Advancing Stem Cell Research through Multimodal Single Cell Analysis

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**Abstract**

Technological advances play a key role in furthering our understanding of stem cell biology, and advancing the prospects of regenerative therapies. Highly parallelized methods, developed in the last decade, can profile DNA, RNA or proteins in thousands of cells and even capture data across two or more modalities (multi-omics). This allows unbiased and precise definition of molecular cell states, thus allowing classification of cell types, tracking of differentiation trajectories and discovery of underlying mechanisms. Despite being based on destructive techniques, novel experimental and bioinformatic approaches enable embedding and extraction of temporal information, which is essential for deconvolution of complex data and establishing cause and effect relationships. Here we provide an overview of recent studies pertinent to stem cell biology, followed by an outlook on how further advances in single cell molecular profiling and computational analysis have the potential to shape the future of both basic and translational research.

**Introduction**

Stem cells are commonly characterized by two, often correlated, properties: extended self-renewal capability and multi-lineage potential. Embryonic development follows a natural progression from the totipotent zygote (able to generate all cell types), to pluripotent cells contributing to the three germ layers which in turn diversify into tissues that will be maintained by more restricted stem and progenitor cells. Establishment of human embryonic stem (ES) cells successfully captured the pluripotent cell state (Thomson et al. 1998), thus raising hopes for the generation of virtually any cell type for therapeutic purposes. In parallel, adult stem cells (e.g., hematopoietic, skeletal, gut) capable of regenerating organs or parts thereof, were pursued, also with a prospect of immediate clinical applications. It quickly became obvious that predicting how a cell state will change (’cell fate’) under certain conditions (Figure 1A is not trivial, limiting the utility of many current stem cell manipulation efforts. Over the past decades, we have made great strides in our understanding of cell fate control, allowing us to direct differentiation of pluripotent cells (Thomson et al. 1998; Ilic and Ogilvie 2017; Montel-Hagen and Crooks 2019; Bernareggi et al. 2019), switch between two differentiated states (transdifferentiation) (Davis et al. 1987; Graf and Enver 2009), or even dedifferentiate mature cells into a pluripotent state (induced pluripotent stem cells iPS) (Gurdon 1962; Takahashi et al. 2007). Despite this progress, our efforts remain largely imprecise and inefficient due to an incomplete understanding of the intricate mechanisms involved. The lack of predictive models forces us to rely on broad screening approaches, followed by subsequent refinement of efficiency, accuracy and quality to ensure efficacy and safety in the clinical setting. Complex single cell measurements are now revolutionizing our view of cell types and hold great promise for dissecting the molecular circuits governing cell transitions and cell fate engineering. In this review we will consider how recent advances in single cell analyses, in particular multi-omics, are helping us to achieve this goal.

**Defining the single cell state**

Cell type definitions are constantly evolving as addition of new cell surface markers allows further subdivision of bulk cell populations. Nevertheless, transcriptomic analyses show that populations isolated using several surface markers typically are heterogenous and more complete molecular information is required for correct cell classification (Treutlein et al. 2014; Shalek et al. 2014; Kumar et al. 2014; Lawson et al. 2018). This realization was particularly impactful in the hematopoietic field, where previously defined progenitor populations are in fact a mixture of cells at various stages of differentiation (Paul et al. 2015; Pietras et al. 2015; Nestorowa et al. 2016; Rodriguez-Fraticelli et al. 2018). Lineage choice decisions are therefore initiated earlier than previously thought. Somewhat perplexingly however, single cell molecular profiling of the immature stem/progenitor compartment suggested a rather flat landscape, without obvious bifurcations corresponding to stepwise commitment. Instead of transitioning through such discrete states, cells appear to be smoothly distributed among stem cells and progenitors expressing lineage commitment markers. It is possible that we have not yet measured the correct molecules, or do not yet comprehend how molecular differences reflect fate choices. Alternatively, cell potential may be regarded as a probability distribution, with each small molecular change in cellular state contributing to the cell fate likelihoods. Nevertheless, it is becoming clear that cell positions in these flat landscapes are related to functional cell types (Hamey and Gottgens, 2019) or reflect cell potential (Weinreb et al. 2018a).

The cell state can be seen as a large set of features (parameters), accommodating over 20,000 genes encoding RNAs and proteins as well as a range of other molecules such as lipids or metabolites. Similarly, extracellular conditions influence cell state and comprise cell neighbors, extra-cellular matrix interactions, endocrine signaling and many other factors. It is impossible to list all the components as we are still discovering new aspects of cell biology (Figure 1A,B). In order to predict and manipulate cell fates we need to precisely define cell state, which in turn requires a wide coverage of different parameters, which is now becoming possible with the development of highly parallel single cell measurements (multi-omics). A detailed list of relevant methods is provided in Table 1, below we will focus on discussing how these methods further our understanding of stem cell biology. Currently, single cell RNA sequencing (scRNA-Seq) profiles RNAs genome-wide across millions of cells in parallel (Svensson et al. 2018). This repertoire can be expanded with detection of individual proteins and their modifications using nucleotide-barcoded antibodies (Stoeckius et al. 2017; Peterson et al. 2017; Mimitou et al. 2019). As mRNA abundance explains only a fraction of protein levels (Genshaft et al. 2016; Darmanis et al. 2016; Gong et al. 2017), the unexplained variance is likely to reflect functional differences between cell types. Indeed, adding information about surface markers to the transcriptomic data has already helped distinguishing T lymphocyte subpopulations (Stoeckius et al. 2017; Peterson et al. 2017). Furthermore, scRNA-Seq can be supplemented with chromatin accessibility information (Buenrostro et al. 2015; Cao et al. 2018) (to provide putative DNA regulatory elements), DNA methylation status (information about gene silencing) (Hou et al. 2016; Angermueller et al. 2016; Clark et al. 2018), protein-chromatin binding (transcription factor activity) (Moudgil et al. 2019), or simply the DNA sequence itself (Macaulay et al. 2015; Dey et al. 2015). These multi-layered data can be used to systematize cell states or mined for molecular mechanisms through analysis of feature-feature and feature-cell state relations (Figure 1C).

**Charting single cell maps**

Tissues consist of numerous cell types, which often exist at greatly varying frequencies, for instance haematopoietic stem cells constitute less than 1 out of 20,000 cells in the adult mouse bone marrow (Kent et al. 2009). Their characterisation requires appropriate sampling, i.e. capture of sufficient number of single cells. Subsequently, through systematic comparison gene expression patterns, we can chart a map of observed states in the high-dimensional feature space (Figure 1C). In the context of scRNA-Seq, these are often referred to as transcriptomic landscapes. Navigating these maps provides an unbiased classification of cell types, and based on observed progressions, inference of putative transitions and cell origins, while providing markers for prospective cell isolation. These approaches have been quickly employed in stem cell biology, for instance to identify human adult skeletal stem cells (Chan et al. 2018) or a new bipotential alveolar progenitor (Treutlein et al. 2014). scRNA-Seq analyses revealed also a new population of self-renewing progenitor-like macrophages in atherosclerotic plaques (Lin et al. 2019) and showed that during skin regeneration a fraction of skin fibroblasts and adipocytes share a common hematopoietic origin (Guerrero-Juarez et al. 2019). Precise reference maps also help avoiding misclassifications. The CD45-, Ter119-, CD31- population has been widely regarded as stromal bone marrow cells, based on absence of typical blood lineage markers (Morikawa et al. 2009; Méndez-Ferrer et al. 2010). Only recently has it become apparent that 85% of these cells are in fact of hematopoietic origin and represent a mixture of pro-B and pre-proerythroblast progenitors (Boulais et al. 2018). Large scale initiatives, such as the Human Cell Atlas (Regev et al. 2017), are on their way to provide global transcriptomic reference maps for many human tissues and we predict that as sequencing costs decrease, scRNA-Seq will become a routine diagnostic technique.

The combination of transcriptomics and imaging enables generation of literal tissue maps, where cell locations and morphological features are overlaid with gene expression information. Several platforms have been developed for this purpose, each with specific advantages. Arrays of immobilized oligonucleotides allow rapid expression profiling across large tissue sections, suitable for histological analysis, but with a limited spatial resolution and number of genes assayed (Ståhl et al. 2016; Salmén et al. 2018; Rodriques et al. 2019, Vickovic et al. 2019). A much higher resolution is delivered by methods relying on in-situ sequencing (Lee et al. 2015) or hybridisation (Femino et al. 1998). Although these methods capture RNAs more efficiently than the typical scRNA-Seq protocols (Chen et al. 2015), measurement of multiple genes had been challenging due to microscopy limitations. Recently developed sophisticated encoding schemes enable detection of 100-1000 pre-selected mRNAs both in cells grown *in vitro* (MERFISH)(Chen et al. 2015) and in tissue sections (seqFISH, STARMAP) (Shah et al. 2018; Wang et al. 2018b). The second iteration of the seqFISH protocol (seqFISH+) employs super-resolution and pseudo-color encoding to read out 10,000 transcripts in parallel, with the number of detected molecules per cell approaching the level of the droplet-based scRNA-Seq methods (Eng et al. 2019). STARMAP, although not yet capable of detecting quite as many genes, enables imaging of 150*µ*m thick tissue sections capturing a true three-dimensional view of the tissue. Considerable progress has also been made to detect proteins while preserving spatial information. Most methods employ antibody-based detection, either through iterative sequential labelling (MxIF and CycIF) (Gerdes et al. 2013; Lin et al. 2015) or through labelling antibodies with barcodes (CODEX, DEI, Immuno-SABER) (Wang et al. 2017; Goltsev et al. 2018; Saka et al. 2018). So far, these techniques are limited to fewer than 100 proteins. New imaging approaches, such as imaging mass cytometry (Giesen et al. 2014), vibrational imaging (Wei et al. 2017) or multiplexed ion beam imaging (Angelo et al. 2014) should permit acquisition of more targets, as well as multimodal analysis (Schulz et al. 2018). Until now spatial transcriptomics focused on brain tissue. In the future we expect that imaging ’omics’ technologies will provide great insight into stem-cell niche communication (Ferraro et al. 2010) and will be instrumental for the ongoing efforts of 3D tissue engineering (Camp et al. 2018).

The maps currently generated contain a large number of missing values and gaps among cell states. This is due to limitations of the number of cells analyzed, measurement efficiency (leading to drop-outs) and simultaneous detection of only 2-3 types of molecules. Increasing effort has been placed into the integration of datasets spanning various platforms, cell compositions, species or modalities. This is particularly useful when certain samples are difficult or impossible to source, such as translating findings from animal to human models (Lotfollahi et al. 2018). Recently developed techniques successfully join sparse droplet-based scRNASeq datasets with the higher-coverage plate-based methods (Welch et al. 2018; Stuart et al. 2019; Haghverdi et al. 2018; Alpert et al. 2018) and integrate gene methylation data or spatial FISH with RNA-Seq (Welch et al. 2018; Stuart et al. 2019). Therefore, even though it may not be possible to measure a complete single cell state, computational approaches will help bridge the gaps. The resulting maps will serve not only as an important reference for many areas of biology but also serve as discovery platforms for new processes and molecular mechanisms.

**Inference of molecular mechanisms**

While cell state classification is evidently useful, in order to control cell fate we need to understand the molecular mechanisms driving cell transitions. As we measure a large number of features, we can correlate them and ask how these relations control the respective cell state (Figure 1C). This has been widely used to construct gene regulatory networks, with particular focus on transcription factors (TFs) (The FANTOM Consortium et al. 2009; Cahan et al. 2014; D’Alessio et al. 2015; Göttgens 2015; Rackham et al. 2016; Suo et al. 2018). Recently developed networks successfully recover multiple factors used for transdifferentiation assays (e.g. fibroblasts to hepatocytes) (Cahan et al. 2014; Huang et al. 2011; Rackham et al. 2016), can improve existing assays like the conversion of B-cells to macrophages (Morris et al. 2014) and identify new pathways, such as a combination of 7 TFs converting fibroblasts to keratinocytes (Rackham et al. 2016). Originally based on RNA-Seq datasets, the field now benefits from vast scRNA-Seq data spanning multiple tissues and cell types (Suo et al. 2018). Although useful for guiding research, these networks lack specific molecular mechanisms, such as the DNA regulatory elements and target genes regulated by the TFs.

Data spanning multiple modalities help address these issues. Covariance of mRNA levels (scRNA-Seq) and chromatin accessibility (scATAC-Seq) facilitate linking putative DNA regulatory elements to their gene targets (Cao et al. 2018). In parallel, enrichment of DNA motifs within the ATAC-Seq data highlights putative TFs driving the observed expression patterns. Until recently it was not possible to observe TF-DNA binding events genomewide at single cell resolution. Two recent studies, however, were able to take advantage of transposase tethered to DNA-binding proteins to capture these events in single cells (KayaOkur et al. 2019). Together with mRNA levels (Moudgil et al. 2019), this allows comparison of TF binding patterns with complex cell state maps. Moreover, multiple new techniques allow combined measurement of: chromatin accessibility and CpG methylation (scNOMESeq, COOL-Seq) (Pott 2017; Guo et al. 2017), methylation and gene expression (scM&T, scTRIO-Seq) (Angermueller et al. 2016; Hou et al. 2016), or combining all three features (scNMT-seq) (Clark et al. 2018). These approaches in turn show which methylation sites may affect chromatin conformation and expression (or vice versa). Interestingly, these associations are found, depending on the site, as either positive or negative, and dynamically evolve during differentiation *in vitro* (Clark et al. 2018) or in the embryo (Guo et al. 2017). Besides transcription, multi-omics can also reveal higher modes of regulation. Co-sequencing of both mRNA and small RNAs enables discovery of miRNA-mRNA pairs regulating mRNA stability and translation (Wang et al. 2018a). Simultaneous measurement of proteins and mRNA provides information about translational and post-translational regulation, which are key for modulation of protein levels (Genshaft et al. 2016; Darmanis et al. 2016; Gong et al. 2017).

Arguably, one of the most clinically relevant aspects of multi-omics is the combined analysis of single cells gene expression and mutation status. Navin et al. (2011) demonstrated that genomic sequence can be used to dissect clonal composition in breast cancer, and that a single clone can initiate metastasis. Furthermore, aneuploid changes occur early and remain stable during cancer evolution, whereas point mutations occur later expanding the clonal diversity (Wang et al. 2014). Therefore, simultaneous analysis of genomic DNA and RNA in the same cell appears promising for understanding mechanisms controlling clonal behavior of cancerous cells. Although several techniques offering such capabilities have been developed (Macaulay et al. 2015; Dey et al. 2015; Han et al. 2018), single cell genome sequencing remains expensive and does not offer sufficient coverage to reliably score small mutations. As a result, approaches for detection of specific mutations have been devised. Giustacchini et al. (2017) adapted the Smart-Seq2 protocol to both obtain the transcriptomic profiles and detect the BCR-ABL fusion gene in >2,000 cells from chronic myeloid leukemia patients. The BCR-ABL+ cells contain a quiescent subgroup which selectively survives treatment and is characterized by a specific molecular programme involving Wnt/*β*-catenin, TGF-*β*, NF-*κ*B and HIF1A pathways. Interestingly, the BCR-ABL- stem cells are transcriptionally distinct from normal HSCs, supporting the idea that CML cells affect also the non-mutated cells either through signaling or microenvironment changes. A second iteration of this method, TARGET-SEQ (Rodriguez-Meira et al. 2019), was used to profile transcriptomes and mutational status for several loci in >4500 cells from patients with myeloproliferative disorders (MPN). TARGET-SEQ revealed mutation-dependent stem cell subpopulations together with their molecular features and was also able to define the order of mutation acquisition, previously reported as an important factor in MPN progression (Ortmann et al. 2015; Nice et al. 2018). A less sensitive but more scalable method employed nanowells and long read sequencing technologies to profile >38,000 cells and profile 27 mutation loci in acute myeloid leukemia cells (AML) (van Galen et al. 2019). This yielded a detailed classification of clonal AML subtypes and contrasted differentiation states between the FLT3-ITD and FLT3-TKD leukemias. Altogether, the simultaneous analysis of genetic background and gene expression, although still in its infancy, sheds new light on the molecular programmes underlying carcinogenesis, previously hindered by the need to isolate specific cell types. Importantly, new discoveries will not be limited to cell autonomous programmes within the malignant cells but will also uncover new non-cell-autonomous effects on the host cells contributing to the pathology.

A lack of temporal trajectories connecting cell states and dependence on correlations can make establishing cause and effect difficult. Gata1 and Spi1 (aka PU.1) had been considered critical factors for the choice between erythroid and myeloid fates, based on their expression patterns in committed cells and the ability to drive cells down a respective path upon overexpression (Miyamoto et al. 2002; Graf and Enver 2009). However, only temporally resolved data offers direct insight into the decision process itself, which still remains a contentious issue. Hoppe et al. (2016) used live imaging of mouse hematopoietic stem cells (HSCs) to show that Gata1 and Spi1 do not coexist at the decision point but instead become upregulated soon after, most likely to ensure strict lineage commitment. Conversely, mass cytometry detects Gata1 and Spi1 proteins co-expressed at the common myeloid progenitor stage following human stem and progenitor cell differentiation sampled at multiple timepoints (Palii et al. 2019). The latter study also highlights Klf1 and Fli1 co-expression in bipotent progenitors and their opposing roles in erythroid and megakaryocytic differentiation, reinforcing the idea of antagonistic TF interactions controlling fate decisions. Another interesting example is that of Tal1, a mesodermal marker thought to be responsible for the choice between cardiomyocyte and blood/endothelial fates (Org et al. 2015). As evident from scRNA-Seq analysis of the mouse embryos, cells affected by Tal1 loss do not immediately express cardiac genes, but instead accumulate certain cardiac features only later during development, possibly due to a secondary induction (Scialdone et al. 2016; Pijuan Sala et al. 2019). Tal1 itself appears to be mainly responsible for driving the blood programme. Both of these examples show that the time is a critical variable for understanding molecular functions.

**In Search of Lost Time**

As we are traveling through time in one direction, time observation is problematic. Ideally, one would like to take repeated measurements of the same object (cell) to unambiguously reconstruct a chain of events, assuming the object is not destroyed in the process. This is possible with live imaging microscopy, but only for a few markers at the time. Destructive techniques, on the other hand, provide a huge amount of information in a single measurement, e.g. genome-wide coverage of chromatin state or gene expression pattern for a single cell (Figure 2A). Interestingly, the single time-point data is not devoid of temporal information. In this section we will thus discuss how we can extract time information from the raw -omic data themselves or embed additional temporal data by specific experimental designs (Figure 2B).

Cell differentiation is continuous and largely asynchronous, hence a single snapshot may contain cells at different stages of differentiation. With sufficient sampling, one can observe a continuum of cell states leading from a stem cell into a fully mature cell type. Relying on the similarity between the neighboring cell states (often regarded mathematically as ’distance’), it is possible to arrange cells along a ’pseudotime’ axis (Trapnell et al. 2014; Bendall et al. 2014), reflecting progressive stages of differentiation. This idea immediately attracted large attention and reconstructed differentiation trajectories were often consistent with real time observations (Trapnell et al. 2014; Setty et al. 2016; Tusi et al. 2018). While the pseudotime inference is relatively simple for a unidirectional trajectory (i.e. when all cells differentiate into a single cell type), following branched cell fates is more complicated. This ’trajectory inference’ problem is an area of active development, with dozens of methods already available. We refer readers to other reviews for a comprehensive discussion of assumptions, possibilities and limitations of pseudotime inference (Weinreb et al. 2018b; Watcham et al. 2019) and a benchmark of available methods (Saelens et al. 2019).

The notion of pseudotime has quickly proved useful in *in vitro* settings, beginning with ES and iPS cells. ES cells fluctuate in expression of pluripotency genes, reflecting variable potential (Singh et al. 2007; Chambers et al. 2007; Hayashi et al. 2008). This behavior is dependent on miRNA biogenesis (Kumar et al. 2014) and DNA methylation (Singer et al. 2014) and can be stabilized if cells are cultured in 2i medium conditions (Kumar et al. 2014), which through inhibition of Erk and Gsk3 signaling promote self-renewal and inhibit differentiation (Ying et al. 2008). Similarly, considerable heterogeneity has been observed among cultured human induced pluripotent stem cells (hiPSCs), where there are distinct subpopulations: core, proliferative, and early and late primed for differentiation ordered in a specific hierarchy (Nguyen et al. 2018). Transcriptome-wide analyses characterized the multiple metastable subpopulations in detail (Kumar et al. 2014; Klein et al. 2015; Kolodziejczyk et al. 2015) but these models lacked the conversion rates. The transitions between NanogLo and NanogHi were quantified using time lapse imaging and FISH (Singer et al. 2014) and later extended to epiblast-like, inner cell mass-like and 2C-like (similar to 2 cell stage embryo) states (Hormoz et al. 2016). Thus, analysis of genome-wide snapshot data can identify and characterize cell state transitions but time information is critical for comprehending their dynamics.

An example of *in vivo* application of the pseudotime concept has been the analysis of the hematopoietic hierarchy. The canonical hematopoietic tree, established using selected surface markers, starts with self-renewing multipotent hematopoietic stem cell (long term HSCs), which give rise to multipotent cells with limited self-renewing capacity (short-term HSCs and multipotent progenitors) which in turn lose their potential in a stepwise manner creating more restricted oligopotent and unipotent progenitors leading finally to mature cell types (Eaves 2015; Laurenti and Göttgens 2018). scRNA-Seq analyses revealed that the classically defined progenitor populations do not reflect defined molecular states. Instead the progenitors form a smooth and continuous landscape with no obvious discrete oligopotent progenitor populations (Velten et al. 2017; Laurenti and Göttgens 2018; Nestorowa et al. 2016; Dahlin et al. 2018; Rodriguez-Fraticelli et al. 2018; Tusi et al. 2018; Watcham et al. 2019). Even the relatively well-defined long-term HSCs are heterogenous and molecular markers can further segregate them by self-renewing capacity (Wilson et al. 2015; Kowalczyk et al. 2015; Cabezas-Wallscheid et al. 2017). The pseudotime inspired approaches have been used to reconstruct as many as 8 trajectories, connecting stem cells to: monocytes, neutrophils, megakaryocytes, erythrocytes, basophils, mast cells, eosinophils and lymphoid cells (Velten et al. 2017; Dahlin et al. 2018; Rodriguez-Fraticelli et al. 2018; Tusi et al. 2018). Similar work has also been done in other systems, including olfactory stem cell (Fletcher et al. 2017) and cardiac progenitor cell trajectories (Jia et al. 2018). More work is however needed to pinpoint the fate restriction stages and understand the underlying molecular mechanisms controlling them.

*In silico* reconstruction of differentiation trajectories shows great promise for development of reprogramming and differentiation protocols and engineering of 3D organoid culture conditions (Camp et al. 2018). Transcriptomic landscapes reveal the spectrum of generated cell types and help to validate their identity (Quadrato et al. 2017). Pseudotime trajectories, in turn, can be used to locate undesired cell transitions and improve efficiency. For instance, inhibition of BDNF-NTRK2 drastically reduces the number of undesired non-renal cells during kidney organoid generation and prolonged culture deteriorates organoids rather than allows full cell type maturation (Wu et al. 2018). In a related manner, *in vitro* cell reprogramming is generally inefficient, with only a small fraction of cells acquiring the desired state. scRNA-Seq data often show that a large proportion of cells are funneled into a ’dead-end’, either a different cell type or cell death. Indeed, chemical reprogramming of mouse embryonic fibroblasts (MEFs) into iPSC cells bifurcates into two branches and supplementation of VPA (a histone deacytelase inhibitor) skews the intermediate programme towards the desired pluripotent state (Zhao et al. 2018). Furthermore, during transdifferentiation of MEFs into endoderm progenitors, most cells initially respond to reprogramming but, as revealed by lineage tracing, only a small subset become endodermal while other cells begin reexpressing fibroblast genes (Biddy et al. 2018). The successful route is marked by expression of Mettl7a1, upregulation of which increases reprogramming efficiency. In some cases, it may not be immediately obvious what is the desired cell differentiation trajectory, as comparisons across experimental systems are not trivial. To overcome this issue, Cacchiarelli et al. (2018) developed a ’dynamic time warping’ , a method to align trajectories across two datasets (by pairing the most transcriptomically similar cells) enabling superposition of inferred *in vitro* and *in vivo* differentiation pathways.. A comparison of MyoD-mediated transdifferentiation of human fibroblasts into myotubes with differentiation of primary muscle myoblasts revealed that insulin addition and inhibition of BMP signaling can be used to promote myogenic conversion (Cacchiarelli et al. 2018).

Differentiation trajectories are often highly complex and multiple possible differentiation routes may lead to the same cell state, as showed by Briggs et al. (2017) who compared protocols for ES cell differentiation into motor neurons. In one of the protocols, cells transition through states ordered similarly to the normal embryonic spinal development, whereas the other protocol forces cells to take ’a shortcut’, during which cells express forebrain genes but eventually end up in the same transcriptional and functional motor neuron state. Alternative paths are utilized also *in vivo*, e.g. in early platelet biogenesis. It appears that megakaryocytes arise either directly from single lineage biased HSCs or through a longer route involving multipotent progenitor cells (Rodriguez-Fraticelli et al. 2018).

Unaided pseudotime analysis may be insufficient to resolve the chain of events in complex scenarios. Part of the problem is that pseudotime does not contain directions, thus establishing start and end points requires known cell markers and prior knowledge of the analyzed populations. These issues can be addressed by including multiple time-points. To this end, Schiebinger et al. (2019) provided a mathematical framework - Waddington-OT, which optimizes transport of cells between subsequent timepoints by assigning the most probable ancestors in the past and most likely fates in the future. When applied to a comprehensive dataset containing >250,000 cells across 39 timepoints during MEF to iPS reprogramming, the method captured trajectories leading to iPSCs and a range of off-target cell types like neuronal, stromal, epithelial and trophoblast cells as well as intermediate stages resembling mesenchymal to epithelial transition (EMT). At the same time putative ’sinks’ were highlighted, where trajectories lead to increased apoptosis or senescence. Comparison of two reprogramming protocols confirmed that the serum culture conditions are less efficient than the 2i conditions and identified a possible bottleneck of the process followed by a rapid transition into the pluripotent state. It appears that only a small proportion of cells transitioning out of the EMT region has the potential to become iPSCs and expand only upon arrival at the final state. Characterization of this small subpopulation highlighted multiple putative molecular factors, of which Obox6, Zfp42 and Gdf9 were capable of boosting reprogramming efficiency. Altogether, even a seemingly simple *in vitro* cell conversion protocol exhibits complex cell states and transitions. Timepoint measurements, although not following the cells live, prove invaluable in resolving dynamics and mechanisms of these processes.

*In vivo* embryo development presents a staggering complexity of differentiation pathways. Fortunately, the development follows natural progression through well-characterized embryonic stages. In the zebrafish, analysis across the first 12 hours of development identified 25 different trajectories, including the endoderm, several mesodermal structures and specific spinal and brain regions as well as a rare population of germ cells. Exploiting knowledge about spatial expression patterns in the embryo allowed the authors to provide putative locations for certain progenitor populations (Farrell et al. 2018; Wagner et al. 2018). A conceptually similar study of *Xenopus* embryogenesis (Briggs et al. 2018), in addition to trajectory reconstruction, also showed that cell hierarchies are broadly conserved between the frog and the zebrafish (Wagner et al. 2018). Similarly, TF expression patterns were conserved, with wide expression in the early stages, and are subsequently more restricted in specific lineages, often reusing the same TFs in combinatorial patterns.

Similar approaches have been used to dissect differentiation pathways during mouse embryogenesis (Scialdone et al. 2016; Ibarra-Soria et al. 2018; Pijuan Sala et al. 2019; Cao et al. 2019a). Initially, the focus had been on a single timepoint or specific aspects of embryo development, such as the molecular programme of mesodermal and blood programme specification (Scialdone et al. 2016; Ibarra-Soria et al. 2018) or cardiac development (Lescroart et al. 2018). More recent studies offer a comprehensive view of >115,000 cells spanning embryonic stages E6.5 to E8.5 (Pijuan Sala et al. 2019) and >2,000,000 cells covering E9.5 to E13.5 (Cao et al. 2019a) with sufficient sampling to cover nearly all cell types. These large datasets classified hundreds of cell types and dozens of differentiation trajectories. The combined use of real-time and pseudo-time inference provided insight into myogenesis (Cao et al. 2019a), origins of the gut endoderm (visceral and definitive endoderm), highlighted differences between the primitive and definitive waves of hematopoiesis and identified the putative origin for early myeloid cells (Pijuan Sala et al. 2019). Importantly, embryo scRNASeq analysis is amenable to functional assays of gene function as exemplified by the analysis of mutant cells in zebrafish (*Nodal* and *Chordin*) (Wagner et al. 2018; Farrell et al. 2018) and mouse (*Tal1*)(Scialdone et al. 2016; Pijuan Sala et al. 2019) embryos. Additionally, the demonstrated use of chimeric embryos streamlines analysis of genes with lethal phenotypes (Pijuan Sala et al. 2019) and permits assessment of non-autonomous effects. Unlike the classical fixed time-point analysis, the time-resolved view of embryonic differentiation reveals immediate and secondary consequences of gene mutation and pinpoints the precise cell state where the phenotype manifests. Of note, some human embryonic data have already become available (Cui et al. 2019) and we expect comprehensive maps of human embryogenesis to appear soon.

**The molecular time**

Pseudotime, even when supplemented with multiple "real" experimental timepoints, does not capture the history of actual individual cells, and may thus not always be sufficient to reconstruct the chain of events. In such cases we might want to encode additional time information (a single event or a continuous signal) within the cell machinery, which is then decoded when the cell state is harvested.

Although in both *Xenopus* and zebrafish embryos a tree structure appears to provide a good approximation of cell trajectories, pseudotime struggles to resolve cyclic processes such as somite generation (Wagner et al. 2018; Briggs et al. 2018; Pijuan Sala et al. 2019; Cao et al. 2019a) or other forms of closed loops in the gene expression space. A notable example in the *Xenopus* embryo is a convergent behavior, where two alternative trajectories of mesodermal and neural crest cells lead to a similar pharyngeal arch molecular state (Wagner et al. 2018). To prove that indeed cells of separate origins contribute to the pharyngeal arches, the authors combined scRNA-Seq and lineage tracing readouts within the same cell. A range of techniques is now available (Kester and van Oudenaarden 2018) for parallel analysis of transcriptomes and genetic barcodes to unambiguously assign lineage history for each cell state (Lyne et al. 2018). However, further improvements in time-controlled labelling, complexity and detection efficiency are required. Unfortunately, such invasive techniques cannot be used for studying humans, except for gene therapy transplantations and analysis of viral insertion sites (Biasco et al. 2016). Instead, two recent studies demonstrate that naturally accumulating mutations can be used to infer genetic relationships. Lee-Six et al. (2018) demonstrated that analysis of somatic mutations unravels clonal relationships of hematopoietic progenitors and estimated the number of stem cells maintaining blood in the human body. Ludwig et al. (2019) exploited multiple copies of the smaller mitochondrial genome, which accumulate mutations at a faster rate per cell cycle. When applied to chronic myeloid leukemia samples mitochondrial DNA lineage tracing improves stratification of malignant and benign cases, consistent with the BCR-ABL status. Altogether, lineage tracing techniques combined with genome-wide readouts provide a clearer definition of cell states, hold great promise for understanding not only embryonic development but also aberrant clonal behaviors during aging or cancer formation.

New techniques using CRISPR/Cas9 offer a more general approach for recording information in the genome, not only limited to cell ancestry. Perli et al. (2016) demonstrated the use of self-targeting guide RNAs allowing continuous recording of input signal with high complexity. When connected with an NF-*κ*B responsive element, the method records TNF*α* in a time and dose-dependent manner. Another technique, known as MEMOIR (Frieda et al. 2017), also allows recording of custom information (e.g. cell ancestry or Wnt signaling) on CRISPR/Cas9 scratchpads but preserves cell position in the tissue as it utilizes single molecule FISH readout. Maintaining spatial information may prove invaluable when reading out the ancestry information or responses to the external stimuli in context of morphogenetic cell movement in the embryo or in pathogenetic changes in cancer.

Remarkably, short term information about the future state of a given cell can also be extracted from the nuclear transcriptome. La Manno et al. (2018) developed a method called ’RNA-velocity’ which detects nascent RNAs by scoring intronic reads (typically just exon-spanning reads are analyzed). These newly synthesised RNAs can also be detected experimentally using a recently developed protocol (Erhard et al. 2019, Cao et al. 2019b). The information about nascent RNAs appears to predict the state of the cell in the near future, thus providing directionality to the pseudotime, a great improvement in dissecting complex differentiation trajectories.

Finally, the fact that two very similar cells are often the result of a fairly symmetrical cell division, permits capture of two linked states at different timepoints. For instance, one of the sister cells may be analyzed soon after mitosis marking the original state, while the other sister can be analyzed at a later time-point to characterize the target state. Importantly, the degree of sister cell similarity can also be estimated through transcriptomic analysis of both cells soon after the mitosis. This approach has been introduced by Weinreb et al. (2018a) to follow cell fates of lentivirally barcoded hematopoietic progenitors cultured *in vitro* or after transplantation *in vivo*. With scRNA-Seq readout providing unambiguous cell state information, the analysis of thousands of linked sister pairs systematically inferred cell potentials across the hematopoietic landscapes. Consistent with previous assumptions based on gene expression patterns alone, this functional data proves that cell positions in the reconstructed gene expression landscapes largely reflect lineage potentials and that cells move through the landscape in a largely coordinated, unidirectional manner. Future technologies will offer an even greater ability to embed and read time-resolved information, allowing temporal reconstruction of molecular events. These will unlock systematic functional characterization of stem and progenitor landscapes and construction of predictive, mechanistical models.

**Concluding remarks**

Thanks to recent technological developments we are now able to define cell states and transitions with unprecedented precision, an essential step towards dissecting cell fate control mechanisms in stem and progenitor cells. Cells oscillate between distinct gene expression states associated with different cell potentials, as seen clearly in ES cells followed live *in vitro* (see section *In search of lost time*). At least in some circumstances this process seems to be noise-driven (conceptually similar to the ’brownian motion’) and reversible, hence based on reversible molecular processes, such as TF-enhancer or TF-promoter binding leading to transient bursts of transcription.

It has been proposed that cells straying away from the metastable multipotent state accumulate more stable molecular changes in a ratchet like mechanism (MacArthur et al. 2009; Furusawa and Kaneko 2012; Okamoto et al. 2018) thus favoring a unidirectional journey (Figure 3). These more stable molecular events are likely to be epigenetic in nature, for instance involving chromatin state changes via DNA methylation or histone modifications. Accordingly, if multiple molecular events are accumulated, the cell potential will appear as a gradient of fate probabilities along the state landscape, whereas a dramatic, rapid molecular event may appear step-like. For instance the hematopoietic landscape seems to exhibit smooth changes of gene expression and cell potentials. Conversely, a sharp increase in Bcl11b expression extinguishes all undesired fates during T cell thymic maturation (Longabaugh et al. 2017), suggesting a step-like behavior. In future, we ought to embrace this probabilistic nature of stem and progenitor cell potentials using the new technologies to our advantage. Single cell multi-omics are able to capture the landscape shapes and pinpoint cell states, while various time-resolved approaches will enable mapping of cell transition rates (’fluxes’) through the landscape and the associated cell potential probabilities. Altogether, these models will provide the groundwork enabling control of the fates in a wide range of clinical settings including cell therapy and regenerative medicine.

**Authorship**

IK and BG wrote the paper together. IK generated the diagrams. Both authors approved the final manuscript. The authors declare that there are no conflicts of interest.

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**Figure legends**

**Figure 1. Multimodal molecular analysis of single cells.** (A) A schematic of a cell fate choice: how will the cell state change (self-renewal or differentiation into muscle or neuron cells) under certain conditions? (B) Key molecular features defining the cell state, amenable to multimodal analysis. (C) Multimodal analysis of single cells yields a large matrix (features by cells), which can be used to: (i) compare cell states and chart maps of cell types and transitions (ii) correlate features and infer putative molecular mechanisms, (iii) predict how molecular mechanisms regulate cell states.

**Figure 2. Approaches to obtain time-resolved information.** (A) Comparison between live imaging and destructive observation techniques (such as multi-omics). (B) Overview of strategies providing temporal information when using destructive techniques. Timepoints and pulse chase experiments supplement the observed cell states with real time information. Capture of asynchronous cell state transitions allows reconstruction of differentiation trajectories (pseudotime) and direction of differentiation (RNA velocity). Analyses of experimentally introduced barcodes (lineage tracing) or naturally occurring mutations (acquired mutations) provide ancestry information. A cell’s history, such as exposure to signaling cues or ancestry, can be recorded using tailored genetic constructs (genetic recorders).

**Figure 3. Diagram depicting a cell potential landscape and the molecular events shaping it.** A bipotent progenitor cell exists in a metastable state (top of the hill), with equal propensity towards both fates (green and purple). Transient events, such as stochastic bursts of transcription (illustrated by factors binding to DNA), drive progenitor oscillations between two opposite fates. More stable events, such as chromatin modifications (illustrated by coiled DNA), drive unidirectional progression towards a specific fate. Global accumulation of molecular events (array of green/purple dots) shape the probabilities of cell transition in each direction (green/purple gradient).

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