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Spatial transcriptomics: paving the way for tissue-level systems biology

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The tissues in our bodies are complex systems composed of diverse cell types that often interact in highly structured repeating anatomical units. External gradients of morphogens, directional blood flow, as well as the secretion and absorption of materials by cells generate distinct microenvironments at different tissue coordinates. Such spatial heterogeneity enables optimized function through division of labor among cells. Unraveling the design principles that govern this spatial division of labor requires techniques to quantify the entire transcriptomes of cells while accounting for their spatial coordinates. In this review we describe how recent advances in spatial transcriptomics open the way for tissue-level systems biology.

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Introduction

The field of Systems Biology has made a profound impact on our ability to reverse engineer the biological networks that govern cellular behavior. Fueled by biotechnological developments over the past two decades, researchers have been able to obtain a detailed description of regulatory, signaling and metabolic networks [1–3]. Remarkably, many of these networks exhibited features that were common to engineered systems, such as modularity, robustness and recurring building blocks [4]. The techniques used for these studies required 'bulk' analyses of extracts from many cells, be it RNA [5,6], proteins [7] or chromatin [8]. While highly informative for unicellular organisms or cultured cells, these techniques provide only partial information when the biological systems are heterogeneous.

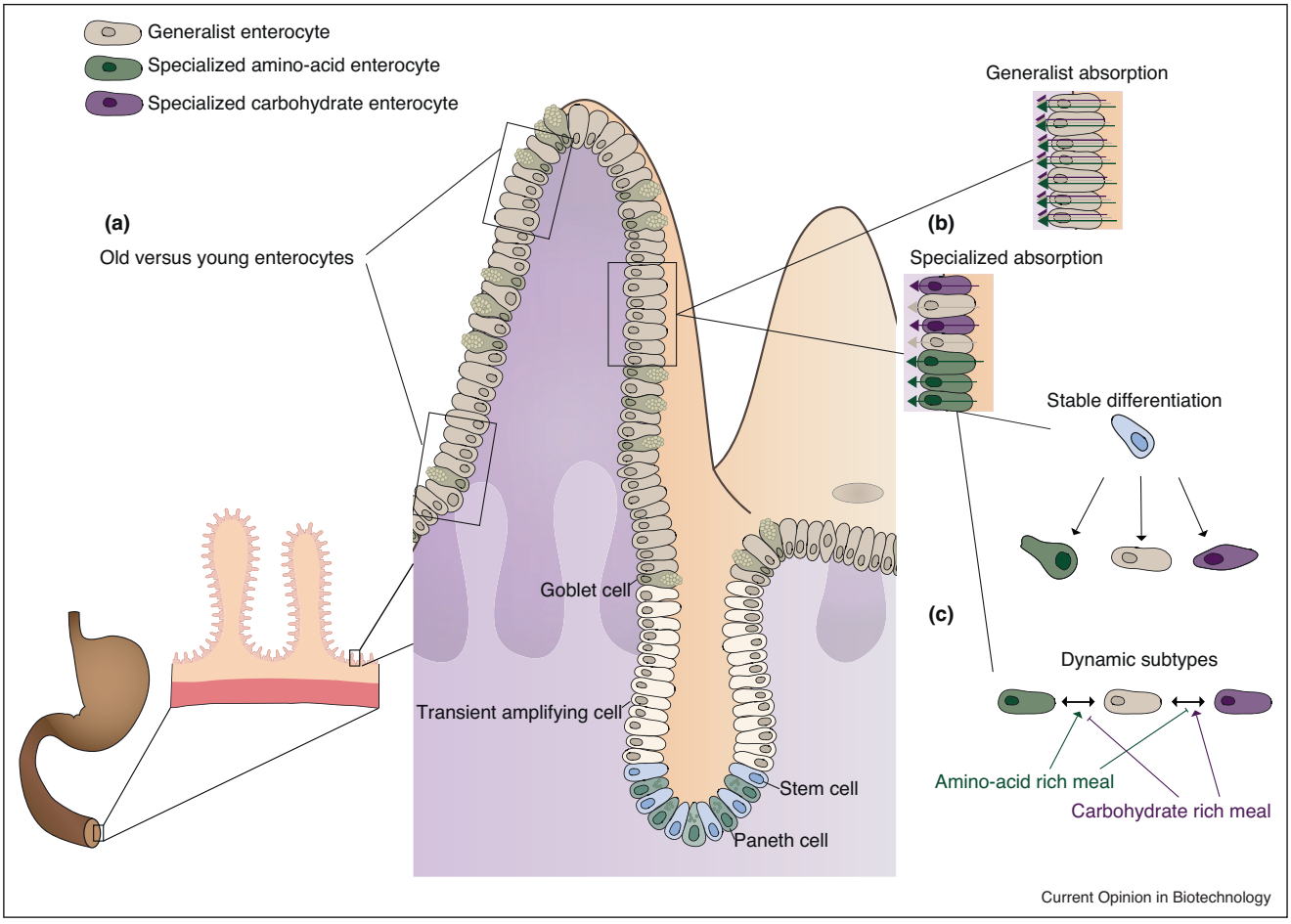
The tissues in our bodies consist of diverse cell types and subpopulations; their molecular identities would be lost in bulk measurements. Single cell RNA sequencing [9–13] has revolutionized our ability to identify and characterize such subpopulations. However, these techniques require tissue dissociation, thus losing the original tissue coordinates. Since the physical location of a cell within the tissue is a key determinant of its molecular identity, tissue-level systems biology requires obtaining whole-genome measurements while accounting for the spatial localization of cells. Several methods for spatial transcriptomics have been comprehensively reviewed in [14], herein we will focus on more recent advances in the field and their potential use in advancing our understanding of design principles of tissue organization.

Spatial context is a key determinant of cellular identity in mammalian tissues

To motivate the importance of spatial transcriptomics, we will shortly describe open questions in two typical heterogeneous, yet structured mammalian tissues—the intestine and the liver. The mammalian small intestine is lined with a highly folded epithelial sheet composed of deep pits called crypts, and larger protrusions called villi (Figure 1). Stem cells and progenitors within the crypt constantly feed the villus with secretory goblet cells and absorptive enterocytes. These differentiated cells rapidly migrate along the villus walls as they operate for a few days, until they are shed off from the villi tips. The positions of cells along the crypt villus axis correlate with their age, making this a classic system to study processes of cell differentiation, homeostasis, aging and death.

Extensive studies deciphered the homeostatic mechanisms that operate in the crypts to maintain constant stem cell numbers while ensuring a steady flux of differentiated cells [15–17]. Much less is known about the diverse processes in the differentiated compartments—the intestinal villi. How fast do enterocytes mature upon entering the villi? Do 'old' enterocytes at the villi tips operate less efficiently than 'young' enterocytes at the villi bottoms as a result of accumulated cellular damage acquired due to the hostile lumen environment (Figure 1a)? Are there different subtypes of enterocytes that subspecialize in the absorption of particular nutrients, such as carbohydrates, lipids or amino acids, or are enterocytes all-absorbing generalists (Figure 1b)? If such enterocyte division of labor exists is it a result of clonal subtypes generated in the crypts, or rather a transient division of labor modulated by the constantly changing

Figure 1

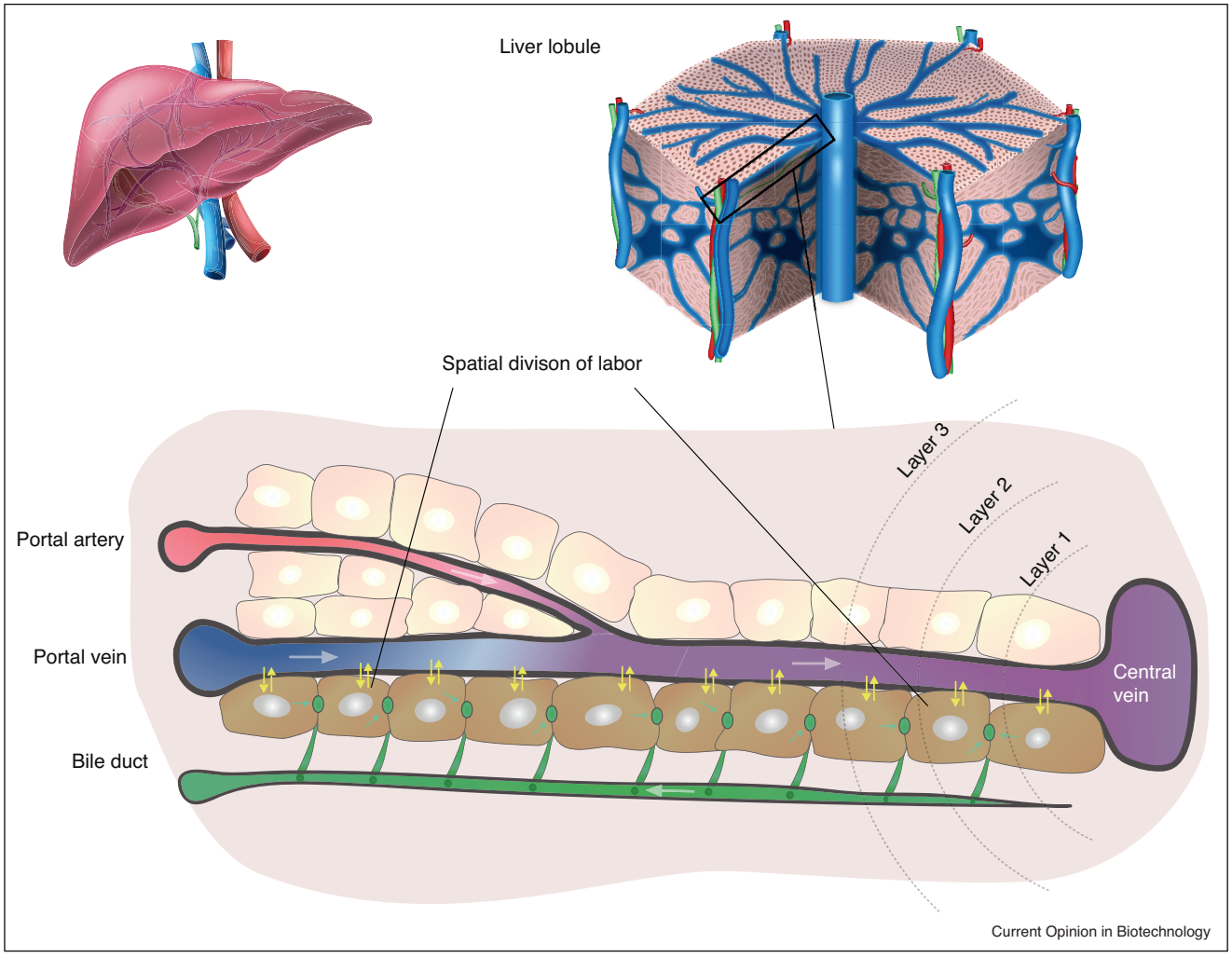


Open questions in the biology of the intestinal epithelium that require spatially resolved single-cell measurements. This illustration depicts a small intestinal crypt-villus unit. Each crypt forms a well-protected adult stem cell niche. Stem cells at the bottom of the crypt, intermingled with supporting Paneth cells, constantly proliferate to generate progenitors, termed 'transit amplifying cells'. These migrate upwards as they continue to divide yielding a constant flux of differentiated secretory goblet cells and nutrient absorbing enterocytes that feed the villi. The differentiated cells migrate upwards along the villi walls as they function for a few days until they are shed off from the villi tops. Several open questions regarding the collective behavior of this key cell population include: **(a)** Are 'old' enterocytes, that have arrived at the tip of the villus, functionally different from 'young' enterocytes that only recently acquired their differentiated function? **(b)** Are enterocytes 'generalists' that absorb each nutrient class equally efficiently or are there subspecialized types of enterocytes that preferably absorb carbohydrates, lipids or amino acids? **(c)** If such subspecialized enterocytes exist, do they differentiate hierarchically from stem cells and maintain their fate in a stable manner? Alternatively, the fractions of these subspecialized cells might fluctuate dynamically depending on extrinsic stimuli, for example, the presence or absence of the respective nutrients. Quantitative measurements of the complete transcriptome of enterocytes at defined coordinates along the villus axis can address these and other open questions.

nutrient composition in the gut (Figure 1c)? Are there spatial domains along the vertical villus axis for such subpopulations that define a hierarchy of absorption? Tools to characterize the complete cellular gene expression signatures of enterocytes along the vertical crypt-villus axis would address these open questions and unravel how the intestine can economically yet efficiently absorb nutrients that only exist for transient periods of time [18].

The mammalian liver is a second example in which the spatial locations of cells are critical for understanding their molecular identities and physiological roles. The liver is a central organ for maintaining organismal homeostasis. Hepatocytes perform a wealth of biological tasks including protein secretion, nutrient storage and release and detoxification. The hepatocytes that perform these tasks operate in repeating hexagonal anatomical units termed 'lobules'. Each lobule consists of around 12–15 concentric layers of hepatocytes and is polarized by blood that flows inward from outer portal nodes toward draining central veins (Figure 2). The absorption and secretion of hepatocytes residing along the radial blood vessels modulates the microenvironment available for more 'downstream'

Figure 2



Unresolved questions regarding the spatial organization of the liver. The liver is orchestrating anabolism and catabolism of nutrients and xenobiotics. It is composed of hexagonal lobules that are polarized by blood flow; Blood originates from portal triads at the corners of the lobule and flows toward a draining central vein. Each portal triad consists of a hepatic artery (red) that carries oxygenated blood, a portal vein (blue) that conveys nutrient-rich blood from the gastrointestinal tract and a bile duct (green). Gray arrows indicate directions of flow, yellow arrows indicate exchange of materials between hepatocytes and blood, green arrows mark bile canaliculi. Various metabolic tasks are spatially distributed in specialized sublobular layers between the central vein and the portal field, an organization termed 'liver zonation'. Where along the radial lobule axis is each liver function localized? What are the optimality principles governing this spatial division of labor? Are there spatially distributed metabolic cycles in which pathway intermediates are transferred among sequential cells? Spatially resolved transcriptomics of the liver could facilitate detailed understanding of this fundamental organ.

hepatocytes, creating a fertile ground for tissue optimization. For example, excessive respiration or nutrient consumption of portal hepatocytes could potentially deplete these inputs to levels that could dangerously compromise the function of central hepatocytes. Indeed, hepatocytes at different lobule layers subspecialize in different tasks in a way that is thought to facilitate optimal overall liver function. For example, the outer highly oxygenated portal layers of the lobules subspecialize in energy demanding tasks such as protein secretion and glucose production, whereas central hepatocytes specialize in detoxification [19,20]. Obtaining the complete gene expression signatures of individual hepatocytes at all lobule coordinates would unravel the design principles underlying this spatial division of labor and facilitate detailed modeling of the liver response to diverse perturbations.

In situ spatial transcriptomics

The age of spatial transcriptomics can be traced back to the development of single molecule fluorescence *in situ* hybridization (smFISH) methods [21,22]. smFISH enables transcript quantification *in situ* (at the tissue site where they reside) by making use of libraries of multiple

short, 20 bp long olignonucleotide probes, each labeled with typically a single fluorophore (Figure 3a1). Through the specific accumulation of these fluorescent probes on the target mRNA, individual transcripts can be visualized as diffraction-limited spots by fluorescence microscopy [22]. smFISH has been applied in cells as well as in mammalian tissues [23], where it enabled characterization of cell subpopulations and spatial heterogeneity [24–26].

An outstanding limitation of smFISH is the small number of transcripts that can simultaneously be identified, typically three or four. This is due to the limited number of fluorophores that are suitable for parallel use. An elegant approach to overcome this limitation uses combinatorial labeling. Levsky *et al.* [27•] were the first to demonstrate combinatorial labeling for single transcript detection. By dividing the library of probes into groups, each coupled to one of three fluorophores, they could differentiate between dots that have single colors, pairs of colors or triplets. This approach increased the number of transcripts that could be detected with n fluorophores to 2^n-1. Lubeck and Cai further increased the number of simultaneously detected transcripts by combining spatial barcodes with super resolution microscopy [28••]. With the higher resolution of 10–20 nm they were able to resolve different physical subparts of the mRNA molecules of interest (Figure 3a2). In this way the transcripts of two different genes combinatorially labeled with three colors could be resolved if one uses a sequence of probes coupled to red, blue and green, whereas the other uses a sequence of blue, red and green. An additional factor that increased the number of simultaneously detected genes was the use of seven photoswitchable dye pairs, as compared to the four fluorescent dyes typically detected in smFISH experiments.

An alternative method for combinatorial smFISH uses the temporal dimension to multiplex probe libraries. Cai and colleagues generated four different versions of a smFISH probe library, each with an identical probe set [29]. Each library was coupled to one of four fluorophores. During each hybridization round only one of the four libraries was utilized, and DNAse treatements removed bound probes between hybridization rounds (Figure 3a3). The recorded sequence of probe signals enabled the identification of the detected RNA species based on the temporal sequence of dots. In theory, this approach allows for the detection of F^n distinct transcripts (F = fluorophores, n = hybridiaztion rounds) [29]. For example, with four fluorophores and eight hybridization rounds one could identify 65 536 different transcripts. However, this approach would currently be prohibitively expensive if applied transcriptome-wide.

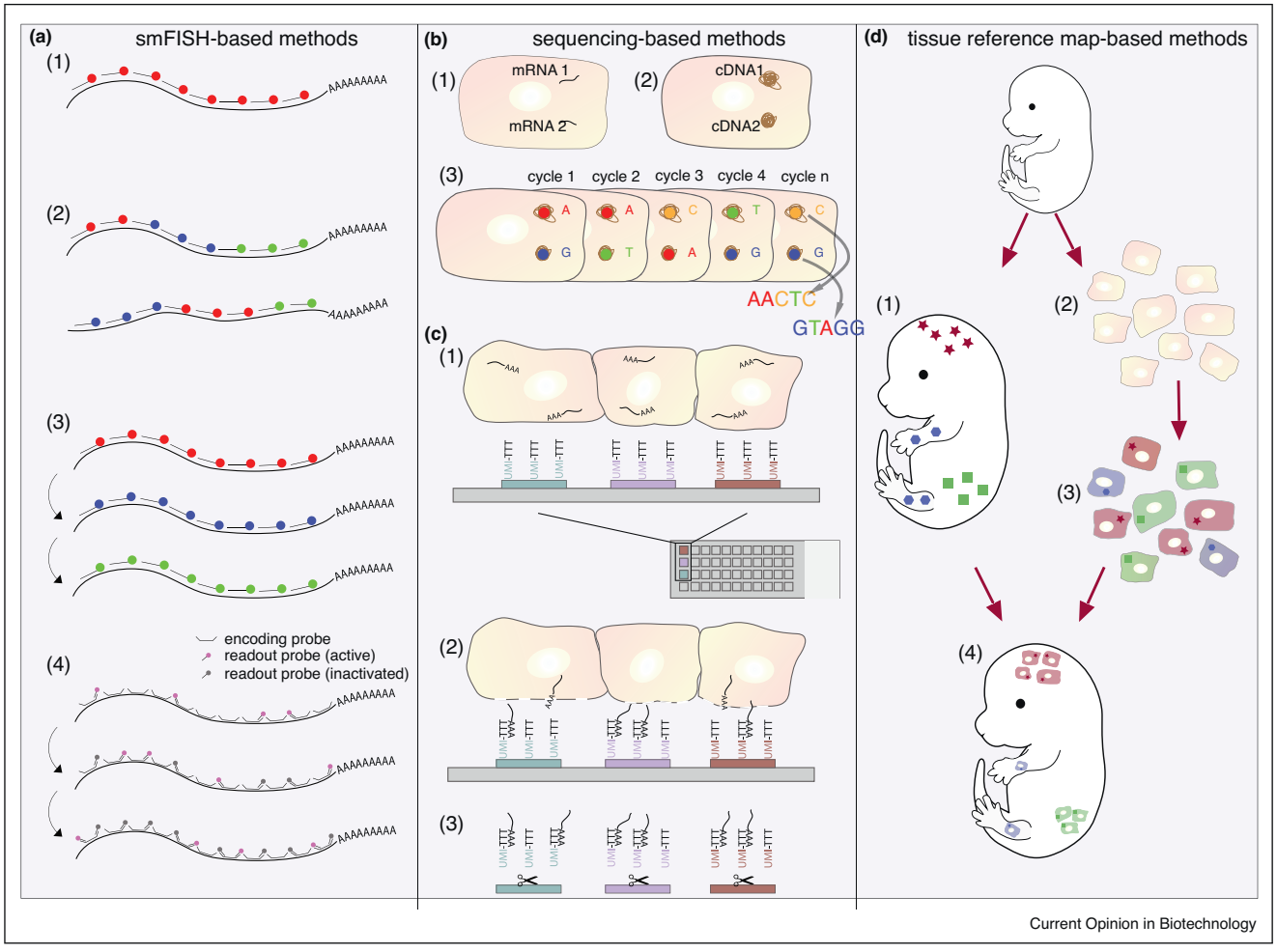
A pending issue with combinatorial smFISH is the robustness to readout errors. Since probe hybridization is an inherently stochastic process, one could erroneously assign an RNA species to the wrong gene if one of the sequential hybridizations failed. This readout error increases exponentially with the number of hybridization rounds. To overcome this, Chen *et al.* developed an elegant barcode assignment scheme termed 'MERFISH', which ensures that the sequence of barcodes for different genes is far enough so that only multiple readout errors would cause miss-assignments [30••,31]. In addition, both the cost of fluorescent probe synthesis and time-consuming hybridization rounds were addressed by MERFISH through a clever two-stage hybridization scheme (Figure 3a4), leading to the accurate detection of 1000 transcripts with 14 hybridization rounds in 100 cells [30••]. A recent improvement of MERFISH enabled the detection of 130 genes in 40 000 cells in one 18 hours experiment [32]. These proof-of concept studies for combinatorial smFISH were applied to cultured single cells. It remains to be seen how well the methods will perform in tissue sections. This could be particularly challenging since tissues have an inherently larger readout noise due to increased auto-fluorescence.

In-situ sequencing

While combinatorial methods have dramatically increased the throughput of *in-situ* transcript detection, they are still targeted approaches, requiring pre-selecting genes of interest for which probes are designed. *In-situ* sequencing is a complementary technique that enables an unbiased census of all RNA molecules while preserving localization. High-throughput sequencing reconstructs the sequence of cDNA molecules tethered to a flow cell by sequential synthesis with fluorescent nucleotides. *In situ* RNA sequencing in essence replaces the flow cell with the original tissue of interest (Figure 3b). It utilizes *in situ* cDNA synthesis, cDNA amplification and cross-linking [33,34••,35]. The resulting circular cDNA amplicons are amenable to SOLID sequencing by ligation; four-color microscopy records the SOLID base information for each pixel, the fluorophore is inactivated and the sequencing reaction is repeated for the next base. Nilsson and colleagues introduced targeted *in situ* RNA sequencing by making use of padlock probes to initiate targeted cDNA synthesis *in situ* [33]. They sequenced four base-pair fragments and detected 31 different transcripts in parallel in fixed tissue sections. Lee *et al.* further developed fluorescent *in situ* RNA sequencing (FISSEQ) by generating 150 000 short 30 bp reads that were mapped to 8100 genes in fibroblasts [34••]. They also demonstrated the *in situ* cDNA library generation in intact tissues, such as drosophila embryos and mouse brain sections. Some inconsistencies in the correlations between FISSEQ reads and traditional RNAseq remain to be further explored [35].

A recent promising *in-situ* sequencing method was developed by Stahl *et al.* They used a glass slide that spatially captures mRNA for library assembly when overlaid with a tissue section [36•]. Each polyA-capturing feature

Figure 3



Methods for spatial transcriptomics. **(a)** *In situ* hybridization methods. 1) single molecule RNA fluorescence *in situ* hybridization enables identifying individual transcripts as fluorescent dots, by making use of libraries of multiple 20 bp long oligonucleotide probes, each labeled with a single fluorophore. 2) The use of super resolution microscopy in combination with smFISH stainings enables the identification of fluorophore sequences along single transcripts [28••]. Multiple transcripts can be identified by making use of differing fluorophore orders. 3) Temporal barcoding is based on sequential hybridizations and imaging steps that are followed by the digestion of the imaged probe oligonucleotides. Subsequent reactions make use of probe sets that are labeled with differing fluorophores, the resulting temporal barcode of fluorophores for each smFISH dot enable the identification of multiple transcripts [29]. 4) The MERFISH approach makes use of a two-stage hybridization scheme [30••,31,32]. Encoding probes are hybridized to RNA molecules *in situ*, these probes encompass landing sequences for the rapid subsequent assembly of fluorescent readout probes. The fluorescent dyes that are coupled to the readout probes can be deactivated chemically and allow for the repeated hybridization cycles with different readout probes. The rapid hybridization cycle duration of this protocol allows for a sophisticated barcode assignment scheme that requires four readout errors for barcode miss-assignments; single errors can be unequivocally corrected. **(b)** Fluorescent *in situ* sequencing (FISSEQ) [34••,35]. 1) mRNA molecules are reverse-transcribed *in situ* and circularized. 2) Resulting cDNA molecules are amplified with rolling circle amplification. Cross-linking of modified cDNA residues prevents diffusion of the resulting amplicons. 3) Repeated sequencing reactions by ligation and subsequent microscopic imaging and fluorophore cleavage yield a nucleotide sequence for each imaged pixel; sequences that are retrieved from RNA molecules can be mapped to a reference transcriptome while background fluorescence is not retrieved. **(c)** Spatial mRNA capture slide [36•]. 1) Commercially available mRNA capturing slides incorporate polyT-nucleotides with embedded positional barcodes. 2) Tissue sections are permeabilized and mRNA molecules are captured by the polyT-nucleotides of the glass slide. 3) Captured mRNA molecules are eluted from the glass slide and feature a positional barcode. Subsequent library preparation and massively parallel sequencing enables the retrieval of the corresponding positional barcode and originating tissue coordinates for each resulting library. **(d)** Reference map-based single cell RNA sequencing. 1) Spatial expression patterns of landmark genes are mapped in two or three dimensional space within the intact tissue. 2) In parallel, the tissue of interest is dissociated into single cells; these cells are profiled in depth by scRNAseq [41••,42••,43,44,45••] or single cell qPCR [40]. 3) The expression levels of the landmark genes that were used for the assembly of positional tissue maps in 1) are retrieved for all sequenced single cells. 4) The original position of the sequenced single cells is inferred based on their expression levels of all marker genes. This consequently enables identifying the spatial domain of expression of any gene of interest.

consists of a positional barcode, unique molecular identifier (UMI), and library adaptor sequences (Figure 3c). RNA is eluted and cDNA synthesis and library preparation are performed in bulk reactions for the whole slide. Importantly, spatial information is preserved by the positional barcodes. This results in a considerably simpler workflow compared to FISSEQ and sequential FISH. The method currently utilizes 1000 capture features of 100 μm diameter, spread across a 6 × 6 mm area. It is a promising approach that could enable non-specialized laboratories to robustly perform spatial transcriptomics in tissues, albeit with a currently low resolution and spatial coverage.

Both sequential FISH and FISSEQ methods suffer from densities of labeled transcripts that surpass the resolution capacity of the optical system. Because of the diffraction limit of ~0.3um, a typical mammalian cell with a diameter of 10 μm would only contain on the order of 10^4 'pixels'. Since there are around 10^6 mRNA molecules per cell, each pixel thus contains about 100 mRNA molecules, rendering efficient optical detection impossible. To overcome this limitation Coskun and Cai have applied local image correlation patterns to quantify highly abundant transcripts with conventional fluorescence microscopy in both cultured single cells and tissue sections [37]. An alternative approach to saturated transcript density consists of tissue expansion. Expansion FISH (ExFISH) links RNA molecules to a swellable gel and enables effectively super resolution RNA imaging with diffraction-limited microscopes in thick tissues [38•]. The combination of ExFISH with self-amplifying smFISH probes [39] led to bright dots in thick tissues that can be rapidly acquired with lightsheet microscopy [38•].

Combination of single cell RNA sequencing with tissue reference maps

A complementary approach for obtaining spatial transcriptomics is to infer the tissue coordinate from the single cell expression patterns of dissociated tissues (Figure 3d). Single cell RNA sequencing (scRNAseq) methods generate simultaneous gene expression data of thousands of cells [9–13]. A given cell's positional origin within the tissue of interest is lost, however, during the dissociation process. Several laboratories have utilized pre-established tissue reference maps, which consist of the spatial expression patterns of a selected subset of marker genes, to infer the positional information of cells in the scRNAseq data (Figure 3d). The laboratory of Stefan Heller generated single cell qRT-PCR data of several hundred sorted otocyst cells [40]. Subsequently they used expression patterns of a few genes that were previously mapped out using RNA *in-situ* hybridization, to establish a three-dimensional model of the otocyst in the shape of a sphere. This elegant approach enabled mapping the single cell expression dataset back to its positional origin by using 3D principal component analysis [40]. Ensuing studies generated *in situ* maps and used them to infer the position of scRNAseq data in the zebrafish embryo [41••], the developing brain of a bristle worm [42••], and gastrulating mouse embryos [43,44].

A recent study combined scRNAseq with tissue reference maps that were generated with smFISH to reconstruct the global spatial expression profiles along the mammalian liver lobule [45••]. The higher precision and dynamic range of smFISH, compared to traditional *in-situ* hybridization techniques enables precise spatial inference with significantly smaller number of marker genes. The use of smFISH to create tissue reference maps becomes critical in tissues where genes are expressed in a graded, rather than in a binary manner. The tissue reference map-based methods combine the advantages of two worlds: the ability of sequencing methods to perform deep unbiased profiling of large amounts of single cells and preservation of positional information of *in situ* methods. Central to these methods are sophisticated algorithms for inferring the positional information while accounting for different experimental sources of variability.

Theory and outlook

The diverse methods for spatial transcriptomics are expected to generate highly detailed maps of single cell gene expression at any tissue coordinate. As with other previous revolutions in systems biology we expect a parallel development of theoretical frameworks to integrate these measurements and identify tissue design principles. One central goal of mammalian systems biology is to understand a tissue's metabolic response to diverse inputs. Large-scale reconstructions of liver metabolic networks [46] enable simulating such responses, however these networks model the liver as a homogenous well-mixed pool of cells. Given the intricate spatial division of labor along the liver lobule radial axis there is a need to develop metabolic modeling approaches that will consist of connected cellular subnetworks, which exchange substrates and products [47–49]. Key to the success of these methods is an integration of the single cell spatial measurements with advanced tissue imaging techniques to define the interactions between the relevant tissue cellular components [50,51].

A second avenue to explore is the modeling of how cell circuits achieve tissue homeostasis. Interactions between cells in a tissue, through juxtacrine and paracrine signaling, are crucial for tuning overall tissue function and for maintaining homeostatic balance of biomass. Theoretical works that analyze the performance of diverse cell circuits will be instrumental to understanding how cells in tissues jointly achieve physiological goals [52–55]. Another exciting theoretical avenue is the analysis of the conditions when division of labor among cells is more optimal for organ function compared to homogenous tissues with generalist cells. This question has analogies in ecology, economics and engineering, for example, the mathematical

analysis of castes systems in social insect colonies [56], and the theory of pareto optimality [57,58]. The astonishing speed of current developments in imaging and sequencing technologies promises better resolution, better transcriptome coverage and cheaper assays in the near future. Integrating this flood of high quality data with theory will dramatically advance the field of tissue systems biology and could expose the underlying principles of tissue organization.

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