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REVIEW

Exploring single cells in space and time during tissue development, homeostasis and regeneration

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ABSTRACT

Complex 3D tissues arise during development following tightly organized events in space and time. In particular, gene regulatory networks and local interactions between single cells lead to emergent properties at the tissue and organism levels. To understand the design principles of tissue organization, we need to characterize individual cells at given times, but we also need to consider the collective behavior of multiple cells across different spatial and temporal scales. In recent years, powerful single cell methods have been developed to characterize cells in tissues and to address the challenging questions of how different tissues are formed throughout development, maintained in homeostasis, and repaired after injury and disease. These approaches have led to a massive increase in data pertaining to both mRNA and protein abundances in single cells. As we review here, these new technologies, in combination with *in toto* live imaging, now allow us to bridge spatial and temporal information quantitatively at the single cell level and generate a mechanistic understanding of tissue development.

KEY WORDS: Gene regulatory networks, Local interactions, Single cell, Multiplexed imaging, Cell-to-cell variability

Introduction

The cell is the smallest structural and functional unit of living organisms (Schwann, 1839). During development, populations of cells interact and coordinate their behaviors in space and time to generate, bottom up, tissues and organs without a pre-defined blueprint (Bryant and Mostov, 2008; Gilmour et al., 2017; O'Brien et al., 2002). In particular, cells integrate complex intracellular and extracellular cues, both chemical and mechanical, and make individual decisions with respect to cell proliferation, differentiation or migration that, at the population level, lead to emergent processes such as tissue morphogenesis, homeostasis and regeneration (Bryant and Mostov, 2008; Chau et al., 2012; Sasai, 2013; Xavier da Silveira Dos Santos and Liberali, 2018). To achieve this, single cells have evolved different molecular and cellular mechanisms to sense neighboring cells and their local environment, and to regulate numerous biological features such as the cell cycle, cell shape, gene expression and polarization (Bryant and Mostov, 2008; Kim et al., 2018; Snijder and Pelkmans, 2011). It is thus essential, in order to dissect the complexity of coordinated events such as development and regeneration in health and disease, to measure the multivariate phenotypic and genetic states of single cells and to also place each single cell in its environmental context at a specific moment in time. A number of recently developed technologies now allow us to probe cells in this detailed manner. In this Review, we explore these approaches, highlighting how single cell methods that provide spatial and temporal resolution can be used to analyze tissue organization quantitatively and to ask fundamental questions about development, regeneration and disease.

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Cell state changes during development, homeostasis and regeneration: integrating signals in time and space

During development, single cells become specified and cell lineages develop, thereby allowing tissues consisting of multiple functional cell types to form. Cell lineages are often described as discrete populations of cells undergoing progressive differentiation steps: stem cells, progenitors, transient amplifying cells, and differentiated cells; however, this hierarchy is not always linear and irreversible (Clevers, 2015; Clevers and Watt, 2018; Sánchez Alvarado and Yamanaka, 2014). During regeneration, for example, this hierarchy can be reverted and single differentiated cells can reprogram, often acquiring embryonic pluripotent states that confer extensive plasticity on the system. It is therefore often difficult to distinguish cell type from cell state. Currently, a 'cell type' is frequently defined by static and abundant cell features, such as functional molecular markers and landmark genes, whereas a 'cell state' is explained by temporary traits (Wagner et al., 2016; see also Morris, 2019, in this issue). As we briefly summarize below, these 'cell state' traits are often regulated rhythmically by the cell cycle and circadian rhythm, and may be spatially induced by local signaling, nutrients, mechanics and microenvironments, giving rise to fractions of cells with different probabilities of differentiating. Even in environmentally controlled cell culture conditions, cell-to-cell variability in cellular states is observed and can be accurately predicted by considering the local population context and cell cycle phases (Altschuler and Wu, 2010; Battich et al., 2013; 2015; Frechin et al., 2015; Gut et al., 2015; Snijder et al., 2009, 2012; Spencer et al., 2009).

Spatial regulation of cell states

Spatial regulation of cellular states has been observed during developmental processes but also during homeostasis and regeneration (Chacón-Martínez et al., 2018; Nowotschin et al., 2019; Page et al., 2013; Ritsma et al., 2014). One example of how cell position can influence cell state comes from the adult intestinal epithelium. In this context, tissue homeostasis is controlled by the whole intestinal stem cell compartment, but each cell contributes to tissue self-renewal to a different extent depending on its location in the stem cell niche (Ritsma et al., 2014). This also highlights the presence of variability within apparently uniform populations (Vermeulen and Snippert, 2014). Moreover, it has also been shown that differentiating progenitors in the intestinal epithelium can revert to a proliferative state and repopulate the stem cell niche

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following injury (Buczacki et al., 2013; Tetteh et al., 2016; Tian et al., 2011; van Es et al., 2012). This suggests that 'stemness' is a cell state that can be reached by progenitors or more mature cells in specific environments or under certain conditions. Notably, this feature characterizes several tissues that display regeneration and a high degree of plasticity, such as the epidermis and liver, lung, kidney, intestine and stomach epithelia (Donati and Watt, 2015; Evans et al., 2013; Michalopoulos, 2011; Michalopoulos et al., 2005; Qiao et al., 2007; Stange et al., 2013; Tata et al., 2013; Tetteh et al., 2015; Vogetseder et al., 2007; Yan et al., 2017; Yanger et al., 2013).

Some of the factors that control the spatial regulation of cell state have been identified. Indeed, it is known that the stem cell niche, which consists of extracellular matrix, stem cells and their progeny, creates an environment that induces and maintains stemness. For instance, Paneth cells in the crypts of the intestine produce and release Wnt, express the Notch ligand Dll1 on their cell surface, and produce lactate, which sustains the stemness of neighboring Lgr5-positive cells (Clevers, 2013; Pellegrinet et al., 2011; Rodríguez-Colman et al., 2017; Sato and Clevers, 2013; Sato et al., 2011). Cells moving away from this location lose stem-like properties and start to differentiate (Chacón-Martínez et al., 2018; Sato et al., 2011). Progenitor cells in hair follicles similarly influence stem cell state, positively regulating stem cell proliferation by paracrine Sonic hedgehog (Shh) signaling (Hsu et al., 2014). The newly generated cells then compete with progenitor cells for space in the niche, thus separating the inducing progenitor cells further from the stem cells. By contrast, stem cells exposed to reduced Shh stimulation by distant progenitor cells divide less frequently and shift to a less proliferative state. It is therefore becoming clear that cell state is not hard-wired per se but is determined by the coordination of intracellular and extracellular inputs from neighboring cells and the environment (Chacón-Martínez et al., 2018; Hsu et al., 2014).

Spatial stimuli are not only chemical but can also be mechanical, arising for instance from changes in tissue architecture. Indeed, crowding in the basal layer of the embryonic epidermis regulates the state of progenitor cells and, accordingly, tissue homeostasis. In this context, crowded cells show reduced cortical tension and increased cell-to-cell adhesion and, thereby, exhibit reduced contact with the substrate, leading to differentiation and delamination of the crowded cells from the basal layer (Miroshnikova et al., 2018). In the adult epidermis, the mechanism monitoring cell state and the balance between proliferation and differentiation changes such that differentiating and delaminating cells trigger compensatory cell divisions in neighboring cells and exit from the G1 phase of the cell cycle (Mesa et al., 2018). These findings again highlight that cells sense their environment and change state depending on neighboring cells and spatial constraints.

Mechanical inputs also play a role in defining the spatial context and organization of cells and highlighting when this organization is perturbed. For example, cells flanking a wound after tissue injury are exposed to altered substrate stiffness, which induces reprogramming to a transitory progenitor-like state that restores the homeostatic condition. This phenomenon has been extensively studied in the epidermis but is also observed in heart repair (Evans et al., 2013; Morikawa et al., 2015). Important factors in this process are the cellular mechanosensors YAP/TAZ, which are co-transcriptional activators able to transduce changes in the spatial environment of a cell into different gene regulatory programs and cell behaviors (Dupont et al., 2011; Panciera et al., 2017). During homeostasis, YAP/TAZ are found in proliferating cells of the stem cell niche of the intestine, epidermis, mammary gland, and airway epithelia and maintain their cellular state. More importantly, their activity is necessary during tissue regeneration *in vivo* and organoid formation *in vitro* (Dupont et al., 2011; Elbediwy et al., 2016; Evans et al., 2013; Gjorevski et al., 2016; Gregorieff et al., 2015; Gregorieff and Wrana, 2017; Morikawa et al., 2015; Panciera et al., 2017, 2016; Patel et al., 2017; Schlegelmilch et al., 2011; Serra et al., 2019; Tan et al., 2017; Tremblay and Camargo, 2012; Xin et al., 2013; Yui et al., 2018; Zhao et al., 2014). In the context of intestinal organoid development, which mimics intestinal regeneration, Yap1 acts as a sensor of tissue integrity and its transient and heterogeneous activation is essential for the regulation of cellular states and the induction of symmetry breaking (Serra et al., 2019).

In conclusion, these findings highlight the importance of the environment and neighboring cells in defining cell state. Different environments increase the propensity of a cell to transition towards a specific state and this transition can further influence the states of adjacent cells and eventually of the whole cell population, creating a feedback loop oriented towards a dynamic equilibrium.

Temporal regulation of cell states

Time is also an important cue that influences the state of a cell. In this regard, modulation of the cell cycle and the circadian clock are well-known mechanisms that temporally control cell states. For example, in several systems it has been argued that cells in the G1 phase of the cell cycle have a higher propensity to differentiate (Calegari and Huttner, 2003; Coronado et al., 2013; Lange and Calegari, 2010; Lange et al., 2009). During G1, cells integrate information from their spatial context, signaling pathways, metabolic and stress stimuli, among others, and decide whether to divide or stop proliferating and differentiate. It has been suggested that the longer the G1 phase is, the more time cells are exposed to factors that can accumulate and induce a cell-state conversion once they reach a specific threshold (Calegari and Huttner, 2003).

The role of time in regulating cell state has also been explored in human embryonic stem cells (hESCs), revealing that hESCs differentiate towards endoderm or neuroectoderm depending on whether they are in early G1 or late G1, respectively (Pauklin and Vallier, 2013). This effect is mediated by the transcriptional activity of Nodal/Activin signaling, which is high in early G1 and prompts the expression of endoderm-specific genes; by contrast, this activity is inhibited in late G1, when the concentration of cyclin D proteins is high and prevents nuclear localization of the Nodal/Activin downstream effectors Smad2/3, leading to neuroectoderm differentiation (Pauklin and Vallier, 2013).

Cellular states are also affected by the oscillatory activity of the circadian clock machinery. The circadian clock is connected to the cell cycle and regulates proliferation in several mammalian tissues and organs, such as the bone marrow, brain, skin, and oral mucosa (Bjarnason et al., 2001; Dickmeis and Foulkes, 2011; Granda et al., 2005). For example, quiescent neural progenitors (qNPs) of the mouse adult hippocampus exhibit oscillating proliferation events that peak during the night. Indeed, the Per2 and Bmal1 (Arntl) components of the clock machinery set a permissive time-frame in which qNPs can enter the cell cycle and divide (Bouchard-Cannon et al., 2013). These findings suggest that cells rhythmically experience varying cell states, based on the circadian clock, that affect their probability of transitioning to a differentiated state.

In summary, it is clear that individual cells within a tissue or organ are subjected to multiple factors, both spatially and temporally, that can influence their state, their probability to differentiate and hence their behavior (Fig. 1), ultimately affecting their form and function. Therefore, a single cell approach that

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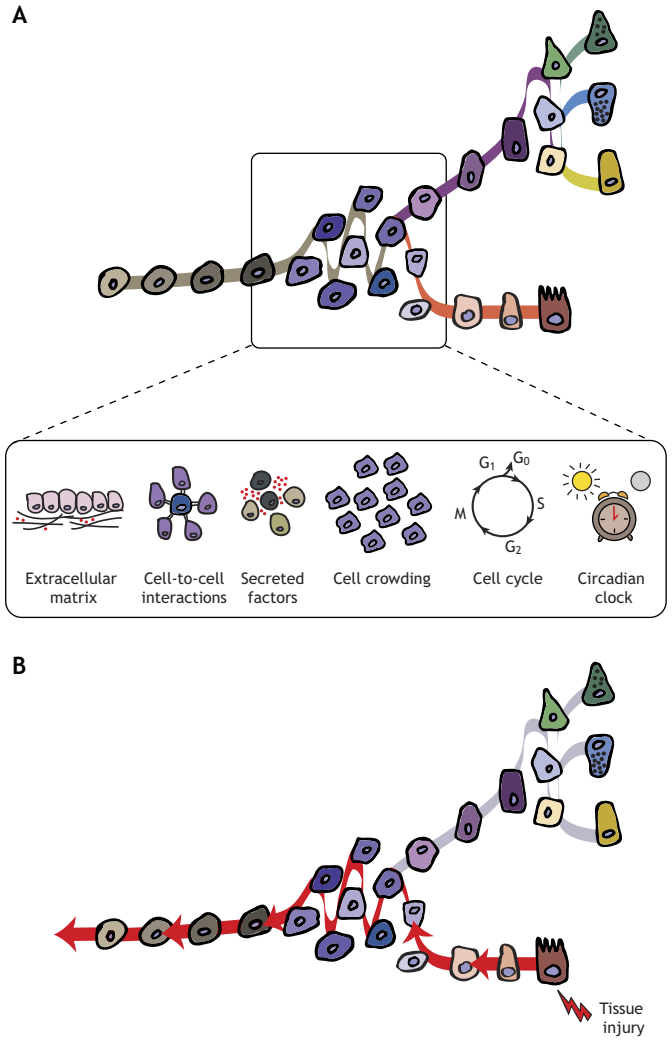

Fig. 1. Single cells in space and time. Single cells integrate multifactorial cues, both chemical and mechanical, to adapt their state in a timely manner and drive tissue morphogenesis, maintenance of homeostatic stem cell niches and repair of injured tissues. (A) Intestinal cells transition between diverse states and differentiate into distinct cell types, as visualized here on a pseudotime trajectory. Several factors can influence these cell states, especially at bifurcations and decision-making moments in the trajectory. These factors include the extracellular matrix, cell-to-cell interactions, secreted factors, cell crowding, the cell cycle and the circadian clock, among others. (B) After tissue injury, cells can revert their fate (indicated by red arrows), de-differentiate and adopt different cell states to re-establish tissue homeostasis.

considers these spatial and temporal localizations of a cell within a collectivity is important for understanding how tissue homeostasis is preserved and how development and regeneration are regulated. In the next section, we review recently developed single cell technologies that can provide us with such spatial and temporal resolution and discuss how their combination can bring us closer to an understanding of the key biological processes that govern development and regeneration.

Spatially resolved single cell methods

Current technologies that offer single cell resolution of transcriptomes and proteomes fall into two general categories: single cell RNA sequencing (scRNAseq) approaches and imaging-based approaches. Major progress in increasing the throughput and depth of scRNAseq has been achieved in recent years (Hashimshony et al., 2016, 2012; Islam et al., 2014; Jaitin et al., 2014; Klein et al., 2015; Macosko et al., 2015; Picelli et al., 2013; Ramsköld et al., 2012; Rosenberg et al., 2018), allowing the quantification of cellular states as well as heterogeneity between individual cells (for recent reviews on the advantages and disadvantages of different methods, as well as the challenges of data analysis, see Papalexi and Satija, 2018; Stegle et al., 2015; and Tritschler et al., 2019, in this issue). Although accounting for complexity and noise in scRNAseq experiments remains an important technical hurdle (Tanay and Regev, 2017), we would argue that the major limitation of biological interpretation lies in the lack of spatial and temporal resolution provided by these techniques. Because these techniques require that tissues need to be first dissociated into single cells, the local microenvironment and social context of each cell is lost and, as we have highlighted above, it is this local context that is the key determinant of the cellular state and accounts for much of the observed heterogeneity between cells (Altschuler and Wu, 2010; Pelkmans, 2012; Snijder and Pelkmans, 2011). To understand the functional importance of cell-to-cell variability and to characterize processes that lead to cellular decision-making events, it is important to account for spatial information when analyzing single cell states. As we discuss below, this can be achieved either by mapping scRNAseq data onto spatial reference maps, or by directly visualizing transcriptomes and proteomes within intact cells, tissues and organs.

Mapping scRNAseq to spatial reference maps

An interesting approach to indirectly infer cell-to-cell interactions from scRNAseq data without explicit knowledge of spatial context is to map receptor expression in one cell to ligand expression in other cells (Camp et al., 2017; Kumar et al., 2018; Zepp et al., 2017). However, this only allows the identification of potential interactions and does not reveal whether pairs of cells are actually associated in space.

In parallel, different approaches have been developed to link single cell transcriptome information from scRNAseq data to the original spatial coordinates of the corresponding cell in its tissue context (Fig. 2A) (for reviews on single cell genomics expanding to spatial context, see Giladi and Amit, 2018; Lein et al., 2017; Moor and Itzkovitz, 2017; Tanay and Regev, 2017). This can be achieved by mapping cells computationally to known expression profiles of landmark genes (spatial reference maps) generated by *in situ* hybridization (Achim et al., 2015; Halpern et al., 2017; Satija et al., 2015). Such an approach has been applied successfully to study zebrafish embryos (with the aid of a computational approach called Seurat) (Satija et al., 2015), the brain of a marine annelid (Achim et al., 2015), *Drosophila* embryos (Karaiskos et al., 2017) and the mammalian liver (Halpern et al., 2017).

To allow mapping in the absence of pre-established landmark genes, scRNAseq has also been combined with laser capture microdissection (Moor et al., 2018). For example, analysis of the zonation of enterocytes along the villus axis of the small intestine has been performed by laser capture microdissection of equally spaced compartments of the villus to infer *de novo* landmark genes from bulk-RNAseq; the landmark genes were then used to map the positions of sequenced single cells onto the villus (Moor et al., 2018). Intriguingly, the extended heterogeneity measured between individual enterocytes could be explained by functional sub-specialization along the villi axis. Unfortunately, however, not all model systems are reproducible enough to show stereotypical spatial organization in order to assign landmark genes. This is often the case, for example, for *in vitro* organoid systems that show non-reproducible spatial variability (Huch et al., 2017).

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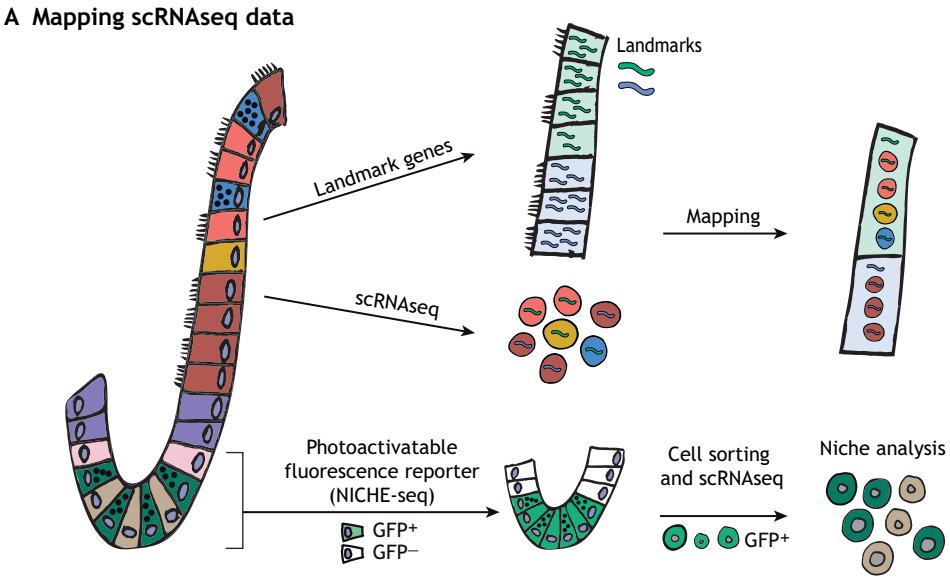

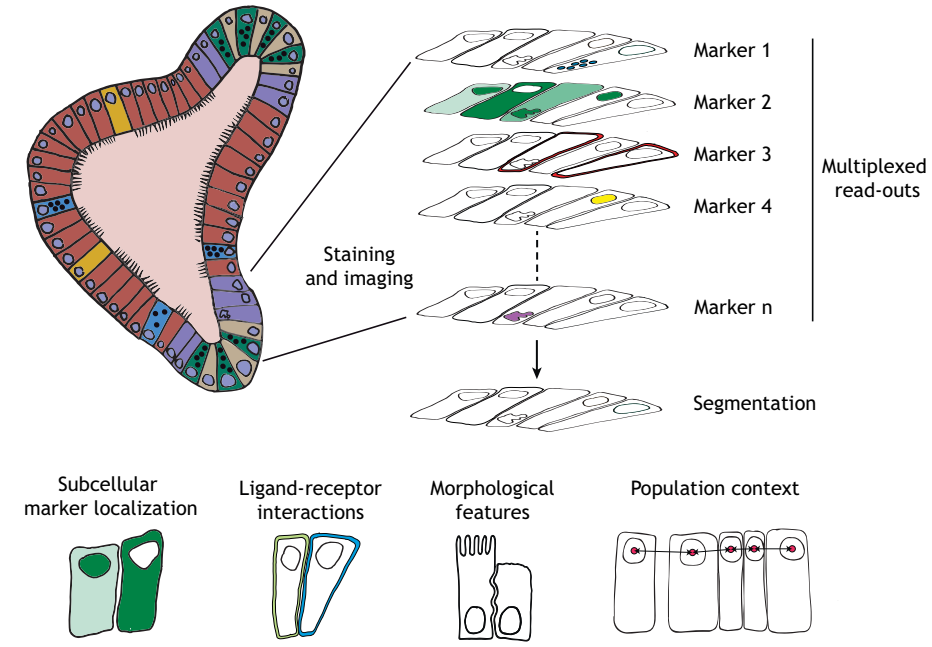

Fig. 2. Spatially resolved single cell methods. (A) Sequenced single cells (i.e. scRNAseq data) can be mapped to spatial coordinates within the tissue using known expression patterns of landmark genes. Landmark genes are often detected by *in situ* hybridization or can be identified by laser capture microdissection coupled to bulk sequencing. Alternatively, cells from a known and restricted spatial location can be analyzed with scRNAseq. In NICHE-seq, for example, cells within a precisely defined tissue location are fluorescently labeled and analyzed by scRNAseq. (B) Image-based methods allow the analysis of cells in their intact spatial context and microenvironment. Combining multiplexed read-outs with single cell segmentation allows the simultaneous quantification of protein and RNA expression together with information about localization, cell morphology and local environment (e.g. the number and identity of neighbors, local crowding, etc.).

As an alternative to mapping cells to pre-established reference maps, an approach termed de novo spatial reconstruction (novoSpaRc) has been used to computationally infer positional information based on the underlying spatial organization of gene expression with or without knowledge about landmark genes (Nitzan et al., 2018). The scRNAseq-based analysis of cells in a known area of origin has also been used to preserve spatial information (Chen et al., 2017; Medaglia et al., 2017; Nichterwitz et al., 2016). For example, in an approach named NICHE-seq (Medaglia et al., 2017), cells expressing photoactivatable green fluorescent proteins were labeled within the lymph nodes and spleens of mice using two-photon microscopy; the subsequent analysis of labeled cells by scRNAseq allowed the characterization of T and B cell-specific niches after viral infection.

Although already very powerful, current methods for spatial mapping have their limitations. Landmark genes extracted from small regions of tissue [e.g. via laser capture microdissection or non-single cell fluorescence *in situ* hybridization (FISH)] are still relatively coarse, often representing an average of gene expression over a population of cells. At the moment, this prevents mapping with high spatial resolution to a single cell and does not allow the inference of direct cell-to-cell interactions. It will be interesting to see how reference maps with higher resolution, for example acquired with multiplexed single cell approaches, in combination with new computational frameworks (Nitzan et al., 2018) can be used to further alleviate this limitation. The power of mapping based on multiplexed landmark genes inferred from sequential single-molecule *in situ* hybridization data has recently been demonstrated by dissecting the contribution of cell type and spatial factors to variations in cell state within heterogeneous cellular populations (Zhu et al., 2018).

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Image-based transcriptome and proteome analysis

As an alternative to relating scRNAseq-generated information to positional information, it is possible to use image-based approaches to analyze cells directly in their native context. Ideally, understanding how individual cells within the same spatial region communicate and interact requires the simultaneous assessment of cellular state, local environment, and cell-to-cell contacts (Fig. 2B). An intuitive approach to combined analysis of cell morphology, gene expression and protein localization is the fluorescence imaging of fixed samples combined with computer vision-based cell segmentation (Battich et al., 2015; Liberali et al., 2014; Snijder et al., 2009). This allows extraction of a large number of quantitative features describing multiple molecular factors and their subcellular localization, as well as providing information about cell morphology and population context. Single molecule *in situ* hybridization (smFISH) (Raj et al., 2008) and immunohistochemistry/immunofluorescence are widely used standard methods that allow the quantification of RNA and protein expression and localization with subcellular resolution, without destroying the local context of the cells. In a series of interesting publications, it has been demonstrated that the combined analysis of phenotypic states (as indicated by the cell cycle or marker expression) and the microenvironment (as indicated by local cell density) can predict cell-to-cell variability in mRNA or protein expression levels (Battich et al., 2015; Sero et al., 2015; Snijder et al., 2009).

Classically, these approaches allow the simultaneous quantification of only a small number of markers, because spectral overlap of the available fluorophores limits the number of simultaneously resolvable colors to between two and five. Various approaches have therefore been developed to increase the number of simultaneously resolvable colors. For example, the number was increased up to six using quantum dots (Han et al., 2001), up to seven with Fourier spectroscopy and singular value decomposition (Tsurui et al., 2000), up to nine using spectrally resolved fluorescence lifetime imaging microscopy (Niehorster et al., 2016), and even up to 32 by combining combinatorial labeling and super-resolution imaging (Lubeck and Cai, 2012). The downsides of these techniques, however, include the need for specialized and expensive equipment, a high sensitivity to signal-to-noise ratio (which can confound computational convolution), and limited scalability for higher throughput. As an alternative, methods using sequential read-out of colors have been developed for detecting multiple RNAs and proteins. As we move on to discuss below, these 'multiplexing' techniques have provided a marked increase in throughput using the standard color spectra of available fluorophores and therefore represent promising approaches.

Multiplexing transcriptomes

In situ sequencing

A very powerful approach, which is closely related to RNAseq of isolated cells, is *in situ* sequencing. In RNAseq, RNAs are extracted from the tissue for amplification and detection, whereas with *in situ* sequencing, enzymatic reactions are conducted directly within the original tissue (Ke et al., 2013; Lee et al., 2015, 2014) (for an overview of existing methods and limitations, see Crosetto et al., 2015; Lein et al., 2017; Moor and Itzkovitz, 2017). During this process, each of the four bases of DNA is encoded by one fluorescence color and bases of multiple target sequences are then read out sequentially, directly within the original tissue with subcellular resolution. For certain methods, such as fluorescence *in situ* sequencing (FISSEQ), this potentially allows scaling up to whole genome coverage with single-nucleotide resolution (Lee et al., 2014). However, implementation within complex cellular environments has so far proved difficult. This is especially true of deep tissues in which auto-fluorescence masks fluorescently labeled molecules, and light scattering hampers reliable signal read-out, resulting in low efficiency and accuracy (Lein et al., 2017). In a recently developed method called spatially resolved transcript amplicon read-out mapping (STARmap; Wang et al., 2018b), Wang et al. combined hydrogel-tissue chemistry (Gradinaru et al., 2018) with *in situ* sequencing to facilitate sequencing of targeted sequences within intact 3D-tissues. In this approach, the cross-linking of selected intracellular biomolecules to a network of polymer allowed the preservation of three-dimensional tissue-structural relationships whilst also improving optical properties by clearing lipids and proteins. STARMap was shown to map up to 1000 genes in sections of the mouse brain at single cell resolution, revealing the spatial arrangement and self-clustering organization of different neuronal subtypes. As an alternative to reading out DNA bases directly within the tissue, spatial transcriptomics (Stahl et al., 2016) and Slide-seq (Rodriques et al., 2019) involve transferring mRNAs from the tissue to either spatially barcoded oligonucleotides (Stahl et al., 2016) or DNA-barcoded microparticles (beads) encoding spatial information (Rodriques et al., 2019). Sequencing is then performed outside of the tissue context and spatial information is reconstructed based on the spatial barcodes.

Multiplexed smFISH

Complementary to *in situ* sequencing, multiplexed smFISH methods have been established that allow quantification of the abundance of RNA molecules with high sensitivity and accuracy at subcellular resolution. Compared with normal smFISH, most multiplexed smFISH methods achieve a massive increase in throughput by applying multiple rounds of sequential hybridization and imaging. Similar to normal smFISH, these methods rely on detecting RNA molecules by hybridizing multiple fluorescent probes to transcripts in cells. This results in a single diffraction-limited fluorescence spot per transcript, which can then be resolved by conventional microscopy and accurately quantified. In seqFISH (Lubeck et al., 2014) (Fig. 3A), the identity of each targeted transcript is encoded as a unique color sequence (a barcode) that is sequentially read out over multiple rounds of imaging. However, because each color of each round needs to be identified correctly, and because images between subsequent rounds need to be aligned precisely to allow decoding of the transcript, this leads to the drawback that encoding becomes more prone to misidentification with increasing number of rounds. To overcome this, different error-correction schemes, such as Hamming distance-based error correction in MERFISH (Chen et al., 2015; Moffitt et al., 2016b) (Fig. 3A), have been introduced.

Similar to *in situ* sequencing, multiplexed smFISH is limited by factors such as auto-fluorescence and spatial crowding within cells, when transcripts are too close for simultaneous optical resolution. To overcome this, clearing (Moffitt et al., 2016a) and signal amplification (Choi et al., 2014; Shah et al., 2016a; Kishi et al., 2019) approaches have been developed. In HCR-seqFISH, for example, seqFISH is combined with single-molecule hybridization chain reaction (smHCR) to achieve signal amplification (Shah et al., 2016a); this approach was applied successfully to quantify single cell transcription profiles within the mouse hippocampus (Shah et al., 2016b). To increase the density of RNAs profiled, expansion microscopy (Chen et al., 2016a; Wang et al., 2018a) or a coding schema combining pseudocolors with barcoding (Eng et al., 2017) have been used. Indeed, using the latter method, the profiling of 10,421 nascent transcripts (Shah et al., 2018) as well as the imaging of RNAs for 10,000 genes in single cells has been demonstrated (Eng et al., 2019). As alternatives to sequential barcoding, other methods have used

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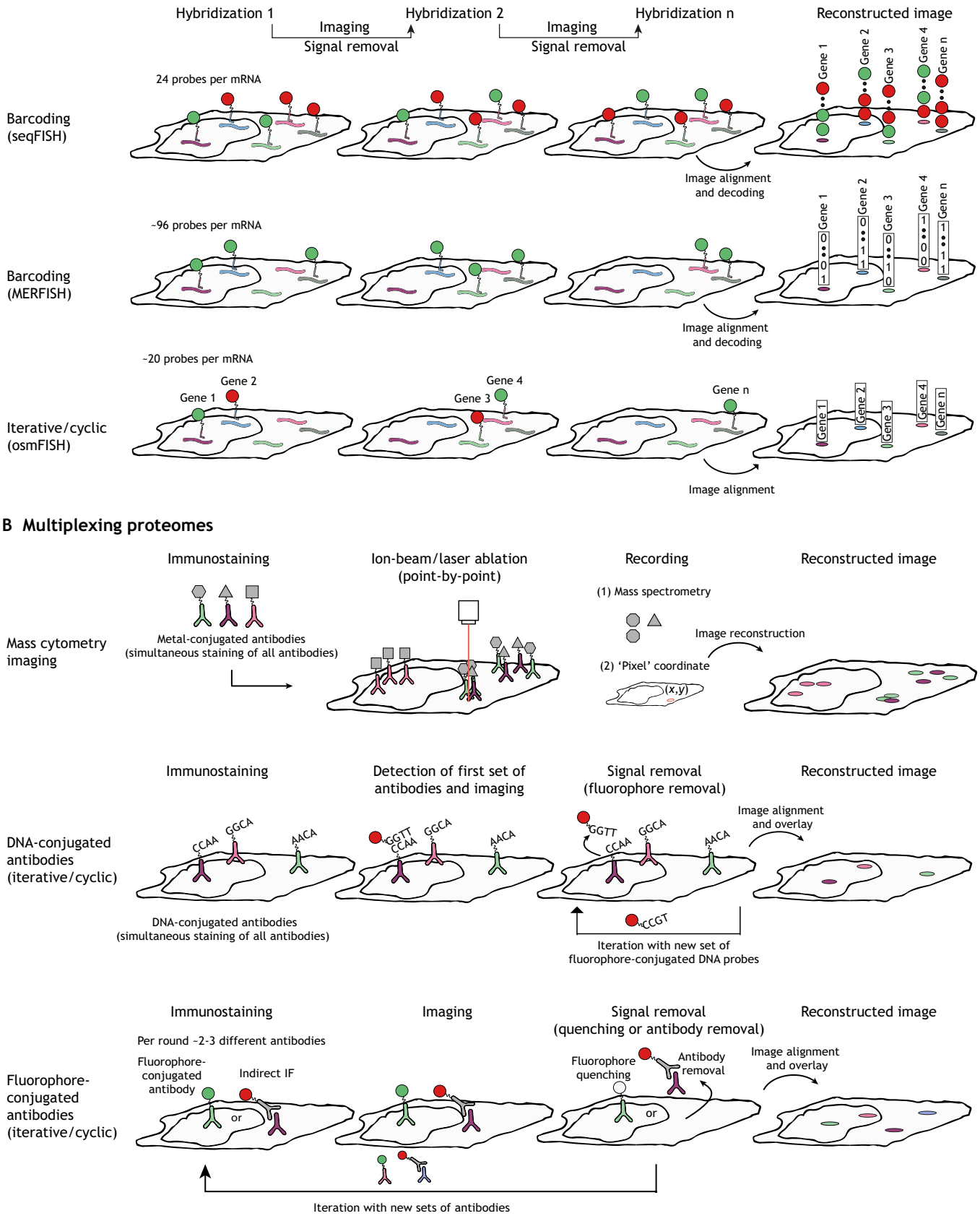

Fig. 3. See next page for legend.

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Fig. 3. Schematics outlining multiplexed RNA and protein detection methods. (A) Multiplexing transcriptomes. In barcoding-based methods (e.g. seqFISH and MERFISH), RNA identity is encoded over sequential hybridization rounds. In seqFISH, RNA identity is barcoded as a color sequence. In MERFISH, RNA identity is barcoded as a binary string that allows error correction based on a modified Hamming code. In iterative/cycle multiplexing (e.g. osmFISH), RNA identity is distinguished by a unique color within each hybridization round. (B) Multiplexing proteomes. Mass cytometry imaging allows the labeling of all target proteins with heavy-metal-conjugated antibodies in one incubation step. Protein abundance is quantified by point-by-point ablation of the sample coupled to mass spectrometry. An image is reconstructed based on the ablation coordinates and mass data. In protein multiplexing with DNA-conjugated antibodies, all target proteins are labeled simultaneously and antibody identity is revealed by iterative exchange of fluorophore-conjugated DNA strands or with *in situ* incorporation of fluorophore-labeled nucleotides (not shown in the schematics). For fluorophore-conjugated antibodies, multiplexing is achieved by iterative rounds of staining, imaging and signal removal.

sequential hybridization whereby each RNA molecule is directly encoded by a unique color in each round, and multiplexing is achieved by multiple rounds of hybridization (Codeluppi et al., 2018; Kishi et al., 2019; Shah et al., 2016b) (Fig. 3A).

Multiplexing proteomes

Although the analysis of mRNA content and localization can provide significant insight, it alone is not sufficient to fully characterize cellular states and microenvironments, and therefore cellular decision making is, as most cellular functions are, ultimately, executed by proteins. Although mRNA often serves as a good estimator of protein abundance, the correlation may not always hold true (Edfors et al., 2016) and the ratio between mRNA and protein is itself dependent on the context of a cell (Popovic et al., 2018). Moreover, many signaling pathways involved in fate decisions or mechanosensing rely on direct protein interactions with membranes, or on the subcellular localization and post-transcriptional modifications of proteins. The same is true for many transcription factors involved in fate decisions, where nuclear or cytoplasmic localization often determines functionality. Thus, the detection and analysis of specific proteins within cells is key for understanding cell states. Compared with RNA detection in cells, however, multiplexed detection of proteins poses additional challenges. Protein detection relies on high-affinity reagents (most often antibodies), which, compared with nucleotide detection of RNAs, impairs flexibility of probe design (Baker, 2015). In addition, proteins, in contrast to RNA molecules, which are sparse, often occur in highly crowded intracellular environments and have dynamic ranges of molecule abundances several orders of magnitude higher than those of mRNA (Liu et al., 2016). Two general type of approaches have been used to achieve multiplexed detection of proteins within cells: mass cytometry imaging and multiplexed fluorescence imaging. As we highlight below, these methods now allow for a marked increase in throughput and predictability of cellular states.

Mass cytometry imaging

In mass cytometry imaging (Bodenmiller, 2016), antibodies are conjugated to heavy-metal isotopes and quantified by mass spectrometry (Fig. 3B). As these heavy-metal isotopes each have a unique atomic mass, mass spectrometry is used to discriminate isotopes with high accuracy and high multiplicity over a large dynamic range of molecule abundance. Two main mass cytometry imaging approaches exist, coupling either ion beam imaging [multiplexed ion beam imaging (MIBI); Angelo et al., 2014] or laser ablation [imaging mass cytometry (IMC); Giesen et al., 2014] to mass cytometry. In contrast to regular mass cytometry (e.g. CyTof) (Spitzer and Nolan, 2016), in which suspension samples are analyzed, mass cytometry imaging involves point-by-point ablation of the sample out of its natural context. Computer reconstruction then allows the generation of images of protein expression, with spatial information, based on mass data. For instance, MIBI (Angelo et al., 2014) currently allows simultaneous measurement of the spatial expression of up to 36 proteins with subcellular resolution (Keren et al., 2018). Interestingly, using MIBI to study the tumor-immune microenvironment in triple-negative breast cancer patients, Keren et al. (2018) found that some cells from different lineages but with similar expression profiles tended to be enriched in spatial proximity. This suggests, again, that some phenotypic features of cells may be driven more by their microenvironment rather than being an intrinsic property of cell type. IMC allows the measurement of up to 32 proteins with a resolution of ∼1 µm (Giesen et al., 2014) and can be used for the simultaneous measurement of mRNA and protein levels (Schulz et al., 2018). Overall, these mass cytometry imaging techniques allow quantification of antibody abundance without the confounding factor of autofluorescence observed in fluorescence imaging and allow simultaneous staining of all antibodies in a single experimental step. However, the latter comes with the limitation that high-abundance antibodies may mask epitopes of low-abundance proteins. In addition, antibodies need to be labeled using costly and labor intense methods, and the technique generally involves low throughput by slow point-by-point scanning.

Multiplexed fluorescence imaging

Similar to multiplexed smFISH, various technologies have applied sequential fluorescence imaging to detect multiple proteins using standard fluorescence microscopy equipment. Promising methods, such as co-detection by indexing (CODEX) (Goltsev et al., 2018), DNA exchange imaging (DEI) (Wang et al., 2017) and Immuno-SABER (Saka et al., 2018 preprint), rely on DNA-barcoded antibodies (Fig. 3B). Similar to mass cytometry imaging, these methods involve a single round of immunostaining. The identity of each antibody is then revealed via sequential read-out of antibodies, either with *in situ* incorporation of fluorophore-dye-labeled nucleotides (CODEX) or by exchange of short fluorophore-conjugated DNA strands (for DEI and Immuno-SABER). Immuno-SABER also allows additional signal amplification steps.

Alternative methods, based on iterative antibody labeling, can increase the number of proteins that can be detected via the iteration of staining, signal removal, and re-staining with a new set of antibodies (Gerdes et al., 2013; Gut et al., 2018; Lin et al., 2015, 2018) (Fig. 3B). This allows direct use of a large palette of commercially available antibodies but has the disadvantage that many slow primary antibody incubation steps are required. These methods have had to overcome several previously encountered limitations such as incomplete antibody elution (Gendusa et al., 2014) or marked sample degradation due to harsh antibody elution protocols. Signal removal is usually achieved by chemical inactivation of fluorophore dyes (Gerdes et al., 2013; Lin et al., 2015, 2018) or by complete elution of primary and secondary antibodies (Gut et al., 2018). In CycIF (Lin et al., 2015, 2018), primary antibodies that are directly conjugated to fluorophore dyes are inactivated by hydrogen peroxide oxidation at high pH. This allows the quantification of up to 60 proteins (Lin et al., 2018) and has been demonstrated to work in high-throughput plate formats (Lin et al., 2015) as well as for formalin-fixed, paraffin-embedded (FFPE) samples (Lin et al., 2018). However, primary antibodies

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that are already conjugated to the dye are often not commercially available or are dim. Using indirect immunofluorescence overcomes this limitation but has the drawback that primary antibodies produced in identical host species need to be removed after each imaging round (to avoid cross-labeling). In a variant of the CycIF protocol, this removal is achieved by enzymatically digesting the antibodies (Lin et al., 2015). An alternative indirect immunofluorescence approach, termed iterative indirect immunofluorescence imaging (4i) (Gut et al., 2018), has been optimized to work with standard unconjugated primary antibodies in combination with fluorophore dye-conjugated secondary antibodies. Building on previous antibody elution approaches (Pirici et al., 2009; Schubert et al., 2006; Toth and Mezey, 2007) and in particular by introducing an essential step to prevent photo-induced cross-linking of antibodies to the sample, this method allows efficient elution of primary and secondary antibodies. Indeed, by combining multiplexed imaging of more than 40 proteins in ∼20,000 single cells with a data-driven computer vision approach, Gut et al. explored subcellular protein distributions in different cellular states (Gut et al., 2018). More recently, 4i multiplexing has been applied to study the 3D mouse intestinal organoid system. By combining multiplexed imaging time-course experiments with time-resolved scRNAseq data, the molecular mechanism underlying symmetry-breaking events during organoid development was characterized (Serra et al., 2019). This example demonstrates how the combination of single cell technologies with spatial and temporal resolution can quantitatively bridge single cell behavior to collective properties of a developing tissue.

Temporally resolved single cell methods

Another inherent component of biological systems is their dynamics. However, studying tissue dynamics in relation to single cells is a daunting task. Biological processes occur over timescales ranging from seconds to hours or even years. The challenge, therefore, is to plan appropriate time points for sampling at sufficient resolution to collect enough information to reconstruct the process under investigation. Currently, no single technology allows the plotting and capture of complex processes spanning several temporal and spatial scales with sufficient resolution in terms of time-point sampling and cellular state read-outs. However, the combination of multiple technologies is now paving the way for a more comprehensive –in terms of both time and space – understanding of tissue development.

It is clear that highly multiplexed spatially resolved single cell methods can provide snapshots of cell and microenvironment states with an unprecedented depth of information, allowing the study of spectrums of cell types and their spatial organization within tissues (Wang et al., 2018b). Such methods also allow correlation of cellular states and microenvironmental factors (Goltsev et al., 2018; Keren et al., 2018). Importantly, however, information about cell state transitions, the history of a cell, and how temporal events regulate cellular transitions is lost or hidden. Nonetheless, powerful computational inference frameworks have emerged that support the move from descriptive studies of cellular states to models of dynamic events (Bendall et al., 2014; Chen et al., 2016b; Guo et al., 2017; Haghverdi et al., 2016; Herring et al., 2018; Qiu et al., 2017; Setty et al., 2019; Setty et al., 2016; Shin et al., 2015; Trapnell et al., 2014; Weinreb et al., 2018; Wolf et al., 2019). These methods assume that single cells transit from one cellular state to another in a continuous fashion, and that all necessary cellular states for the process under investigation are sampled with sufficient depth, allowing the ordering of cells along a pseudotime trajectory of cellular progression. This process of ‘trajectory inference’ has been applied successfully to various imaging (Gut et al., 2015; Herring et al., 2018; Serra et al., 2019), CyTof (Bendall et al., 2014; Setty et al., 2016) and sequencing (Chen et al., 2016b; Guo et al., 2017; Haghverdi et al., 2016; Qiu et al., 2017; Setty et al., 2019; Shin et al., 2015; Trapnell et al., 2014; Weinreb et al., 2018; Wolf et al., 2019) datasets. However, trajectory inference solely on cellular states has its limitations, as reviewed recently elsewhere (Kester and van Oudenaarden, 2018; Wagner et al., 2016; and, also in this issue, Tritschler et al., 2019).

As an alternative approach to studying cell state transitions, the clonal history of single cells (Alemany et al., 2018; Biddy et al., 2018; Frieda et al., 2017; Raj et al., 2018; Rodríguez-Fraticelli et al., 2018; Spanjaard et al., 2018; Wagner et al., 2018; Yao et al., 2017) or the history of dynamic molecular events (Frieda et al., 2017; Perli et al., 2016) can be recorded in the genome of each cell. Various methods allow cellular states and cellular history to be monitored simultaneously by using multiplexed end-point measurement. For example, Spanjaard et al. used CRISPR/Cas9-induced genetic scars to devise a genetic barcoding system that allows cell-lineage reconstruction based on recorded clonal history and cellular states extracted by scRNAseq (Spanjaard et al., 2018). In another approach, named MEMOIR (Frieda et al., 2017), barcoded recording elements called scratchpads are introduced into mouse ESCs and, by using CRISPR/Cas9-based targeted mutagenesis, the state of those scratchpads is altered in a stochastic fashion as cells proliferate, thus creating a heritable barcode. At an endpoint measurement, scratchpad states, cellular states and spatial information can then be read out by multiplexed smFISH. By using additional independent scratchpads targeted with orthogonal gRNAs expressed in response to specific signals, this system could, in principle, allow the storage of not only clonal history into the genome of each cell but also of dynamic and stimulus-triggered events.

As an alternative to storing events for later read-out, the combination of high temporal resolution time-lapse imaging (to record dynamic events in real time) with cellular endpoint measurements is a powerful tool to study biological processes on different scales. Recent studies have begun to exploit this possibility. Although live imaging may not always be possible because of physical inaccessibility or sample opaqueness, technological improvements in high-throughput confocal microscopy, and especially the introduction of light-sheet microscopy (Huisken et al., 2004; Reynaud et al., 2015), open up major possibilities for live imaging cell populations. Light-sheet microscopy combines high-speed acquisition with low phototoxicity and good optical sectioning at subcellular resolution. Tracking evolving biological processes over a long-term timescale (in days) and using high-speed recordings (seconds or minutes) can bridge different spatial and temporal scales (de Medeiros et al., 2016; Höckendorf et al., 2012). Indeed, light-sheet imaging has been applied to the study of calcium dynamics in plants (Costa et al., 2013), clathrin dynamics, organelle reorganization and cell migration in zebrafish (Liu et al., 2018), division dynamics in tumor spheroids (Lorenzo et al., 2011), mouse intestinal organoid development (Serra et al., 2019) and mouse embryo development, both from pre-implantation embryos (Strnad et al., 2016) and from gastrulation to organogenesis with single cell resolution (McDole et al., 2018). In combination with automated cell tracking, this method also opens up the intriguing possibility of constructing high-resolution fate maps for individual cells over the course of development (McDole et al., 2018; Strnad et al., 2016). However, high-resolution live-imaging data demands efficient ways to segment and visualize data, and major initiatives that tackle this challenge are under way. For example, the Allen Institute for Cell Science (Horwitz, 2016) is developing high-throughput

imaging approaches along with data visualization and integration tools to understand and predict cellular behavior (Chen et al., 2018 preprint; Ounkomol et al., 2018). Furthermore, although these imaging approaches can provide impressive insight into morphological changes, cell movements and cell divisions, the cellular-state read-outs for live imaging are limited to a maximum of six to seven colors (Cutrale et al., 2017; Valm et al., 2017). However, this limitation can be alleviated by combining live imaging with additional end-state measurements (Filipczyk et al., 2015; Hormoz et al., 2016; Takei et al., 2017). In a method called kin correlation analysis (KCA) (Hormoz et al., 2016), single cell tracking was combined with end-state measurements of three cell state markers measured by smFISH; this method was used to show that mouse ESCs traverse along a linear chain of metastable states in a stochastic and reversible fashion, in contrast with the prevailing view that ESC heterogeneity is mostly attributed to random noise. In a recent approach, Gehart et al. used a bi-fluorescent timer reporter to measure the time from the onset of enteroendocrine differentiation and combined it with end point scRNA-seq to build a time-resolved lineage tree of enteroendocrine differentiation in the small intestine (Gehart et al., 2019).

In addition to providing live dynamics for molecular markers, which can help unravel transitions between different cell states inferred from fixed samples, combining live imaging with inferred trajectories from multiplexed snapshots opens up a powerful opportunity to map events in pseudotime back to real time or vice versa. Pseudotime conveys stepwise progression of molecular markers along a trajectory. However, this may not necessarily represent dynamics in real time, which can make purposeful interpretation difficult. By simultaneously acquiring stereotypic features such as marker expression or morphological features from live-imaging and fixed samples, it is possible to infer the respective position of the observed state in both real time and pseudotime. This was recently demonstrated for intestinal organoids where a trajectory inferred from fixed imaging samples was mapped with growth dynamics extracted by light-sheet microscopy data (Serra et al., 2019).

Conclusions and perspectives

Thousands of RNAs and hundreds of proteins can now be quantified in single cells in their spatial context. Combined with powerful computational frameworks and live-imaging technologies, this now allows the mapping of cellular states and the inference of dynamic transitions between these states. Another important layer of information that will be essential to integrate with spatial information is the chromatin landscape (as reviewed in this issue by Ludwig and Bintu, 2019). Therefore, to understand how tissue organization and function emerges, we must continue to move forward from a view of single cells as isolated entities to one in which cellular functions are governed by the dynamic interactions between connected cells within a changing environment. The combination of multiscale spatial and temporal technologies is now enabling the quantitative morphological and molecular description of cells in their natural context and the study of their interactions over time. This will pave the way for fundamental discoveries in cell and developmental biology. For example, we will hopefully be able to obtain predictive models on how cell-to-cell variability emerges, and its functional implications in an evolving biological system. Moreover, these approaches will no doubt provide insights into how probabilistic and metastable cellular states transition to more stable cell fates and how these states are reversed during regeneration and diseases.

Combining datasets spanning multiple spatial and temporal scales will be a formidable task and will require new computational and experimental frameworks. Whereas in the past the limiting number of biological read-outs often hampered interpretation of biological results or prevented study of the full complexity of the process, we now face the challenge of identifying and extracting meaningful conclusions from the plethora of data generated by new technologies. It will, therefore, be important to fit experimental design closely to the biological question of interest and to develop ways to quantitatively reduce data dimensionality and make data interpretation amiable. Moreover, computational frameworks will be required to efficiently handle, normalize, visualize and connect large datasets spanning different scales, with the ultimate aim of understanding decision making at single cell resolution and revealing the design principles of tissue organization during development, regeneration and disease.

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Competing interests

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