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Review Article

Progress in multifactorial single-cell chromatin profiling methods

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Chromatin states play a key role in shaping overall cellular states and fates. Building a complete picture of the functional state of chromatin in cells requires the co-detection of several distinct biochemical aspects. These span DNA methylation, chromatin accessibility, chromosomal conformation, histone posttranslational modifications, and more. While this certainly presents a challenging task, over the past few years many new and creative methods have been developed that now enable co-assay of these different aspects of chromatin at single cell resolution. This field is entering an exciting phase, where a confluence of technological improvements, decreased sequencing costs, and computational innovation are presenting new opportunities to dissect the diversity of chromatin states present in tissues, and how these states may influence gene regulation. In this review, I discuss the spectrum of current experimental approaches for multifactorial chromatin profiling, highlight some of the experimental and analytical challenges, as well as some areas for further innovation.

Introduction

In multicellular organisms, a single common genomic DNA sequence is decoded in a multitude of different ways to give rise to the vast array of different cellular states that are present in the organism. A fundamental question is how this diversity of cell states is enabled through gene regulation. The biochemical state of chromatin plays a critical role in determining the transcriptional output of genes, and ultimately in determining cellular states and fates. These biochemical states are incredibly complex and inherently multifactorial. They involve the chemical modification of histone proteins, the distribution of histone variants across the genome, nucleosome positioning, chemical modification of the DNA, protein-DNA binding, the physical three-dimensional structure of chromatin fibers, and potentially other factors yet to be discovered.

Large-scale global efforts have succeeded in mapping the genome-wide distribution of many different individual aspects of chromatin states [1,2]. However, these datasets have been mostly limited to bulk-cell samples. Many of these assays are now being converted into single-cell-resolution versions, enabled by technological improvements in various aspects of the experimental workflows. DNA methylation profiling methods were the first chromatin assays to be adapted to a single-cell format [3,4], followed by chromatin accessibility using microfluidics or combinatorial indexing approaches [5,6]. Since then, many new methods have been developed that enable mapping the genome-wide distribution of different histone modifications [7–10] and 3D chromatin structure [11,12]. These new approaches are able to resolve the heterogeneity in cell states that exist within tissues and are now paving the way towards a deepened understanding of the diversity of chromatin states that exist across cells.

More recently, new methods have been developed that pair chromatin state measurement with other cellular assays, such as gene expression profiling, to gain a more comprehensive view of the cellular state and reveal how chromatin states may impact other cellular modalities. Recent reviews have extensively covered methods for measuring individual chromatin marks at single-cell resolution, or

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measuring a single aspect of chromatin state alongside other modalities such as gene or protein expression [13–16]. As there is a complex interplay between different chromatin modifications (marks), it is essential that we can observe multiple aspects of chromatin state together in a single experiment [17]. This would enable the identification of combinations of marks that may direct functional changes in chromatin states, and how these marks vary in relation to each other across cell states. Here, I focus on a new frontier of methods aiming to measure multiple aspects of chromatin state together in single cells, discuss the current state-of-the-art, and discuss some of the unique challenges that these experiments present both in data generation and analysis.

Experimental methods for multifactorial chromatin analysis

A variety of new molecular methods have been developed over the past few years that now enable the simultaneous measurement of multiple aspects of chromatin state with single-cell resolution. These methods can be broadly grouped into different approaches according to the molecular strategies used (Table 1). One family of methods focus on the detection of DNA methylation and use chemical or enzymatic conversion methods to encode multiple types of chromatin state information into the DNA sequence itself (Figure 1). Another set of methods uses affinity reagents, mostly antibodies, to guide the insertion of DNA-barcoded information into the genome, which can later be read out through sequencing (Figure 2). A third strategy relies on imaging to read out multifactorial chromatin information, rather than high-throughput DNA sequencing, and develop ways to encode multiple aspects of chromatin state into a fluorescent signal (Figure 3).

DNA methylation-based methods

DNA methylation and chromatin accessibility

Within vertebrates, 5-methylcytosine (5mC) occurs mainly in the CG dinucleotide context [18,19]. Cytosines in the GC sequence context are rarely methylated (only at GCG and sometimes GCA sites) [20]. This observation has enabled the development of methods such as NOMe-seq (nucleosome occupancy and methylome sequencing) that encode additional information through the deposition of GC methylation using the *Escherichia coli* GC DNA methyltransferase M.CviPI [21]. Critically, these GC methylation marks are added in regions of open chromatin, as these sites are more accessible to the M.CviPI enzyme (Figure 1A). Following M. CviPI treatment, whole-genome bisulfite sequencing (WGBS) is used to determine the genome-wide presence of 5mC bases. By mapping the sequence context of these methylated bases, sites that were endogenously methylated in the cell can be distinguished from induced GC methylation sites that mark open chromatin. Recent methodological improvements have adapted NOMe-seq to a single-cell format through the development of scNOMe-seq and scCOOL-seq [22,23]. Further iterations on the this approach (scNMT-seq, scNOMeRe-seq, snmCAT-seq) have coupled multifactorial chromatin measurement with simultaneous detection of mRNA abundance, allowing gene expression profiles to be studied in the context of multifactorial chromatin states [24–26]. These studies have been applied to the human brain, mouse embryos, and to mouse embryonic stem cells and helped to reveal the co-ordinated shifts in DNA accessibility, nucleosome positioning, and DNA methylation that occur across cell states. Although these innovative methods can provide highly informative chromatin data, they are still limited in the number of cells that can be profiled due to the need for processing in microtitre plates. While the use of liquid handling robots has enabled the generation of larger datasets, further adapting these methods to leverage droplet microfluidics or combinatorial indexing would greatly improve the scalability of these approaches.

DNA methylation and hydroxymethylation

Whereas 5mC is the most abundant DNA modification in vertebrate genomes, the presence of 5hmC can be uniquely informative as it is produced by the oxidation of 5mC by TET2 that occurs during active DNA demethylation [27]. 5hmC is typically found in the promoters and enhancers of actively transcribed genes, in contrast with 5mC which correlates with transcriptionally repressed regions of the genome. Standard bisulfite conversion methods cannot distinguish between 5mC and 5hmC, presenting some limitations in the interpretation of these data. Two methods [28,29] were recently developed that are capable of detecting both 5mC and 5hmC together in the same cell (Figure 1B). These methods enable insight into active DNA demethylation processes and are particularly powerful approaches for studying developmental dynamics.

Bai et al. [28] developed simultaneous profiling of epigenetic cytosine modifications by sequencing (SIMPLE-seq). SIMPLE-seq uses an orthogonal labeling approach to record both 5mC and 5hmC on the same

Table 1. Overview of experimental methods for multifactorial chromatin profiling

Assay	Modalities	Approach	Single-cell capture	Reference
DNA methylation-based methods				
scNOMe-seq	DNA accessibility and DNA methylation	GC methyltransferase treatment and whole genome bisulfite sequencing	Microtitre plate	[22]
scCOOL-seq	DNA accessibility and DNA methylation	GC methyltransferase treatment and whole genome bisulfite sequencing	Microtitre plate	[23]
scNMT-seq	DNA accessibility, DNA methylation, mRNA abundance	GC methyltransferase treatment and whole genome bisulfite sequencing, physical separation of cytosol and nucleus	Microtitre plate	[25]
scNOMeRe-seq	DNA accessibility, DNA methylation, mRNA abundance	GC methyltransferase treatment and whole genome bisulfite sequencing, physical separation of cytosol and nucleus	Microtitre plate	[24]
snmCAT-seq	DNA accessibility, DNA methylation, mRNA abundance	GC methyltransferase treatment and whole genome bisulfite sequencing. Reverse transcription with 5'-methyl-dCTP.	Microtitre plate	[26]
scMethylHi-C	DNA methylation and 3D structure	Hi-C and whole genome bisulfite sequencing	Microtitre plate	[34]
snm3C-seq	DNA methylation and 3D structure	Hi-C and whole genome bisulfite sequencing	Microtitre plate	[33,36]
SIMPLE-seq	DNA methylation and hydroxymethylation	Orthogonal chemical labelling of DNA	Combinatorial indexing	[28]
Joint-snhmC-seq	DNA methylation and hydroxymethylation	Physical separation of DNA after bisulfite treatment	Microtitre plate	[29]
Affinity binding-based methods				
multiCUT&Tag	Multiple histone	Pre-complexing pA-Tn5 with primary antibodies	10x Chromium	[42]
MulTI-Tag	Multiple histone	Conjugation of Tn5 adapter DNA to antibody, sequential tagmentation	Takara ICELL8	[43]
CUT&Tag2for1	H3K27me3 + RNAPII	CUTAC tagmentation and computational deconvolution	Takara ICELL8	[45]
uCoTarget	Multiple histone	Pre-complexing pA-Tn5 with primary antibodies	Split-pool ligation	[44]
nanoCUT&Tag	Up to three histone, DNA accessibility	Nanobody-Tn5 fusion proteins	10x Chromium	[49]
scNTT-seq	Up to three histone, protein expression	Nanobody-Tn5 fusion proteins	10x Chromium	[48]
scGET-seq	H3K9me3 + DNA accessibility	HP1-a chromodomain-Tn5 fusion protein	10x Chromium	[52]
MAbID	Multiple histone	Proximity ligation of antibody-conjugated DNA	Microtitre plate	[54]
Dam&ChIC	Dual chromatin target	Combination of DamID and ChIC-seq	Microtitre plate	[53]
Imaging-based methods				
SCEPTRE	Multiple histone	Expansion microscopy and DNA FISH	Microscopy	[55]
DNA seqFISH+	Multiple histone	Multiplexed FISH	Microscopy	[56]

DNA molecule. First, 5hmC is converted to 5-formylcytosine (5fC) using oxidation with potassium ruthenate, followed by indanedione labeling of 5fC. This creates a C-to-T transition at sites that were originally 5hmC, but not at unmodified cytosines or 5mC. Importantly, a single primer extension step is performed using a primer containing a synthetic 5-carboxylcytosine (5caC) base that serves as an indicator, revealing which strand is the newly synthesized strand after 5hmC conversion. Following the primer extension step incorporating the 5caC indicator, TET-mediated oxidation is used to convert 5mC in the unreplicated strand to 5caC.

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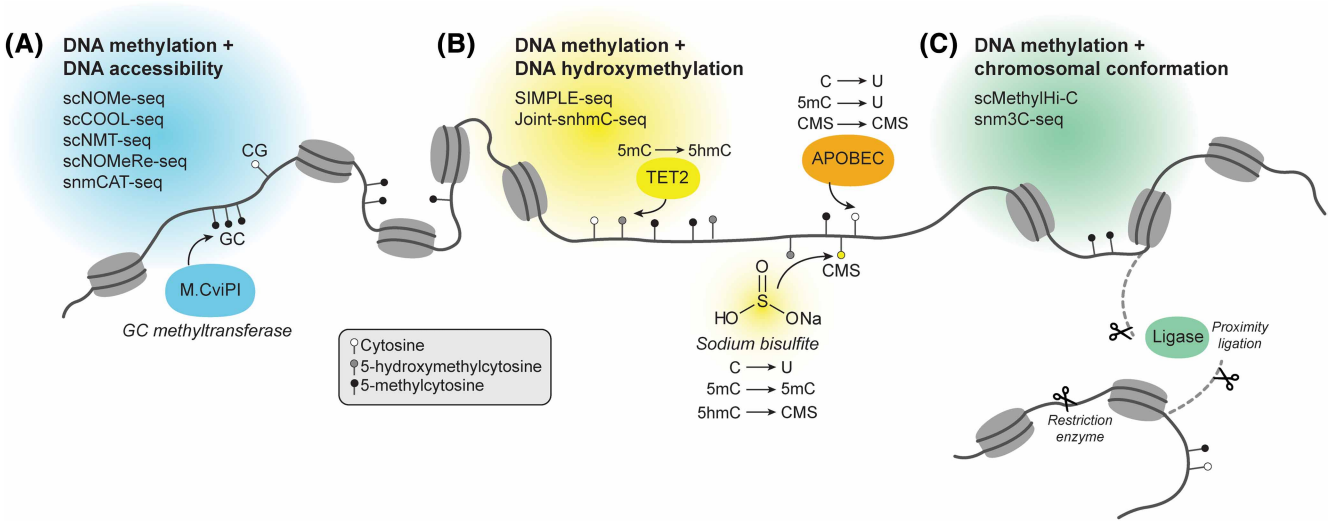

Figure 1. DNA methylation-based methods for multifactorial chromatin profiling.

(**A**) Methods for capturing DNA methylation alongside DNA accessibility in single cells. These approaches use the GC methyltransferase M.CviPI to add DNA methylation in the GC dinucleotide context. Subsequent profiling using Bisulfite sequencing can reveal the genome-wide presence of methylated cytosine, and the surrounding sequence context used to infer which sites were methylated M.CviPI signifying open chromatin sites. (**B**) Methods for profiling both DNA methylation and DNA hydroxymethylation. SIMPLE-seq uses different labelling steps to label 5hmC and 5mC on separate DNA strands, whereas Joint-snhmC-seq uses physical separation of Bisulfite-treated DNA for parallel detection of 5hmC and 5mC sites, with APOBEC3A used to convert 5mC to and C to uracil. (**C**) Methods for capturing DNA methylation alongside chromosomal conformation. These methods use restriction enzyme digestion of the DNA followed by proximity ligation to combine DNA strands in physical proximity. Bisulfite treatment of the DNA is then used to encode information about the DNA methylation status of DNA bases, in addition to the conformational information encoded by DNA ligation events.

This is followed by a reduction to dihydrouracil (DHU) and results in a second C-to-T transition at sites that were originally 5mC. Critically, the 5caC indicator base present on the newly synthesized strand is also converted to DHU, providing a C-to-T transition at a known site that reveals which strand underwent 5mC-to-T conversion and which underwent 5hmC-to-T conversion, thus encoding both chemical modifications in the same double-stranded DNA molecule [28]. The authors developed SIMPLE-seq as a single-cell-resolution assay by incorporating Tn5 tagmentation and ligation-based combinatorial indexing that enable cell-specific barcodes to be added to each molecule.

Fabyanic et al. [29] developed another approach for simultaneous profiling of 5hmC and 5mC in single cells, named Joint-snhmC-seq. This approach utilized the ability of bisulfite treatment to simultaneously fragment DNA, deaminate cytosine to uracil, and to convert 5hmC to cytosine-5-methylenesulfonate (CMS). Subsequent treatment of the DNA with APOBEC3A enzyme allows conversion of 5mC and C positions to uracil, while CMS-converted sites (originally 5hmC) are protected due to the presence of a bulky 5-position substitute. This enables profiling of 5hmC positions using a simplified experimental setup in an approach the authors named snhmC-seq. To profile both 5mC and 5hmC in the same cell, bisulfite-treated DNA was split for parallel processing by standard bisulfite sequencing (mapping 5mC positions) and snhmC-seq (mapping 5hmC positions). This allowed the identification of true 5mC sites and 5hmC sites within the same cell. However, in contrast with SIMPLE-seq, snhmC-seq was not able to identify both modifications in the same DNA molecule due to the physical separation of molecules in the assay.

The ability to measure both 5mC and 5hmC on the same DNA molecule presents unique opportunities to study the co-occurrence of these marks. The SIMPLE-seq authors identified a subset of genomic sites where 5hmC and 5mC appear to coexist together, while the Joint-snhmC-seq authors demonstrated improved multimodal integration performance when considering true 5mC sites, and variation in genome-wide 5hmC abundance across mouse neuronal cell types. These new methods provide intriguing opportunities to study DNA methylation dynamics and the processivity of TET2 dioxygenase.

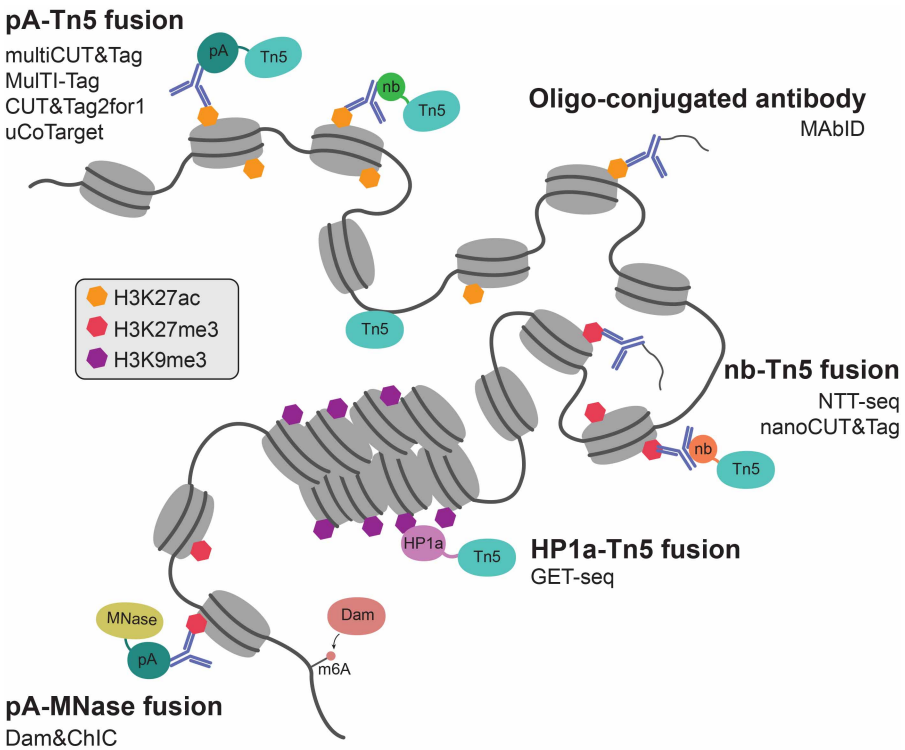

Figure 2. Affinity binding-based methods for multifactorial chromatin profiling.

Affinity binding-based methods use a binding reagent such as an antibody or protein binding domain to specifically recognize different chromatin targets. These targets typically encompass histone posttranslational modifications or other proteins such as RNA polymerase or CTCF. Different strategies can then be used to incorporate barcoded DNA into the genome to enable a readout of the genome-wide distribution of these targets, such as DNA tagmentation or proximity ligation.

DNA methylation and 3-dimensional chromatin architecture

The 3D structure of chromatin in the nucleus is known to play an important role in regulating gene expression and genome stability. At a macro scale, the position of chromosomal domains at the nuclear periphery versus the interior of the nucleus is associated with large differences in the transcriptional activity of genes, with genes located at the nuclear periphery being more lowly expressed [30]. At a finer scale the precise looping of chromatin can cause the physical colocalization of DNA regulatory elements such as promoters and enhancers, and play an important role in the transcriptional regulation of individual genes [31]. Chromosome conformation capture methods such as Hi-C use cross-linking followed by restriction enzyme cutting of the DNA and ligation to join DNA molecules according to their physical proximity [32]. High-throughput DNA sequencing is then used to map chromosomal contacts genome-wide. As these methods rely on ligation, the DNA methylation marks present in the nucleus remain intact.

Multiple approaches have now been developed that combine Hi-C with DNA methylation profiling using WGBS (Figure 1C). These methods are also amenable to single-cell-resolution profiling by isolating individual nuclei into wells of a microtitre plate. Both scMethylHi-C and snm3C-seq use similar workflows to simultaneously generate chromosomal conformation and DNA methylation information from the same single cells [33,34]. These approaches enable the relationship between chromatin architecture and DNA methylation patterns to be studied, and have revealed that spatially proximal DNA sequences often exhibit coordinated DNA methylation patterns. Furthermore, the binding of CTCF is known to be sensitive to 5mC within the CTCF binding motif [35]. Joint DNA methylation and chromatin conformation studies found that CTCF sites with variable DNA methylation status across cell types were also more likely to have variable chromosomal interactions, suggesting a relationship between methylation of the CTCF site and chromosomal looping [33]. While these methods typically have lower throughput due to the need for well-based cell processing, a landmark study

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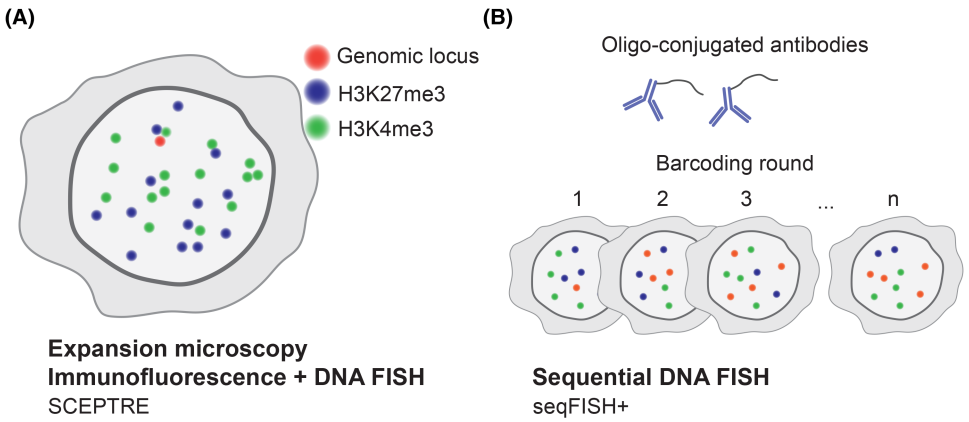

Figure 3. Imaging-based methods for multifactorial chromatin profiling.

(**A**) Combining immunofluorescence, DNA FISH, and expansion microscopy, SCEPTER allows the visualization of different multiplexed antibodies targeting histone posttranslational modifications at a specific genomic locus. (**B**) DNA seqFISH+ provides a highly multiplexed approach for imaging-based chromatin profiling. It leverages sequential DNA FISH encoding methods together with DNA-conjugated antibody staining of the cell.

used liquid handling robotic automation to profile over 176 000 single cells from 117 different regions of the mouse brain [36].

Affinity binding-based methods

Histone posttranslational modifications are known to have important roles in determining the functional state of chromatin, both through their ability to physically alter the packing of chromatin and by providing recognition binding sites for other proteins in the cell [37]. The precise combination of histone modifications that colocalize on a nucleosome or genomic region are also understood to correlate with functionally distinct states. For example, the presence of both H3K27ac and H3K4me1 indicates active enhancer elements, whereas the presence of H3K4me1 without H3K27ac indicates a poised state [38]. Relationships between histone modifications and chromatin structure exist too. The acetylation of lysine can neutralize the positive charge normally present on the lysine residue, causing a more open chromatin structure permissive to protein binding [39]. The ability to co-assay several histone marks within the same cell, as well as other aspects of chromatin state such as DNA accessibility, is an important experimental goal fundamental to advancing our understanding of how these marks co-ordinate to guide chromatin states.

ProteinA-Tn5 fusions

Several approaches were recently developed that employ antibody-guided DNA tagmentation using a Protein A (pA)-Tn5 fusion protein to profile the genome-wide distribution of histone modifications (Figure 2) [7–10]. Since these methods use Tn5 transposase to simultaneously cut the DNA and insert sequencing adapters, they are relatively easy to adapt to a single-cell-resolution format using approaches originally developed for scATAC-seq. This first generation of methods enabled measurement of one antibody target in each experiment. More recently, several new methods have further built on the concept of antibody-guided DNA tagmentation and enable the simultaneous measurement of two or more targets. As Tn5 will preferentially tagment open chromatin regions, methods that aim to use Tn5 in epigenomic assays must develop careful strategies to avoid open chromatin bias in the resulting data [40,41]. The original CUT&Tag method used a high-salt wash and tagmentation buffer to alleviate this open chromatin bias [7], and most subsequent methods have incorporated similar steps in their protocols.

The multiCUT&Tag method used a pre-complexing approach to first associate pA-Tn5 loaded with barcoded Tn5 adapters with different primary antibodies [42]. Once these pA-Tn5-antibody complexes are formed they are relatively stable, due to the binding strength of pA to IgG antibodies. This enabled the authors to pool different pA-Tn5-antibody complexes in a single experiment targeting multiple different histone modifications, and allowed profiling of two histone targets in a single experiment. Meers et al. [43] developed

another pA-Tn5 multiplexing approach based on the covalent attachment of Tn5 adapter DNA directly to the primary antibody, named MulTI-Tag. This provided a stronger coupling of Tn5 and antibody and resulted in very low blending of signal between the different chromatin marks profiled, although with the requirement of a more complex experimental workflow requiring sequential staining and tagmentation [43]. Xiong et al. [44] used a similar similar approach in the development of uCoTarget, and found that a pre-complexing approach similar to that used by multiCUT&Tag was sufficient to couple pA-Tn5 and the antibody together when used in combination with sequential tagmentation. The authors profiled up to five different histone targets simultaneously in human cell lines using uCoTarget, and also demonstrated the co-profiling of the transcription factor RUNX1 alongside three different histone modifications [44].

Janssens et al. [45] took a different approach utilizing the observation that pA-Tn5 tagmentation in a low-salt buffer causes tagmentation to be directed to more accessible chromatin sites nearby the bound nucleosome, and results in a shift in the fragment length distribution for active histone marks profiled [46]. Here the authors developed a novel approach, CUT&Tag2for1, that used a mixture of antibodies targeting H3K27me3 and RNA Polymerase II (RNAPII), and computationally deconvolve the resulting reads using the fragment length distribution information [45]. However, this approach is limited to profiling one repressive mark and one active mark, and it is unclear how the method would perform in cases where the same genomic region occupies different states across different cell types.

Overall, while the use of pA-Tn5 has proven a powerful method for directing Tn5 to certain regions of the genome, this approach becomes challenging when attempting to profile multiple antibody targets simultaneously due to the lack of antibody specificity in pA binding.

Alternative Tn5 fusions

Two new and related methods recently built on the idea of antibody-guided DNA tagmentation introduced by CUT&Tag and related methods, and replaced the pA-Tn5 fusion with different secondary nanobody (nb)-Tn5 fusion proteins. As the secondary nanobodies are able to bind with high specificity and affinity to primary antibodies from different species or IgG isotypes [47], this enabled a simplified multiplexing workflow involving different primary antibodies [48,49]. These approaches, named nanobody-tethered transposition followed by sequencing (NTT-seq) and nanoCUT&Tag, enabled profiling of up to three different antibody targets per experiment and are compatible with droplet microfluidics methods for the generation of single-cell-resolution data. The authors applied these methods to generate multifactorial datasets from the human blood and bone marrow [48] or mouse brain [49]. Furthermore, these methods are compatible with the simultaneous measurement of other cellular modalities too. NTT-seq was demonstrated with simultaneous detection of cell-surface protein abundance using DNA-conjugated antibodies, similar to ASAP-seq or CITE-seq [48,50,51]. NanoCUT&Tag demonstrated co-detection of chromatin accessibility by performing tagmentation with Tn5 enzyme in the ATAC-seq tagmentation buffer prior to nanoCUT&Tag, allowing open chromatin sites to be captured [49].

Other Tn5 fusions have been constructed to enable profiling of multiple chromatin targets. Genome and epigenome by transposase sequencing (GET-seq) was recently reported by Tedesco et al. [52], and involves the use of a novel fusion protein containing the Tn5 transposase and the chromodomain of HP1a, which binds to H3K9me3. The authors named this fusion protein TnH and assayed both chromatin accessibility and heterochromatin simultaneously through the sequential tagmentation of nuclei with Tn5 followed by TnH [52]. Importantly, this removes the need for an antibody targeting the H3K9me3 mark, further simplifying the experimental protocol. GET-seq was compatible with commercial droplet microfluidics methods for single-cell capture, enabling a streamlined workflow for single-cell-resolution profiling. The authors applied GET-seq to various biological samples including patient derived xenografts and iPSC reprogramming. The method was shown to faithfully reflect genome-wide patterns of H3K9me3 and DNA accessibility and further improved the identification of copy number variants due to the increased genomic coverage per cell. Notably, this approach should be compatible with other single-cell histone profiling methods that use pA-Tn5 or nb-Tn5, and future studies may aim to combine these methods to further increase the number of chromatin targets able to be assayed in a single experiment.

ProteinA-MNase fusions

Although many multifactorial chromatin profiling approaches have focused on the use of Tn5 transposase, a recent study took a very different approach and combined scDamID and ChIC-seq to create a new assay,

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Dam&ChIC, capable of measuring two aspects of chromatin state simultaneously [53]. Dam&ChIC uses inducible expression of the Dam DNA methyltransferase enzyme fused to a protein of interest to add m6A modifications to the genomic DNA at GATC sites, a modification that does not naturally exist in the vertebrate genome. Next, cells are fixed and permeabilized and an antibody added for the desired epitope. A pA-MNase fusion protein is then used to cut genomic DNA surrounding sites where the antibody is bound, releasing the DNA. Digesting the released fragments with DpnI, which selectively cuts GATC motifs containing m6A, allows fragments originating from DamID and ChIC-seq to be separated *in silico* based on the presence of GATC at the end of the DNA fragment. The authors demonstrated simultaneous measurement of LMNB1 and different histone modifications, including H3K4me3, H3K27me3, and H3K9me3 [53]. Furthermore, untethered Dam enzyme can be expressed to enable profiling of open chromatin regions using the DamID aspect of the method, in place of Dam fused to a protein of interest. Importantly, the Dam DNA methyltransferase must be expressed in a living cell. This limits the application of Dam&ChIC to systems that can be genetically engineered to express Dam, but also creates an opportunity to profile chromatin marks across time. The developers of Dam&ChIC demonstrated how the method can be used to measure past and present genome-lamina interactions by profiling LMNB1 using both DamID, creating a past snapshot of LMNB1 sites, and ChIC-seq, capturing LMNB1 at the time of cell fixation. This enabled profiling the temporal dynamics of laminar associated domains during interphase. This is a unique capability that will likely provide interesting opportunities to study the temporal dynamics of chromatin marks over time.

DNA-antibody conjugation

While nb-Tn5 or pA-Tn5 methods have proven extremely useful in generating multifactorial chromatin profiles, they do reach an upper limit in the number of targets that can be profiled. For nb-Tn5 methods, the development of additional nanobodies specific for different primary antibodies would be needed to increase the current multiplexing capacity. Lochs et al. [54] took a different approach and developed Multiplexing Antibodies by barcode Identification (MAbID). This method uses DNA-conjugated antibodies containing a barcode sequence and substantially raises the upper limit for the number of possible multiplexed combinations of targets. MAbID applies restriction enzyme digestion of the DNA and antibody oligo to create compatible sticky ends, followed by proximity ligation to incorporate the antibody oligo into nearby genomic DNA sequences. These sequences can then be amplified and sequenced, revealing the genome-wide binding profiles for many different antibody targets simultaneously. The authors demonstrated single-cell profiling of mouse embryonic stem cells with simultaneous measurement of six different chromatin targets, including histone modifications and Lamin B1 [54]. This is a powerful and promising new approach capable of yielding high-quality data for many different simultaneous targets. However, due to the use of plate-based processing for single-cell-resolution data, the cell throughput is low. Future methods that aim to combine proximity-ligation based barcoding with higher-throughput cell profiling using droplet- or combinatorial indexing-based approaches would provide an ideal combination of target multiplexity and cell throughput.

Imaging-based methods

Innovative new imaging-based methods for chromatin profiling present some notable advantages over DNA sequencing-based methods. One key distinction is that by relying on microscopy these approaches are inherently single-cell-resolution. Furthermore, the number of targets able to be profiled simultaneously using imaging is potentially very high with the use of multiplexed fluorescence methods. However, imaging-based methods are only capable of profiling a predefined set of genomic loci, in contrast with genome-wide DNA sequencing-based assays. Single Cell Evaluation of Post-TRanslational Epigenetic Encoding (SCEPTRE) combined immunofluorescence and DNA fluorescence *in situ* hybridization (FISH) to simultaneously profile multiple different histone marks at a locus (Figure 3A) [55]. SCEPTRE leveraged expansion microscopy to achieve 75 nM spatial resolution, corresponding to ~10 kb of genomic DNA. By combining immunofluorescence measurement of chromatin targets with DNA FISH, the presence of different histone modifications at a genomic locus could be measured by fluorescence. The authors applied SCEPTRE to simultaneously measure H3K4me3 and H3K27me3 at the *GAPDH* locus in human cells. However, this method was limited in the number of genomic loci able to be viewed simultaneously.

Another imaging-based method, DNA seqFISH+, has greatly expanded the number of loci able to be profiled using a multiplexed FISH encoding approach (Figure 3B) [56]. In developing DNA seqFISH+, the authors use DNA oligonucleotide-conjugated antibodies for different chromatin targets to enable the multiplexed readout

of antibody binding alongside DNA and RNA FISH. They demonstrated the detection of 3660 different genomic loci, spanning the whole genome at 1 Mb resolution, as well as 17 chromatin targets and 70 mRNA targets [56]. By identifying overlapping voxels, the authors constructed multifactorial chromatin state profiles for each cell. Furthermore, sub-nuclear localization information could also be leveraged to infer the proximity of each genomic locus to the nuclear lamina, speckles, and the nucleolus, providing additional contextual information not available from other multifactorial chromatin profiling approaches. These methods present a promising alternative to high-throughput DNA sequencing for multifactorial chromatin profiling. However, in contrast with most sequencing-based approaches, genomic loci of interest must be carefully selected prior to the experiment, and the resulting chromatin profiles do not provide base-resolution information.

Analyzing multifactorial chromatin data

A fundamental challenge in all single-cell analysis is to identify the sub-populations of cells present based on their measured signal for the various features measured in the assay. For multifactorial chromatin data, this requires computational methods that are capable of integrating signals across multiple assays to leverage all the available information in defining cell-cell similarities. A variety of computational methods have now been developed that enable a so-called vertical integration [57], incorporating multiple sources of information measured for the same set of cells to produce a low-dimensional embedding space. Many of these approaches are assay-agnostic and can be readily applied to new multifactorial chromatin assays to incorporate all available information in creating a low-dimensional representation of the data. The weighted nearest neighbor method builds a single graph that represents all modalities, with a set of modality weights learned for each individual cell [58]. This joint graph can be used for downstream clustering, visualization, and trajectory analysis. MOFA+ constructs a shared low-dimensional space for cells with observations across several different modalities using a matrix factorization approach [59]. This reduced dimension space incorporates information from all input data modalities and can be used for various downstream analysis tasks, such as clustering. Neural network methods such as MultiVI also present powerful and flexible approaches for the analysis of multifactorial single-cell chromatin datasets, as separate data encoders can be trained for different input assays [60]. Past studies have demonstrated how applying such vertical integration methods to multifactorial chromatin data can improve the ability to identify the distinct states present [44,48].

Getting the most out of multifactorial chromatin datasets still presents an ongoing problem that is hampered by multiple challenges. The unique aspects of these datasets often require the development of new quality control metrics that are tailored to capture the different sources of technical noise, and in some cases these metrics must be modality-specific. For example, Tn5 tagmentation-based assays are prone to open chromatin bias, and Tn5 also has a complex DNA sequence insertion bias. These sources of error need to be carefully accounted for in the analysis. Promising new methods have been developed to more accurately model the Tn5 insertion bias [61,62], but it will take time for these to be integrated into the analysis pipelines for Tn5-based multifactorial assays.

Multifactorial assays also measure a massive number of features per cell, with each of these being extremely sparsely sampled. This compounds a problem already present in most unimodal chromatin assays, where datasets can routinely detect over 100 000 peak regions, and pushes the limit of current software implementations for analyzing these data [63–66]. While some of these issues may be solved with large amounts of memory, new approaches are still needed that enable analysis methods to scale more effectively while keeping memory requirements low. The number of features measured also presents a large multiple testing burden in a differential testing analysis. Some studies have applied feature-based clustering approaches to first identify groups of highly related peaks, and subsequently aggregate information across these peaks to greatly reduce the number of statistical tests performed [67].

New computational methods that contextualize information spanning the entire genome are needed to fully leverage the information present in chromatin datasets for dimensionality reduction, clustering, and annotation of cells. Ideally, these methods should also be highly scalable to datasets containing many cells and features.

Challenges and future opportunities

Alongside the exciting new opportunities to advance our understanding of gene regulation that multifactorial chromatin methods present, these methods come with substantial challenges that still need to be overcome. Some of these challenges are shared by other types of single-cell assays — for example, the need to carefully optimize protocols for each tissue or cell type and for high-quality antibodies. Other challenges are simply

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exacerbated when trying to measure additional chromatin modalities within the same cell. As more chromatin targets are added, the sparsity of each measurement tends to increase. This increases the need for improved sensitivity and cell throughput in these assays. Promising new experimental approaches to address both of these challenges have recently been developed for scATAC-seq through s3-ATAC-seq (increasing sensitivity) and EasySci-ATAC (increasing throughput) [68,69]. Both of these methods are compatible with Tn5-based assays, not limited to scATAC-seq, and are yet to be applied to enhance the collection of multifactorial chromatin data.

As a result of the rapid development of methods in this field, users are now faced with the challenge of selecting the best method for their research problem. This will depend on a variety of factors specific to the needs of the research question, particularly the number and types of marks to be captured (for example, DNA methylation or histone modifications), and the amount of starting material available. The desired number of cells is also a key consideration, and different profiling methods use very different approaches for single-cell capture (Table 1), resulting in lower or higher cell throughput. Users should also be conscious that many of the methods discussed in this review do not have commercially available kits or reagents. Some methods will require enzymes to be custom-made in the laboratory, presenting an additional barrier for their adoption.

With the substantial progress in multifactorial chromatin assays that has been made over the past few years, opportunities also exist to combine these approaches with other multimodal assays to enable co-assay of gene expression, protein expression, lineage information, or perturbation status. Blending these experimental approaches will provide exciting new opportunities to build comprehensive molecular views of tissues at single-cell resolution and to identify how multifactorial chromatin states guide or are influenced by other cellular modalities. Key to achieving these goals will be the parallel development of tailored computational methods capable of modeling these unique datasets. In particular, gene regulatory network inference methods may be enhanced through the incorporation of multifactorial chromatin state information through a more precise measurement of regulatory states. Similarly, methods such as MultiVelo [70], developed to model the temporal relationship between chromatin and transcriptional states, may be improved through the extension to multifactorial chromatin measurements. While the major focus so far in the field has been on method development, a transition to applying these single-cell assays to diverse biological models and clinical samples is needed, and will require the development of robust and scalable experimental protocols.

Perspectives

- Tn5 tagmentation-based methods have notable advantages due to their simplicity and amenability to single-cell-resolution profiling. However, these methods present different types of bias that needs to be carefully accounted for in the analysis.
- Computational methods for analyzing multifactorial single-cell chromatin data are still in early stages. Improvements are needed both in the scalability of existing software for analyzing single-cell chromatin data, and in the development of new computational methods specialized for the analysis of multifactorial chromatin assays.
- Measurement of transcription factor binding in single-cells still presents a major challenge. New methods are required that enable scalable measurement of these binding events in single cells.

Competing Interests

The author declares that there are no competing interests associated with this manuscript.

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Abbreviations

CMS, cytosine-5-methylenesulfonate; DHU, dihydrouracil; FISH, fluorescence *in situ* hybridization; GET-seq, genome and epigenome by transposase sequencing; MAbID, Multiplexing Antibodies by barcode Identification; SCEPTRE, Single Cell Evaluation of Post-TRanslational Epigenetic Encoding; WGBS, whole-genome bisulfite sequencing.

References

1 ENCODE Project Consortium, Moore, J.E., Purcaro, M.J., Pratt, H.E., Epstein, C.B., Shoresh, N. et al. (2020) Expanded encyclopaedias of DNA elements in the human and mouse genomes. *Nature* **583**, 699–710 https://doi.org/10.1038/s41586-020-2493-4
2 Roadmap Epigenomics Consortium, Kundaje, A., Meuleman, W., Ernst, J., Bilenky, M., Yen, A. et al. (2015) Integrative analysis of 111 reference human epigenomes. *Nature* **518**, 317–330 https://doi.org/10.1038/nature14248
3 Guo, H., Zhu, P., Wu, X., Li, X., Wen, L. and Tang, F. (2013) Single-cell methylome landscapes of mouse embryonic stem cells and early embryos analyzed using reduced representation bisulfite sequencing. *Genome Res.* **23**, 2126–2135 https://doi.org/10.1101/gr.161679.113
4 Smallwood, S.A., Lee, H.J., Angermueller, C., Krueger, F., Saadeh, H., Peat, J. et al. (2014) Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. *Nat. Methods* **11**, 817–820 https://doi.org/10.1038/nmeth.3035
5 Buenrostro, J.D., Wu, B., Litzenburger, U.M., Ruff, D., Gonzales, M.L., Snyder, M.P. et al. (2015) Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature* **523**, 486–490 https://doi.org/10.1038/nature14590
6 Cusanovich, D.A., Daza, R., Adey, A., Pliner, H.A., Christiansen, L., Gunderson, K.L. et al. (2015) Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science* **348**, 910–914 https://doi.org/10.1126/science.aab1601
7 Kaya-Okur, H.S., Wu, S.J., Codomo, C.A., Pledger, E.S., Bryson, T.D., Henikoff, J.G. et al. (2019) CUT&tag for efficient epigenomic profiling of small samples and single cells. *Nat. Commun.* **10**, 1930 https://doi.org/10.1038/s41467-019-09982-5
8 Carter, B., Ku, W.L., Kang, J.Y., Hu, G., Perrie, J., Tang, Q. et al. (2019) Mapping histone modifications in low cell number and single cells using antibody-guided chromatin tagmentation (ACT-seq). *Nat. Commun.* **10**, 3747 https://doi.org/10.1038/s41467-019-11559-1
9 Wang, Q., Xiong, H., Ai, S., Yu, X., Liu, Y., Zhang, J. et al. (2019) CoBATCH for high-throughput single-cell epigenomic profiling. *Mol. Cell* **76**, 206–216.e7 https://doi.org/10.1016/j.molcel.2019.07.015
10 Harada, A., Maehara, K., Handa, T., Arimura, Y., Nogami, J., Hayashi-Takanaka, Y. et al. (2019) A chromatin integration labelling method enables epigenomic profiling with lower input. *Nat. Cell Biol.* **21**, 287–296 https://doi.org/10.1038/s41556-018-0248-3
11 Ramani, V., Deng, X., Qiu, R., Gunderson, K.L., Steemers, F.J., Disteche, C.M. et al. (2017) Massively multiplex single-cell Hi-C. *Nat. Methods* **14**, 263–266 https://doi.org/10.1038/nmeth.4155
12 Arrastia, M.V., Jachowicz, J.W., Ollikainen, N., Curtis, M.S., Lai, C., Quinodoz, S.A. et al. (2022) Single-cell measurement of higher-order 3D genome organization with scSPRITE. *Nat. Biotechnol.* **40**, 64–73 https://doi.org/10.1038/s41587-021-00998-1
13 Preissl, S., Gaulton, K.J. and Ren, B. (2022) Characterizing cis-regulatory elements using single-cell epigenomics. *Nat. Rev. Genet.* **24**, 21–43 https://doi.org/10.1038/s41576-022-00509-1
14 Harada, A., Kimura, H. and Ohkawa, Y. (2021) Recent advances in single-cell epigenomics. *Curr. Opin. Struct. Biol.* **71**, 116–122 https://doi.org/10.1016/j.sbi.2021.06.010
15 Henikoff, S. and Ahmad, K. (2022) In situ tools for chromatin structural epigenomics. *Protein Sci.* **31**, e4458 https://doi.org/10.1002/pro.4458
16 Ludwig, C.H. and Bintu, L. (2019) Mapping chromatin modifications at the single cell level. *Development* **146**, dev170217 https://doi.org/10.1242/dev.170217
17 Janssen, S.M. and Lorincz, M.C. (2022) Interplay between chromatin marks in development and disease. *Nat. Rev. Genet.* **23**, 137–153 https://doi.org/10.1038/s41576-021-00416-x
18 Klose, R.J. and Bird, A.P. (2006) Genomic DNA methylation: the mark and its mediators. *Trends Biochem. Sci.* **31**, 89–97 https://doi.org/10.1016/j.tibs.2005.12.008
19 Klughammer, J., Romanovskaia, D., Nemc, A., Posautz, A., Seid, C.A., Schuster, L.C. et al. (2023) Comparative analysis of genome-scale, base-resolution DNA methylation profiles across 580 animal species. *Nat. Commun.* **14**, 232 https://doi.org/10.1038/s41467-022-34828-y
20 He, Y. and Ecker, J.R. (2015) Non-CG methylation in the human genome. *Annu. Rev. Genomics Hum. Genet.* **16**, 55–77 https://doi.org/10.1146/annurev-genom-090413-025437
21 Kelly, T.K., Liu, Y., Lay, F.D., Liang, G., Berman, B.P. and Jones, P.A. (2012) Genome-wide mapping of nucleosome positioning and DNA methylation within individual DNA molecules. *Genome Res.* **22**, 2497–2506 https://doi.org/10.1101/gr.143008.112
22 Pott, S. (2017) Simultaneous measurement of chromatin accessibility, DNA methylation, and nucleosome phasing in single cells. *Elife* **6**, e23203 https://doi.org/10.7554/eLife.23203
23 Guo, F., Li, L., Li, J., Wu, X., Hu, B., Zhu, P. et al. (2017) Single-cell multi-omics sequencing of mouse early embryos and embryonic stem cells. *Cell Res.* **27**, 967–988 https://doi.org/10.1038/cr.2017.82
24 Wang, Y., Yuan, P., Yan, Z., Yang, M., Huo, Y., Nie, Y. et al. (2021) Single-cell multiomics sequencing reveals the functional regulatory landscape of early embryos. *Nat. Commun.* **12**, 1247 https://doi.org/10.1038/s41467-021-21409-8
25 Clark, S.J., Argelaguet, R., Kapourani, C.-A., Stubbs, T.M., Lee, H.J., Alda-Catalinas, C. et al. (2018) scNMT-seq enables joint profiling of chromatin accessibility DNA methylation and transcription in single cells. *Nat. Commun.* **9**, 781 https://doi.org/10.1038/s41467-018-03149-4
26 Luo, C., Liu, H., Xie, F., Armand, E.J., Siletti, K., Bakken, T.E. et al. (2022) Single nucleus multi-omics identifies human cortical cell regulatory genome diversity. *Cell Genomics* **2**, 100107 https://doi.org/10.1016/j.xgen.2022.100107
27 Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y. et al. (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* **324**, 930–935 https://doi.org/10.1126/science.1170116
28 Bai, D., Zhang, X., Xiang, H., Guo, Z., Zhu, C. and Yi, C. (2024) Simultaneous single-cell analysis of 5mC and 5hmC with SIMPLE-seq. *Nat. Biotechnol.* https://doi.org/10.1038/s41587-024-02148-9

29 Fabyanic, E.B., Hu, P., Qiu, Q., Berríos, K.N., Connolly, D.R., Wang, T. et al. (2024) Joint single-cell profiling resolves 5mC and 5hmC and reveals their distinct gene regulatory effects. *Nat. Biotechnol.* **42**, 960–974 https://doi.org/10.1038/s41587-023-01909-2
30 Finlan, L.E., Sproul, D., Thomson, I., Boyle, S., Kerr, E., Perry, P. et al. (2008) Recruitment to the nuclear periphery can alter expression of genes in human cells. *PLoS Genet.* **4**, e1000039 https://doi.org/10.1371/journal.pgen.1000039
31 Kadauke, S. and Blobel, G.A. (2009) Chromatin loops in gene regulation. *Biochim. Biophys. Acta* **1789**, 17–25 https://doi.org/10.1016/j.bbagrm.2008.07.002
32 Lieberman-Aiden, E., van Berkum, N.L., Williams, L., Imakaev, M., Ragoczy, T., Telling, A. et al. (2009) Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science* **326**, 289–293 https://doi.org/10.1126/science.1181369
33 Lee, D.-S., Luo, C., Zhou, J., Chandran, S., Rivkin, A., Bartlett, A. et al. (2019) Simultaneous profiling of 3D genome structure and DNA methylation in single human cells. *Nat. Methods* **16**, 999–1006 https://doi.org/10.1038/s41592-019-0547-z
34 Li, G., Liu, Y., Zhang, Y., Kubo, N., Yu, M., Fang, R. et al. (2019) Joint profiling of DNA methylation and chromatin architecture in single cells. *Nat. Methods* **16**, 991–993 https://doi.org/10.1038/s41592-019-0502-z
35 Wang, H., Maurano, M.T., Qu, H., Varley, K.E., Gertz, J., Pauli, F. et al. (2012) Widespread plasticity in CTCF occupancy linked to DNA methylation. *Genome Res.* **22**, 1680–1688 https://doi.org/10.1101/gr.136101.111
36 Liu, H., Zeng, Q., Zhou, J., Bartlett, A., Wang, B.-A., Berube, P. et al. (2023) Single-cell DNA methylome and 3D multi-omic atlas of the adult mouse brain. *Nature* **624**, 366–377 https://doi.org/10.1038/s41586-023-06805-y
37 Musselman, C.A., Lalonde, M.-E., Côté, J. and Kutateladze, T.G. (2012) Perceiving the epigenetic landscape through histone readers. *Nat. Struct. Mol. Biol.* **19**, 1218 https://doi.org/10.1038/nsmb.2436
38 Creyghton, M.P., Cheng, A.W., Welstead, G.G., Kooistra, T., Carey, B.W., Steine, E.J. et al. (2010) Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc. Natl Acad. Sci. U.S.A.* **107**, 21931–21936 https://doi.org/10.1073/pnas.1016071107
39 Nitsch, S., Zorro Shahidian, L. and Schneider, R. (2021) Histone acylations and chromatin dynamics: concepts, challenges, and links to metabolism. *EMBO Rep.* **22**, e52774 https://doi.org/10.15252/embr.202152774
40 Wang, M. and Zhang, Y. (2021) Tn5 transposase-based epigenomic profiling methods are prone to open chromatin bias. bioRxiv https://doi.org/10.1101/2021.07.09.451758
41 Kaya-Okur, H.S., Janssens, D.H., Henikoff, J.G., Ahmad, K. and Henikoff, S. (2020) Efficient low-cost chromatin profiling with CUT&Tag. *Nat. Protoc.* **15**, 3264–3283 https://doi.org/10.1038/s41596-020-0373-x
42 Gopalan, S., Wang, Y., Harper, N.W., Garber, M. and Fazzio, T.G. (2021) Simultaneous profiling of multiple chromatin proteins in the same cells. *Mol. Cell* **81**, 4736–4746.e5 https://doi.org/10.1016/j.molcel.2021.09.019
43 Meers, M.P., Llagas, G., Janssens, D.H., Codomo, C.A. and Henikoff, S. (2022) Multifactorial profiling of epigenetic landscapes at single-cell resolution using MulTI-Tag. *Nat. Biotechnol.* **41**, 708–716 https://doi.org/10.1038/s41587-022-01522-9
44 Xiong, H., Wang, Q., Li, C.C. and He, A. (2024) Single-cell joint profiling of multiple epigenetic proteins and gene transcription. *Sci. Adv.* **10**, eadi3664 https://doi.org/10.1126/sciadv.adi3664
45 Janssens, D.H., Otto, D.J., Meers, M.P., Setty, M., Ahmad, K. and Henikoff, S. (2022) CUT&tag2for1: a modified method for simultaneous profiling of the accessible and silenced regulome in single cells. *Genome Biol.* **23**, 81 https://doi.org/10.1186/s13059-022-02642-w
46 Henikoff, S., Henikoff, J.G., Kaya-Okur, H.S. and Ahmad, K. (2020) Efficient chromatin accessibility mapping in situ by nucleosome-tethered tagmentation. *Elife* **9**, e63274 https://doi.org/10.7554/eLife.63274
47 Pleiner, T., Bates, M. and Görlich, D. (2018) A toolbox of anti-mouse and anti-rabbit IgG secondary nanobodies. *J. Cell Biol.* **217**, 1143–1154 https://doi.org/10.1083/jcb.201709115
48 Stuart, T., Hao, S., Zhang, B., Mekerishvili, L., Landau, D.A., Maniatis, S. et al. (2022) Nanobody-tethered transposition enables multifactorial chromatin profiling at single-cell resolution. *Nat. Biotechnol.* **41**, 806–812 https://doi.org/10.1038/s41587-022-01588-5
49 Bartosovic, M. and Castelo-Branco, G. (2023) Multimodal chromatin profiling using nanobody-based single-cell CUT&Tag. *Nat. Biotechnol.* **41**, 794–805 https://doi.org/10.1038/s41587-022-01535-4
50 Stoeckius, M., Hafemeister, C., Stephenson, W., Houck-Loomis, B., Chattopadhyay, P.K., Swerdlow, H. et al. (2017) Simultaneous epitope and transcriptome measurement in single cells. *Nat. Methods* **9**, 2579 https://doi.org/10.1038/nmeth.4380
51 Mimitou, E.P., Lareau, C.A., Chen, K.Y., Zorzetto-Fernandes, A.L., Hao, Y., Takeshima, Y. et al. (2021) Scalable, multimodal profiling of chromatin accessibility, gene expression and protein levels in single cells. *Nat. Biotechnol.* **39**, 1246–1258 https://doi.org/10.1038/s41587-021-00927-2
52 Tedesco, M., Giannese, F., Lazarević, D., Giansanti, V., Rosano, D., Monzani, S. et al. (2022) Chromatin velocity reveals epigenetic dynamics by single-cell profiling of heterochromatin and euchromatin. *Nat. Biotechnol.* **40**, 235–244 https://doi.org/10.1038/s41587-021-01031-1
53 Kefalopoulou, S., Rullens, P.M.J., de Luca, K.L., de Vries, S.S., Korthout, T., van Oudenaarden, A. et al. (2023) Time-resolved and multifactorial profiling in single cells resolves the order of heterochromatin formation events during X-chromosome inactivation. bioRxiv https://doi.org/10.1101/2023.12.15.571749
54 Lochs, S.J.A., van der Weide, R.H., de Luca, K.L., Korthout, T., van Beek, R.E., Kimura, H. et al. (2023) Combinatorial single-cell profiling of major chromatin types with MAbID. *Nat. Methods* **21**, 72–82 https://doi.org/10.1038/s41592-023-02090-9
55 Woodworth, M.A., Ng, K.K.H., Halpern, A.R., Pease, N.A., Nguyen, P.H.B., Kueh, H.Y. et al. (2021) Multiplexed single-cell profiling of chromatin states at genomic loci by expansion microscopy. *Nucleic Acids Res.* **49**, e82 https://doi.org/10.1093/nar/gkab423
56 Takei, Y., Yun, J., Zheng, S., Ollikainen, N., Pierson, N., White, J. et al. (2021) Integrated spatial genomics reveals global architecture of single nuclei. *Nature* **590**, 344–350 https://doi.org/10.1038/s41586-020-03126-2
57 Argelaguet, R., Cuomo, A.S.E., Stegle, O. and Marioni, J.C. (2021) Computational principles and challenges in single-cell data integration. *Nat. Biotechnol.* **39**, 1202–1215 https://doi.org/10.1038/s41587-021-00895-7
58 Hao, Y., Hao, S., Andersen-Nissen, E., Mauck, III, W.M., Zheng, S., Butler, A. et al. (2021) Integrated analysis of multimodal single-cell data. *Cell* **184**, 3573–3587.e29 https://doi.org/10.1016/j.cell.2021.04.048
59 Argelaguet, R., Arnol, D., Bredikhin, D., Deloro, Y., Velten, B., Marioni, J.C. et al. (2020) MOFA+: a statistical framework for comprehensive integration of multi-modal single-cell data. *Genome Biol.* **21**, 111 https://doi.org/10.1186/s13059-020-02015-1

60 Ashuach, T., Gabitto, M.I., Koodli, R.V., Saldi, G.-A., Jordan, M.I. and Yosef, N. (2023) MultiVI: deep generative model for the integration of multimodal data. *Nat. Methods* **20**, 1222–1231 https://doi.org/10.1038/s41592-023-01909-9
61 Nair, S., Ameen, M., Sundaram, L., Pampari, A., Schreiber, J., Balsubramani, A. et al. (2023) Transcription factor stoichiometry, motif affinity and syntax regulate single-cell chromatin dynamics during fibroblast reprogramming to pluripotency. bioRxiv https://doi.org/10.1101/2023.10.04.560808
62 Hu, Y., Ma, S., Kartha, V.K., Duarte, F.M., Horlbeck, M., Zhang, R. et al. (2023) Single-cell multi-scale footprinting reveals the modular organization of DNA regulatory elements. bioRxiv https://doi.org/10.1101/2023.03.28.533945
63 Stuart, T., Srivastava, A., Madad, S., Lareau, C.A. and Satija, R. (2021) Single-cell chromatin state analysis with Signac. *Nat. Methods* **18**, 1333–1341 https://doi.org/10.1038/s41592-021-01282-5
64 Granja, J.M., Corces, M.R., Pierce, S.E., Bagdatli, S.T., Choudhry, H., Chang, H.Y. et al. (2021) Archr is a scalable software package for integrative single-cell chromatin accessibility analysis. *Nat. Genet.* **53**, 403–411 https://doi.org/10.1038/s41588-021-00790-6
65 Fang, R., Preissl, S., Li, Y., Hou, X., Lucero, J., Wang, X. et al. (2021) Comprehensive analysis of single cell ATAC-seq data with SnapATAC. *Nat. Commun.* **12**, 1337 https://doi.org/10.1038/s41467-021-21583-9
66 Zhang, K., Zemke, N.R., Armand, E.J. and Ren, B. (2023) SnapATAC2: a fast, scalable and versatile tool for analysis of single-cell omics data. bioRxiv https://doi.org/10.1101/2023.09.11.557221
67 Xiong, X., James, B.T., Boix, C.A., Park, Y.P., Galani, K., Victor, M.B. et al. (2023) Epigenomic dissection of Alzheimer's disease pinpoints causal variants and reveals epigenome erosion. *Cell* **186**, 4422–4437.e21 https://doi.org/10.1016/j.cell.2023.08.040
68 Sziraki, A., Lu, Z., Lee, J., Banyai, G., Anderson, S., Abdulraouf, A. et al. (2023) A global view of aging and Alzheimer's pathogenesis-associated cell population dynamics and molecular signatures in human and mouse brains. *Nat. Genet.* **55**, 2104–2116 https://doi.org/10.1038/s41588-023-01572-y
69 Mulqueen, R.M., Pokholok, D., O'Connell, B.L., Thornton, C.A., Zhang, F., O'Roak, B.J. et al. (2021) High-content single-cell combinatorial indexing. *Nat. Biotechnol.* **39**, 1574–1580 https://doi.org/10.1038/s41587-021-00962-z
70 Li, C., Virgilio, M.C., Collins, K.L. and Welch, J.D. (2022) Multi-omic single-cell velocity models epigenome-transcriptome interactions and improves cell fate prediction. *Nat. Biotechnol.* **41**, 387–398 https://doi.org/10.1038/s41587-022-01476-y