EPIGENOMICS
2026, VOL. 18, NO. 1, 117–135
https://doi.org/10.1080/17501911.2025.2571391

Taylor & Francis
Taylor & Francis Group

REVIEW

Recent advances in methodologies of epigenomics

Hiroaki Ohishi [a] and Wan Kin Au Yeung [b]

[a]Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan; [b]Department of Natural Sciences, College of Liberal Arts, International Christian University, Mitaka-shi, Japan

ABSTRACT

Rapid methodological breakthroughs over the past ten years have transformed epigenomics from bulk, population-averaged assays into single-cell, multi-omic, and intracellular spatial investigations. This review surveys the interconnected technology pillars that now map the epigenome with unprecedented breadth and resolution. First, advances in next-generation and long-read sequencing empower investigators to chart chromatin accessibility, histone and DNA modifications, and three-dimensional higher-order chromatin structure in thousands of individual cells while retaining allele-specific information across kilobase-long molecules. Second, live-cell fluorescence probes and multiplexed chromatin tracing enable visualizing the dynamic organization of epigenetic marks and genome architecture of intact nuclei and tissues. Third, integrative platforms merge base-level reads with their native 3D coordinates, providing a holistic view of gene regulation in physiologic context. We distill key biological insights yielded by each methodology, discuss unresolved and persistent limitations, and outline future directions toward routine, cost-effective investigations. Together, these innovations are redefining how we interrogate chromatin biology in health and disease.

PLAIN LANGUAGE SUMMARY

Epigenomics explores how chemical tags and folding of DNA turn genes on or off without changing DNA itself. This review explains three groups of advanced methods to study these marks. First, sequencing reads these marks in single cells and long DNA molecules. This helps find what these cells are and see patterns passed down from parents. Second, imaging watches how these marks change inside living cells. Third, combining sequencing and imaging links DNA information to its physical location inside a cell. Together, these methods provide a clearer picture of development, aging, and disease. The article also outlines how these methods could be widely used in future research and medicine.

ARTICLE HISTORY
Received 29 May 2025
Accepted 3 October 2025

KEYWORDS
Epigenetics; sequencing; long-read; imaging; spatial; single-cell; multi-omics; integrative approach

1. Introduction

Epigenomics investigates chemical modifications on the genome that regulate gene expression without altering the DNA sequence itself, such as DNA methylation, histone (post-translational) modifications, and the related three-dimensional (3D) higher-order chromatin structure. Early epigenomic studies relied on bulk cell measurements, such as bisulfite sequencing for DNA methylation and chromatin immunoprecipitation sequencing (ChIP-seq) for histone modifications [1]. These bulk approaches revealed global epigenetic mechanisms, but obscured cellular heterogeneity and masked local epigenetic regulation. To address these limitations, an explosion of new methodologies developed over the past ten years enable researchers to interrogate epigenetic states at single-cell resolution, detect modifications on long reads without the need for harsh chemical treatments, and integrate multiple data layers (transcriptome, accessibility, and modification states) within the same cell. Furthermore, developments in imaging-based methods, such as super-resolution microscopy and high-plex fluorescent *in situ* hybridization (FISH), have enabled direct visualization of higher-order chromatin structure and epigenetic marks in their intracellular spatial contexts. Integrated with machine learning approaches, these parallel technological revolutions (sequencing-based and imaging-based) offer deeper insights into how epigenetic modifications shape gene regulation.

In this review, we first delve into sequencing-based epigenomic methodologies (Section 2). We then survey imaging-based approaches and their applications for the intracellular spatial and dynamic analysis of the epigenome (Section 3). Next, we explore integrative frameworks that combine sequencing and imaging to link genomic and epigenomic data with intracellular spatial organization information at high resolution (Section 4). Finally, we discuss key challenges and future prospects (Section 5) before concluding (Section 6). By focusing on innovations in recent years, we aim to provide a current perspective on the methodological advances and biological insights that are advancing this research field (Figure 1) (See [2] for an extensive review of the technical basis of these advancements.).

2. Sequencing-based methods

The evolution of sequencing-based methodologies studying chromatin accessibility, histone modifications, DNA

CONTACT Wan Kin Au Yeung auyeung.wankin@icu.ac.jp Department of Natural Sciences, College of Liberal Arts, International Christian University, Troyer Memorial Arts and Sciences Hall, 3-10-2 Osawa, Mitaka-shi, Mitaka-shi, Tokyo 181-8585, Japan

Article highlights

- Single-cell and long-read sequencing reveal cell-specific chromatin states and allele-specific epigenetic patterns.
- Spatialtemporal imaging techniques map spatial epigenome patterns within intact nuclei, linking chromatin organization to transcriptional regulation.
- Integrated sequencing-imaging platforms connect nucleotide data with spatial coordinates, creating holistic single-cell epigenetic maps.
- Remaining hurdles include data sparsity, high reagent costs, and integrating complex multi-omic datasets.

methylation, and the related 3D higher-order chromatin structure, has been driven by three core scientific aspects: resolving cell-to-cell variability achieving long-range allelic phasing, and capturing multiple molecular layers simultaneously. We can now move beyond population averages to dissect the cell-to-cell heterogeneity that regulates complex development and disease processes. This high-resolution view enables the characterization of rare but functionally critical cell subpopulations, such as drug-resistant cancer cells or specific progenitor lineages, opening new opportunities to investigate their precise roles. Concurrently, long-read technologies provide a comprehensive view of the genome's linear structure. By preserving the contiguity of DNA molecules, these methods enable the direct phasing of epigenetic marks with genetic variants across long genomic distances. This capability is crucial for deciphering the allelic-specific gene regulation, particularly the long-range interactions between genes and their distal enhancers, and raises new questions about how parental alleles are differentially controlled. Furthermore, by capturing multiple molecular layers from the same cell, multi-omic approaches are transforming our understanding of gene regulation from correlational to causal. Integrating these datasets provides a holistic picture of the dynamic interplay between the epigenome, transcriptome, and other molecular events, allowing us to ask fundamental questions about how these layers are coordinated to define cellular identity and function.

This section provides an overview of these three major developments: (i) single-cell epigenomics, (ii) long-read sequencing technologies for epigenetic modifications, and (iii) multi-omic approaches that simultaneously measure multiple epigenetic layers. Each category has advanced our understanding of epigenetic heterogeneity, dynamics, and

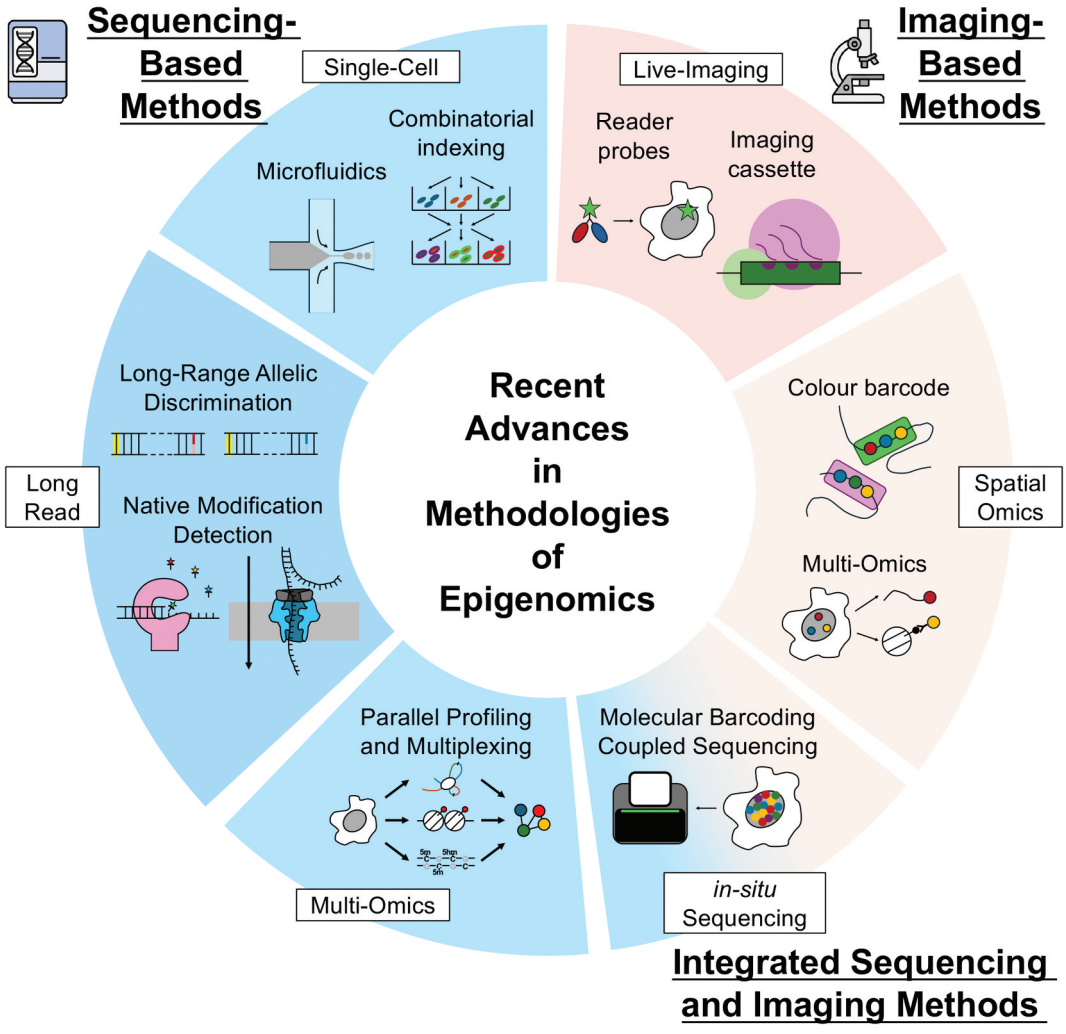

Figure 1. Overview of recent epigenomic methodologies: sequencing-based, imaging-based, and integrative approaches. Key technological concepts are summarized.

regulatory function in unprecedented depth. An overview of technological principles, compatible sample types, and key features of the methods described in this section is summarized in Table 1 and Figure 2.

2.1. Single-cell epigenomics

2.1.1. Single-cell chromatin accessibility

Sequencing of deoxyribonuclease I digestion products (DNase-seq) and of transposase-accessible chromatin (ATAC-seq) are the main techniques for mapping open chromatin regions that likely harbor active enhancers, promoters, and other regulatory elements [3]. While conventional DNase-seq and ATAC-seq require tens of thousands of cells and yields their averaged profile which masks cellular heterogeneity, single-cell ATAC-seq (scATAC-seq) emerges more prominently as a high-resolution approach to overcome this limitation [4,5]. Two major strategies are employed to isolate individual cells for analysis. In the first, single cells are sealed by microfluidic devices into oil droplets as independent reactions, to minimize sample loss and to increase reaction efficiency [4]. Commercial microfluidics systems like 10x Genomics streamline the library preparation and, combined with RNA-seq, enable revealing the effect of chromatin accessibility on transcript abundance at single cell levels. Alternatively, in the second strategy, the chromatin is labeled with unique cellular barcodes by combinatorial indexing (also called a repeated 'split-pool' strategy) of cell nuclei, followed by pooling for DNA amplification [5]. Further improvement of combinatorial indexing and development of other microfluidic-free droplet approaches will enable wider adoption of single-cell chromatin accessibility assays without requiring expensive hardware [6].

By providing a cell-by-cell view, single-cell ATAC-seq enables simultaneous annotation of rare cell populations in heterogeneous mammalian tissues [28,29]. At the same time, the cell-specific open chromatin signature are the footprints of regulatory transcription factors during mouse forebrain development [30], human spermatogenesis [31], and rice virus infection [32]. These insights are often masked in bulk ATAC-seq analysis [33].

2.1.2. Single-cell histone modification and transcription factor binding

ChIP-seq revolutionized histone modification and transcription factor mapping [34,35], but its requirement for millions of cells makes it unsuitable for rare cell populations, and adapting it to single cells using microfluidics is still challenging due to limited resolution [36,37]. To address this input requirement, new approaches like Cleavage under targets and release using nuclease (CUT&RUN) and Cleavage under targets and tagmentation (CUT&Tag) directly tether a micrococcal nuclease (MNase) or Tn5 transposase, respectively, to the antibody-bound region [8,38–40]. Instead of immunoprecipitating large chromatin fragments, these methods selectively cleave and release DNA only at antibody-bound sites, dramatically improving efficiency. CUT&RUN increases the signal-to-noise ratio and is suitable as a low-cell-input alternative, working with as few as 100–1,000 cells. CUT&Tag is often combined with combinatorial indexing, to further lower its limit to single cell applications. Amplifying Tn5-tagged DNA by *in vitro* transcription demonstrated an alternative way to recover single-cell DNA fragments [41].

Single-cell CUT&Tag provided an unbiased and unsupervised view of epigenetic dynamics in biological processes without prior knowledge, demonstrating the ability to identify cell heterogeneity during *in vivo* brain development, *in vitro* endoderm differentiation and tumor progression [42,43]. Another study identified tumor-subtype-specific sites of aberrant chromatin regulation induced in mixed-lineage leukemia [44]. While highly effective for histone modifications, CUT&Tag can be less robust for profiling transcription factors compared to histone marks, but can still be used in single cells, enabling cell type identification [42].

2.1.3. Single-cell DNA methylation

While whole-genome bisulfite sequencing (WGBS) provides a gold standard for mapping cytosine methylation (the predominant form 5-methylcytosine (5mC) in eukaryotes and its oxidized form 5-hydroxylmethylcytosine (5hmC) [45,46], its adaptation to single cells has been challenging. The core issue is that the underlying principle of unmethylated cytosine-to-uracil conversion by sodium bisulfite treatment damages DNA and leads to significant sample loss, a critical problem when starting with picograms of material. Despite this, single-cell approaches like single cell bisulfite sequencing (scBS-seq) has only recently become feasible due to optimized protocols and improved library preparation efficiency [9], with robust population-scale data only emerged in recent years with refinements in amplification and barcoding strategies [47,48]. This single-cell approach identified differential methylation status of cell-type-specific regulatory elements in mammalian frontal cortex [47]. To overcome the DNA damage inherent to bisulfite treatment, an alternative enzymatic conversion (EM-seq) method uses a combination of Ten-eleven translocation dioxygenase (TET) and Apolipoprotein B mRNA editing enzyme subunit (APOBEC) to convert unmethylated cytosine to uracil without damaging the DNA template. Carrier-assisted base-conversion by enzymatic reaction with end-tagging (Cabernet) is a single cell technique based on EM-seq and incorporates tagmentation to minimize sample loss [10]. Furthermore, running parallel reactions that omit TET activity [49] as Cabernet-H method allows unified distinction between the two forms of cytosine methylation [10]. Applying Cabernet revealed the degree of methylation heterogeneity among the blastomeres during the epigenetic reprogramming of mouse embryos.

2.1.4. Single-cell analysis of higher-order chromatin structure

While the aforementioned methods profile linear epigenetic features, understanding gene regulation requires knowledge of the genome's higher-order chromatin structure. Chromosome conformation capture (3C) and its genome-wide derivative, Hi-C, are foundational for mapping these 3D interactions. Although they do not directly measure chemical modifications, they are essential for epigenomics as they reveal the spatial proximity between elements like enhancers and promoters and define structural units such as

Table 1. Summary of sequencing-based methods. The principles, compatible sample types (including frozen and FFPE-fixed samples), and key features of the methods introduced are summarized. See also Figure 2 for technical principles.

Method Name	Epigenetic and Transcriptomic Features Covered	Principle	Sample Type	Frozen Compatibility	FFPE Compatibility	Strength	Limitation	Ref
Single-cell epigenomics								
scATAC-seq (Single-cell Assay for Transposase-Accessible Chromatin using sequencing)	Open chromatin regions	A Tn5 transposase is used to insert sequencing adapters into open chromatin regions.	Single cells	Yes	No report (Bulk ATAC-seq is compatibile.)	Resolves cellular heterogeneity and identifies rare cell populations. Can detect cell-type-specific regulatory factor footprints that are masked in bulk analysis.	Requires expensive microfluidic devices in the standard protocol. Inherently sparse data.	[3–6]
CUT&Tag (Cleavage under targets and tagmentation)	Histone modifications, transcription factor binding	An antibody guides a Protein A-Tn5 transposase fusion protein to insert sequencing adapters near target sites.	Low-cell-number to single-cell samples	Yes	Yes	Achieves high-efficiency, high signal-to-noise data from very small input amounts. Ideal for rare cell populations and single-cell analysis. Rapid workflow (1–2 days). Can combine with spatial labeling of histology sections.	Less robust when profiling transcription factors. Uses unfixed cells in standard protocols.	[7–9]
scBS-seq (Single-cell bisulfite sequencing)	DNA methylation	Bisulfite treatment converts unmethylated cytosines to uracil. Adaptor tagging and random priming lowers cell number requirement.	Single cells	Yes	Difficult (Bisulfite treatment is DNA damaging.)	Directly assesses heterogeneity in methylation status between cells. Has been used to identify methylation differences in cell-type-specific regulatory elements in the mammalian cortex.	Critical DNA damage and sample loss from bisulfite treatment when starting with picogram-level DNA from a single cell.	[9]
Cabernet(–H) (Carrier-assisted base-conversion by enzymatic reaction with end-tagging)	DNA methylation (5mC, 5hmC)	Sequential enzymatic reactions with TET and APOBEC enzymes convert unmethylated cytosines to uracil, minimizing DNA damage. A Tn5 transposase is used to insert sequencing adapters to minimize sample loss.	Single cells	Yes	Yes	Avoids the DNA damage associated with bisulfite treatment. Can detect 5hmC in parallel by omitting the TET enzyme reaction.	Different genomic coverage profiles then bisulfite treatment methods. May take time for standardization and widespread adoption as a newer technology.	[10]
scHi-C/Dip-C (Single-cell Hi-C)	3D high-order chromatin structure	3D high-order chromatin is randomly digested by restriction enzyme and proximal fragments are ligated inversely proportional to intracellular spatial distance.	Single cells	Yes	No report (Bulk Hi-C is compatibile.)	Reveals heterogeneity in 3D genome architecture between cells. Capture the dynamic nature of structural units like topologically associating domains (TADs).	Sparse data, lacking long-range inter-chromosomal interactions and often contacts around centromeres and nucleoli.	[11,12]
scMicro-C (Single-cell Micro-C)	High-resolution 3D high-order chromatin structure	3D high-order chromatin is randomly digested by micrococcal nuclease (MNase) and proximal fragments are ligated inversely proportional to intracellular spatial distance.	Single cells	Yes	No report (MNase activity is likely affected by formalin cross-linking.)	Detects promoter-enhancer interactions with higher resolution than scHi-C. Has successfully identified multi-enhancer hubs.	Sparse data, lacking long-range inter-chromosomal interactions and often contacts around centromeres and nucleoli.	[13]

(*Continued*)

Table 1. (Continued).

Method Name	Epigenetic and Transcriptomic Features Covered	Principle	Sample Type	Frozen Compatibility	FFPE Compatibility	Strength	Limitation	Ref
scSPRITE (Single-cell split-pool recognition of interactions by tag extension)	3D high-order chromatin structure	3D high-order chromatin is randomly digested by restriction enzyme and fragments are tagged by combinatorial indexing of barcodes. Similar barcodes reflect spatially proximal fragments.	Single cells	Yes	No report	Detects 10x more pairwise contacts and 5x more inter-chromosomal contacts with fewer reads compared to scHi-C. Significantly mitigates the data sparsity issue.	Combinatorial indexing requires a large number of single cells to start with. May take time for standardization and widespread adoption as a newer technology.	[14]
Third generation long-read epigenomics								
scNanoATAC-seq2	Open chromatin regions	Long fragments are sequenced after Tn5 transposition (instead of sequencing short fragments in scATAC-seq), enabling allelic discrimination and long-range co-occurence.	Single cells	Yes	No report (Technologically feasible with length trade-off)	Resolves allele-specific accessibility information and identifies co-accessibility of multiple sites on the same allele. Useful for studying imprinting and X-chromosome inactivation.	May take time for standardization and widespread adoption as a newer technology.	[15]
scNanoSeq-CUT&Tag	Histone modifications	Long fragments are sequenced after Tn5 transposition (instead of sequencing short fragments in CUT&Tag), enabling allelic discrimination and long-range co-occurence.	Single cells	Yes	No report (Technologically feasible with length trade-off)	Resolves allele-specific accessibility information and identifies co-occurrence patterns for histone modifications on the same allele. Can accurately detect modification status on individual copies of repetitive elements.	May take time for standardization and widespread adoption as a newer technology.	[16]
Native Detection of Base Modifications	Native DNA/RNA base modifications (5mC, 5hmC, 6 mA, etc.)	Modified bases cause characteristic delays in complementary base incorporation (PacBio) or produce unique current patterns when passing through a protein nanopore (Oxford Nanopore).	Microgram-scale native high-molecular-weight DNA.	Yes	Yes	Directly detects multiple base modifications without bisulfite or enzymatic conversion. Long reads enable the resolution of complex genomic regions and allele phasing.	Higher error rate compared to short-read platforms. Requires a large amount of input DNA.	[17–19]
Multi-omics in epigenomics								
SHARE-seq (Simultaneous high-throughput ATAC and RNA expression)	Chromatin accessibility, transcriptome (mRNA)	ATAC-seq and RNA-seq are performed simultaneously in permeabilized single nuclei. Tn5 transposase tagged open chromatin and oligo-dT captured mRNA are physically separated for parallel library preparation.	Single cell nuclei	Yes	No report	Directly links chromatin accessibility and gene expression in the same cell, allowing the capture of chromatin state changes that precede transcriptional activation.	Complex protocol due to parallel handling two modalities. Spare data.	[20]

(*Continued*)

Table 1. (Continued).

Method Name	Epigenetic and Transcriptomic Features Covered	Principle	Sample Type	Frozen Compatibility	FFPE Compatibility	Strength	Limitation	Ref
scM&T-seq (Single-cell Methylation and Transcriptome sequencing)	DNA methylation, transcriptome (mRNA)	Streptavidin-conjugated oligo-dT beads physically separates mRNA from genomic DNA for parallel library preparation.	Single cells	Yes	No report	Demonstrates the heterogeneous negative correlation between promoter DNA methylation and transcript abundance at the single-cell level.	Complex protocol due to parallel handling two modalities. DNA damage from bisulfite treatment.	[21]
scNMT-seq (Single-cell Nucleosome, Methylation, and Transcriptome sequencing)	Chromatin accessibility, DNA methylation, transcriptome	Open chromatin regions in permeabilized cells with an exogenous GpC methyltransferase (M.CviPI). Then, RNA and gDNA are then separated for RNA-seq and bisulfite sequencing as in scM&T-seq.	Single cells	Yes	No report	Obtains three layers of regulation from the same cell, enabling direct correlation of chromatin state, DNA methylation, and gene expression during dynamic processes.	Complex protocol due to parallel handling two modalities. Spare data.	[22]
scMulti-CUT&Tag/ scMulTI-Tag	Multiple histone modifications or transcription factors	Multiple rounds of antibody-guided Tn5 transposition guide the insertion of sequencing adaptors with different barcodes, allowing signals from different antibodies to be distinguished during sequencing.	Single cells	Yes	No report	Enables analysis of combinations of histone modfications within the same cell. Allows for a more precise definition of regulatory element function.	Complex protocol due to parallel handling two modalities. Cross-reactivity of antibodies and varible efficiency of different antibodies.	[23,24]
Six-letter-seq	DNA methylation (5mC versus 5hmC)	DNA methyltransferase 5 (DNMT5) copies 5mC in vitro to the synthetic complementary strand but not glycosylation-protected 5hmC. This allows the two to be distinguished after sequencing.	Nanogram-scale DNA.	Yes	No report	Distinguishes between 5mC and 5hmC in minute cell populations or low input DNA sample.	May take time for standardization and widespread adoption as a newer technology.	[25]
scMethyl-HiC	DNA methylation, 3D high-order chromatin structure	Bisulfite treatment is peformed on ligated proximal DNA fragments, providing DNA methylation information versus scHi-C.	Single cells	Yes	No report (Bulk Methyl-HiC is compatibile.)	Can directly correlate pairwise 3D contacts with the methylation status of interacting loci. Has revealed co-regulation of spatially proximal distal regions.	Complex protocol due to parallel handling two modalities. DNA damage from bisulfite treatment.	[26]
Pore-C	3D high-order chromatin structure, native DNA methylation	Different from Hi-C, ligated proximal DNA fragments are sequenced directly to capture high-order chromatin strcuture and native DNA base modications at the same time.	Millions of cells	Yes	No report	Can directly correlate multiway 3D contacts with the allele-specific methylation status of interacting loci.	May take time for standardization and widespread adoption as a newer technology.	[27]

topologically associating domains (TADs) and A/B compartments, which are intimately linked to epigenetic states. The major idea of Hi-C is to fragment genome by restriction enzymes, and ligate spatially proximal fragments to create chimeras. To dissect cellular heterogeneity in chromatin structure, single-cell Hi-C (scHi-C) methods were developed to map 3D contacts in thousands of individual cells, including the original protocol [11] and the expanded Dip-C protocol to map haplotype-resolved structure [12]. These studies have revealed cell-to-cell variability in TAD structures. To detect promoter-enhancer interactions, single-cell Micro-C (scMicro-C) uses micrococcal nuclease in place of restriction enzymes to increase the data resolution up to 5 kb, identifying multi-enhancer hubs in human lymphoblastoid cell lines [13]. However, a major limitation of scHi-C is its extreme data sparsity in long-range contacts across chromosomes (inter-chromosomal contacts) around nuclei aggregates (centromeres, nucleoli and nuclear speckles). An alternative method called single-cell split-pool recognition of interactions by tag extension (scSPRITE) uses the combinatorial indexing to tag spatially proximal fragments [14]. scSPRITE detects ten times more pairwise contacts in a fraction of reads compared to scHi-C, at the same time detecting five times more inter-chromosomal contacts in mouse embryonic stem cells.

2.2. Third generation long-read epigenomics

The above short-read methods offered by next-generation sequencing, although powerful, present challenges in phasing epigenetic marks (i.e., determining which alleles carry certain modifications) and accurately mapping repetitive or structurally complex regions. In addition, detecting base modifications requires chemical conversion, which limits the application to only well-defined modifications. Third generation sequencers from Pacific Biosciences (PacBio) and Oxford Nanopore Technologies address these issues by offering two key advantages.

2.2.1. Allelic discrimination and long range co-occurrence

Third generation sequencers read intact molecules up to tens or hundreds of kilobases long, dramatically improving the chances to distinguish alleles by single nucleotide polymorphism and insertion/deletion. At the same time, reading long molecules allows the detection of epigenetic information on the same allele in single molecule or single-cell levels [15,17,50]. Phased epigenetic datasets can then be compared to reveal differentially DNA methylated regions in DNA methyltransferase triple knockout mouse embryonic stem cells [17] and imprinted loci in human breast cancer cell lines [50]. Similarly, long-read chromatin accessibility assay (scNanoATAC-seq2) not only resolves allele-specific accessibility information, but demonstrates the ability of identifying co-occurrence of accessible sites on the same allele of individual cells [15]. Similar benefits exist for histone modifications using scNanoSeq-CUT&Tag [16]. While the biological significance of detecting allelic DNA methylation is well recognized on genomic imprinting and X chromosome inactivation, the power to detect chromatin co-accessibility requires further investigation including multi-omic approaches.

2.2.2. Native detection of base modifications

Third generation sequencers can also directly measure modifications to nucleotides [51]. PacBio sequencers use the single molecule real-time (SMRT) technology to monitor the incorporation rate of complementary bases, and the polymerase kinetics for a complementary base to a modified base are different from those for an unmodified base, producing a characteristic delayed incorporation [18]. In contrast, Nanopore sequencers from Oxford Nanopore Technologies record the electric current patterns when a single DNA strand passes through tiny protein channels called nanopores, and a modified base produces a characteristic electric current pattern different from its unmodified counterpart [17,19]. These two methods convert time-domain fluorescent or electric signal patterns to modified bases using machine learning models trained on synthetic DNA standards (Figure 2B). While machine learning is also used in next-generation sequencers for assigning bases without modifications, the third generation sequencers can detect multiple modifications in one sequencing run. Moreover, the sequencing (signal acquisition) and base calling can be separated, meaning that a future machine learning model trained on a new DNA modification can be used to analyze a previously sequenced sample. However, because this native information is lost during *in vitro* amplification, these methods require a microgram scale of native DNA as the starting material. Thus, third generation sequencing cannot yet profile the native chromatin of single cells or rare cell populations. However, this approach is suitable in combination with other omic layers like higher-order chromatin structure [27]. (For further explanation please see Section 2.3.3 below.)

2.3. Multi-omics in epigenomics

Single-omics assays, even at single-cell resolution, capture only one facet of cellular regulation. Multi-omic approaches have emerged in recognition that epigenetic modifications, chromatin accessibility, chromatin structure and transcription crosstalk and collectively shape phenotypes. While the integration of separate omics data from bulk cell provides the overview of developmental dynamics, single cell and single molecule multi-omic approaches, offered by next-generation sequencing and third generation sequencing, reveal temporal cause-effect relationships among omic layers and developmental trajectories of cell populations.

2.3.1. Single-cell profiling of chromatin, DNA methylation, and transcriptome

A major goal of multi-omics is to link the epigenome to the transcriptome within the same cell. Several methods achieve this by physically separating molecules for parallel profiling. Simultaneous high-throughput ATAC and RNA expression (SHARE-seq) merges chromatin accessibility and transcriptomes in single nuclei, enabling the dissection of accessible chromatin preceding transcription activation during mouse skin differentiation [20]. Single-cell Methylation and Transcriptome sequencing (scM&T-seq) uses streptavidin-coupled oligo-dT primer to isolate RNA for parallel profiling

of DNA methylation and transcriptome, and identified at the single-cell level the heterogenous negative correlation between promoter DNA methylation and transcript abundance [21]. Moreover, single-cell Nucleosome, Methylation, and Transcriptome sequencing (scNMT-seq) builds upon scM&T-seq by adding a step to label accessible chromatin through an *in vitro* GpC methyltransferase treatment, thus capturing three modalities [22]. This method identified the establishment of a genome-wide negative correlation between DNA methylation and chromatin accessibility along mouse *in vitro* embryoid differentiation.

2.3.2. Multiplex profiling of histone and DNA modifications

Beyond transcriptomes, other methods focus on profiling multiple epigenetic marks simultaneously. This is crucial because the combinatorial patterns of histone modifications, or "histone code," often define regulatory element function more accurately than any single mark. The original techniques of studying histone modifications and transcription factors recognize only one type of protein at a time. Instead of splitting the bulk sample into multiple batches for multiple proteins, there are multiplexed protocols to insert protein-target-specific tag sequences to the genomic DNA of single cells. Methods like scMulti-CUT&Tag [23] and scMulTI-Tag [24] use different antibodies in successive rounds, each coupled with a unique barcoded adapter, to profile multiple proteins in the same cell. For example, scMulTI-Tag was used to analyze the co-occurrence of multiple histone modifications (H3K4me1, H3K27me3 and H3K36me3) at the single-cell level during the *in vitro* differentiation of human embryonic stem cells.

In a different approach targeting DNA modifications, a novel technique called six-letter-seq was developed to study single-molecule DNA methylation status of the two common forms 5mC and 5hmC. This approach uses DNA methyltransferase 5 (DNMT5) to copy 5mC but not glycosylated 5hmC to the synthetic complementary strand, allowing their distinction after sequencing [25].

2.3.3. Multi-omics with higher-order chromatin structure

Finally, integrating chromatin structure with other epigenetic layers provides the complete intracellular spatial information for gene regulation. Methods that simultaneously profile chromatin contacts and DNA methylation, such as scMethyl-HiC, revealed the co-regulation of distal regions that are spatially close in mouse embryonic stem cells [26]. This allows for direct correlation between 3D structure and the methylation state of interacting loci in single cells. Similarly, combining long-read sequencing with conformation capture, as in Pore-C, enables the simultaneous detection of higher-order chromatin contacts and native DNA modifications on the same long DNA molecule, linking multi-way interactions directly to their underlying allelic methylation patterns without PCR amplification [27].

2.4. Bioinformatics of sequencing-based methods

The vast and complex datasets generated by modern sequencing methods necessitate specialized bioinformatic approaches. Key challenges include handling data sparsity in single-cell assays, accurately aligning long, error-prone reads, and integrating diverse data types from multi-omic experiments.

A major challenge in single-cell epigenomics is data sparsity. DNA sample loss during experimental procedures is inevitable. Due to the minute (picogram level) amount of DNA in single cells, single cell sequencing data is always sparse and noisy compared to bulk sequencing. Moreover, handling the data from a large number of single cells pose computational challenges. Bioinformatic pipelines are needed for cell clustering, trajectory inference, motif enrichment, and missing data imputation. Statistical clustering tools like ArchR integrate single-cell ATAC-seq data for cell clustering, trajectory inference, and motif enrichment [52], while matrix factorization tools like scOpen and scCASE can enhance the resolution of single cell data, using the general rule learnt from large numbers of sparse single cell data to predict the missing data in each single cell [53,54]. Deep learning approaches are powerful tools to summarize non-linear rules hidden in the genomic sequences coupled to epigenomic profiles. Models trained on bulk data that estimate confidence by only single or a few, dense (broad genome coverage), and deep (genome sequences being read multiple times) datasets are not directly applicable to sparse single-cell data. These conditions are distinct from the nature of single cell epigenomics data, requiring novel modeling approaches. A deep learning approach called Scover learns from scATAC-seq data to infer cell-type-specific transcription factor activity in mouse tissues and human kidney [55]. DeepCpG imputes single-cell DNA methylation profiles at single nucleotide resolution, which was used to estimate the average and heterogenous regulation of DNA methylation in mouse embryonic stem cells [56]. Higashi learns the cell characteristics through graph neural network on scHi-C data, aiding cell-type-specific clustering, A/B compartment and TAD detection in human prefrontal cortex [57].

For long-read epigenomics, the initial critical step is aligning long, and often noisy, reads to a reference genome. Vendors provide integrated analysis platforms, such as EPI2ME (Oxford Nanopore Technologies), which offer user-friendly, cloud-based pipelines that bundle long-read-specific alignment, variant calling, methylation analysis and higher-order chromatin structure into automated workflows. Alternatively, the alignment tool in EPI2ME called minimap2 can be used separately, offering high speed and accuracy for both genomic and spliced RNA reads [58]. Once aligned, downstream analyses can often re-use or adapt tools developed for short-read data.

The analysis of multi-omic data aims to find a joint low-dimensional representation that captures the primary sources of variation across different molecular layers (See [59] for an extensive review of the fast-evolving nature of multi-omic

analysis tools.). The most recent version of single-cell analysis tool Seurat v5 expands beyond the well-known transcriptomics to modalities such as epigenomics and proteomics to uncover coordinated biological processes [60].

3. Imaging-based methods

Genome-wide information obtained from bulk sequencing methods for histone modification or DNA methylation provides a population-averaged view of the epigenome. While single-cell sequencing can overcome this averaging problem, it introduces another critical limitation: the loss of spatial context, both histologically and intracellularly. Because these methods require cell dissociation, crucial information about a cell's position and its interactions within the native tissue and cellular architecture is lost. This is a significant drawback, as cellular processes are often spatially regulated. This limitation is particularly consequential for the study of intracellular events, which is the focus of this review. For instance, gene expression is a dynamic process. At any given moment, a specific gene might be actively transcribed in some cells while being silenced in others [61].

To overcome this limitations of sequencing and investigate the epigenetic mechanisms governing such cellular heterogeneity, imaging-based approaches that preserve spatial context are indispensable. Direct visualization of the epigenome enables the elucidation of the complex interplay between higher-order chromatin structure, its dynamic rearrangements, and gene function. Advanced imaging techniques provide insights across multiple scales. For instance, live-cell fluorescence strategies enable the real-time tracking of histone marks and transcriptional regulators, revealing how chromatin structure responds dynamically during processes like transcription or DNA repair. This provides critical temporal information that sequencing-based methods cannot capture. Concurrently, multiplexed chromatin tracing techniques allow for the high-resolution mapping of the genome's spatial organization in fixed cells. These methods empower researchers to directly observe the physical proximity of distal enhancers to their target promoters, visualize the co-localization of active and repressive marks, and dissect the higher-order structures that orchestrate gene regulatory networks. Furthermore, by physically overcoming the diffraction limit, super-resolution imaging methods provide unprecedented nanoscale views of chromatin ultrastructure, such as the compaction state of individual nucleosome clusters. This allows for the direct correlation of these physical properties with functional outcomes. Collectively, these advances provide a powerful framework for establishing causal links between the physical organization of the epigenome and the regulation of cellular identity and function.

This section provides an overview of these three major developments of intracellular imaging methods: (i) live-cell fluorescence strategies for real-time tracking of histone

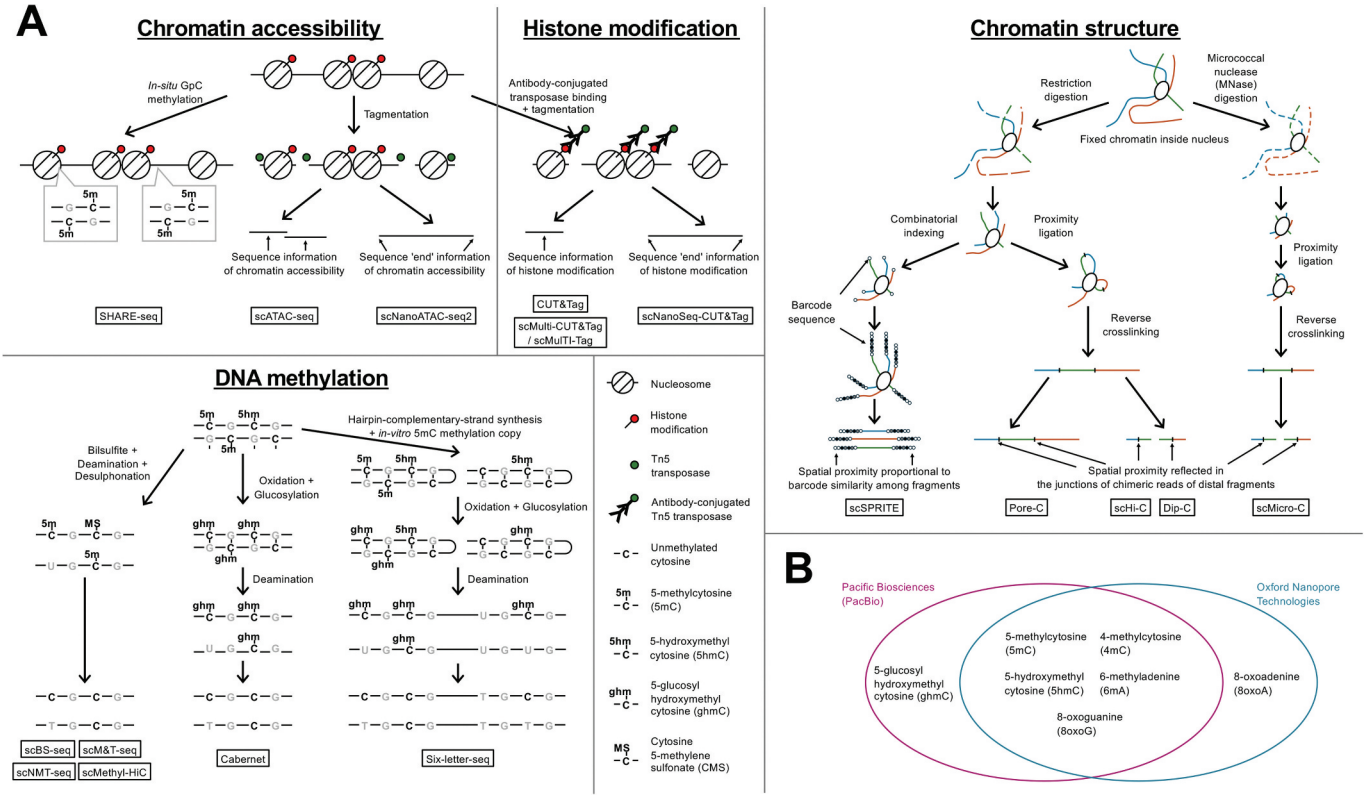

Figure 2. Technical principles of sequencing-based methods and native detection of base modifications. (A) the key technical principles of indirectly detecting chromatin accessibility, histone modification, DNA methylation, and chromatin structure are shown. The respective methods described in this review are labelled at the end of each workflow. The legends of the key components are explained near the center panel. (B) the types of DNA base modifications that can be natively detected by third generation sequencers are shown. Note that chemical modifications also occur on RNA and can be detected at the transcript level (epitranscriptomics) with direct RNA sequencing by third generation sequencers.

marks and transcriptional regulators, (ii) multiplexed chromatin tracing techniques for high-throughput spatial mapping on fixed cells, and (iii) expansion microscopy – coupled methods that physically overcome the diffraction limit to boost spatial resolution. For a comparative overview of the techniques introduced in this section, refer to Table 2, which summarizes their compatible sample types, resolution, and other features.

3.1. Epigenome analysis using live cells

3.1.1. Analysis by visualizing the epigenome across the entire nucleus

One method for visualizing the epigenome is immunofluorescence, which utilizes antibodies that specifically recognize histone modifications or DNA methylation. Traditionally, immunofluorescence has been widely applied to fixed cells for detecting histone post-translational modifications at the single-cell level. By using antibodies against specific modifications such as H3K9me3 (a heterochromatin mark), H3K27ac (an active enhancer mark), and H3K4me3 (an active promoter mark), their spatial distribution within the nucleus can be observed with high precision. For example, regions intensely stained for H3K9me3 correspond to constitutive heterochromatin (such as pericentromeric regions), whereas H3K27ac tends to be distributed in more diffuse euchromatin regions [62,63].

Recently, efforts have advanced to apply this fluorescence labeling technology to living cells. This enables the analysis of the impact of epigenome dynamics on genome function with high temporal resolution. For instance, by simultaneously visualizing fluorescent probes for specific histone modifications along with fluorescently labeled antibody fragments that specifically recognize H3K27ac and RNA polymerase II (e.g., FabLEM: Fab-based live endogenous modification labeling) within living cells, the relationship between those epigenome state can be directly observed [64]. Thus, visualizing target modifications and proteins with fluorescence and tracking their dynamics makes it possible to capture changes in the epigenome state at the single-cell level. However, when using standard fluorescence microscopy, limitations exist, such as restricted resolution due to the diffraction limit of light (approximately 200 nm) and constraints on multiplexing (the number of targets that can be simultaneously observed) due to the spectral overlap of fluorescence channels. Despite these constraints, fluorescence imaging plays a crucial role as a foundational technology in single-cell epigenetics research.

3.1.2. Techniques for epigenome analysis in live cells

Visualizing specific epigenetic modifications within live cells requires the introduction of labeled molecular probes. The probes (e.g., FabLEM) are loaded into the cells and bind to the targeted marks in living cells [64,65]. While these macromolecule probe loading techniques face procedural limitations and probe degradation during cell culture, which create hurdles for the stable analysis of multiple cells, the Mintbody (Modification-specific intracellular antibody) technology represents a powerful alternative approach to overcome these challenges [66]. This technology involves constructing plasmids that encode probes composed of the complementarity-determining regions (CDRs) from antibodies recognizing specific histone modifications (or other targets), fused to a fluorescent protein (e.g., GFP). Transfection of these plasmids into cells enables the visualization of the distribution and dynamics of target epigenetic modifications within living cells. Combining the visualization of epigenetic modifications using Mintbody with methods for visualizing transcriptional activity (e.g., using MS2-MCP and TetO/TetR system) has demonstrated that the dynamics of factors involved in transcriptional regulation and specific post-translational modifications are closely correlated with transcriptional dynamics [67]. However, developing functional Mintbody probes posed a challenge, as reconstructing stable and specific probes from antibody CDR sequences is not straightforward, making the development process time-consuming. Recently, the application of protein structure prediction tools such as AlphaFold2 and ProteinMPNN, combined with protein engineering techniques, has accelerated the design and development of functional probes, advancing the creation of probes targeting a broader diversity of epigenetic modifications [68]. Mintbody technology is a highly valuable tool for real-time tracking of epigenome dynamics in live cells and is expected to significantly contribute to elucidating the dynamic aspects of genome function regulation.

Alternatively, probe technologies utilizing naturally occurring protein domains ('reader domains') that specifically recognize epigenetic modifications have also been advanced (for a general overview about reader proteins, see e.g., Musselman et al. [69]). For instance, probes created by fusing reader domains such as methyl-CpG binding domains (MBDs) or bromodomains that bind to acetylated lysine residues to fluorescent proteins allow for the observation of the distribution and enrichment of specific epigenetic modifications in live cells. Examples include fusion probes of MBDs with fluorescent proteins called MethylRO for detecting DNA methylation [70] and sets of probes combining various histone modification recognition domains with fluorescent proteins (e.g., ChromID; H3K9me3 Sensors) [71,72]. Furthermore, approaches combining these reader domain-based probes with CRISPR imaging technology have been reported, enabling the labeling and visualization of the dynamics of the epigenetic state at specific genomic loci [73]. These tools hold the potential to evolve into technologies capable of tracking dynamic changes in epigenetic states at the single-gene level in real-time.

3.2. Spatial intracellular epigenome analysis using fixed samples

3.2.1. Challenges and advances in spatial epigenome analysis using fixed cells

Recent technical advances in cellular imaging have enabled significant progress, particularly through fluorescence *in situ* hybridization (FISH) technology, which allows simultaneous imaging of multiple molecule species in fixed cells with imaging-based spatial multi-omics emerging as a standout approach [88]. However, current epigenome analysis methods face distinct limitations: live-cell imaging struggles with the diffraction limit and spectral overlap issues; conventional immunofluorescence on fixed cells reveals only average

nuclear epigenome states without genomic specificity; and while sequence-based approaches like ChIP-seq and ATAC-seq have identified epigenetic modifications at functional genomic regions such as promoters and enhancers, they lack spatial information within the nuclear context.

To overcome these challenges, novel imaging techniques targeting fixed cells are being developed that bridge the critical gap between sequence and spatial information. These innovative approaches not only enable observation with high spatial resolution but also, through integration with genomic sequence data, allow for acquiring genome sequence-specific epigenome information. This capability is unattainable by conventional fluorescence imaging alone. By linking precise genomic locations with their spatial context within the nucleus, these techniques greatly expand the analytical scope of epigenome research, offering new insights into nuclear organization and the regulatory mechanisms underlying gene expression.

3.2.2. Integrated spatial epigenomics using multiplexed chromatin tracing technologies

DNA seqFISH+, a sequential DNA FISH approach from the Long Cai group, offers remarkable capabilities for the integrated analysis of the epigenome and higher-order chromatin structure in single cells (Figure 3) [74–76]. A standout feature is its ability to interactively acquire multi-layered information. This means that alongside mapping numerous genomic loci with high spatial resolution using its sequential DNA FISH with barcoding strategies, DNA seqFISH+ can simultaneously reveal important epigenome information, such as the distribution of specific epigenetic marks, and RNA expression patterns. This makes it an exceptional technique for investigating how epigenetic landscapes are established and maintained in relation to the genome's spatial conformation and transcriptional activity at the single-cell level.

DNA seqFISH+ was first reported in 2021 (Figure 3A) [74]. Using multi-step barcoded DNA FISH, they visualized approximately 3660 genomic regions in mouse embryonic stem cells (2,460 regions at 1 Mb intervals genome-wide, and 1200 regions at 25 kb intervals within specific domains). Furthermore, by combining this with RNA seqFISH for detecting 70 mRNAs and immunofluorescence for 17 chromatin marks and nuclear structural proteins within the same cells, they achieved integrative mapping of higher-order chromatin structure, transcriptome, and epigenome information. This is in sharp contrast to conventional FISH techniques, which are limited to visualizing only tens of loci. From these omics data, they identified differences of chromatin compartments between active and inactive genes, and cell heterogeneity of epigenomes. Similar approaches include DNA MERFISH, which utilizes barcoding based on Hamming distance, but reports integrating epigenome information using DNA MERFISH are currently limited [89].

In a subsequent study, the same group applied DNA seqFISH+ to mouse brain tissue sections, comparing the higher-order chromatin structures of diverse cell types, such as neurons and glial cells, at the single-cell level [75]. After optimizing the technique for efficient probe penetration even in thick tissue sections and identifying cell types based on RNA expression profiles, they compared the higher-order chromatin structure and histone modifications in each cell type, achieving the first large-scale visualization of cell-type-specific chromatin structures in the brain. This study demonstrated that the spatial positioning of nucleoli and heterochromatin differs among cell types and that this correlates with cell-type-specific gene expression patterns.

Furthermore, a "Two-layer DNA seqFISH+" strategy was introduced, significantly enhancing the scalability of the technology (Figure 3B) [76]. This enables the visualization of approximately 100,000 genomic loci (at 25 kb intervals across all chromosomes) per cell. This strategy divides the conventional barcoding method into two stages: first, chromosomes are divided into 1.5 Mb blocks, and probes targeting loci at 25 kb intervals within each block are detected through 60 rounds of hybridization. Subsequently, through 36 rounds of pseudo-color barcoding, identification information is assigned to each 1.5 Mb block. This two-layer strategy avoids the simultaneous fluorescence of adjacent loci within the same hybridization round, thereby mitigating optical signal crowding and achieving genome-wide mapping at 25 kb resolution. Applying this technique to mouse cerebellar tissue and other samples revealed that while the organization of repressive chromatin compartments (regions marked by H3K27me3 or H4K20me3) differs significantly between cell types, the organization of active chromatin compartments is relatively similar. Additionally, novel nuclear structures (compartments) where RNA polymerase II accumulates were discovered around large genes exceeding 200 kb, suggesting their potential function as transcription "factories" for mega-genes. These findings specifically illustrate how differences in cell-type-specific nuclear organization relate to the regulation of gene expression, highlighting the importance of integrated analysis of spatial genomics and epigenomics. However, seqFISH technology does not inherently analyze DNA modifications like DNA methylation and DNA hydroxymethylation. Thus, other techniques and ongoing developments are being explored to achieve a multi-layered analysis that includes both these DNA modifications alongside histone modifications.

3.2.3. Spatial epigenomics using other multiplexed chromatin tracing technologies

As discussed previously, chromatin tracing techniques are pivotal for investigating higher-order chromatin structure at higher resolution, revealing intricate details of genome organization. Building upon the foundational insights provided by methods like DNA seqFISH+, this section explores other key multiplexed technologies that offer alternative or complementary strategies for spatial epigenomic analysis. These approaches further expand the repertoire of tools available to researchers, enabling a more comprehensive understanding of the dynamic chromatin landscape.

Using super-resolution microscopy, chromatin tracing which visualized across approximately 46 regions reveals that genomic regions characterized by repressive epigenetic states like H3K27me3 are not simply compact structures but instead exhibit a high degree of chromatin intermixing within the domain [90]. This finding challenges simpler models of chromatin condensation. Furthermore, ORCA (Optical

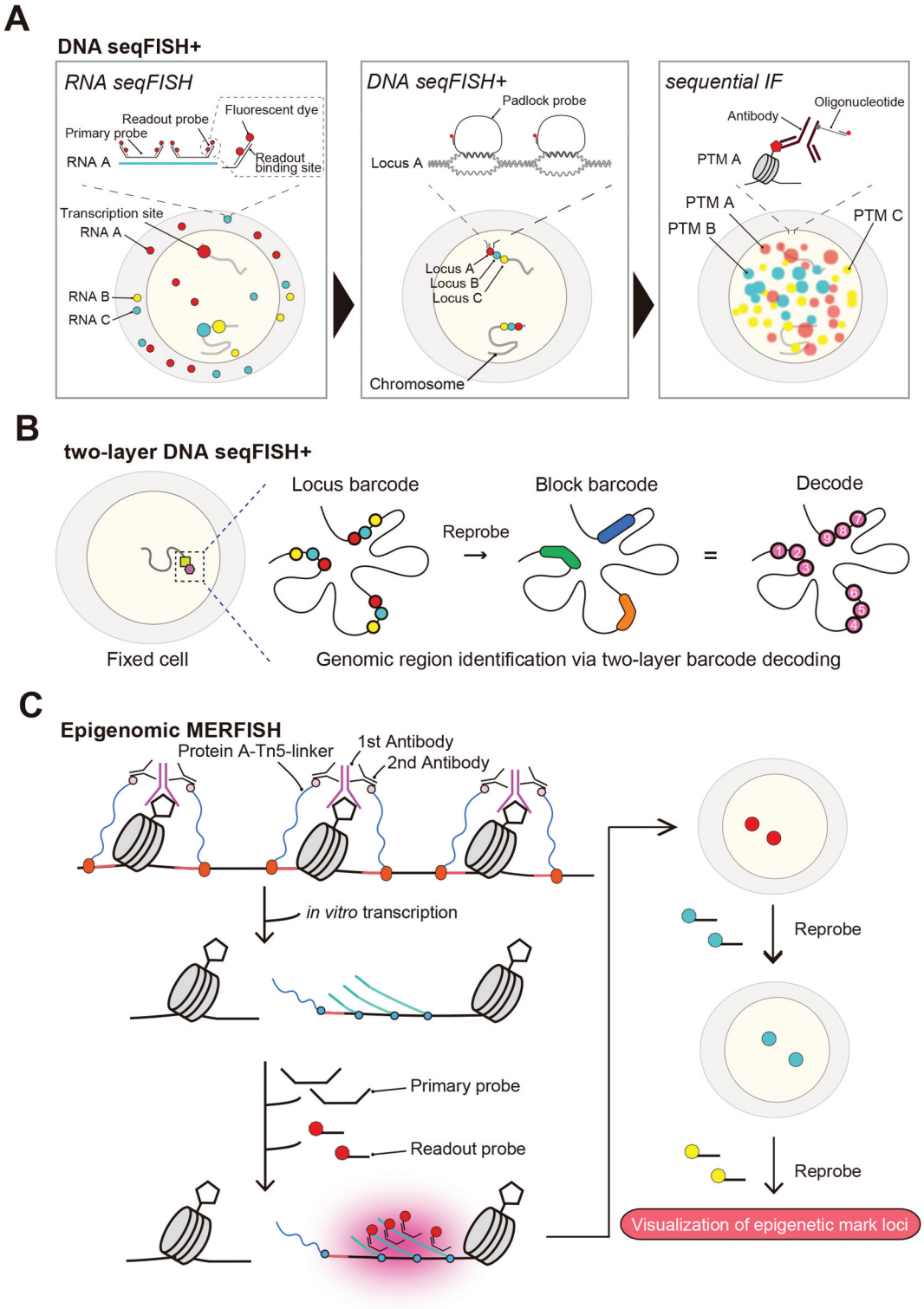

Figure 3. Multi-omic imaging of higher-order chromatin structure and epigenetic marks using seqFISH+ (A, B) and epigenomic MERFISH (C) technologies. (A) schematic of the DNA seqFISH+ workflow. The method sequentially visualizes RNA localization (via RNA seqFISH), the positions of numerous genomic loci (via multi-step barcoded DNA seqFISH), and the distribution of proteins and PTMs (via sequential IF), enabling integrated imaging of the transcriptome, genome architecture, and epigenome in a single cell. (B) principle of the two-layer DNA seqFISH+ strategy. To visualize a vast number of genomic regions, this approach uses a two-stage barcoding process. First, locus barcodes identify specific genomic positions within larger chromosomal blocks. These blocks are subsequently identified by a second layer of block barcodes. The combined barcode information is then decoded to assign a unique identity to each target locus. (C) workflow of the epigenomic MERFISH method. An 1^{st}- and 2^{nd}-antibodies-pA-Tn5 complex targets a specific histone modification and inserts a T7 promoter into the adjacent DNA. This positional information is then amplified into multiple RNA molecules via in situ transcription. The spatial locations of these RNA signals are subsequently resolved using the MERFISH method, enabling multiplexed mapping of epigenetic marks.

Table 2. Summary of imaging-based and sequencing-integrated methods. The principles, compatible sample types (including frozen and FFPE-fixed samples), and key features of the methods introduced are summarized.

Method Name	Principle	Sample Type	Live/ Fixed	Genomic Resolution	FFPE/Frozen Compatibility	Spatiotemporal Resolution & Key Features	Ref
Immunofluorescence (IF)	Employs specific antibodies to visualize histone modifications and nuclear proteins.	Cells, tissue sections	Fixed	Non-sequence-specific	Compatible with both (FFPE requires antigen retrieval protocols).	Spatial resolution is diffraction-limited (~200 nm). Maps general nuclear distribution of epitopes. Limited multiplexing capacity.	[62,63]
FabLEM/Mintbody	Genetically encodes antibody fragments (scFv) or intrabodies fused to fluorescent proteins for live-cell visualization.	Live cells, tissue sections	Live	Non-sequence-specific	Not applicable (strictly live-cell method).	High temporal resolution for real-time tracking of epigenetic dynamics.	[64–68]
Reader Domain Probes	Uses naturally occurring protein domains ('readers') that recognize specific modifications.	Primarily in vitro, also live-cell use	Live	Non-sequence-specific	Not applicable.	The methodology offers a dual capability: visualizing the target in situ in live cells and identifying its interaction partners through pull-down assays.	[69–73]
DNA seqFISH+	Sequential hybridization of barcoded oligonucleotide probes enables spatial mapping of numerous genomic loci.	Fixed cells, tissue sections	Fixed	25 kb – 1 Mb	Restricted to fixed cells and frozen sections. FFPE compatibility remains limited and requires further validation.	Achieves super-resolution imaging. Enables simultaneous mapping of ~10^5 genomic loci with concurrent RNA and protein visualization in single cells.	[74–76]
ORCA	Sequential labeling and imaging of short genomic segments to reconstruct three-dimensional chromatin paths at high resolution.	Fixed cells, embryo sections	Fixed	as fine as 2 kb	Restricted to fixed cells and frozen sections. FFPE compatibility remains limited and requires further validation.	Reconstructs chromatin topology at kilobase resolution. Compatible with concurrent nascent RNA detection. Extensively validated with open-source analysis tools.	[77]
MINA/Hi-M	Multiplexed oligopaint FISH methodology for visualizing multi-scale chromatin organization from loops to territories. Hi-M incorporates optimizations for thick tissue imaging.	Fixed embryos, tissues	Fixed	as fine as 5 kb	Restricted to fixed cells and frozen sections. FFPE compatibility remains limited and requires further validation.	Captures hierarchical chromatin folding across scales. Demonstrates high correlation with Hi-C data ($r > 0.8$). Compatible with concurrent imaging of nuclear landmarks.	[78–80]
GOLD FISH	Employs Cas9-superhelicase fusion for targeted DNA denaturation, enabling probe hybridization without thermal denaturation.	Fixed cells, tissue sections	Fixed	Locus-specific (2.3-kb locus)	Optimized for lightly fixed cells; FFPE compatibility not established.	Enables visualization of non-repetitive sequences while preserving cellular architecture.	[81]
Epigenomic MERFISH	Antibody-directed Tn5 transposase deposits adapters at histone modification sites, followed by rolling circle amplification and MERFISH detection.	Fixed cells, tissue sections	Fixed	Profiles hundreds of loci	Requires fresh-frozen sections for optimal antibody binding and enzymatic activity. FFPE compatibility remains limited and requires further validation.	Spatially resolves histone modifications with sequence specificity. Currently limited to one epigenetic mark per experiment due to technical constraints.	[82]
ATAC-see/NicE-seq	Transposase (ATAC-see) or nicking enzyme (NicE-seq) deposits fluorescent oligonucleotides at accessible chromatin regions.	Fixed cells	Fixed	Sequence-specific when coupled with NGS	Incompatible with FFPE due to formaldehyde cross-linking that impedes enzyme accessibility. NicE-seq shows partial compatibility with lightly fixed samples.	Direct visualization of chromatin accessibility patterns. NicE-seq demonstrates superior performance on fixed samples compared to ATAC-based methods.	[83,84]
Expansion Microscopy (ExM)	Physical expansion of polyacrylamide gel-embedded specimens enables sub-diffraction imaging with conventional microscopes.	Fixed cells, tissue sections	Fixed	Effective resolution: ~75 nm	Compatible with both FFPE and frozen (protocol-specific optimization required). **Critical limitation**: Chromatin ultrastructure preservation is incomplete.	Suitable for large-scale spatial organization (μm scale) but compromises fine chromatin structure.	[85]
IGS/ExIGS	In situ genome sequencing via sequencing-by-synthesis within intact specimens. ExIGS incorporates expansion microscopy for enhanced resolution.	Fixed cells, embryos	Fixed	Single-base sequence information, with genomic loci mapped at a resolution of 10 kb over a range of up to 1 Mb (ExIGS), and at a resolution of 10 Mb (IGS).	Restricted to fixed cells and embryos, frozen sections. FFPE compatibility remains limited and requires further validation.	Directly correlates DNA sequence with spatial coordinates. ExIGS achieves super-resolution through physical expansion.	[86,87]

Reconstruction of Chromatin Architecture) sequentially labels and images tens to hundreds of short genomic segments to reconstruct the 3D path of chromatin at kilobase-level resolution [77]. This method achieves high genomic resolution (approximately 2 kb) in *Drosophila* embryos to trace the 3D folding of the Hox gene cluster, revealing cell-type-specific physical boundaries between active and Polycomb-repressed domains. Researchers simultaneously measured nascent RNA, linking higher-order chromatin structure with gene expression.

Other chromatin tracing technologies also play important roles in understanding nuclear architecture and epigenetic states [78,79,91]. For instance, multiplexed imaging of nucleome architectures (MINA) visualized an entire chromosome at approximately 30 kb intervals, enabling detailed analysis of topologically associating domain (TAD) boundaries and cooperative interactions between enhancers. Studies using MINA have also demonstrated simultaneous detection of multi-scale chromatin folding (from loops to entire chromosome territories) along with nuclear components like the nuclear lamina and RNA. Furthermore, Hi-M is another similar strategy for imaging early *Drosophila* embryos, where high-numerical-aperture objective lenses with water immersion can access samples at greater depths [80]. Additionally, the genome editing tool CRISPR-Cas9 system can be used to visualize genome organization. Genome oligopaint via local denaturation fluorescence *in situ* hybridization (GOLD FISH) is an advanced DNA imaging technique that utilizes a modified Cas9 enzyme to introduce a single-strand nick at a specific genomic location. This nick serves as an entry point for a superhelicase, which unwinds the DNA locally, allowing fluorescent probes to hybridize to the exposed single-stranded regions without the need for global heat denaturation. This approach enables high-specificity visualization of non-repetitive genomic loci under physiological conditions, preserving cellular structures and reducing background noise [81]. These techniques are particularly valuable for studying the relationship between higher-order chromatin structure and gene expression regulation, such as the mechanisms of enhancer-promoter contact formation.

3.2.4. Region-specific epigenome analysis using Epigenomic MERFISH

Although DNA FISH-based spatial omics analyses enable visualizing histone accumulation at targeted DNA spots within cells, these spots may not solely represent the intended genomic regions. Due to the optical diffraction limit, signals can also encompass adjacent genomic regions or other proximal molecules. This inherent limitation can reduce the accuracy of DNA FISH-based methods for precise epigenomic mapping when compared to sequence-based methods like ChIP-seq and CUT&Tag. To overcome this challenge, Epigenomic MERFISH has been developed (Figure 3C) [82]. It is a technique that enables spatial analysis of genome region-specific epigenetic states by using antibody-Tn5 transposase-mediated labeling to amplify the epigenetic signal into RNA molecules *in situ*. These RNA molecules are then detected and quantified using the MERFISH method.

The basic principle of Epigenomic MERFISH begins with binding antibodies specific to target histone modifications (e.g., H3K4me3, H3K27ac, H3K27me3) within tissue or cell samples. Subsequently, a fusion protein of Protein A and Tn5 transposase (pA-Tn5) is added, which binds to the antibodies. This fusion protein cuts the DNA near the antibody binding site and simultaneously inserts (via tagmentation) a DNA adapter containing a T7 promoter sequence. Next, *in situ* T7 RNA polymerase transcription generates RNA copies containing sequence information from the region around the adapter insertion site. These RNA molecules are then detected and quantified using the MERFISH method with pre-assigned binary barcodes for each genomic region [92].

Application of Epigenomic MERFISH to mouse brain tissue successfully enabled the detection of histone modification states at 100–200 different genomic regions simultaneously within single cells. As an example, the authors imaged 90 distinct H3K27ac-marked enhancer regions within single nuclei simultaneously and revealed their spatial positioning relative to nuclear speckles.

Compared to DNA seqFISH+, Epigenomic MERFISH differs fundamentally in that it does not hybridize directly to the genomic sequence itself but rather detects the presence of histone modifications by converting this information into an RNA signal. This approach allows direct mapping of specific chromatin to their underlying DNA sequence. While DNA seqFISH+ can be combined with simultaneous detection of multiple immunofluorescence signals, Epigenomic MERFISH is, in principle, relatively limited in the number of epigenetic marks that can be assayed concurrently in a single experiment.

3.2.5. Fluorescence imaging and sequencing of open chromatin

One significant advancement in epigenome analysis in fixed cells involves techniques for directly visualizing open chromatin regions. Assay for Transposase-Accessible Chromatin with visualization (ATAC-see), a method based on the ATAC-seq concept [3], uses Tn5 transposase loaded with fluorescently labeled DNA adapters [83]. This transposase inserts adapters *in situ* into open chromatin regions accessible to transcription factors and other proteins within fixed cells, thereby visualizing these regions as fluorescent signals. The advantage of this approach lies in the ability to amplify and sequence the DNA using the inserted adapter sequences, allowing the genomic locations of the imaged open chromatin regions to be identified. ATAC-see has been applied to studies such as the process of neutrophil NETosis (a form of cell death involving large-scale chromatin decondensation) and changes in chromatin accessibility associated with the cell cycle. Furthermore, in combination with CRISPR screening, ATAC-see identified a new chromatin modulator TFDP1 for chromatin accessibility [93]. A similar approach is Nicking Enzyme-assisted sequencing (NicE-seq). In this method, instead of Tn5 transposase, a nicking enzyme (single-strand endonuclease) is used to introduce nicks into accessible DNA regions. Fluorescently labeled nucleotides are then incorporated at these sites, labeling and

visualizing the open chromatin regions. Analysis of samples before sequencing has shown that the NicE-seq signal co-localizes with regions exhibiting low staining intensity for DAPI staining [84]. Thus, combinations of seeing and sequencing are a powerful tool for analyzing new players and cell activity.

3.2.6. High-resolution epigenome visualization using expansion microscopy (ExM)

A notable recent development is the application of Expansion Microscopy (ExM) to sequencing technologies for epigenome analysis. ExM is a technique that achieves super-resolution imaging by embedding tissue samples in a swellable hydrogel and physically expanding them, thereby surpassing the diffraction limit of conventional light microscopy [94,95]. This physical expansion increases the distance between molecules like DNA, enabling the analysis of genomic sequences and epigenetic states at high resolution using standard optical microscopes.

Expansion microscopy has been applied to epigenome analysis in combination with DNA-FISH [85]. In this technique, known as SCEPTRE, samples are first immunostained, then embedded in a gel matrix. Following gel digestion and expansion, DNA FISH probes are hybridized to the samples. When SCEPTER is used to stain histone modifications, histone accumulation can be analyzed with enhanced resolution. For example, in these expanded samples, the signal intensity of histone modifications at GAPDH regions identified by DNA-FISH correlates with gene activity: H3K27me3 is low while H3K4me3 is high at active loci. High-resolution imaging with expanded samples enables more accurate assessment of epigenome status, although only a limited number of loci can be analyzed by DNA-FISH at one time. By implementing multiple rounds of detection techniques similar to DNA-seqFISH, we would gain a more comprehensive understanding of the epigenome's role in genome regulation in detail.

4. Integrated sequencing and imaging methods

If the DNA sequence information could be read in intact cells with spatial information, it would provide new mechanistic insights into epigenome regulation across various biological processes. To this end, technical advances are required to link unique sequence information with its precise DNA position and integrate these data with NGS sequence. Although FISH-based omics technology cannot distinguish allele information until now, sequence integrated-methods allow for allele- and sequence-level information with spatial context. A summary of the techniques introduced in this section can be found in Table 2.

In situ genome sequencing (IGS) has been developed as an innovative method for simultaneously analyzing DNA sequence and its higher-order chromatin structure within cells. Research reported by Fei Chen's group demonstrated a method to obtain DNA sequence information while preserving tissue architecture [86]. The core of this technology lies in performing sequencing reactions (sequencing-by-synthesis) directly on DNA within the cells and embryos, simultaneously reading the base sequence and recording its spatial position. Specifically, sequencing adaptors are inserted into the genome of fixed cells using Tn5 transposase. Subsequently, DNA oligos containing a Unique Molecular Identifier (UMI) barcode and a sequencing primer site are hybridized to these adaptor-ligated genomic regions. After performing rolling circle amplification (RCA) on the UMI barcode regions, the base sequence is detected during DNA polymerase-mediated extension reactions using fluorescently labeled nucleotides. The fluorescent signals incorporated in each cycle are imaged by microscopy, allowing for the concurrent recording of sequence information and its 3D positional data. Furthermore, the resulting DNA molecules, which contain both the UMI and the captured genomic region, can be sequenced using NGS technology. The integration of in situ sequencing and NGS data allows for the identification of genomic positions with their corresponding sequences, thereby providing allelic information. Application of IGS to mouse preimplantation embryos has revealed that sister blastomeres share an epigenetic memory of global chromosome positioning.

Expansion *in situ* genome sequencing (ExIGS) combines ExM and *in situ* genome sequencing (IGS) and was used to analyze the link between nuclear abnormalities and hotspots of aberrant euchromatin repression at high resolution [87]. This study demonstrated a strong association between nuclear morphological abnormalities in cancer cells and epigenetic alterations, specifically showing that aberrant repression of euchromatin in certain chromosomal regions correlates with nuclear morphological defects. ExM-based technologies open new avenues for high-resolution analysis of epigenetic states in specific genomic regions within single cells by physically overcoming the resolution limits of conventional light microscopy.

The key innovation of these methods is its ability to identify the genome sequence while simultaneously analyzing the structure of the chromatin containing that sequence and its spatial organization within the nucleus at high resolution. While traditional sequencing methods require DNA extraction from tissues, leading to the loss of spatial information, IGS/ExIGS overcomes this problem. Furthermore, distinguishing between alleles, which is difficult with conventional FISH-based spatial omics techniques, is possible with IGS/ExIGS due to its single-base resolution. This capability opens up the possibility to integrate multi-omic methods (including epigenomics) with immunostaining through IGS/ExIGS.

5. Key challenges and future perspectives

Despite the impressive array of modern epigenomic tools, several challenges remain before these approaches become routine and universally accessible. Addressing these issues will shape the next 10 years of epigenomic research.

5.1. Resolution and data sparseness

Single-cell sequencing methods inherently interrogate only minute amounts of DNA or RNA, which leads to dropout events and bias. Advances in amplification chemistry and microfluidics have begun to mitigate these limitations but have not yet eliminated them. Current analytical pipelines

typically assume that cells from the same sample share underlying physical and biological principles and impute missing data with generalized models. Developing robust models that tolerate noise and sample-to-sample variability is therefore critical for extracting reliable insights from single-cell sequencing data.

In imaging, spatial resolution is fundamentally constrained by the optical diffraction limit, and the genomic regions accessible for observation are heavily dependent on probe design and labeling density. While genome-scale imaging analysis is theoretically feasible, the scale of required probe sets, labeling costs, and data acquisition time present practical barriers to research implementation. Discriminating the two alleles in diploid nuclei remains a technical challenge, yet it is essential for dissecting allele-specific epigenetic asymmetry. Additionally, addressing the sparsity inherent in single-cell imaging data, developing imputation algorithms for missing values, and establishing integrated analysis pipelines remain inadequately developed. Live-cell imaging faces similar optical constraints along with intrinsic limitations such as the number of simultaneously observable targets and phototoxicity of fluorescent labels, but it promises to reveal the temporal dynamics of chromatin reorganization during normal physiology and disease progression.

5.2. Data integration and systematic analysis

Intracellular spatiotemporal techniques that unify epigenomics, transcriptomics, proteomics, and metabolomics in single cells, while preserving higher-order structure information, represent a major frontier. At present, no single experimental approach captures all these dimensions simultaneously, yet such breadth is essential for a truly comprehensive view of how the epigenome interacts with other omic layers. Until universal approaches emerge, efficient computational pipelines are needed to integrate disparate information from multiple epigenetic modifications and even multiple omic layers, normalizing for technical and biological variation across samples and approaches.

6. Conclusion

Rapid progress in single-cell, long-read, imaging and spatial multi-omic technologies has transformed epigenomics into an integrative, high-resolution discipline that links molecular detail with cell identity and 3D chromatin structure. These methods are already uncovering novel chromatin regulatory mechanisms in development and disease, yet widespread adoption is still limited by data sparsity, cost and analytical complexity. Paired with open standards for data sharing, continued innovation in chemistry, optics and computation will be essential to translate next-generation epigenomic profiling from research laboratories to routine biomedical and clinical practice.

Acknowledgments

We thank Dr. Julien Richard Albert at Institut Jacques Monod (Paris) for critical reading of the manuscript.

ChatGPT o3 (OpenAI) and Claude 4 (Anthropic) were used for the linguistic revision of the manuscript.

Disclosure statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Funding

This work was supported by The Japan Society for the Promotion of Science (JSPS) KAKENHI grants to Hiroaki Ohishi [JP22K15084 and JP25K02255] and Wan Kin Au Yeung [JP23K14166]; The Japan Science and Technology Agency (JST), PRESTO grant to Hiroaki Ohishi [JPMJPR24O3] and JST, ACT-X grant to Wan Kin Au Yeung [JPMJAX24L3]; The Nakajima Foundation, Nakatani Foundation, Astellas Foundation for Research on Metabolic Disorders to Hiroaki Ohishi; Hirose Foundation grant and Mochida Memorial Foundation for Medical and Pharmaceutical Research grant to Wan Kin Au Yeung. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

Hiroaki Ohishi: Investigation, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition. Wan Kin Au Yeung: Conceptualization, Investigation, Project administration, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

ORCID

Hiroaki Ohishi http://orcid.org/0000-0001-6672-4093
Wan Kin Au Yeung http://orcid.org/0000-0002-0312-1073

References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Abascal F, Acosta R, Addleman NJ, et al. Expanded encyclopaedias of DNA elements in the human and mouse genomes. Nature. 2020;583(7818):699–710. doi: 10.1038/s41586-020-2493-4
2. Baysoy A, Bai Z, Satija R, et al. The technological landscape and applications of single-cell multi-omics. Nat Rev Mol Cell Biol. 2023;24(10):695–713. doi: 10.1038/s41580-023-00615-w
3. Buenrostro JD, Giresi PG, Zaba LC, et al. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods. 2013;10(12):1213–1218. doi: 10.1038/nmeth.2688 .

•• **Flagship paper as the gold standard of chromatin accessibility analysis, at the same time forming the basis of single cell whole-genome epigenomic analysis with Tn5 transposases.**

4. Buenrostro JD, Wu B, Litzenburger UM, et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature. 2015;523(7561):486–490. doi: 10.1038/nature14590
5. Cusanovich DA, Daza R, Adey A, et al. Multiplex single-cell profiling of chromatin accessibility by combinatorial cellular indexing. Science. 2015;348(6237):910–914. doi: 10.1126/science.aab1601

6. Clark IC, Fontanez KM, Meltzer RH, et al. Microfluidics-free single-cell genomics with templated emulsification. Nat Biotechnol. 2023;41(11):1557–1566. doi: 10.1038/s41587-023-01685-z
7. Deng Y, Bartosovic M, Kukanja P, et al. Spatial-CUT&Tag: spatially resolved chromatin modification profiling at the cellular level. Science. 2022;375(6581):681–686. doi: 10.1126/science.abg7216
8. Kaya-Okur HS, Wu SJ, Codomo CA, et al. CUT&Tag for efficient epigenomic profiling of small samples and single cells. Nat Commun. 2019;10(1):1930. doi: 10.1038/s41467-019-09982-5
9. Smallwood SA, Lee HJ, Angermueller C, et al. Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. Nat Methods. 2014;11(8):817–820. doi: 10.1038/nmeth.3035
10. Cao Y, Bai Y, Yuan T, et al. Single-cell bisulfite-free 5mC and 5hmC sequencing with high sensitivity and scalability. Proc Natl Acad Sci U States Am. 2023;120(49):e2310367120. doi: 10.1073/pnas.2310367120
11. Nagano T, Lubling Y, Stevens TJ, et al. Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. Nature. 2013;50(7469):59–64. doi: 10.1038/nature12593
12. Tan L, Xing D, Chang CH, et al. Three-dimensional genome structures of single diploid human cells. Science. 2018;361(6405):924–928. doi: 10.1126/science.aat5641.
    - **Flagship paper in single-cell chromatin structure that boost the power to detect not only cell heterogeneity but also allele heterogeneity.**
13. Wu H, Zhang J, Tan L, et al. Single-cell Micro-C profiles 3D genome structures at high resolution and characterizes multi-enhancer hubs. Nat Genet. 2025;57(7):1777–1786. doi: 10.1038/s41588-025-02247-6
14. Arrastia MV, Jachowicz JW, Ollikainen N, et al. Single-cell measurement of higher-order 3D genome organization with scSPRITE. Nat Biotechnol. 2021;40(1):64–73. doi: 10.1038/s41587-021-00998-1
15. Li M, Jiang Z, Xu X, et al. Chromatin accessibility landscape of mouse early embryos revealed by single-cell NanoATAC-seq2. Science. 2025;387(6741):eadp4319. doi: 10.1126/science.adp4319
16. Li Q, Guo Y, Wu Z, et al. scNanoseq-CUT&Tag: a single-cell long-read CUT&Tag sequencing method for efficient chromatin modification profiling within individual cells. Nat Methods. 2024;21(11):2044–2057. doi: 10.1038/s41592-024-02453-w
17. Haggerty C, Kretzmer H, Riemenschneider C, et al. Dnmt1 has de novo activity targeted to transposable elements. Nat Struct Mol Biol. 2021;28(7):594–603. doi: 10.1038/s41594-021-00603-8
18. Flusberg BA, Webster DR, Lee JH, et al. Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods. 2010;7(6):461–465. doi: 10.1038/nmeth.1459
19. Simpson JT, Workman RE, Zuzarte PC, et al. Detecting DNA cytosine methylation using nanopore sequencing. Nat Methods. 2017;14(4):407–410. doi: 10.1038/nmeth.4184
20. Ma S, Zhang B, LaFave LM, et al. Chromatin potential identified by shared single-cell profiling of RNA and Chromatin. Cell. 2020;183(4):1103–1116.e20. doi: 10.1016/j.cell.2020.09.056
21. Angermueller C, Clark SJ, Lee HJ, et al. Parallel single-cell sequencing links transcriptional and epigenetic heterogeneity. Nat Methods. 2016;13(3):229–232. doi: 10.1038/nmeth.3728
22. Clark SJ, Argelaguet R, Kapourani C-A, et al. scNMT-seq enables joint profiling of chromatin accessibility DNA methylation and transcription in single cells. Nat Commun. 2018;9(1):781. doi: 10.1038/s41467-018-03149-4
23. Gopalan S, Wang Y, Harper NW, et al. Simultaneous profiling of multiple chromatin proteins in the same cells. Mol Cell. 2021;81(22):4736–4746.e5. doi: 10.1016/j.molcel.2021.09.019
24. Meers MP, Llagas G, Janssens DH, et al. Multifactorial profiling of epigenetic landscapes at single-cell resolution using MulTI-Tag. Nat Biotechnol. 2023;41(5):708–716. doi: 10.1038/s41587-022-01522-9
25. Füllgrabe J, Gosal WS, Creed P, et al. Simultaneous sequencing of genetic and epigenetic bases in DNA. Nat Biotechnol. 2023;41(10):1457–1464. doi: 10.1038/s41587-022-01652-0
26. Li G, Liu Y, Zhang Y, et al. Joint profiling of DNA methylation and chromatin architecture in single cells. Nat Methods. 2019;16(10):991–993. doi: 10.1038/s41592-019-0502-z
27. Deshpande AS, Ulahannan N, Pendleton M, et al. Identifying synergistic high-order 3D chromatin conformations from genome-scale nanopore concatemer sequencing. Nat Biotechnol. 2022;40(10):1488–1499. doi: 10.1038/s41587-022-01289-z
28. Cusanovich DA, Hill AJ, Aghamirzaie D, et al. A single-cell Atlas of in vivo mammalian chromatin accessibility. Cell. 2018;174(5):1309–1324.e18. doi: 10.1016/j.cell.2018.06.052
29. Zhang K, Hocker JD, Miller M, et al. A single-cell atlas of chromatin accessibility in the human genome. Cell. 2021;184(24):5985–6001.e19. doi: 10.1016/j.cell.2021.10.024
30. Preissl S, Fang R, Huang H, et al. Single-nucleus analysis of accessible chromatin in developing mouse forebrain reveals cell-type-specific transcriptional regulation. Nat Neurosci. 2018;21(3):432–439. doi: 10.1038/s41593-018-0079-3
31. Wu X, Lu M, Yun D, et al. Single-cell ATAC-Seq reveals cell type-specific transcriptional regulation and unique chromatin accessibility in human spermatogenesis. Hum Mol Genet. 2021;31(3):321–333. doi: 10.1093/hmg/ddab006
32. Li M, Li J, Zhang Y, et al. Integrated ATAC-seq and RNA-seq data analysis identifies transcription factors related to rice stripe virus infection in Oryza sativa. Mol Plant Pathol. 2024;25(3):e13446. doi: 10.1111/mpp.13446
33. Gur ER, Hughes JR. scATAC-seq generates more accurate and complete regulatory maps than bulk ATAC-seq. Sci Rep. 2025;15(1):3665. doi: 10.1038/s41598-025-87351-7
34. Barski A, Cuddapah S, Cui K, et al. High-resolution profiling of histone methylations in the human genome. Cell. 2007;129(4):823–837. doi: 10.1016/j.cell.2007.05.009
35. Johnson DS, Mortazavi A, Myers RM, et al. Genome-wide mapping of in vivo protein-DNA interactions. Science. 2007;316(5830):1497–1502. doi: 10.1126/science.1141319
36. Rotem A, Ram O, Shoresh N, et al. Single-cell ChIP-seq reveals cell subpopulations defined by chromatin state. Nat Biotechnol. 2015;33(11):1165–1172. doi: 10.1038/nbt.3383
37. Grosselin K, Durand A, Marsolier J, et al. High-throughput single-cell ChIP-seq identifies heterogeneity of chromatin states in breast cancer. Nat Genet. 2019;51(6):1060–1066. doi: 10.1038/s41588-019-0424-9
38. Janssens DH, Greene JE, Wu SJ, et al. Scalable single-cell profiling of chromatin modifications with sciCUT&Tag. Nat Protoc. 2024;19(1):83–112. doi: 10.1038/s41596-023-00905-9
39. Skene PJ, Henikoff S. An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. Elife. 2017;6:e21856. doi: 10.7554/elife.21856
40. Hayashi R, Inoue A. Low-input CUT&RUN for mouse oocytes and preimplantation embryos. Methods Mol Biol Clifton N J. 2022;2577:83–92. doi: 10.1007/978-1-0716-2724-2_6
41. Harada A, Maehara K, Handa T, et al. A chromatin integration labelling method enables epigenomic profiling with lower input. Nat Cell Biol. 2018;21(2):287–296. doi: 10.1038/s41556-018-0248-3
42. Bartosovic M, Kabbe M, Castelo-Branco G. Single-cell CUT&Tag profiles histone modifications and transcription factors in complex tissues. Nat Biotechnol. 2021;39(7):825–835. doi: 10.1038/s41587-021-00869-9
43. Wu SJ, Furlan SN, Mihalas AB, et al. Single-cell CUT&Tag analysis of chromatin modifications in differentiation and tumor progression. Nat Biotechnol. 2021;39(7):819–824. doi: 10.1038/s41587-021-00865-z
44. Janssens DH, Meers MP, Wu SJ, et al. Automated CUT&Tag profiling of chromatin heterogeneity in mixed-lineage leukemia. Nat Genet. 2021;53(11):1586–1596. doi: 10.1038/s41588-021-00941-9
45. Cokus SJ, Feng S, Zhang X, et al. Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. Nature. 2008;452(7184):215–219. doi: 10.1038/nature06745
46. Lister R, O'Malley RC, Tonti-Filippini J, et al. Highly integrated single-base resolution maps of the epigenome in Arabidopsis. Cell. 2008;133(3):523–536. doi: 10.1016/j.cell.2008.03.029
47. Luo C, Keown CL, Kurihara L, et al. Single-cell methylomes identify neuronal subtypes and regulatory elements in mammalian cortex. Science. 2017;357(6351):600–604. doi: 10.1126/science.aan3351

48. Mulqueen RM, Pokholok D, Norberg SJ, et al. Highly scalable generation of DNA methylation profiles in single cells. Nat Biotechnol. 2018;36(5):428–431. doi: 10.1038/nbt.4112
49. Schutsky EK, Nabel CS, Davis AKF, et al. APOBEC3A efficiently deaminates methylated, but not TET-oxidized, cytosine bases in DNA. Nucleic Acids Res. 2017;45(13):7655–7665. doi: 10.1093/nar/gkx345 .
• **Pioneer work demonstrating an efficient bisulfite-free reaction to study DNA methylation 5-methylcytosine.**
50. Sakamoto Y, Zaha S, Nagasawa S, et al. Long-read whole-genome methylation patterning using enzymatic base conversion and nanopore sequencing. Nucleic Acids Res. 2021;49(14):e81–e81. doi: 10.1093/nar/gkab397
51. Lucas MC, Novoa EM. Long-read sequencing in the era of epigenomics and epitranscriptomics. Nat Methods. 2023;20(1):25–29. doi: 10.1038/s41592-022-01724-8
52. Granja JM, Corces MR, Pierce SE, et al. ArchR is a scalable software package for integrative single-cell chromatin accessibility analysis. Nat Genet. 2021;53(3):403–411. doi: 10.1038/s41588-021-00790-6
53. Li Z, Kuppe C, Ziegler S, et al. Chromatin-accessibility estimation from single-cell ATAC-seq data with scOpen. Nat Commun. 2021;12(1):6386. doi: 10.1038/s41467-021-26530-2
54. Tang S, Cui X, Wang R, et al. scCASE: accurate and interpretable enhancement for single-cell chromatin accessibility sequencing data. Nat Commun. 2024;15(1):1629. doi: 10.1038/s41467-024-46045-w
55. Hepkema J, Lee NK, Stewart BJ, et al. Predicting the impact of sequence motifs on gene regulation using single-cell data. Genome Biol. 2023;24(1):189. doi: 10.1186/s13059-023-03021-9
56. Angermueller C, Lee HJ, Reik W, et al. DeepCpG: accurate prediction of single-cell DNA methylation states using deep learning. Genome Biol. 2017;18(1):67. doi: 10.1186/s13059-017-1189-z
57. Zhang R, Zhou T, Ma J. Multiscale and integrative single-cell Hi-C analysis with Higashi. Nat Biotechnol. 2022;40(2):254–261. doi: 10.1038/s41587-021-01034-y
58. Li H. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics. 2018;34(18):3094–3100. doi: 10.1093/bioinformatics/bty191
59. Baião AR, Cai Z, Poulos RC, et al. A technical review of multi-omics data integration methods: from classical statistical to deep generative approaches. arXiv [Preprint]. 2025. p. 2501.17729. doi: 10.48550/arXiv.2501.17729
60. Hao Y, Stuart T, Kowalski MH, et al. Dictionary learning for integrative, multimodal and scalable single-cell analysis. Nat Biotechnol. 2024;42(2):293–304. doi: 10.1038/s41587-023-01767-y
61. Ohishi H, Shinkai S, Owada H, et al. Transcription-coupled changes in genomic region proximities during transcriptional bursting. Sci Adv. 2024;10(49):eadn0020. doi: 10.1126/sciadv.adn0020
62. Muramatsu D, Kimura H, Kotoshiba K, et al. Pericentric H3K9me3 formation by HP1 interaction-defective histone methyltransferase Suv39h1. Cell Struct Funct. 2016;41(2):145–152. doi: 10.1247/csf.16013
63. Hayashi A, Yamauchi N, Shibahara J, et al. Concurrent activation of acetylation and tri-methylation of H3K27 in a subset of hepatocellular carcinoma with aggressive behavior. PLoS One. 2014;9(3):e91330. doi: 10.1371/journal.pone.0091330
64. Stasevich TJ, Hayashi-Takanaka Y, Sato Y, et al. Regulation of RNA polymerase II activation by histone acetylation in single living cells. Nature. 2014;516(7530):272–275. doi: 10.1038/nature13714
65. Hayashi-Takanaka Y, Yamagata K, Wakayama T, et al. Tracking epigenetic histone modifications in single cells using Fab-based live endogenous modification labeling. Nucleic Acids Res. 2011;39(15):6475–6488. doi: 10.1093/nar/gkr343
66. Sato Y, Takenoshita M, Ueoka M, et al. Visualizing histone H4K20me1 in knock-in mice expressing the mCherry-tagged modification-specific intracellular antibody. Histochem Cell Biol. 2024;162(1–2):41–52. doi: 10.1007/s00418-024-02296-8
67. Ohishi H, Shimada S, Uchino S, et al. STREAMING-tag system reveals spatiotemporal relationships between transcriptional regulatory factors and transcriptional activity. Nat Commun. 2022;13(1):7672. doi: 10.1038/s41467-022-35286-2
68. Galindo G, Maejima D, DeRoo J, et al. AI-assisted protein design to rapidly convert antibody sequences to intrabodies targeting diverse peptides and histone modifications. bioRxiv [Preprint]. 2025. p. 2025:2025.02.06.636921. doi: 10.1101/2025.02.06.636921
69. Musselman CA, Lalonde ME, Côté J, et al. Perceiving the epigenetic landscape through histone readers. Nat Struct Mol Biol. 2012;19(12):1218–1227. doi: 10.1038/nsmb.2436
70. Ueda J, Maehara K, Mashiko D, et al. Heterochromatin dynamics during the differentiation process revealed by the DNA methylation reporter mouse, MethylRO. STEM Cell Rep. 2014;2(6):910–924. doi: 10.1016/j.stemcr.2014.05.008
71. Villaseñor R, Pfaendler R, Ambrosi C, et al. ChromID identifies the protein interactome at chromatin marks. Nat Biotechnol. 2020;38(6):728–736. doi: 10.1038/s41587-020-0434-2
72. Sánchez OF, Mendonca A, Min A, et al. Monitoring histone methylation (H3K9me3) changes in live cells. ACS Omega. 2019;4(8):13250–13259. doi: 10.1021/acsomega.9b01413
73. Brenner LM, Meyer F, Yang H, et al. Repeat DNA methylation is modulated by adherens junction signaling. Commun Biol. 2024;7(1):286. doi: 10.1038/s42003-024-05990-4
74. Takei Y, Yun J, Zheng S, et al. Integrated spatial genomics reveals global architecture of single nuclei. Nature. 2021;590(7845):344–350. doi: 10.1038/s41586-020-03126-2
75. Takei Y, Zheng S, Yun J, et al. Single-cell nuclear architecture across cell types in the mouse brain. Science. 2021;374(6567):586–594. doi: 10.1126/science.abj1966
76. Takei Y, Yang Y, White J, et al. Spatial multi-omics reveals cell-type-specific nuclear compartments. Nature. 2025;641(8064):1037–1047. doi: 10.1038/s41586-025-08838-x .
•• **Flagship method for multi-omic epigenome analysis using sequential FISH imaging.**
77. Mateo LJ, Murphy SE, Hafner A, et al. Visualizing DNA folding and RNA in embryos at single-cell resolution. Nature. 2019;568(7750):49–54. doi: 10.1038/s41586-019-1035-4
78. Liu M, Lu Y, Yang B, et al. Multiplexed imaging of nucleome architectures in single cells of mammalian tissue. Nat Commun. 2020;11(1):2907. doi: 10.1038/s41467-020-16732-5
79. Liu M, Yang B, Hu M, et al. Chromatin tracing and multiplexed imaging of nucleome architectures (MINA) and RNAs in single mammalian cells and tissue. Nat Protoc. 2021;16(5):2667–2697. doi: 10.1038/s41596-021-00518-0
80. Cardozo Gizzi AM, Cattoni DI, Fiche JB, et al. Microscopy-based chromosome conformation capture enables simultaneous visualization of genome organization and transcription in intact organisms. Mol Cell. 2019;74(1):212–222.e5. doi: 10.1016/j.molcel.2019.01.011
81. Wang Y, Cottle WT, Wang H, et al. Genome oligopaint via local denaturation fluorescence in situ hybridization. Mol Cell. 2021;81(7):1566–1577.e8. doi: 10.1016/j.molcel.2021.02.011
82. Lu T, Ang CE, Zhuang X. Spatially resolved epigenomic profiling of single cells in complex tissues. Cell. 2023;186(10):2275–2279. doi: 10.1016/j.cell.2023.04.006
83. Chen X, Shen Y, Draper W, et al. ATAC-see reveals the accessible genome by transposase-mediated imaging and sequencing. Nat Methods. 2016;13(12):1013–1020. doi: 10.1038/nmeth.4031 .
• **Profiling region-specific epigenomes at high resolution by detecting histone mark-encoded RNAs with MERFISH.**
84. Ponnaluri VKC, Zhang G, Estève PO, et al. NicE-seq: high resolution open chromatin profiling. Genome Biol. 2017;18(1):122. doi: 10.1186/s13059-017-1247-6
85. Woodworth MA, Ng KKH, Halpern AR, et al. Multiplexed single-cell profiling of chromatin states at genomic loci by expansion microscopy. Nucleic Acids Res. 2021;49(14):e82. doi: 10.1093/nar/gkab423
86. Payne AC, Chiang ZD, Reginato PL, et al. In situ genome sequencing resolves DNA sequence and structure in intact biological samples. Science. 2021;371(6532):eaay3446. doi: 10.1126/science.aay3446

87. Labade AS, Chiang ZD, Comenho C, et al. Expansion in situ genome sequencing links nuclear abnormalities to aberrant chromatin regulation. Science. 2025 24;389(6758):eadt2781. doi: 10.1126/science.adt2781 .

•• **An approach integrating expansion microscopy with in situ genome sequencing for scalable, allele-resolved mapping of epigenetic states.**

88. Vandereyken K, Sifrim A, Thienpont B, et al. Methods and applications for single-cell and spatial multi-omics. Nat Rev Genet. 2023;24(8):494–515. doi: 10.1038/s41576-023-00580-2

89. Su JH, Zheng P, Kinrot SS, et al. Genome-scale imaging of the 3D organization and transcriptional activity of chromatin. Cell. 2020;182(6):1641–1659.e26. doi: 10.1016/j.cell.2020.07.032

90. Boettiger AN, Bintu B, Moffitt JR, et al. Super-resolution imaging reveals distinct chromatin folding for different epigenetic states. Nature. 2016;529(7586):418–422. doi: 10.1038/nature16496

91. Bintu B, Mateo LJ, Su JH, et al. Super-resolution chromatin tracing reveals domains and cooperative interactions in single cells. Science. 2018;362(6413):eaau1783. doi: 10.1126/science.aau1783

92. Chen KH, Boettiger AN, Moffitt JR, et al. RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. Science. 2015;348(6233):aaa6090. doi: 10.1126/science.aaa6090

93. Ishii S, Kakizuka T, Park SJ, et al. Genome-wide ATAC-see screening identifies TFDP1 as a modulator of global chromatin accessibility. Nat Genet. 2024;56(3):473–482. doi: 10.1038/s41588-024-01658-1

94. Wassie AT, Zhao Y, Boyden ES. Expansion microscopy: principles and uses in biological research. Nat Methods. 2019;16(1):33–41. doi: 10.1038/s41592-018-0219-4

95. Zhuang Y, Shi X. Expansion microscopy: a chemical approach for super-resolution microscopy. Curr Opin Struct Biol. 2023;81:102614. doi: 10.1016/j.sbi.2023.102614