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Abstract
Genome-wide analysis of transcription factors and epigenomic features is instrumental to shed light on DNA-templated regulatory processes such as transcription, cellular differentiation or to monitor cellular responses to environmental cues. Two decades of technological developments have led to a rich set of approaches progressively pushing the limits of epigenetic profiling towards single cells. More recently disruptive technologies using innovative biochemistry came into play. Assays such as CUT&RUN, CUT&Tag and variations thereof show considerable potential to survey multiple TFs or histone modifications in parallel from a single experiment and in native conditions. These are in the path to become the dominant assays for genome-wide analysis of TFs and chromatin modifications in bulk, single-cell, and spatial genomic applications. The principles together with pros and cons are discussed.

Keywords
Transcription factors, chromatin, epigenetics, ChIP-Sequencing, CUT&RUN, spatial epigenomics

Abbreviations
ATAC, Assay of Transposase Accessible Chromatin; ChIP, Chromatin ImmunoPrecipitation; CUT&RUN, Cleavage Under Target and Release Using Nuclease; CUT&Tag, Cleavage Under Targets and Tagmentation; DHS, DNAse I hypersensitivity; MERFISH, Multiplexed Error Robust Fluorescence *In Situ* Hybridization, MNase, Micrococcal Nuclease; Ser5P-RNAPII, Serine 5 phosphorylated RNA polymerase II; TF, Transcription Factor.

Introduction

Binding of transcription factors (TFs) to regulatory DNA is a universal feature of gene regulation in living organisms. TFs have the ability to target specific genomic sites and to 'read' the DNA code in order to control the transcriptional levels of virtually all genes. TFs come in two flavors: DNA-binding TFs, which recognize specific DNA motifs, and transcriptional co-factors that interact with the DNA-binding TFs and control or mediate their activity. Transcriptional co-factors may act by stabilizing TF on DNA, by modifying chromatin structure to control accessibility of chromatin regions or by regulating positively or negatively the transcription machinery. For simplicity we will use here the term TF throughout the text to refer to both DNA-binding TFs and non-DNA binding co-factors.

The fundamental roles played by TFs have been highlighted by numerous knock-out studies throughout the past decades showing that disrupting TF function can completely impair tissue formation, cellular differentiation or can induce dramatic changes in cell fate (e.g. [1–4]). Enforcing TF expression may also reprogram differentiated cells towards stem cell-like state or can induce a switch towards a different lineage identity, further underscoring their critical functions [5,6]. Understanding how TFs shape the cellular epigenetic landscape during development or differentiation and in response to extracellular cues represents a central question in modern biology. Out of the thousands known and putative TFs encoded by the human genome [7], only a minority have been thoroughly characterized. In this review we present some of the most popular and emerging technologies to study TFs and chromatin modifications including (native) high-throughput profiling assays, single-cell and spatial epigenomic technologies.

Unraveling TF chromatin occupancy: from *in vitro* to genome-scale *in vivo* assays

Indirect assays

It has long been observed that the position of active cis-regulatory elements (e.g. enhancers, promoters) is associated with a local depletion of nucleosomes, and increased DNA accessibility. Such regulatory elements are typically occupied by TFs and other regulatory proteins. Assays have therefore been designed to measure chromatin accessibility profiles, the DNAse I hypersensitivity (DHS) assay being one of the most popular. It is based on the fact that partial/moderate digestion of chromatin by DNAse I primarily results in digestion at open and accessible chromatin regions, as they are more easily targeted by DNAse I compared to condensed chromatin regions [8–11]. Although originally used in low throughput applications, using DNA Southern blotting as a readout of DNAse cleavage [8], DHS assay has been widely used to identify gene regulatory elements and has for instance been instrumental to define the globin distal enhancer cluster, known as the Locus Control Region (LCR) [9,12].

A simpler and high-throughput alternative to DNAse I hypersensitivity, the FAIRE (formaldehyde-assisted isolation of regulatory elements) assay was subsequently developed, based on increased recovery of accessible genomic regions from phenol/chloroform extraction of sheared chromatin [13,14]. When coupled to high-throughput sequencing, FAIRE-Seq provides a genome-wide overview of regulatory elements matching data obtained by alternative assays such as DHS, or ChIP-Seq (**Figure 1**) (see below). Importantly, DHS has also been coupled to deep sequencing to produce genome-wide mapping of accessible chromatin sites and regulatory elements. Although the DHS procedure is more labor intensive than FAIRE, very deep sequencing of the small DNAse I cleavage fragments allows precise mapping of DNA-binding proteins footprints within regulatory elements and can help

determining the grammar of DNA motifs usage within enhancers [10,11,15–17]. It was instrumental for instance to demonstrate that genomic variants associated with phenotypic traits in humans are enriched in regulatory DNA [16], and that functional variants frequently localize within TF footprints, where they can alter TF occupancy [11]. Such DHS-associated footprinting assay was also used in a multitude of primary human acute myeloid leukemia samples to unravel the aberrant signaling and transcriptional networks established by various oncogenic alterations to sustain AML maintenance [15]. In summary, this technique represents a very powerful tool to unravel protein occupancy at the genome scale, however it requires high sequencing depth and sophisticated bioinformatic tools for accurate analysis.

More recently, an even simpler approach was designed based on the use of transposases that preferentially target open chromatin regions. In this assay, named ATAC-Seq (Assay of Transposase Accessible Chromatin), transposase-mediated direct release of the accessible DNA regions is used to construct libraries for deep sequencing, resulting in the genome-wide detection of open chromatin regions [18] (**Figure 1**). This technique has been extensively used to dissect the regulatory features of physiological and pathological hematopoiesis. This includes characterization of the accessible chromatin landscape changes during human erythropoiesis, including the terminal stages of differentiation [19], or the regulation of the alpha-globin locus and the impact of single nucleotide variants in alpha-thalassemia patients [20]. It is also a very popular tool for the fine mapping of epigenetic alterations associated with leukemia development or resistance to treatments, as exemplified by recent analyses of B-ALL resistance to CD19 CAR T-cell therapy [21], or oncogenic cooperativity in pure erythroid leukemia [22]. One advantage of ATAC-Seq is that transposase-mediated accessible DNA release is an efficient process generating fragments combined with sequencing adaptors directly usable for deep sequencing. ATAC-Seq is therefore adapted for use on low cell numbers (within the range of few tens to hundred thousands as compared to millions of cells for DHS), and has been adapted for use in single cells [23], including in human fetal liver [24], bone marrow and blood [25], and in spatial genomic approaches (see below) [26]. Due to its simplicity and applicability to low cell numbers, ATAC-Seq has become a widely used and highly popular method to highlight open chromatin regions. Although the commonly used Tn5 transposase possesses DNA sequence biases, strategies have been designed to correct for such bias during data processing and detect footprints corresponding to sites of TF occupancy [27]. As it is the case for digital footprinting of DHS, deeply sequenced ATAC-Seq libraries therefore offer the advantage of allowing detection of TF footprints, in a simple and straightforward experimental set-up, with the need of advanced bioinformatic tools.

Direct targeted assays for endogenous transcription factors

From in vitro EMSA to in vivo ChIP-Seq: high quality and genome-wide coverage of TF occupancy

One of the earliest techniques used to study protein-DNA interactions is the electrophoresis mobility shift assay (EMSA). EMSA is based on the migration properties of nucleic acid molecules (probes), which when bound by a sequence-specific DNA binding protein will migrate more slowly compared to the free probe. As such, appearance of a higher molecular weight band on a gel indicates the presence of a protein-DNA complex. Although straightforward, the *in vitro* nature of this assay presents some disadvantages and is not amenable to high-throughput and genome-scale analyses. Another popular assay addressing

some of these limitation came into play in the late 80's early 90's, and is called chromatin immunoprecipitation (ChIP) [28] (see [29] for a historical perspective). In this assay, cells are fixed using a crosslinking agent, usually formaldehyde. The chromatin is then extracted, fragmented and subjected to immunoprecipitation. Immunoprecipitated protein/DNA complexes are then decrosslinked and the recovered DNA sequences are analyzed by qPCR or high-throughput sequencing for genome-wide analyses (ChIP-Seq) (**Figure 1**). The development of ChIP-Seq (i.e. combination of ChIP to high-throughput sequencing) has truly be a game changer in the field, allowing for the first time to highlight genome-wide chromatin occupancy at an unprecedented pace and resolution. Several major discoveries originated from the use of ChIP-Seq, for instance the discovery that gene regulatory elements (e.g. enhancers) are spread along the non-coding genome and are almost exclusively distally located in respect to their target gene promoters, confirming at the whole genome level what was already known for a handful of loci including the globin genes. ChIP-Seq has become the dominant assay to study chromatin occupancy, and has been extensively used to study TFs, chromatin modifications, and their dynamics in a multitude of tissues and cell types including hematopoietic stem cells during ontogeny and self-renewal (e.g. [30,31]), erythroid (e.g. [32,33]), myeloid (e.g. [34,35]) and lymphoid cells (e.g. [36,37]), and in disease situations, such as acute leukemia of myeloid (e.g. [38,39]) and lymphoid (e.g. [40,41]) origins. ChIP-Seq has been the method of choice in large international consortia aiming at deciphering cell- and tissue-specific epigenomes and cancer-associated epigenetic alterations (e.g. ENCODE, BluePrint).

Advantages of ChIP-Seq

ChIP-Seq allows the analysis of direct DNA-binding TFs and histones, as well as proteins binding indirectly to chromatin, for instance through protein-protein interactions as part of large multiprotein complexes (e.g. the Mediator complex). Robust protocols are readily available, and a large number of ChIP-grade antibodies are now accessible for numerous TFs and chromatin complexes, or their post-translationally modified forms (e.g. phosphorylated RNA polymerase II or modified histone tails), which facilitates the implementation of ChIP-Seq in a wide range of laboratories. In addition, strategies have been devised to ensure proper signal quantification for cross-sample and cross-conditions comparisons (i.e. upon different drug treatments, or when comparing KO versus WT situations), through the use of spike-in material [42] or internal normalizing control [43]. Finally, robust and widely accessible bioinformatic tools and pipelines for quality control of sequencing reads, read alignments, peak calling and comparative analyses are now available, facilitating ChIP-Seq analysis (see **Table 1** for a summary of the most popular tools). In addition, one of the crucial challenges in bioinformatics analyses is to provide a way to define the steps of a pipeline, specify the dependencies between steps, and execute the pipeline in a reproducible and portable way. In this aspect, several solutions have been developed: Nextflow [44], Snakemake [45], and nf-core [46] which are all open-source workflow management systems that have been widely adopted in the bioinformatics community and have been used to automate the execution of computational pipelines. All these solutions support parallel execution and are designed to run on various computer environments, including clusters, cloud and also personal machines.

Quality control of sequencing reads		
Fastqc	Reads quality control tool	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Fastq_screen	Screening a library of sequences against a set of genomes sequences to identify cross species contaminations	https://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/
Trimming		
Cutadapt	Trimming and filtering out low quality reads	Martin 2011 [47]
Trimmomatic		Bolger et al. 2014 [48]
Trim Galore		https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
Alignment and mapping		
STAR	Aligning and mapping reads to a reference genome	Dobin et al. 2013[49]
HISAT2		Kim et al. 2019 [50]
Bowtie2		Langmead et al. 2012 [51]
Peak calling		
MACS	Detection of reads enrichment over local background	Zhang et al. 2008 [52]
SICER		Xu et al. 2014 [53]
F-Seq		Boyle et al. 2008 [54]
HOMER		Heinz et al. 2010 [55]
SEACR	Peak caller optimized for CUT&RUN	Meers et al. 2019 [56]
CUT&RUNTools	Peak caller optimized for CUT&RUN	Zhu et al. 2019 [57]
EChO	Peak caller optimized for CUT&RUN, nucleosomes, and TF co-dependency	Meers et al. 2019 [58]
LanceOtron	Machine learning based peak caller	Hentges et al. 2022 [59]
Annotation		
HOMER	Assignment of peaks to target genes and genomic features	Heinz et al. 2010 [55]
ChIPseeker		Yu et al. 2015 [60]
rGREAT		Gu et al. 2023 [61]
Bioinformatic pipeline management		
NextFlow	Creation of automated, reproducible, and scalable data analysis pipelines	Di Tommaso et al. 2017 [44]
SnakeMake		Koster et al. 2012 [45]
Nf-Core	Collaborative, peer-reviewed, best-practice analysis pipelines	Ewels et al. 2020 [46]

Table 1: widely used tools for bioinformatic processing of ChIP-Seq, ATAC-Seq, or CUT&RUN sequencing reads. The most popular tools used to control reads quality, perform optimal alignments, peak calling and annotations are indicated. Examples of pipeline management solutions are also shown.

Limitations of ChIP-Seq

Although robust protocols have been established over the years, key considerations when using ChIP-Seq should be considered. First, the crosslinking step should be carefully controlled in order to ensure efficient protein-protein and protein-DNA fixation, and to minimize biases originating from over-crosslinking [62,63]. It is also important to bear in mind that fixation may alter or partially mask epitopes in target proteins making the immunoprecipitation step highly inefficient. Second, chromatin fragmentation to a size range of ~200-800bp is another critical step for efficient recovery of ChIP DNA. Oversonicated chromatin may be inefficiently immunoprecipitated, and sonication for long periods of time to improve chromatin fragmentation may be deleterious for large size protein stability. On the other hand, insufficient chromatin fragmentation leads to the persistence of large fragments

(>1-5 kb), which increase the non-specific background and may blur the specific ChIP enrichment signals. Sonication conditions therefore need to be carefully but empirically defined, as they are highly dependent on the type of sonicating instrument used, on the tissue or cell types under study, and on the quality of the crosslinking. Alternatives to sonication employ an enzymatic fragmentation procedure through the use of micrococcal nuclease (MNase) which cuts double strand DNA at positions devoid of nucleosomes or between nucleosomes.

Low input ChIP-Seq for analysis of small cell numbers

ChIP-Seq has been routinely performed using millions of cells as input, precluding the analysis of rare cell populations. Efforts have therefore been undertaken to adapt ChIP-Seq to low cellular inputs (summarized in **Table 2**). Various steps of the ChIP procedure were subjected to optimization in these assays (e.g. cell lysis, MNase digestion versus sonication for chromatin fragmentation, use of carrier chromatin to avoid sample loss) and several tricks were used to simplify the procedure, amplify the signal and multiplex the assay for increased robustness and cross-sample consistency. These various optimizations include chromatin barcoding, sample multiplexing, T7-mediated linear amplification of ChIP DNA, use of Tn5 transposase tagmentation and optimizations in library constructions, use of microfluidics or any combinations thereof. These various technical improvements led to the generation of several ChIP-Seq variants methods including ULI-ChIP-Seq [64], iChIP [65], Mint-ChIP-Seq [66], HT-ChIP [67], LinDA [68], MOWChIP-Seq [69], ChIPmentation [70], FARP-ChIP [71], TAF-ChIP [72], ChIL-Seq [73]. All these advances led to decreased input requirements, which in the best cases could be brought down to ~100 – 500 cells depending on the protocols used (see **Table 2**). Such improvements paved the way towards the first epigenetic analyses of scarce cell populations, including hematopoietic stem (HSCs) and progenitor cells. The use of iChIP for instance allowed for the first time the multiplexed profiling of four histone modifications in 16 different hematopoietic cell types including both short-term (ST-) and long-term (LT-) HSCs, immature progenitors (MPP, CMP, CLP), and maturing erythroid, myeloid and lymphoid cells from mouse bone marrow [65]. It provided a characterization of chromatin landscape and enhancer repertoire of differentiating hematopoietic cells at an unprecedented level, and unraveled the transcription factor networks controlling *in vivo* chromatin dynamics within the hematopoietic hierarchy. Soon thereafter, the use of FARP-ChIP or MINT-ChIP on sorted HSC subpopulations allowed dissection of the H3K4me3 chromatin modification changes associated with LT-HSCs and ST-HSCs (FARP-ChIP) or the profiling of H3K27ac, H3K27me3 and H3K36me3 in HSCs (Mint-ChIP) with a similar or increased precision [66,71]. Fetal liver hematopoietic stem and progenitor cells (HSPCs) were also successfully profiled for both H3K4me3 and H3K27ac with MOW-ChIP which led to the characterization of putative enhancer regions active in this tissue [69]. Such technological developments led to the first in-depth dissection of the epigenetic landscape of HSCs, reaching a resolution that was never achieved before. It is however worth noting that efficient ChIP-Seq on very low cellular inputs mostly apply to histone marks, which are usually highly abundant and tightly associated to DNA. On the other hand, downsizing input requirements for TF ChIP-Seq has been notoriously more difficult as TF are usually less abundant and may be only transiently bound to their DNA target sites, making them more difficult to detect. Successful low-input TF ChIP-Seq were nonetheless reported using as low as 10^3-10^4 cells [65,73], although some optimized procedure could reach the limit of 100 cells (e.g. [74]). In that respect, the development of transposase-mediated addition of sequencing adaptors directly to ChIP DNA frements, a

process known as 'tagmentation' has dramatically improved sequencing library construction. The original procedure called ChIPmentation [70] used the Tn5 transposase to add sequencing adapters on the immunoprecipitated DNA, which eliminated the need to go through the several steps of library construction thereby preventing sample loss [74]. ChIPmentation has been (and continues to be) widely used for TF and histone marks in various experimental contexts, including for instance for the analysis of chronic lymphocytic leukemia [75] or T cell acute lymphoblastic leukemia [76], for the characterization of the epigenetic landscape of human αβ and γδ T cell development [77], or during the reprogramming of mouse bone marrow–derived pre-B cells into pluripotent stem cell-like cells, to follow the precise epigenetic transitions underlying this process [78]. An updated version of the procedure called TAF-ChIP directly combines the Tn5 activity to the immunoprecipitation step to directly fragment the chromatin during immunoprecipitation [72]. It offers in one step the double advantage of (i) eliminating the need to sonicate very small cell amounts, and (ii) to directly generate sequencing-compatible fragments as the Tn5 transposomes are preloaded with Illumina sequencing adaptors. TAF-ChIP therefore offers several advantages and in the original report, efficient genome-wide detection of H3K27me3 and H3K9me3 was achieved using as low as 100 cells [72].

Single cell ChIP-Seq: a true genome-wide assay?

Although chromatin accessibility assays such as ATAC-Seq have been adapted to single cell profiling [23,79], adapting ChIP-Seq to single cells has long been a bottleneck suffering either from insufficient coverage or throughput. The first report of single cell ChIP-Seq (Drop-ChIP) leveraged the power of drop-based microfluidics to manipulate single cells and perform cell lysis, MNase chromatin digestion and chromatin indexing in single drops [80]. In this landmark study, ChIP was performed on a pool of 100 indexed single-cells to yield average profiles of H3K4me3. Although innovative, this approach initially suffered from low coverage. After applying stringent sequencing reads filtering, an average of 500 to 10,000 Drop-ChIP reads were obtained per single cell, corresponding to ~800 peaks per cell [80]. It is worth noting that the small numbers of detected H3K4me3 were nonetheless strongly correlated to signals obtained by conventional bulk ChIP-Seq, and sufficient to define cell identity. These limitations were specifically addressed in a recent, groundbreaking microfluidic-based approach combining real-time live monitoring of droplets (for automated quality control of encapsulation of uniquely barcoded beads and single cells), photocleavage and ligation-based barcode transfer to nucleosomes from thousands of single cells [81]. This high-throughput procedure led to a ~10 fold improvement in coverage per cell (10,000 loci versus less than 1,000 in the original report) [81]. This impressive improvement pushed the limits of scChIP-Seq and allowed defining key epigenomic features associated with treatment resistance in breast cancer. At about the same time, another low input ChIP-Seq variant applicable to single cell analysis, called indexing and tagmentation-based ChIP-seq (itChIP-seq) was reported [82]. Compared to Drop-ChIP and scChIP-Seq, single cell itChIP-Seq presents the great advantage of being devoid of microfluidic-based handling steps, and therefore should more easily be implemented in 'classical' molecular biology laboratory environments and clinical diagnosis laboratories. Although all these progresses are very encouraging, full genome-wide coverage has not been attained yet, underscoring the need for further technological improvements or for alternative methods.

Low-input ChIP-Seq variant	Chromatin: fixed / native	Chromatin fragmentation	Chromatin handling procedure	DNA amplification step prior to library preparation ?	Multiplexing	Cell number for histone modifications	Cell number for TF	Coverage	Reference to protocol
LinDA	Fixed	n/a	n/a	*in vitro* T7 linear amplification	n/a	5.10^3-10^4	5.10^3	Genome-wide	[68]
Mint ChIP	Fixed	MNase	Indexing and pooling	*in vitro* T7 linear amplification	yes	500	n/a	Genome-wide	[66]
iChIP	Fixed	Sonication	Indexing and pooling	none	Yes, high	500	10^4	Genome-wide	[65]
HT-ChIP	Fixed	Sonication	Multiplexed ChIP in 96-well plates	none	Very High, up to 96 parallel ChIP experiments	~10^4	~10^5-10^6	Genome-wide	[67]
ULI-NchIP	Native	MNase	Library pooling	none	yes	10^3	n/a	Genome-wide	[64]
ChIPmentation	Fixed	Sonication	One-step transposase-mediated ligation of sequencing adapters to ChIP DNA	none	possible	10^4	10^5	Genome-wide	[70]
HT-ChIPmentation	Fixed	Sonication	Combined tagmentation, reverse crosslink and library preparation	none	possible	100-500	100-500	Genome-wide	[74]
TAF-ChIP	Fixed	Transposase-mediated fragmentation	Direct chromatin tagmentation and indexing	none	possible	100	n/a	Genome-wide	[72]
MOW-ChIP	Fixed	Sonication	Microfluidic processing of sonicated chromatin	none	n/a	100	n/a	Genome-wide	[69]
CHIL-Seq	Fixed	Antibody-tethered Tn5	Immunoprecipitation-free	*in vitro* T7 linear amplification	n/a	100 - 10^5 (possible use in	1000	Genome-wide	[73]

		transposase	procedure			single cells)			
FARP-ChIP-Seq	Fixed	Sonication	Use of biotinylated carrier DNA to prevent sample loss	none	possible	500	n/a	Genome-wide	[71]
Single cell ChIP-Seq									
Drop-ChIP	Native	MNase	Microfluidics	yes	Very high (hundreds to thousands of single cells)	1	n/a	~800-1,000 loci	[80]
scChIP-Seq	Native	MNase	Microfluidics	yes	Very high (thousands of single cells)	1	n/a	~5,000-10,000 loci	[81]
itChIP-Seq	Fixed	Transposase-mediated fragmentation	FACS-sorted cells, single-tube procedure	yes	Very high (thousands of single cells)	1	100-500	~5,000-10,000 loci	[82]

Table 2. Low-input and single cell ChIP-Seq variants.
The different low-input ChIP-Seq variants are shown, with the associated chromatin processing and the lowest cellular inputs reported for histone modifications and TF profiling. The single cell adaptations are also highlighted.

Native assays for genome-wide TF occupancy: the CUT&RUN and CUT&Tag revolution

Whereas several ChIP-Seq protocols rely on chromatin crosslinking to retain proteins bound to DNA, some are performed using native conditions (for instance in some protocols using MNase digestion for chromatin fragmentation [64,81]). These assays mostly apply to the study of histone modifications due to their tight association to DNA. Therefore, protocols allowing the study of TF chromatin binding in a native context were lacking, until a new assay named CUT&RUN (Cleavage Under Targets and Release Using Nuclease) came into play [83–85]. As it is the case for ChIP-Seq, CUT&RUN allows genome-wide profiling of chromatin modifications and TFs. However, it relies on a different principle based on an enzyme-tethering strategy using unfixed cells (**Figure 2**). Live cells or cell nuclei are first immobilized on a solid phase (concanavalin beads), permeabilized and incubated with an antibody against a TF or a chromatin mark of interest. A recombinant protein A/G fused to MNase (pAG-MNase) is then added and allowed to bind to the primary antibody. Activation of MNase activity by Ca2+ addition induces cleavage of the DNA surrounding the TF binding site (or histone modification), releasing the immunocomplex with the underlying DNA sequence, which freely diffuses out of the nucleus and can be recovered for direct library preparation. CUT&RUN presents multiple advantages compared to ChIP-Seq. First, the assay is performed under native conditions, avoiding any crosslinking artefacts or epitope masking due to fixation. Second, only the DNA binding sequences are released from the nuclei after MNase activation (as it is targeted towards the antibody-bound TF binding sites) whereas the remaining large excess of unbound genomic DNA is retained in the nucleus (and hence not further processed). Sequencing reads therefore mostly account for informative DNA binding sites, rather than an enrichment over unspecific background genomic DNA, which usually results in very high

signal-to-noise ratios. As a consequence, the sequencing depth required for CUT&RUN is much lower compared to ChIP-Seq (where bulk genomic DNA is present, resulting in the need for higher sequencing depth to detect the emergence of enriched TF binding sites over a high background). Third, the CUT&RUN procedure is routinely performed on relatively low cell numbers (in the range of ~100,000 to 500,000 which is on average 10 times lower than for ChIP-Seq), and it is faster (it can be performed in ~1 to 1.5 day) [83–85]. As a consequence, CUT&RUN combines several advantages over ChIP and may become the dominant assay in the coming years as it is faster, cheaper and provides surprisingly high signal to noise genome-wide quality datasets. CUT&RUN is becoming increasingly popular in the hematopoietic field. It was instrumental for instance to show that the fetal globin (γ-globin) repressor BCL11A directly targets the γ-globin promoters (and not distally located elements) to suppress fetal hemoglobin production in adult erythroid cells [86]. The high-resolution signals derived from CUT&RUN were advantageous to precisely map the TF footprints allowing to unravel the interplay between activating (e.g. NF-Y) and repressive (e.g. BCL11A, NFIA/NFIX) TFs controlling the developmental switch in fetal globin expression [87,88], and to characterize additional key mechanisms controlling the switch, mediated by the hypoxia-inducible HIF1α TF [89], or HIC2-mediated control of BCL11A developmental expression [90]. It is worth noting that in our hands, genome-wide profiling of hard-to-ChIP TFs was uniquely achieved using CUT&RUN, when ChIP was inefficient.

Given the success of CUT&RUN, the strategy was further improved by substituting the pAG-MNase by a protein A/G fused to the Tn5 transposase pre-loaded with Illumina adapters [91–93]. The resulting assay called CUT&Tag (Cleavage Under Targets and Tagmentation) uses a similar 'cut and release' experimental procedure, with the advantage of directly generating sequencing-ready DNA fragments thanks to the addition of sequencing adaptors by the Tn5 transposase, as it is the case in ATAC-Seq (**Figure 2** and **Table 3**). The full procedure from cells to indexed sequencing-ready libraries can be performed in one day, in a single tube, and due to very high signal-to-noise ratio, multiple libraries can be pooled prior to sequencing, dramatically decreasing the associated costs [91–93]. Of note, a similar strategy dubbed ACT-Seq, based on the same principle was reported at about the same time [94], and ChIL-Seq also uses antibody-tethered Tn5 but differs in the subsequent processing steps (and is performed on fixed cells) [73]. Importantly, both CUT&RUN and CUT&Tag have been adapted to robotic automation offering increased throughput and consistency, providing a unique opportunity to implement these assays in routine diagnostic labs [95,96]. With the successful decreased input requirements and facilitated workflow, CUT&Tag offers the possibility to work with primary cells available in limiting amounts, exemplified by the modeling of AMKL using induced human pluripotent stem cells engineered to contain various combinations of AMKL-specific oncogenic hits such as GATA1s, *SMC3* haploinsufficiency, *MPL* ^{W515K}, and trisomy 21, differentiated along the megakaryocytic lineage [97].

Single cell Cut&Run/Cut&Tag

Successful downsizing of the cellular material required for efficient CUT&Tag and CUT&RUN profiling was demonstrated using as low as ~60-100 cells [84,91,93]. The strategy was further improved to be adapted to single cells [91,96,98][99], offering the unique opportunity to probe epigenome variability among seemingly uniform cell types or within tissues (**Table 3**). To achieve single cell CUT&RUN, several adjustments to the original protocol were introduced, including library preparation (for detailed procedure see [98]). In this configuration, individual ES cells were FACS-sorted into single wells of 96-well plates prior to

processing, whereas the scCUT&Tag uses bulk antibody binding and pA-Tn5 tethering prior to dispatching into single wells [91,96], or loading into a lane of 10X Genomics microfluidic chip [100]. Importantly, these assays allowed the analysis of TFs (e.g. CTCF, SOX2, NANOG) and chromatin modifiers (e.g. KMT2A) in addition to histone marks (e.g. H3K27me3), demonstrating so far superior pliability as compared to scChIP-Seq. Further adaptations of these procedures include coupling antibody labeling of cell surface markers with detection of chromatin modifications in single cells, a technique dubbed sortChIC (for sort-assisted single-cell chromatin immunocleavage) to link cell lineage identity with specific epigenome information [101]. This strategy was used to profile the active (H3K4me1, H3K4me3) and repressive (H3K27me3, H3K9me3) epigenome, of bone marrow cells in the mouse. It led to the very interesting finding that whereas active chromatin is cell type-specific and displays divergent changes along the different lineages (as was shown before [24,25,65]), repressive chromatin seems surprisingly far less divergent within different cell types from a same lineage (i.e. myeloid or lymphoid). This finding suggests that changes in active chromatin delineate cell types specificities within a given lineage, whereas repressive chromatin may rather act as a common 'road block' restricting alternate lineage potential (i.e. myeloid potential in lymphoid cells and vice versa) in different cell types belonging to a common lineage [101].

Parallel analysis of multiple chromatin features in single assays

The observation that performing transposase activation under low salt conditions during CUT&Tag results in preferential tagmentation of nearby accessible DNA in a narrow window size has motivated the development of strategies aiming at exploiting this property. In CUTAC, low salt activation of Tn5 transposition leads to high resolution profiles of active histone modifications (H3K4me2 and H3K4me3) instead of the broader signal distribution observed in classical CUT&Tag [93]. As developmentally repressed heterochromatin regions marked by H3K27me3 typically span more than 10kb and are more condensed in terms of nucleosome spacing, associated chromatin fragmentation in CUT&Tag or CUTAC results in larger size fragments when compared to active regions where small (<120 bp) size fragments are preferentially generated (**Figure 3**). By leveraging this property, CUT&Tag2for1 was designed by combining antibodies directed against active regions i.e. through the use of initiating RNA polymerase II (Ser5P-RNAPII) and antibodies directed against the H3K27me3 repressive mark [102]. The DNA fragments generated after tagmentation show a typical bi-modal distribution (small to high fragment average) that could be deconvoluted so that fragments corresponding to active regions (i.e. small size <120 bp) or repressed chromatin (i.e. larger fragments) could be correctly inferred to the correct antibodies in a single assay (**Figure 3**). Importantly, CUT&Tag2for1 is efficient in single cell assays and was shown to identify both active and repressive chromatin features in the same single cells [102].

Further developments used combined (multi-CUT&Tag, [103]) or sequential (MulTI-Tag [104], ACT2-Seq [105]) incubation of barcoded primary antibodies directed against distinct histone modifications in the same sample (**Figure 3**). In these approaches, each antibody is complexed with barcoded pA-Tn5 leading to tagmentation of several different chromatin marks in the same assay. The localization and abundance of each histone modification or chromatin binding protein (e.g. H3K27me3, H3K4me1/2, H3K36me3, H3K27ac, RNAPII) is therefore recovered using the antibody-specific barcodes allowing simultaneous detection of several chromatin marks in the same sample. These approaches were successfully adapted to single cells allowing for instance to identify cell type identity in cell mixtures, or to reconstruct pseudo-time developmental trajectories and highlight continuous transitions in chromatin

modifications along a differentiation path [103,104]. Importantly it was shown that typical bivalent chromatin domains (i.e. labelled by both H3K27me3 and H3K4me1/2) or co-localizing chromatin proteins could be detected in single cell MulTI-Tag and multi-CUT&Tag indicating that combinatorial profiling of multiple histone marks or protein binding sites co-occurring in close vicinity can be readily identified in single cells without confounding effects [103,104].

These innovative assays pave the way towards single cell multiplexed analysis of chromatin features in the same sample offering unprecedent opportunities to directly characterize the epigenomic landscape complexity at vastly improved cellular resolution. However, it should be noted that data analysis requires advanced bioinformatics.

Assay name	Native conditions	Single or simultaneous multiple detections?	Antibody	Chromatin fragmentation	Sequencing library ready material?	Minimal cell number requirement	References to original protocols	Amenable to single cells ?	Adaptability to spatial epigenomics
CUT&RUN	yes	single	Primary	MNase	no	1-100	[83–85]	Yes [98]	no
CUT&Tag	yes	single	Primary (+/- secondary)	Illumina adapter loaded Tn5	yes	1-60	[91–93]	Yes [91,96, 100]	Yes
CUTAC	yes	single	Primary (+/- secondary)	Illumina adapter loaded Tn5	yes	1-60	[93]	likely	Not tested
CUT&Tag2for1	yes	Multiple (2 features)	Primary	Illumina adapter loaded Tn5	yes	1		Yes	Not tested
Multi-CUT&Tag	yes	Multiple (3 or more features)	Pre-loaded antibody/pAG-Tn5 complexes	Illumina adapter loaded Tn5	yes	1	[103,106]	Yes [103]	Not tested
Multi-Tag	yes	Multiple (3 or more features)	Pre-loaded Primary (+/- secondary)/pAG-Tn5 complexes	Illumina adapter loaded Tn5	yes	1	[104]	Yes [104]	Not tested
ACT-Seq / ACT2-Seq	yes	Multiple (in ACT2-Seq, at least 2 features)	Pre-loaded Primary /pAG-Tn5 complexes	Illumina adapter loaded Tn5	yes	1	[94,105]	Yes [94]	Not tested

scChIC-Seq	yes	single	Primary directly coupled to MNase	MNase	no	1-100	[99]	yes	Not tested
sortChIC	no	Single detection of chromatin marks combined with multiple cell surface marker detection	Primary	MNase	No	1	[101]	Yes	Not tested

Table 3. CUT&RUN and CUT&Tag variants.
The various adaptations of CUT&RUN and CUT&Tag are listed, together with their specificities and their use in spatial approaches.

Tagging transcription factors for genome-wide analyses

Both ChIP-Seq and CUT&RUN/CUT&Tag and related assays are highly sensitive to the quality of the antibody used. Whereas high quality antibodies are available for the most commonly studied histone modifications (e.g. H3K4me3, H3K4me1, H3K27Ac, H3K27me3), antibodies directed against TFs vary enormously in terms of quality and specificity (e.g. [107,108]). The use of affinity tags may circumvent the lack of ChIP-grade or CUT&RUN-grade antibodies, or could be useful to target a specific isoform when no specific antibodies are available. The development of CRISPR-Cas9 technologies facilitate the introduction of tags in endogenous TF genes avoiding any overexpression artifacts. Common tags such as FLAG, HA, MYC, GFP or V5 have successfully been used in genome-wide assays. Some hard-to-ChIP TFs could be analyzed through the use of tags as in the case of the KLF family TF KLF3 fused to the ER (estrogen receptor) moiety [109], or the GATA2 TF fused to an HA tag in CUT&Tag experiments [110]. Some leukemia-specific fusion oncoproteins (e.g. ETO2-GLIS2) were also profiled genome-wide using GFP as a tag in ChIP-Seq experiments [111]. One additional benefit of affinity tags is that they are associated with low background, providing good signal-to-noise ratios. Of note, the Bio tag, also known as AVI tag, is particularly 'clean' as it provides genome-wide datasets with extremely low background [32,112]. It relies on the addition of biotin by a bacterial enzyme BirA on the Bio tag protein sequence. TF pull down is performed using magnetic streptavidin beads instead of an antibody, thereby dramatically reducing non-specific chromatin pulldown and hence experimental background noise. However, this system requires both tagging of the TF of interest and (constitutively) expressing the BirA enzyme [112]. This may be difficult when working on primary cells, although BirA expressing mice have been produced allowing to perform *in vivo* Bio pulldown assays [113].

Of note, enzymatic tags may be used to address specific questions. The bacterial Dam methyltransferase is able to carry-out adenine methylation. When fused to a TF it locally methylates DNA, leaving an imprint where the TF was bound [114,115]. The resulting DamID assay was thoroughly used to highlight lamina-associated regions in mammalian cells. It was also instrumental to characterize sites of rapidly exchanging TFs in the developing hematopoietic system, which otherwise could not be detected using conventional ChIP approaches when TF binding was too transient [116]. The choice of specific tags may therefore

open-up additional ways to characterize TF function, depending on the biological question to address.

The era of spatial epigenomics

A major hurdle of epigenome profiling technologies, including single-cell approaches is the lack of spatially defined information. Defining where chromatin is decorated with specific marks or TFs within complex tissues or within a tumor sample is instrumental to understand developmental and pathophysiological processes (e.g. cancer cells and their microenvironment). By leveraging the power of transposase mediated chromatin tagmentation (ATAC-Seq/CUT&Tag), spatial epigenomic technologies were recently developed to specifically address these points [26,117]. Spatial profiling was carried-out by applying ATAC or CUT&Tag procedures on fixed tissue sections. After activating the Tn5 transposomes, adapters containing a ligation linker were integrated at sites of accessible chromatin (spatial-ATAC-Seq) or at the histone mark antibody recognition sites (spatial-CUT&Tag). Next, two waves of barcoding schemes were applied (A and B), resulting in the ligation of barcode adapters to accessible/targeted chromatin, such that every region of the tissue section is defined by a specific A(x)B(y) barcode combination (**Figure 4**). Sequencing libraries could then be generated and reads could be aligned and mapped in a spatially resolved manner. Epigenome information could be derived at an unprecedented resolution (below 20µm) i.e. at the range of cellular and subcellular scale. Data derived from spatial epigenomics matched previous single cell epigenome datasets, with the additional information of in-tissue spatial distribution. During the same year, another original approach was published, dubbed epigenomic MERFISH (multiplexed error robust fluorescence *in situ* hybridization), which combined epigenomic labeling with imaging offering the possibility to visualize the spatial and intranuclear distribution of more than 100 chromatin loci (i.e. promoters, enhancers, repressed loci) on a tissue slide [118]. As in spatial CUT&Tag, this method uses antibody-mediated tethering of Tn5 transposase to chromatin regions of choice (e.g. H3K4me3, H3K27Ac, H3K27me3). It differs by the fact that the Tn5 transposomes are loaded with a T7 promoter sequence in addition to sequencing adaptors. After tagmentation, the target loci sequences can be locally amplified by *in situ* linear amplification (T7 transcription) and detected by MERFISH using locus-specific probes [118]. By employing epigenomic MERFISH on a whole mouse embryo slide, several known patterns of active promoters and new putative enhancer promoter pairs and enhancer hubs could be unraveled, demonstrating the power of such high-throughput spatial approaches. Both spatial ATAC-Seq/CUT&Tag and epigenomic MERFISH aim at providing similar spatial information, and are complementary. On one hand, spatial ATAC-Seq and CUT&Tag offer large-scale chromatin accessibility and modification in a spatially resolved manner, on the other hand, epigenomic MERFISH is limited so far to the range of a hundred of loci (due to limitations in the spectral coloring combinations during sequential imaging), but achieves a much higher spatial resolution, allowing to spatially localize loci in single nuclei. More recently, a strategy combining spatial epigenomic (spatial ATAC/spatial CUT&Tag) and spatial transcriptomic analysis in a single assay at near-single-cell resolution was reported, offering the possibility to explore tissue composition and organization at an unprecedented level of refinement [119]. These innovative and extremely powerful approaches require microfluidic device for cross-flow barcode ligations, or state of the art imaging systems and powerful bioinformatics to cluster the epigenetic information into putative cell types, and to correlate the epigenomic maps with gene activity. These requirements currently limit the widespread use of spatial

epigenomics to fully equipped laboratories, but pave the way towards a wide range of discoveries and applications in the fields of developmental biology and biomedicine. Although significantly more complex, these assays have the potential to become the gold standards in the spatial biology era in the future.

Conclusions

Technological developments for genome-wide analysis of transcription factors and chromatin have progressed at an incredible pace. Multiplexed strategies are readily available for simultaneous detection of several epigenetic features in single cells. Looking ahead, the recent breakthroughs in spatial epigenomics lay the foundation of a new dimension of biology and molecular medicine where multi-omics single cell spatial information can be interrogated allowing to decipher the cellular relationships underlying complex tissue homeostasis and pathological situations such as tumoral ecosystems.

Figure legends

Figure 1. Classical methods for epigenome and TF profiling.
The basic principles of DHS, digital footprinting (DFP), FAIRE, ATAC-Seq and ChIP-Seq are depicted. The corresponding outputs are highlighted.

Figure 2. Principles of CUT&RUN and CUT&Tag.
Permeabilized cells are incubated with primary antibodies recognizing TFs or chromatin modifications. Addition and activation of pA/G-MNase (CUT&RUN) or pA/G-Tn5 (CUT&Tag) leads to the cleavage of the underlying DNA sequences, which are used for sequencing library construction or direct sequencing.

Figure 3. Multi-modality chromatin profiling strategies.
(Left) CUT&Tag2for1 involves the use of two distinct primary antibodies directed against active (serine 5 phosphorylated RNA polymerase 2, Ser5P-RNAPII) and repressed (H3K27me3) chromatin marks. Activation of the pA/G-Tn5 under low salt condition leads to narrow cleavage of the DNA sequences around the antibody targets. Repressed chromatin regions lead to higher fragment sizes, allowing reads deriving from the different antibodies to be inferred and identified. (Right) Principle of Multi-CUT&Tag, Multi-Tag and ACT2-Seq. primary antibodies are pre-incubated with barcoded pA/G-Tn5 complexes and used to label various different chromatin regions in parallel. The resulting barcoded DNA is used to infer reads to the proper primary antibodies.

Figure 4. Spatial epigenome mapping.
The technical steps for spatial ATAC-Seq and spatial CUT&Tag are presented. A fixed tissue slide is incubated with Illumina adapter-loaded Tn5 (spatial ATAC-Seq) or with primary antibody assembled with secondary antibodies and pA/G-Tn5 complexes (spatial CUT&Tag) leading to tagmentation. Next, a microfluidic device is used to add sets of barcodes (A and B) on the tagmented tissue slide. The double barcoding scheme generates a pixel-like grid where each single cell epigenome can be mapped back to the original cell location on the tissue slide. After sequencing and mapping, epigenomic clusters can be identified and spatially resolved on the tissue (colored pixels).

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Figure 1

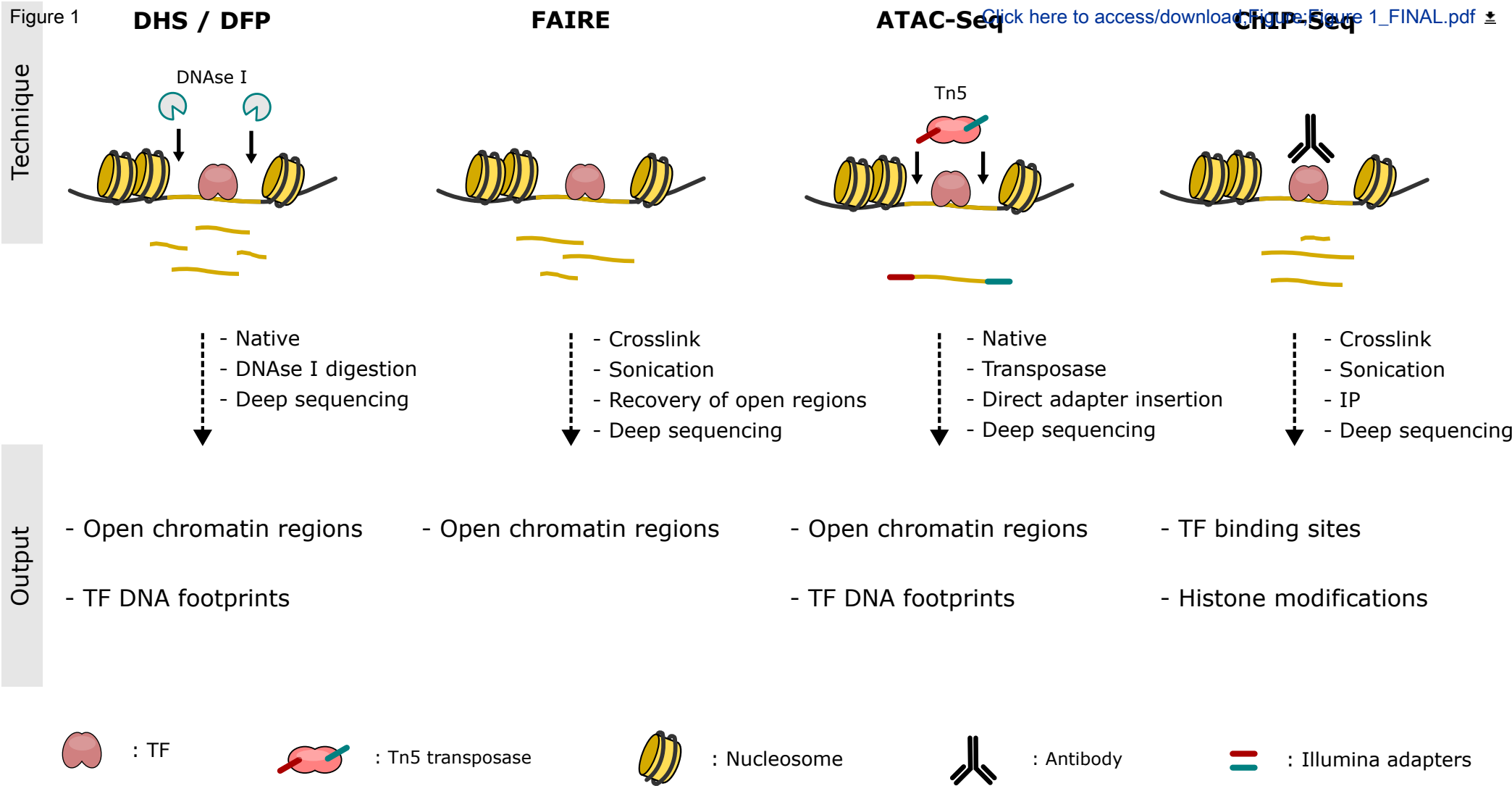

Figure 2

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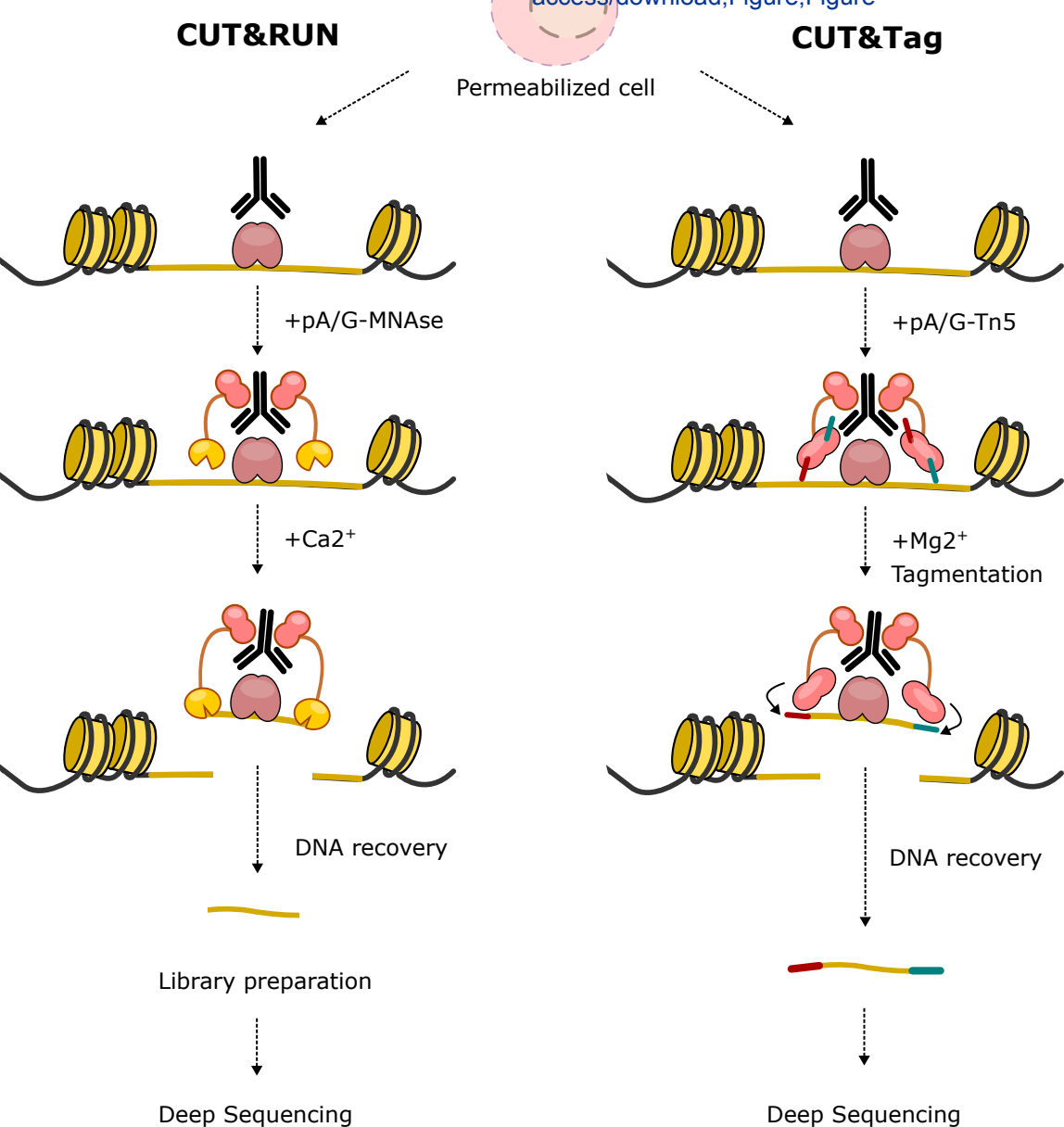
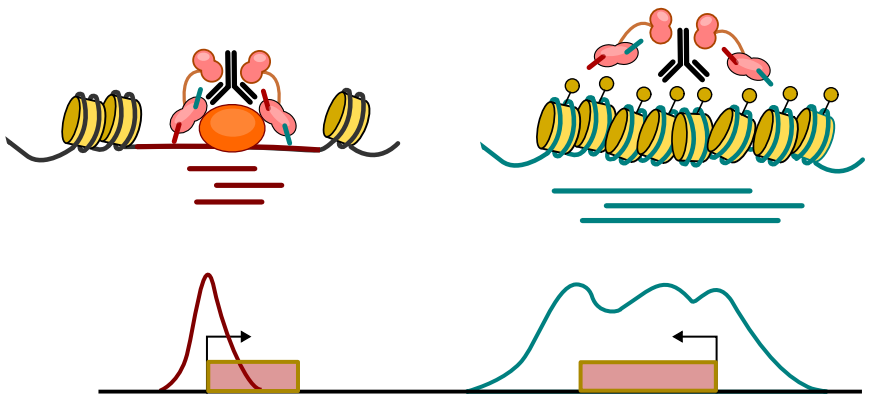
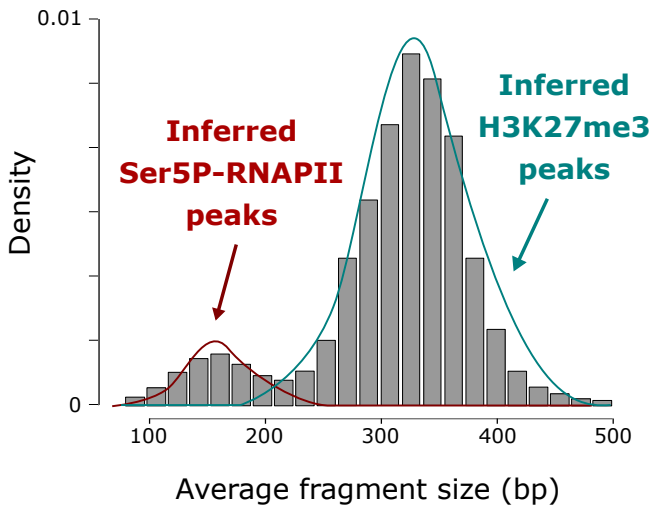


Figure 3

CUT&Tag2for1

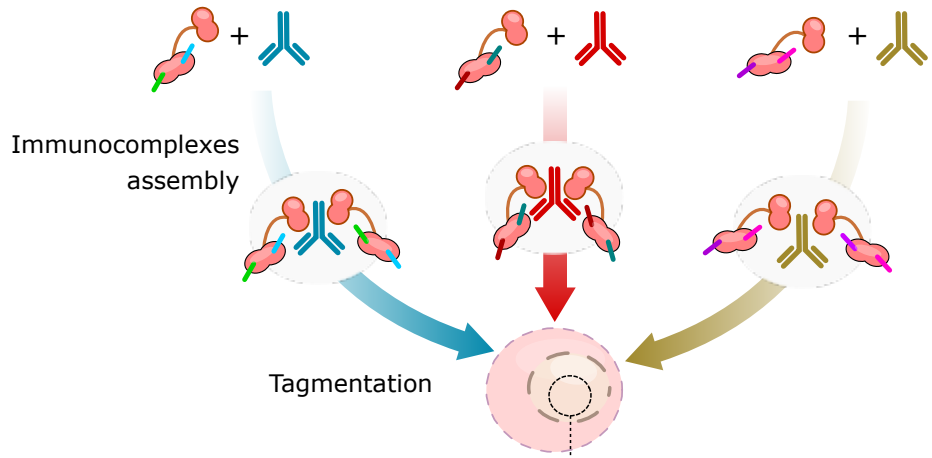
anti-Ser5P-RNAPII + anti-H3K27me3

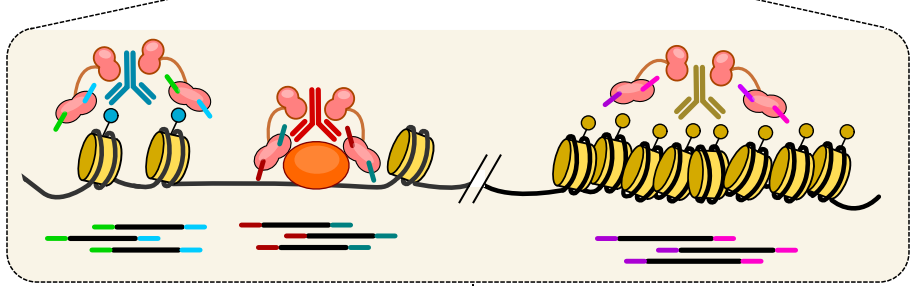




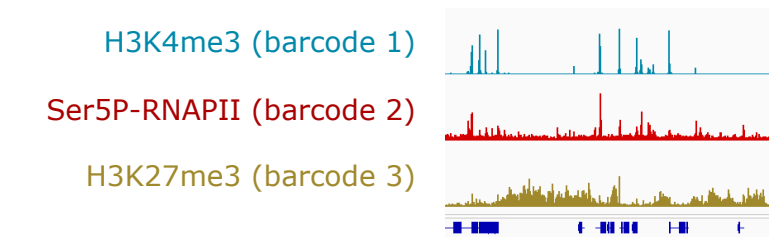
Multi-CUT&Tag / Multi-Tag / ACT2-Seq

anti-H3K4me3 + anti-Ser5P-RNAPII + anti-H3K27me3





Multiple antibody readout in the same sample or single cell



: Ser5P-RNAPII : H3K27me3 nucleosome : H3K4me3 nucleosome : Barcoded & Illumina adaptor-loaded pA/G-Tn5

Figure 4