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Single-cell states versus single-cell atlases—two classes of heterogeneity that differ in meaning and method

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Abstract

Recent advances have created new opportunities to dissect cellular heterogeneity at the -omics level. The enthusiasm for deep single-cell profiling has obscured a discussion of different types of heterogeneity and the most-appropriate techniques for studying each type. Here, I distinguish heterogeneity in regulation from heterogeneity in lineage. Snapshots of lineage heterogeneity provide a cell atlas that catalogs cellular diversity within complex tissues. Profiles of regulatory heterogeneity seek to interrogate one lineage deeply to capture an ensemble of single-cell states. Single-cell atlases require molecular signatures from many cells at a throughput afforded by mass cytometry-, microfluidic-, and microencapsulation-based methods. Single-cell states are more dependent on time, microenvironment, and low-abundance transcripts, emphasizing in situ methods that stress depth of profiling and quantitative accuracy.

Graphical Abstract

Introduction

Cellular heterogeneity is the basis for functional diversity in tissues and organs and defines a characteristic response of cell populations to environmental change [1,2]. The concepts of lineage heterogeneity and regulatory heterogeneity have been transformed by an onslaught of new methods that offer rich molecular details at single-cell resolution [3]. Widespread application of these technologies has begun to blur the distinction between cell type and cell state. This can be good for appreciating the tremendous plasticity of stem cells [4,5] or tumor cells [6,7]. However, there are also many compelling questions about regulatory heterogeneity that are separate from lineage heterogeneity [8] and vice versa [9].

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The objective of this review is to organize the single-cell profiling techniques of today according to their relative strengths at capturing regulatory heterogeneity versus lineage heterogeneity. For brevity, I exclude a third source of heterogeneity—transcriptional bursting—given the accumulating evidence of mechanisms that buffer this intrinsic noise in eukaryotes [10–13]. Insightful applications will be emphasized as much as proof-of-concept studies, because the true value of an approach comes with its implementation. Misapplication of a technique to the wrong heterogeneity type can overwhelm some methods and underpower others. Therefore, it is important to think critically about the heterogeneity of interest first and then dedicate time to master the techniques that are most suitable.

Regulatory heterogeneity versus lineage heterogeneity

The difference between regulatory heterogeneity and lineage heterogeneity is more than mere semantics. It speaks to the driving forces of single-cell transitions and the time scale on which they occur. Conceptually, one can merge lineages and states on a graph of cellular “potential energy” (Figure 1). The energetic barriers between cell types are equivalent to Waddington’s landscape, in which epigenetic modifications normally enforce lineage commitment. Within these valleys lie the states that cells of a single lineage can occupy. In contrast to epigenetic barriers of lineage commitment, the hurdles between regulatory states are much smaller. Thus, environmental change is often sufficient to transition cells between regulatory states, the transitions can happen rapidly, and they are frequently reversible. Whereas cell lineages are crucial for development and tissue homeostasis, aberrant cell states are the major cause of adult-onset disease.

Levine and coworkers [14••] recently provided a nice illustration of the difference between single-cell lineage and regulatory state in acute myeloid leukemia (AML). Using mass cytometry to profile surface markers of lineage concurrently with intracellular phosphoproteins indicating regulatory state, the authors tracked leukemic heterogeneity in response to a panel of perturbations. There was a tight coupling between surface markers of myeloid maturation and phospho-signatures in healthy donors, but this relationship broke down in all AML cases examined. The intracellular profiles enabled definition of a regulatory signature for myeloid maturity, which was nonredundant with surface marker lineage and more generalizable across AML patients. This work suggests that regulatory states may catalog diversity within cell and tissue populations more robustly than markers that have historically correlated with lineage.

Heterogeneities in lineage versus regulation bear directly on the single-cell methods that should be employed. In the above mass cytometry application, for example, Levine et al. [14••] developed PhenoGraph as an unbiased computational alternative to manual gating, in which surface markers are qualitatively assigned to be present (positive, +) or absent (negative, –). Classically, the best lineage markers are those that separate the positive and negative populations unambiguously. Differences in regulatory state are often much less dramatic, prompting their development of a second algorithm (Statistical Analysis of Response Amplitude, SARA) dedicated to quantitative changes in intracellular signaling

[14••]. Similar considerations apply when selecting from the suite of experimental methods now available for single-cell profiling.

Methods for single-cell atlases

How many cell types are there in our lungs [15], or our gut [16], or our brain [17]? We do not really know. Morphology and gross histology underestimate the number of stable cell lineages in any tissue, but how much so remains an open question. The pursuit of undiscovered cell types motivates cell atlasing.

For a molecular profile to be declared characteristic of a new rare cell lineage, it should be undeniably different from the rest of the population. That means looking for outliers. In single-cell transcriptomics, a low outlier of transcript abundance implies “undetected” [18,19], which is not the same as population-level measurements that can declare a gene absent with confidence [20]. Thus, single-cell atlases rely on transcripts that are sporadically expressed at very high levels compared to the rest of the population. Acknowledging this limitation is powerful for RNA sequencing, because it enables lineage designations to be made with very few total reads per cell [21,22•]. Covariation in biomarkers [23] increases the sensitivity of cell-type discrimination even further. Sequencing of fewer than 9000 transcripts per cell was able to identify rare secretory subtypes among intestinal cells [16], which is remarkable considering that the average cell contains $\sim 10^5$ transcripts [24].

For cell atlasing, depth requirements may be minimal, but throughput is critical. The major bottlenecks here are single-cell isolation and cell-specific molecular barcoding. Explicit single-cell methods begin with live cells in suspension, invariably requiring extensive enzymatic dissociation of the starting tissue. Tissue dissociation is finicky business [25], and it presumably dislodges cells from their native regulatory state (Figure 1). However, as long as epigenetic barriers hold strong, the thinking is that enough lineage-specific information will be retained in the single-cell molecular profile. Grün and coworkers [16] cleverly work within this limitation by first culturing intestinal stem cells as 3D organoids, enabling faster isolation of single cells compared to primary tissue. Other techniques have long been available to isolate single cells from snap-frozen tissue [26], but single-cell library preparations have not been reported in this format.

The barcoding bottleneck was recently surmounted by three groups [27••-29••], who independently devised microencapsulation-based workflows for cell lysis, RNA capture, and library preparation. The essence of these methods is to isolate a single cell together with a single microsphere containing custom oligonucleotides for RNA capture. The oligonucleotides contain (from 5' → 3') a universal primer or promoter, a microsphere-specific barcode indicating cell identity, a library of unique molecular identifiers [30], and a common oligo(dT) for binding mRNAs. Isolation can be achieved by microwells [27] or nanoliter water-in-oil emulsions [28••,29••], but goal is the same: to lyse individual cells in a small enough volume such that polyadenylated RNAs hybridize efficiently to the co-encapsulated microsphere. After the RNA has been captured and reverse transcribed onto the bead, libraries can be prepared in bulk and later deconvolved according to the cell-microsphere barcodes and molecular identifiers. Each team sequenced tens of thousands of

unique transcripts per cell across hundreds to thousands of cells, showing proof-of-concept applications to lineage definitions in blood samples [27•], differentiating embryonic stem cells [28•], and the retina [29•]. The microencapsulation techniques substantially increase throughput but still struggle with low-abundance targets because of the low conversion efficiency to RNA to cDNA (~10% or less) that is intrinsic to all methods. Sensitivity is a persistent concern given estimates that 90% of the transcriptome is expressed at fewer than 50 copies per cell [31•].

In pursuit of improved efficiency, Picelli et al. [32,33•] systematically examined the effects of various additives on RNA-to-cDNA conversion of single-cell starting material. Major increases in cDNA yield were achieved with increased concentrations of Mg^{2+} and the addition of the methyl-group donor betaine. These straightforward improvements were incorporated into a template switching-based SMART-seq2 protocol that showed improved sensitivity and reproducibility for detecting full-length transcripts. It would be exciting if such modifications generalized to other single-cell transcriptomic methods like the microencapsulation-based ones described above [27•–29•]. Yet, Fan and coworkers [27•] correctly point out that improving conversion to ~100% would simply shift the bottleneck to sequencing throughput, as tenfold more transcripts would need to be counted per cell. Accommodating throughput, sensitivity, and reproducibility remains the central balancing act of single-cell atlases.

Methods for single-cell states

An adherent cell's regulatory state is largely determined by its microenvironment—local cytokine-hormonal factors, adhesions to extracellular matrix, and juxtacrine signals from neighboring cells [34]. All of these cellular inputs are disrupted upon tissue dissociation, and cell detachment is known to alter signaling and transcriptional regulation within minutes to hours [35–38•]. Several modern methods avoid live-cell handling and can measure single-cell states reliably, albeit at the expense of the breadth of molecules or heterogeneities that can be profiled.

To increase the general utility of mass cytometry, two groups [39•,40•] recently devised different ways to ionize rare earth metal isotopes from tissue sections rather than suspended single cells. For imaging mass cytometry [39•], an ultraviolet beam is raster scanned across a tissue section that has been stained with a cocktail of primary antibodies labeled with rare earth metals. The laser ablation of the tissue ionizes a fraction of the metals, which are fed directly into a commercial mass cytometer. Giesen and coworkers applied this method to perform 32-plex imaging of breast cancer specimens, delineating single-cell regulatory states that occurred with different frequencies across the tumor cohort [39•]. In multiplex ion beam imaging (MIBI) [40•], a dedicated secondary ion mass spectrometry instrument is required, which ionizes the rare earth isotopes with an O^- primary ion beam. Angelo and coworkers used MIBI to perform 10-plex imaging of various breast cancers of different hormone and *HER2* amplification status. Imaging mass cytometry and MIBI are poised to transform immunofluorescence in the same way that mass cytometry has redefined flow cytometry.

The ability to profile transcriptional regulatory states has also improved through multiple advances in single-molecule RNA fluorescence in situ hybridization (smFISH). By replacing conventional probes with a branched DNA alternative that provides massive signal amplification, Battich et al. [41,42••] showed the potential for automated high-throughput smFISH. The authors profiled ~1000 individual transcripts at single-molecule resolution in HeLa cells and keratinocytes and reported that the single-cell abundance of mRNAs was predictable from the contextual features unique to each cell. In lieu of throughput, Chen et al. [43] dramatically expanded multiplexing with the development of MERFISH, an error-robust variant of sequential in situ hybridization. Exploiting a custom-synthesized pool of 100,000 oligonucleotides, the group designed 100–200 nonoverlapping transcript-specific probes, each flanked by a pair of defined readout sequences for transcript calling. MERFISH starts with a typical smFISH hybridization, followed by a series of rapid readout-hybridization and photobleaching cycles that assign a fluorescent code to each transcript discernable within the diffraction limit. The bit depth of the fluorescent code exceeds the number of transcripts profiled, which allows codes to be sufficiently distinct from one another for error detection and correction. In a proof-of-concept study, the authors showed multiplex profiling of 140 transcripts with error detection-correction and ~1000 transcripts with error detection only. The MERFISH and branched DNA methods both struggle with quantifying or accessing nuclear transcripts, but these multiplex and high-throughput variants of smFISH should nonetheless deepen our view of single-cell variability and regulation.

Ultimately, the ideal in situ barcode is the sequence of the transcript itself, and Lee et al. [44] have taken a first foray in this direction with fluorescent in situ RNA sequencing (FISSEQ). The method begins with reverse transcription using a random hexamer that contains a 5' adaptor for later sequencing. The resulting cDNA is then postfixed, circularized, and amplified by rolling circle amplification to generate in situ copies of the reverse transcribed RNA. Last, the amplified products are sequenced by serial hybridization and ligation of fluorescent oligonucleotides to the adaptor. In primary human fibroblasts, Lee and coworkers used FISSEQ to generate ~15,000–150,000 reads of 27 bases, of which 7–45% mapped to mRNA depending on the stimulus conditions. It will be interesting to see whether FISSEQ can follow the trajectories in throughput and read depth that have characterized next-generation sequencing over the past decade.

The above methods compromise breadth or sensitivity of detection to achieve profiles of regulatory states without cell dissociation. An alternative is to restrict the types of regulatory heterogeneities that can be interrogated while achieving transcriptomic scope in situ. This pragmatic compromise is the basis of stochastic profiling [45], a method that repeatedly isolates small 10-cell pools of cells by laser capture microdissection and analyzes statistical fluctuations in the pools to identify regulatory heterogeneities. The pooling provides 10-fold more input material, which yields a qualitative improvement in the accuracy and technical precision of many amplification techniques [24••,32,46]. It also improves the probability of detecting rare cell types (Figure 2A). The analysis presupposes a clean two-state regulatory heterogeneity, which is widely valid within one cell lineage [47] but would break down with pools of cells from diverse lineages. Bajikar and coworkers [48••] exploited the statistical properties of two-state transcriptional regulation to build mixture models that deconvolved

single-cell regulatory states from distributions of 10-cell pools (Figure 2B). Using conventional FISH methods, the authors verified expression frequencies inferred by the models and showed functional importance for a rare transcript that was expressed in less than 3% of the population. Importantly, when the extent of profiling was limited to fewer than 20 samples, 10-cell pools with deconvolution were found to be more accurate at parameterizing the population than an equivalent number of one-cell profiles. Although microencapsulation-based methods [27•–29•] are proficient at handling the thousands of cells required for tissue atlasing [31•], far fewer samples may be needed for capturing regulatory states within a lineage.

Conclusions

Distinguishing lineage heterogeneity and regulatory heterogeneity focuses the goals of a study in single-cell biology. Sometimes, the application is clearly toward an atlas of lineages or a catalog of states (Figure 3). However, several of the most cutting-edge studies involve cells that are just on (or off) the cusp of lineage determination [4–7], which raises the danger of ambiguity. Such work would benefit from a transparent description of which measurements capture lineage and which capture regulation [14•].

The methods reviewed here will undoubtedly continue to improve, but I argue that tradeoffs in single-cell profiling will persist for some time. As with other technological disciplines in biology [49], one cannot get all the advantages without some drawbacks. By understanding the strengths and weaknesses of various profiling methods, I advocate for smart compromises that are tailored to the right class of single-cell heterogeneity.

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Research highlights

- Single cells vary in lineage and in regulatory state.
- Experimental techniques suited for lineage profiling are not appropriate for state profiling and vice versa.
- Some cells, such as stem cells and cancer cells, straddle the distinction between lineage heterogeneity and regulatory heterogeneity.

Figure 1.

Single-cell heterogeneities in lineage (black) and regulatory state (gray). Epigenetic (green) and environmental changes (orange) drive lineage and state transitions respectively. The vertical axis visualizes the extent of gene-regulatory changes between lineages and states as a type of “potential energy”. Black headings provide representative examples of cell lineage and gray subheadings provide examples of regulatory state.

Figure 2.

Stochastic profiling uses the distribution of 10-cell averaged transcriptomic data to extract information about single-cell regulatory states in situ [45,48••]. **(a)** For a theoretically rare cell state present in 1% of the population, 20 samples of individual cells will only capture that cell state 18% of the time on average. By contrast, this cell state is virtually guaranteed to be reflected in 20 averages of 10 cells. **(b)** The theoretical distribution of 10-cell averaged expression profiles is shown in blue (frequency versus 10-cell averaged expression), with modes highlighting the mixture of high and low regulatory states comprising each mode.

Figure 3.

A spectrum of single-cell heterogeneities from lineage to regulation. Clonal populations of unipotent cells (bottom) should predominantly exhibit regulatory heterogeneity, whereas the most-dramatic heterogeneities in tissues (top) arise from cells of different lineages.