglial cell
DMIN	Dopaminergic periglomerular inhibitory neuron
dNBL	Developmental neuroblast
dpEX	Dorsal pallium excitatory neuron
MCG	Microglial cell
mpEX	Medial pallium excitatory neuron
mpIN	Medial pallium inhibitory neuron
MSN	Medium spiny neuron
nptxEX	Nptx+ lateral pallium excitatory neuron
npyIN	Npy+ inhibitory neuron
ntng1IN	Ntng1+ inhibitory neuron
Oligo	Oligodendrocyte
ribEGC	Ribosomal ependymoglial cell
scgnIN	Scgn+ inhibitory neuron
sfrpEGC	Sfrp+ ependymoglial cell
sstIN	Sst+ inhibitory neuron
tlNBL	Telencephalon neuroblast
VLMC	Vascular leptomeningeal cell
wntEGC	Wnt+ ependymoglial cell

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