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PERSPECTIVE

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Single-cell and single-molecule epigenomics to uncover genome regulation at unprecedented resolution

Efrat Shema[1,2,4], Bradley E. Bernstein[1,2] and Jason D. Buenrostro[2,3]*

Recent advances in single-cell and single-molecule epigenomic technologies now enable the study of genome regulation and dynamics at unprecedented resolution. In this Perspective, we highlight some of these transformative technologies and discuss how they have been used to identify new modes of gene regulation. We also contrast these assays with recent advances in single-cell transcriptomics and argue for the essential role of epigenomic technologies in both understanding cellular diversity and discovering gene regulatory mechanisms. In addition, we provide our view on the next generation of biological tools that we expect will open new avenues for elucidating the fundamental principles of gene regulation. Overall, this Perspective motivates the use of these high-resolution epigenomic technologies for mapping cell states and understanding regulatory diversity at single-molecule resolution within single cells.

The activity of genes, and thus the establishment and maintenance of cellular identity, is regulated by a diverse set of transcription factors (TFs), chromatin regulators, noncoding RNAs, factors regulating genome topology and more. Large-scale consortia such as the Encyclopedia of DNA Elements (ENCODE)[1], the National Institutes of Health (NIH) Roadmap Epigenomics Project[2] and the International Human Epigenome Consortium[3] have applied DNase I hypersensitivity[4], chromatin immunoprecipitation (ChIP)[5] and bisulfite sequencing (among others) to characterize chromatin structure, TF occupancy and DNA methylation in many cell types and tissues. These genome-wide assays have identified a large diversity of functional regulatory elements and a plethora of chromatin factors that bind these elements. However, these aggregate maps represent averaged signals over populations of cells and thus mask cellular and regulatory heterogeneity. There is now increasing recognition of the importance of cell-to-cell variation within tissues[6] and also measurements of the physical co-occurrence between different chromatin modifications or chromatin regulators at individual loci[7,8]. Methods for single-cell and single-molecule epigenomic analysis are therefore required to parse the mechanisms of gene regulation across the diverse cellular landscape in development and disease.

Advances in molecular biology, microfluidics and imaging technologies have catalyzed a boom in the number of epigenomic modalities that can be measured at single-cell and single-molecule resolution (Table 1 and refs. [9,10]). Recent reports have described high-throughput single-cell chromatin[11] and DNA methylation[12] analyses powering epigenomic studies to tens of thousands of cells[13–15]. Furthermore, the surge of technological innovations for single-cell transcriptomics promises to further accelerate the development of single-cell epigenomic technologies[6,16,17]. These assays can be used to characterize cell types in complex tissues[13–15,18,19]; however, as single-cell transcriptomics is also a widely accessible and robust technology for de novo discovery of cell states[6], why then are single-cell epigenomic studies a worthwhile endeavor? In this Perspective, we focus on the unique biology that may be uncovered by single-cell analysis of the epigenome (Box 1). We present a number of motivating concepts unique to single-cell epigenomic analysis: for example, the unbiased discovery of cis and trans regulators and their activity profiles across cell states within complex tissues. We also explore how these technologies can be used to answer long-standing questions in cell biology, such as how do cells choose lineage fates and is lineage choice first encoded in the epigenome or in gene expression? Additionally, we investigate the biology underlying epigenomic analysis at the single-molecule scale. Last, we describe what may be the next generation of single-cell and single-molecule epigenomic tools to further progress our understanding of gene regulation.

Single-cell-resolved epigenomic regulation

Single-cell epigenomic assays provide an opportunity to define regulators, and thus mechanisms, of chromatin structure underlying cell identity. Single-cell measures of DNA methylation[20], chromatin accessibility[13–15,18,19] or histone modifications[21] can define cis-regulatory elements that govern the expression of nearby genes (for example, enhancers) and master regulator trans factors, such as TFs, that control the activity of these regulatory elements. In addition, methods to measure genome structure in single cells may be used to define the 3D structure of the genome[22] in order to understand the contribution of topology to gene regulation. Below we draw on examples from single-cell and bulk studies to highlight the future potential for single-cell epigenomics analyses.

Gene expression programs are tightly controlled by the concerted action of TFs, chromatin modifiers and other regulatory factors[23]. Genome-wide epigenomic assays are therefore instrumental for determining key regulators of gene expression and refining gene regulatory network (GRN) models. Many years of work—from classical investigations of TF function to modern genome-wide epigenomic assays—have demonstrated that TFs control cell states in a hierarchical manner, wherein a subset of TFs, the 'master regulators,' control cell fate determination. In extreme cases, for

[1]Department of Pathology and Center for Cancer Research, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA. [2]Broad Institute of MIT and Harvard, Cambridge, MA, USA. [3]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA, USA. [4]Present address: Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel. *e-mail: jason_buenrostro@harvard.edu

Table 1 | Single-cell and single-molecule epigenomic technologies

Technology	Data type	Details	Utility
Sequencing-based technologies			
RRBS and WGBS[12,57–59]	Single-cell, multi-omic	Approaches for reduced representation (RRBS)[57,58], whole-genome (WGBS)[12,57,58] and multi-omic[35,36] profiling of DNA methylation and hydroxymethylation[59]	Nucleotide-specific DNA methylation genome-wide
ATAC-seq[11,60] and DNase-seq[61]	Single-cell	DNase I or Hyperactive Tn5 transposase for mapping chromatin accessibility; these methods have been used for low cell numbers and in single cells to capture the enrichment of nucleosome-free elements of the genome	Nucleotide-specific chromatin accessibility genome-wide
ChIP-seq[21]	Single-cell	Single-cell profiling of histone modifications using immunoprecipitation of modified histones in a microfluidic device	Locus-specific histone modifications genome-wide
Hi-C[22,62,63]	Single-cell	A chromatin conformation approach to determine looping interactions within single cells	Proximity of loci genome-wide
DAM-ID[64]	Single-cell	Mapping nuclear lamin associated domains using a deoxyadenosine (DAM) methylase	Lamin associated domains
Imaging-based technologies			
In vitro analysis of chromatin complexes[65]	Single-molecule	Total internal reflection (TIRF) microscopy, often coupled with single-molecule fluorescence resonance energy transfer (smFRET), is applied for in vitro analysis of nucleosome complexes	Nucleosome configurations and nucleosome remodeling in vitro
TF binding dynamics in live cells[66]	Single-molecule	Single-molecule localization and tracking, as well as fluorescence correlation spectroscopy (FCS), reveal the kinetics of TFs binding to DNA in live cells	TF binding dynamics in vivo
Decoding of combinatorial histone modifications[8]	Single-molecule	Integrated single-molecule analysis of histone modifications on individual nucleosomes coupled with single-molecule sequencing of the associated DNA molecule to identify genomic positions	Multiple histone modifications on single nucleosomes genome-wide
Super-resolution imaging[67]	Single-cell and single-molecule	Application of super-resolution imaging techniques, such as the 3-D stochastic optical reconstruction microscopy (3-D-STORM)[67,68], to study chromatin organization	Super-resolution imaging of chromatin organization and domains

example reprogramming differentiated cells to induced pluripotent stem cells, overexpression of master regulators can induce dramatic changes to cell state[24,25]. Applications of single-cell epigenomic assays to early human hematopoesis[19], human myogenesis[18], human and mouse brain[14,15,20] and *Drosophila* embryogenesis[13] have refined these concepts by identifying key master regulators of discrete cell states within difficult to isolate or rare cell populations in complex tissues.

Epigenomic assays are also essential for relating noncoding genetic variation to regulatory mechanisms underlying phenotypic changes in evolution and disease. Evolutionary studies have identified noncoding mutations at cis-regulatory elements that underlie different phenotypes in a wide variety of species[26,27]. Excitingly, single-cell epigenomic studies comparing profiles across species may be used to track the conservation of noncoding regulatory elements concurrently with the evolution of new cell types[20]. Relevant to human health, the vast majority of common disease-associated variants lie outside of coding regions. Intersection of chromatin accessibility[28], histone ChIP-seq[29] and chromosome conformation capture approaches[30] have enabled systematic dissection of noncoding genetic variation for diseases ranging from diabetes to heart disease[1,2]. Importantly, single-cell epigenomic profiles can be used to map disease-associated genetic variants to active regulatory elements in de novo–defined cell types[15,31], and single-cell genome topology measures may be used to connect noncoding mutations to target genes.

Equally capable and robust computational tools are needed to analyze these data. Single-cell chromatin studies are exceedingly sparse, as normal diploid cells have two genomic copies and thus 0, 1 or 2 reads are observed per locus per cell. Although future work will likely improve throughput, coverage and quality through experimental innovations, advances in computational strategies to analyze these sparse data are needed. Excitingly, during the writing of this Perspective, we have already seen the development of new computational tools, including approaches for pairing single-cell chromatin measurements with single-cell RNA measurements[15,19]. As such, we expect these single-cell epigenomic tools to quickly mature, providing a robust foundation for regulatory analysis of cells in complex tissues to identify master regulators and the function of noncoding genetic variation across evolution and disease.

Are epigenomic and transcriptomic cell states the same?

Although single-cell epigenomics and transcriptomics can de novo identify cell types, a fundamental question remains: are epigenomic and transcriptomic cell states the same? Gene expression is the outcome of the combinatorial activity of regulatory elements. Conversely the regulatory landscape is shaped by the expression of TFs and associated chromatin regulators. Therefore, logic follows that epigenomic and transcriptomic cell–cell differences should be closely related. However, certain biological factors may cause significant discrepancies across epigenomic and transcriptomic studies of single cells. For example, epigenomic investigations may define repressed, poised or primed epigenetic states that may alter the ability of regulatory factors to activate the expression of nearby genes and therefore modulate cell potential at different developmental stages (Fig. 1a). Thus, we expect that memory-encoded processes, such as lineage priming, may be strongly represented in single-cell epigenomic profiles along developmental lineages. Indeed, it

Box 1 | Epilayers of gene regulation

To understand the context of emerging single-cell and single-molecule methods and their distinct utility in identifying underlying features promoting cell states, we first define the information to be assayed. Epigenetic regulation encompasses many more diverse biological processes than can be described here (see review, ref. [10]). For simplicity, in this Perspective we focus on the following:

TF binding to regulatory elements.
Thousands of TFs and chromatin regulators are encoded in eukaryotic genomes. TFs interact with other chromatin-bound proteins and epigenetic layers described below[23,69,70]. TF binding is associated with both activating and repressive regulation.

Chemical modification to genomic DNA.
This includes methylation of cytosines (5-mC) at CpG dinucleotides, as well as other modifications including 5-hydroxymethyl (5-hmC[59]) and more[71]. Regulatory elements generally have low levels of DNA methylation, and DNA methylation has also been shown to actively promote or inhibit the binding of TFs.

Post-translational modifications of histones.
Various chemical groups are attached to histones at specific amino acid positions. The large number and diversity of these modifications as well as the identification of enzymes that deposit, read or erase these histone modifications led to the hypothesis that the combinatorial presence of histone marks specifies distinct regulatory outcomes (the 'histone code')[72]. These modifications are associated with both activating and repressive chromatin features.

Chromatin accessibility.
The genome exists in a continuum ranging between accessible and inaccessible chromatin. Chromatin accessibility is regulated by TF binding and post-translational modifications to histones. Some TFs are described as pioneer factors[39], which can bind and open inaccessible chromatin; however, many transcriptional regulators appear to require accessible chromatin for binding[4].

3-D genome organization.
The genome is hierarchically folded to form 3-D structures at different length scales, ranging from the formation of topologically associating domains (TADs) spanning megabases of the genome to local enhancer–promoter interactions[23]. Genome structure can be both activating and repressive, either by positioning enhancers to target genes or by insulating genomic regions to prevent interactions with activating regions of the genome.

has been shown that mouse lineage-primed hematopoietic stem cells (HSCs) exhibit extensive epigenomic variability with little to no transcriptomic differences[32]. In contrast, other biological phenomena, such as those promoting fast stimulus-response programs, show significant changes in gene expression with few associated chromatin accessibility changes[33].

Changes to the epigenome and transcriptome may occur on vastly different time scales. RNA degradation rates are variable across transcripts, ranging from 3–200 min (or more) per gene[34]. Therefore, gene expression measurements of single cells may represent an integration of dynamic changes in cell state over approximately 1 hour. In contrast, the timescale for epigenetic changes is less well-defined and varies drastically between different epigenetic layers (Box 1) and chromatin states[10]. As such, transcriptomic and epigenomic assays may reflect differing timescales of change and may provide conflicting profiles of cell state, particularly in time-lapse studies of single cells.

To address these questions, methods for measuring DNA methylation, chromatin accessibility and transcriptome changes from the same single cell have been developed[35,36]. However, higher-throughput and additional measurements of the epigenome are needed to associate regulatory factors to changes in gene expression and cell state. Further development of these approaches will provide a unique integrative perspective on the dynamic relationship between the epigenome and transcriptome, providing new insights into the determinants of cellular potential and function.

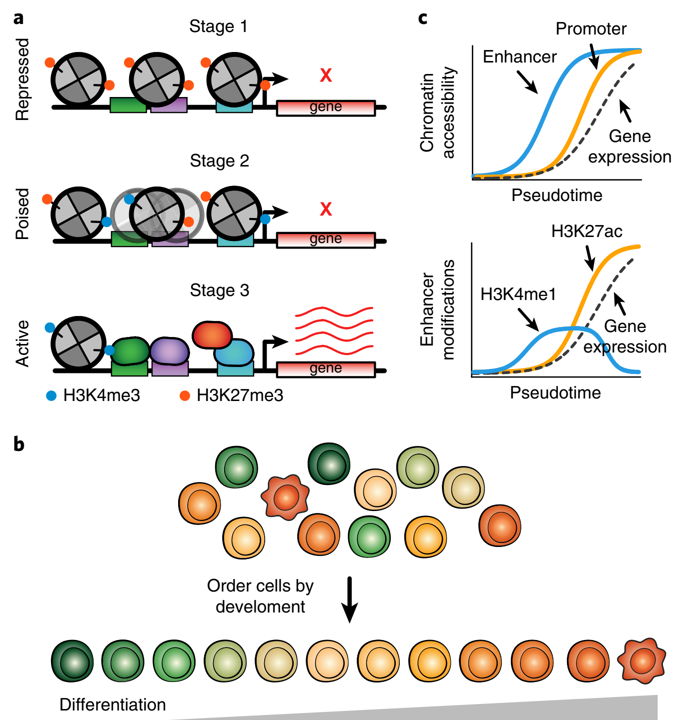

Fig. 1 | Dynamic regulatory changes in development. a, Regulatory promoter configurations showing repression (stage 1), priming (stage 2) and active (stage 3) states. Only stage 3 leads to expression differences. **b**, Single cells can be ordered by their developmental progression through single-cell, genome-wide technologies. **c**, Analysis of chromatin accessibility dynamics (top) or histone modifications of regulatory elements (bottom) leading to gene expression output across a differentiation pseudotime.

Temporal trajectories of the epigenome—cause versus correlation

The relationship between the epigenome and transcriptome is perhaps most relevant to our understanding of the mechanisms underlying cell fate decisions in development. A considerable focus of bulk epigenomic efforts has sought to uncover the regulatory mechanisms at key junctures of multilineage cell fate decisions. Such endeavors aim to model mechanisms of epigenomic change at the earliest moments of cell fate commitment in an effort to address key developmental questions; for example, to what extent are cell fate decisions deterministic or stochastic? And what are the key regulators that lead to lineage choice or lineage fate bias?

Single-cell epigenomics provides a new opportunity to investigate the molecular details defining cell fate decisions. With pseudotemporal ordering algorithms, asynchronous cells can be ordered by their developmental progression[37] (Fig. 1b) to identify the step-wise activation of key cis- and trans-effectors underlying cell differentiation and commitment[13,18,19] without concerns of bulk

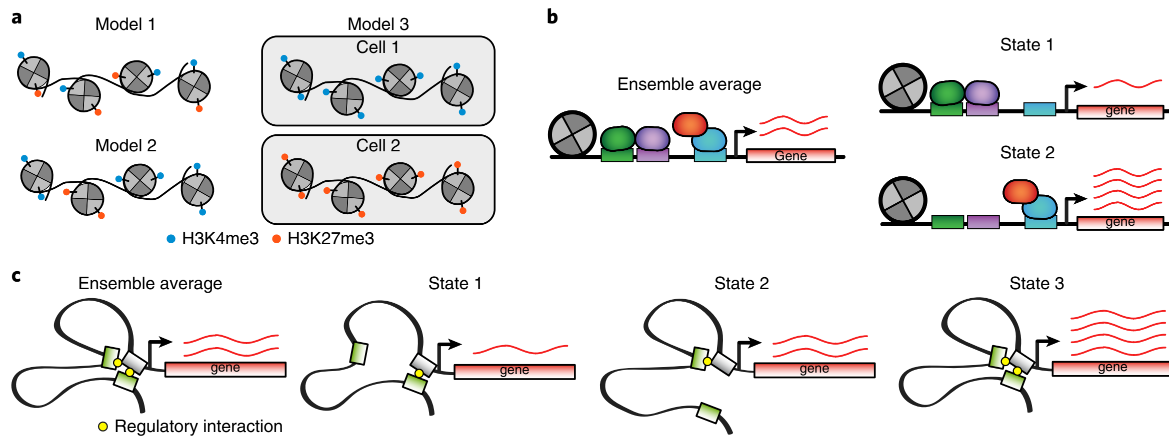

Fig. 2 | Cis and trans modes of regulatory heterogeneity. a, Three models of histone bivalency. Activating (blue) and repressive (red) histone modifications may occur on the same nucleosome (model 1), on different nucleosomes (model 2) or in different cells (model 3). **b**, Variable TF configurations lead to differences in gene expression, specified as the ensemble average (left) and underlying single-cell states (right, states 1 and 2). **c**, Variable looping configurations of specific enhancers (green) to target promoters (gray) leading to differences in gene expression within cells.

cellular asynchrony confounding insights into the regulation of these processes. This pseudotimeline of molecular events may be used to delineate early-initiating and thus likely causal factors of cell fate commitment[38] (Fig. 1c). It may also identify rate-limiting changes leading to functional gene expression differences, allowing us to ask questions such as: what comes first, activation of the enhancer or the promoter? Or does the expression of TF A precede or follow the expression of TF B? As such, single-cell epigenomic assays will provide valuable insight into early initiating and rate-limiting epigenetic changes, furthering the understanding of the regulatory processes underlying cell fate decisions.

Regulatory diversity at the single-molecule level

While single-cell approaches may define regulatory processes underlying cellular heterogeneity, single-molecule-resolved methods are needed to uncover the molecular heterogeneity at individual regulatory elements. Genome-wide maps of TF binding and histone modifications indicate that regulatory elements are associated with highly diverse combinatorial patterns of TFs, chromatin modifiers and histone modifications[1,2]; however, these approaches cannot measure the direct co-occurrence of these epigenomic features and thus only indirectly infer regulatory relationships. For example, bivalent chromatin is characterized by the colocalization of activating (H3K4me3) and repressive (H3K27me3) histone modifications, which poise genomic loci for activation or repression during development[7]. With only bulk observations, several models were possible—these modifications may truly coexist on the same nucleosome or regulatory element, they could represent a step-wise and thus rarely co-occurring process of regulation or they could represent antagonistic chromatin features that originate from different cells or alleles (Fig. 2a). Methods for mapping histone modifications at the single-molecule level were necessary to determine that these modifications indeed co-occur within the same nucleosome[8].

The extent to which combinatorial patterns of TFs, as well as other epigenomic features, physically co-exist on the same regulatory element to impart a unique function remains a daunting but addressable challenge requiring both single-cell and single-molecule methods. As one example, our understanding of 'pioneer factors,' TFs that bind closed chromatin to activate new regulatory elements[39], are obscured by bulk studies. TFs have been shown to bind both active and inactive regulatory elements, resulting in two models: either true binding at inactive loci reflecting their 'pioneering' activity or simply TF binding at open regulatory elements within a small subpopulation of cells. In many cell types, for example in studies of mouse embryonic stem cells (ESCs), careful biochemical studies have shown that a subset of TFs can indeed act as pioneer factors[39,40]; however, single-cell studies have also uncovered dynamic cellular substates within mouse ESCs[41]. More generally, studies in other cell systems have uncovered fluctuating or oscillatory regulatory processes leading to diverse cell subpopulations, either by the dynamic expression of developmental regulators[42] or from the nuclear localization of stimulus response factors[43], further motivating the use of single-cell and single-molecule methods to determine the function of TFs. Single-molecule methods may also be used to better define the interactions between different TFs to determine whether TFs cooperate or compete[44] at regulatory elements to promote different regulatory outcomes (Fig. 2b). Importantly, recent approaches for single-molecule mapping of the interactions between TFs and DNA have shown that there is pronounced cell–cell molecular heterogeneity among regulatory elements, leading to changes in gene expression[45]. Additional tools are needed to characterize the full extent of this molecular heterogeneity and the impact on cell function.

Little is known about the activity dynamics of individual regulatory elements, whether certain epigenomic processes can 'burst' and how these stochastic events relate to expression bursts of nearby genes. RNA expression dynamics is best understood in prokaryotes where transcription is described as an 'all-or-none' event that is modulated by intrinsic (for example, promoter sequence) or extrinsic (for example, cell size) factors[46]. However, less is known about the factors leading to gene expression bursting in eukaryotes. In eukaryotes, genome organization exists as diverse structural ensembles[22], and new evidence may suggest a pervasive role for enhancer–promoter looping dynamics in gene expression bursting (Fig. 2c). Single-molecule approaches have shown that dynamic chromatin loop interactions significantly alter the burst frequency of a gene, as shown for the *XIST* locus in ESCs[47], the β-globin enhancer in erythroid cells[48] and reporter genes in *Drosophila* embryos[49]. Interestingly, it has also been suggested that additional enhancers may serve to buffer gene expression noise to define more robust developmental expression programs[50].

Approaches for single-cell Hi-C and single-molecule DNA fluorescence in situ hybridization (FISH) are beginning to unravel structural diversity of the genome across cells (Table 1). Although these methods suggest significant cell–cell variability in genome organization[22], they also have limited throughput and resolution. Thus, definitive insight into how gene expression bursts relate to

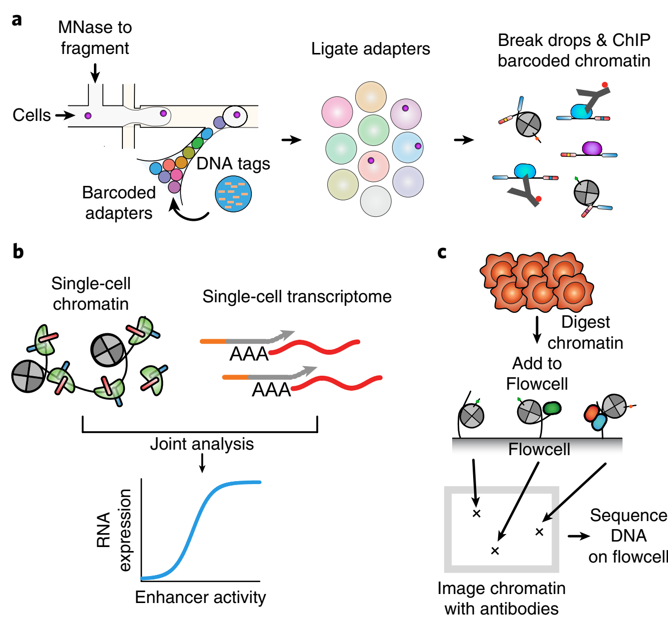

Fig. 3 | Future approaches for uncovering single-cell regulatory principles. **a**, Conceptualization of a high-throughput droplet-based epigenomics platform for single-cell ChIP-seq. MNase, micrococcal nuclease. **b**, High-throughput approach measuring chromatin activity and RNA levels (top) enabling the development of computational tools for linking enhancer activity to changes in gene expression (bottom). **c**, An assay allowing for fixing chromatin to an imaging surface, enabling single-molecule detection of histone modifications and TF binding[8].

stochastic interactions in cis or in trans will likely require new single-molecule methods that couple measures of DNA proximity, TF binding and expression. Toward this goal, recent efforts have begun to significantly improve the throughput of DNA FISH[51,52], which we anticipate may be used to investigate the epigenomic underpinnings of gene expression 'noise.' Altogether, we anticipate that innovations in single-cell and single-molecule technologies will significantly improve the ability to determine regulatory relationships and thus improve our understanding of epigenomic changes and their functional outcomes.

Emerging approaches

With more single-cell measures of the epigenome, a significant challenge for the field will be to experimentally and/or computationally pair diverse single-cell 'omics' methods to enable integrative regulatory models of cells. For simplicity, we have referred to these new technologies as single-cell epigenomic measurements; however, each layer of regulation is unique, and at the moment researchers must choose the most relevant layer of epigenomic information for their biological question (Box 1 and Table 1). As a consequence, we expect continued development of multi-omic approaches, either using experimental tools that directly combine genome-wide assays or computational approaches to optimally pair different single-cell data types. In addition, each layer of epigenomic information may be easier or harder to query at the single-cell level, therefore we should also expect the emergence of computational methods to 'impute'[53] missing epigenomic layers to enable a more complete understanding of regulatory changes across cell types.

One near-term goal for combining high-throughput single-cell omics profiles will be to pair single-cell assay for transposase-accessible chromatin using sequencing (ATAC-seq) or ChIP-seq datasets (Fig. 3a) with single-cell RNA-seq datasets to enable regulatory models of cellular heterogeneity. Regulatory models combining these data may leverage the natural cell–cell variation within tissues to infer (i) enhancer function by correlating change in chromatin activity with the change of expression at nearby genes and (ii) TF function by correlating the expression of TFs to changes of chromatin activity at regulatory elements containing TF-binding sites (Fig. 3b). Further, an integrated approach will likely also allow for more-accurate cell clustering by providing an orthogonal measure of cell type to thus determine sources of confounding batch effects unique to each dataset.

Improvements in imaging probes, resolution and throughput will advance capabilities for measuring regulatory interactions across single molecules within single cells. New imaging assays may allow for combinatorial probing of intact chromatin, which would provide a unique opportunity for a full analysis of diverse epigenetic processes, such as TF binding, histone modifications, chromatin conformation and nascent transcription. As one possible example, extending on our previous work for single-molecule imaging of histone modifications followed by sequencing on a high-throughput platform[8], a similar approach may be used to decode the interplay between TF binding and chromatin structure on a single DNA molecule (Fig. 3c). Further technology development may use larger chromatin fragments and incorporate a cell-identifying DNA barcode to enable single-cell and single-molecule analyses of regulatory factors binding chromatin. Ideally, this approach may be combined with new developments in imaging technologies using either high-throughput DNA FISH[52] or sequencing[8] to accurately map single-molecule interactions to specific loci within the genome. This single-molecule toolset would enable a systematic view of epigenetic regulation, from combinatorial TF binding to the co-occurrence of epigenetic modifications on DNA and histones in single cells.

New tools will enable measurements of the dynamics of epigenetic changes to distinguish whether epigenetic differences among cells are relatively stable or if they dynamically fluctuate over time. Recently a suite of tools have been developed for lineage tracking cells using CRISPR-based methods[54]; new approaches will likely couple these lineage-tracing methods with epigenomic measurements to unravel the dynamic nature of chromatin states and associate long-lived epigenetic configurations with cell potential. We also anticipate a need for time-lapse imaging methods at a much-higher resolution to track cis-regulatory element activation and localization across living cells. Improved tools for tracking chromatin structure, such as imaging genomic loci using CRISPR[55,56], will enable a temporal understanding of how localization of regulatory elements leads to the expression of nearby genes.

Summary

Overall, single-cell and single-molecule epigenomic methods are poised to revolutionize studies of gene regulation within complex tissues. These methods provide an orthogonal genome-wide measure of cell state and invaluable insight into the regulatory mechanisms governing cell function and potential. As such, we eagerly look forward to the continued development of this new generation of epigenomic methods, and as these methods continue to improve in their throughput and quality, we expect these technologies will also reveal many new and surprising insights into the cis- and trans-regulators governing cell function.

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E.S., B.E.B. and J.D.B conceived and wrote the manuscript.

Competing interests

E.S., B.E.B. and J.D.B have filed patents covering single-cell or single-molecule technologies. B.E.B. owns equity in Fulcrum Therapeutics, 1CellBio Inc, Nohla Therapeutics and HiFiBio Inc., and is an advisor for Fulcrum Therapeutics, HiFiBio Inc and Cell Signaling Technologies.

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Correspondence should be addressed to J.D.B.

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