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Review

# Single cell cancer epigenetics

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**Bulk sequencing methodologies have allowed us to make great progress in cancer research. Unfortunately, these techniques lack the resolution to fully unravel the epigenetic mechanisms that govern tumor heterogeneity. Consequently, many novel single cell-sequencing methodologies have been developed over the past decade, allowing us to explore the epigenetic components that regulate different aspects of cancer heterogeneity, namely: clonal heterogeneity, tumor microenvironment (TME), spatial organization, intratumoral differentiation programs, metastasis, and resistance mechanisms. In this review, we explore the different sequencing techniques that enable researchers to study different aspects of epigenetics (DNA methylation, chromatin accessibility, histone modifications, DNA–protein interactions, and chromatin 3D architecture) at the single cell level, their potential applications in cancer, and their current technical limitations.**

## Highlights

The epigenome encompasses several mechanisms controlling gene expression that can be aberrantly regulated during cancer development and progression. Tumors are highly complex and heterogeneous biological systems that require the study of epigenetic alterations at a single cell resolution.

Several single cell technologies developed to study different layers of the epigenome, such as chromatin accessibility or histone modifications, have been developed and applied in cancer research over the past few years, improving our understanding of the mechanisms driving tumorigenesis.

Although these techniques are promising, most are still nascent and present limitations, such as low throughput and limited coverage. In addition, the analysis and integration of the various single cell epigenomic data modalities have challenges and require the development of new computational tools.

## Need for single cell epigenetic approaches to unravel tumor heterogeneity

The importance of epigenetics in both basic and clinical research is indisputable. In the field of cancer, epigenetic alterations have important implications for many aspects of this disease. Indeed, non-mutational epigenetic reprogramming was recently designated as a mechanistic determinant that enables the acquisition of cancer hallmark capabilities [1]. Although it is well established that cancer cells may arise from genetic mutations that drive carcinogenesis, many types of tumor lack strong genetic drivers that could explain important malignant processes, such as tumor progression [2], resistance to therapy [3], and metastasis [4], suggesting that non-genetic determinants have a crucial role in cancer [5]. Thus, alterations of the epigenetic mechanisms affecting both malignant and non-malignant cells in a tumor may act as critical non-genetic determinants of cancer evolution. These epigenetic mechanisms, which regulate the expression of genes without altering the DNA sequence, fall into five main categories: (i) DNA methylation; (ii) chromatin accessibility; (iii) histone modifications; (iv) DNA–protein interactions; and (v) chromatin tridimensional architecture [6,7]. Each type of mechanism can be experimentally studied using several bulk methodologies (Box 1). Unfortunately, due to the complex cellular heterogeneity of many types of tumor, valuable information is lost when using these techniques, since all the possible data that could be retrieved from a single cell point of view are masked by the bulk cell averaging. Nonetheless, with the emergence of single cell-sequencing technologies [5,8], many aspects of this tumoral heterogeneity that were otherwise impossible to assess are now open for exploration.

A tumor is a highly heterogeneous entity comprising malignant and non-malignant cells, each of which has crucial roles in cancer progression [9]. The development of single cell epigenomic sequencing technologies can help to properly dissect non-genetic dependencies of malignant progression and unravel this tumor complexity. There are six important aspects of cancer biology related to tumor heterogeneity in which epigenetic alterations have a key role (Figure 1): (i) clonal heterogeneity; (ii) TME; (iii) spatial organization and intercellular crosstalk; (iv) differentiation and developmental programs (phenotypic plasticity); (v) metastasis; and (vi) the appearance of new resistance mechanisms to therapy. Thus, it is necessary to develop single cell resolution technologies that allow us to understand the epigenetic cues that are otherwise undetectable

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Box 1. Bulk methodologies to analyze epigenetic mechanisms

Various bulk methodologies have been used to understand epigenetic mechanisms: (i) DNA methylation, taking advantage of bisulfite chemistry, can be analyzed by whole-genome bisulfite sequencing (WGBS), reduced representation bisulfite sequencing (RRBS), or 450k/850k Illumina methylation arrays [128]; (ii) DNA accessibility is mainly studied using the assay for transposase-accessible chromatin sequencing (ATAC-seq) [129]; and (iii) histone modifications and (iv) DNA–protein interactions can be studied by chromatin immunoprecipitation sequencing (ChIP-seq) [130]; (v) chromatin 3D architecture can be explored with multiple types of chromosome conformation capture technology, such as 3C, 4C, 5C, Hi-C, promoter-capture Hi-C, and ChIA-PET [131,132]. One important common drawback among these techniques is the need for a considerable sample size, demanding thousands to millions of cells as the minimal input. Thus, these techniques are considered 'bulk methodologies', by which we obtain an average value from the whole-cell bulk [133]. Various epigenetic deconvolution strategies can be applied to bulk data, but with a substantial risk of retrieving artifacts or losing difficult-to-detect minor subclones [134]. Nevertheless, bulk methodologies have been indispensable tools for our current understanding of epigenetics and its relationship with cancer. For example, they allowed for the methylation-based classification of diffuse gliomas (LGm1-LGm6) [135], the potential classification of cancers of unknown primary [123], and the histone modification-based tracking of cell differentiation states [136].

using bulk methodologies. In this review, we catalog current technologies that facilitate the study of different epigenetic characteristics at the single cell level. We classify each technology based on the epigenetic mechanism under study (DNA methylation, chromatin accessibility, histone modifications and DNA–protein interactions, and chromatin 3D architecture), focusing first on mono-omic methodologies (techniques that allow the study of only one epigenetic mechanism on a single cell) and then on multi-omic methodologies (which allow the study of multiple layers of information simultaneously on a single cell). In addition, we summarize currently available single cell spatial epigenomic methodologies and their potential in cancer research. We also highlight recent discoveries and insights gained from these single cell epigenetic technologies, how they can contribute to solve many current challenges in cancer research (mostly derived from tumor heterogeneity), their current limitations, and their potential in translational/clinical scenarios.

## Tumor heterogeneity and its relation to epigenetic alterations

### Clonal heterogeneity

A tumor can comprise multiple malignant subclones, each with unique genetic and epigenetic properties [10,11]. As a cancer population evolves, cells accumulate genetic and epigenetic alterations that contribute to the appearance of new clones that may harbor novel, selective advantages

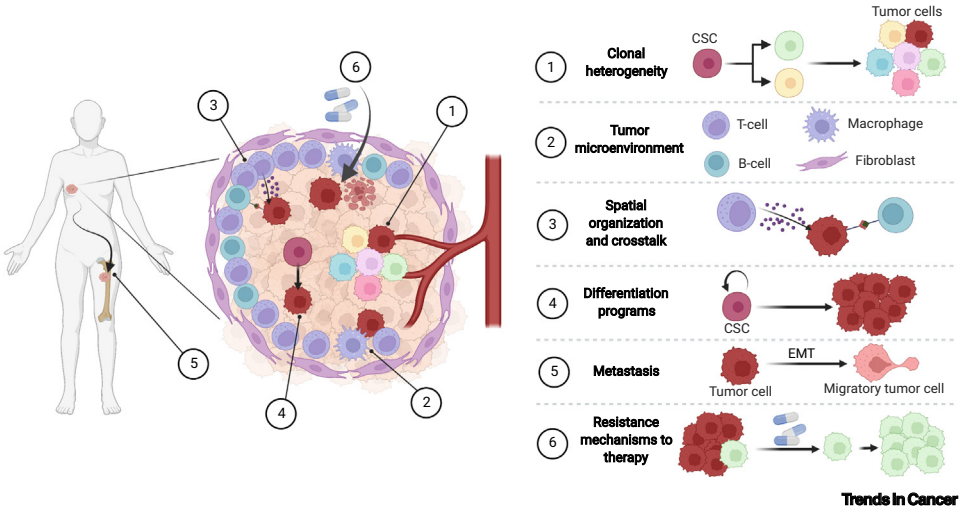


Figure 1. The six main aspects of tumor heterogeneity that are difficult to assess using bulk methodologies.
Abbreviations: CSC, cancer stem cell; EMT, epithelial–mesenchymal transition.

(e.g., enhanced proliferation, resistance to therapy, invasiveness, etc.) [5]. The detection of these clones is crucial to understand tumor progression and its impact on clinical outcome. Single cell methodologies are able to detect each one of these clones (especially minor, difficult-to-detect, subclones), thus revealing valuable prognostic information.

### Tumor microenvironment

A tumor does not comprise solely malignant cells but harbors myriad types of non-malignant cell with distinct roles in cancer progression. The T cell content is directly associated with tumor progression in many cancer types, with cytotoxic T cells (Tc) and helper T cells (Th1, Th2, and Th17) correlating with good prognosis [12]. Tumor-associated macrophages have crucial roles in cancer progression, depending on their M1/M2 differentiation state [13]. Additionally, natural killer (NK) cells, B cells, endothelial cells, fibroblasts, and other cell types participate in this complex interactome [14]. These microenvironmental interactions profoundly modulate the epigenome of both tumoral and nontumoral cells, generating an epigenetic crosstalk that directly determines cancer progression [15]. Thus, studying these epigenetic signals at the single cell level is mandatory to decipher this complex tumoral interactome.

### Spatial organization and intercellular crosstalk

Malignant and non-malignant cells are not randomly distributed inside a tumor, but instead occupy specific positions in the tumoral space, generating discrete cell–cell interactions that impact disease progression [16]. Knowing the spatial distribution of each cell has been crucial for assessing the 'heat' of certain types of tumor (e.g., melanoma), in which the relative quantity and position of cytotoxic T cells are key determinants of cancer progression [17]. In addition, DNA methylation heterogeneity is dependent on the spatial organization of colorectal cancer (CRC) cells in patients with locoregional cancer, correlating with relapse-free and overall survival [18]. Microscopy techniques, such as immunohistochemistry. have enabled great advances in this aspect. Nevertheless, these techniques lack the resolution and specificity to unveil the different epigenetic characteristics for each cell. Thus, single cell-sequencing technologies (including cutting-edge spatial epigenomics) will enable researchers to infer how spatial cues correlate with epigenetic changes inside a tumor, which is of strong clinical value.

### Differentiation and developmental programs (phenotypic plasticity)

The cancer stem cell hypothesis states that tumor growth depends, at least in part, on the asymmetrical divisions of malignant stem cells that differentiate to specific types of committed cancer cell [19]. In addition, depending on their epigenetic background, each malignant cell can follow a specific developmental program that will impact the progression of cancer. For example, in glioblastoma, there are at least four types of malignant cell state program, some related to higher stemness (neural progenitor-like and oligodendrocyte progenitor-like cells), and others related to a more differentiated state (astrocyte-like and mesenchymal-like cells) [20]. The identity of each cell is maintained by epigenetic memory mechanisms (e.g., DNA methylation) that ensure full commitment to specific transcriptional programs [21]. Thus, detecting alterations in this epigenetic machinery at the single cell level may provide valuable information on potential malignant differentiation trajectories, predicting how the tumor may progress and deciding which type of treatment should be applied.

### Metastasis

Some cancer cells acquire the ability to leave their primary site and colonize distant tissues, which is the cause of most cancer-related deaths [22]. From its transformation until its settlement on a new tissue, the metastatic cancer cell experiences drastic changes, such as acquiring a higher motility program (epithelial–to-mesenchymal transition), avoiding immune cell surveillance, and

adapting to the new secondary site [22]. No genetic driver mutations specific to metastasis have yet been identified, suggesting that dynamic epigenetic mechanisms are involved in key steps of metastasis [4,23]. Single cell technologies will be useful to confidently detect in primary and secondary sites those cancer cells that have a metastatic-prone epigenetic background.

### Resistance mechanisms to therapy

Certain malignant subclones that are undetectable by bulk methodologies due to their low abundance may harbor key mutations and epimutations that render them resistant to treatments that otherwise affect other more abundant subclones [24]. These resistant subclones are most likely to become the predominant ones after the first line of treatment, representing the most common cause of relapse. Alterations in epigenetic mechanisms have been strongly linked with antitumoral drug resistance [25]. For example, during bortezomib treatment in multiple myeloma, certain cancer subclones enter a slow-cycling, drug-tolerant reversible state, as a consequence of epigenetic plasticity rather than of genetic determinants. Another case of non-genetically determined resistance to therapy are alterations in histone H3 lysine 4 demethylases, such as KDM5, which contribute to transcriptomic heterogeneity in breast cancer, leading to a decreased sensitivity to antiestrogens [26]. In taxane-resistant triple-negative breast cancer (TNBC), global DNA hypomethylation and relocation of histone H3K27 trimethylation enable an epigenetic state that enables cancer cells to become resistant to paclitaxel, thus creating a new therapeutic vulnerability by using epigenetic inhibitors [27]. There are many more well-documented cases in which non-genetic determinants are the main drivers of the appearance of new resistant subclones [28]. Thus, detecting these resistant subclones early during diagnosis, using single cell epigenetic technologies, would significantly help clinicians to select the best treatment combinations. Additionally, the ability to detect minimal residual disease after treatment is fundamental, because it constitutes a prognostic biomarker that can predict relapse in some cancers [29].

## Single cell mono-omic technologies to explore cancer epigenomics

Single cell techniques encompass a breakthrough methodology that has revolutionized the way in which complex biological systems can be characterized by looking at one cell at a time. Single cell approaches are essential to properly examine the underlying complexity of tumors and explore cellular heterogeneity at several levels. With the advent of single cell RNA-sequencing (scRNA-seq), the transcriptome has become the molecular level most exploited by single cell technologies. It has accelerated our understanding of cancer biology, enabling the characterization of the intratumoral heterogeneity and cellular architecture of several cancer types at unprecedented resolution [20,30–33]. Additionally, there are emerging single cell DNA-sequencing technologies that allow us to profile, in an amplicon-based and targeted manner, recurrently mutated genes, providing the genotype of every cell by detecting single nucleotide variants (SNPs) and copy number variants (CNVs) [34]. Nevertheless, transcriptional cell state diversity among malignant cells in a tumor is often independent of genetic clonal heterogeneity, highlighting the importance of developing epigenetic single cell analysis tools to assess this heterogeneity [35]. Although single cell techniques aimed at studying the epigenome have not evolved as rapidly compared with those for the transcriptome, new approaches are being developed to explore the different epigenetic mechanisms of gene regulation.

### DNA methylation

5-Methylcytosine (5mC) is the most well-known DNA modification. In mammals, this methylation mostly occurs in cytosines that are followed by a guanine, forming a 5′-to-3′ CpG pair. Approximately 70% of all human gene promoters are enriched with multiple clustered CpG pairs, forming ‘CpG islands’ that are prone to 5mC methylation [36]. In these regions, methylation acts as a repressive switch, restricting gene expression. Additionally, 5mC can be found in other genomic

regions, such as gene bodies and distant regulatory regions (enhancers and CTCF sites), regulating their function in *cis*. In most types of cancer, DNA methylation is significantly deregulated. Promoter hyper/hypomethylation in tumor suppressors/oncogenes is a well-established driver of tumoral progression [37]. In addition, deregulation in enhancer methylation and other distant regulatory regions may have crucial implications in cancer by fostering tumoral epigenetic heterogeneity [38].

Bulk methodologies helped revolutionize our understanding in this area. Most of these methodologies are based on the conversion of unmethylated cytosines to uracil after bisulfite treatment of the DNA. This allows the detection of methylated cytosines using sequencing or array-based methods [18]. However, as previously stated, bulk methodologies lack the resolution to unravel the epigenetic variability of a tumoral population. Therefore, in 2013, the first bisulfite-based sequencing methodology was developed to detect DNA methylation at the single cell level, single cell-reduced representation bisulfite sequencing (scRRBS) [39,40], which uses enzymatic cleavage to generate DNA fragments with CpG-rich ends. Since then, multiple bisulfite-based single cell-sequencing technologies have been developed (Table 1): post-bisulfite adaptor tagging (PBAT) technology increases CpG coverage from 3–5% to an average of 18% unique CpGs (up to 48% at saturating sequencing depths) [41], enabling the establishment of new single cell DNA methylation-sequencing technologies, such as scBS-Seq [41], scWGBS [42], and scPBAT [43], with the expense of capturing less-enriched CpG regions and generating more PCR duplicates, especially when applying high-sequencing depths. Whether to use scRRBS-based or whole genome-based approaches depends on the user's preference: scRRBS enables the capture of CpG-island regions (which are usually found in gene promoters), while single cell whole-genome methods capture other, less-enriched, CpG regions, although with higher CpG coverage. Interestingly, there are loci-specific, bisulfite-based alternatives, such as multiplexed-scAEBS, which enable the analysis by PCR of the methylation status of specific CpGs directly from single cells [44]. There are many other bisulfite-based mono-omic methods available, which are beyond the scope of the current review. Nevertheless, the reader can find a comprehensive catalog of all these methods in Table 1. Unfortunately, bisulfite-based single-cell DNA methylation sequencing currently has significantly low coverage, among many other important limitations, summarized in Box 2.

By contrast, unstable DNA modifications, such as 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), are intermediates that serve as useful fingerprints for detecting DNA regions that are being actively demethylated. Single cell detection methods for these intermediates, such as single-cell restriction endonuclease AbaSI sequencing (scAba-Seq, for detecting 5hmC) [45], chemical labeling-enabled C-to-T conversion sequencing (CLEVER-Seq, for 5fC) [46], and single cell methylase-assisted bisulfite sequencing (scMAB-Seq, for both 5fC and 5caC) [47], are promising but have not yet been applied in cancer research.

In addition to bisulfite-based methodologies, novel nonbisulfite-based approaches have been developed, mostly based on methylation-sensitive restriction enzymes (MSREs). The first one developed was the restriction enzyme-based single-cell methylation assay (RSMA), which interrogates the CpG methylation status of specific genomic loci [48]. Some years later, genome-wide MSRE-based single cell technologies were developed, such as single-cell CpG-island sequencing (scCGI-seq) and epigenomics and genomics of single cells analyzed by restriction (epi-gSCAR), achieving up to 18.8% genome coverage [49,50]. Additionally, a novel targeted bisulfite-free method, named single cell-targeted analysis of the methylome (scTAM-seq), directly profiles 650 specific CpG sites in up to 10 000 cells using a commercial microfluidic platform [51]. By avoiding the use of bisulfite, we can ensure better DNA integrity, although nonbisulfite-based technologies still exhibit similar coverage to those that are bisulfite based.

Table 1. Currently available mono-omic methodologies for epigenetic single cell sequencing and spatial epigenomics

| Epigenetic mechanism | Technique | Designed by | Current applications in cancer |
|---|---|---|---|
| DNA methylation | scRRBS | [39,40] | Chronic lymphocytic leukemia [52], glioma [53] |
| | scXRBS | [145] | Acute myeloid leukemia cell lines [145] |
| | scBS-seq | [41] | Circulating tumor cells [146], CRC [54] |
| | scWGBS | [42] | Chronic myeloid leukemia cell lines [42], acute promyelocytic leukemia cell line [42], circulating metastatic tumor cells [56] |
| | scPBAT | [43] | N/A[a] |
| | PBAL | [147] | Acute myeloid leukemia [148] |
| | snmC-seq | [149] | CRC cell line [150] |
| | snmC-seq2 | [151] | N/A |
| | sci-MET | [152] | N/A |
| | MID-RRBS | [153] | N/A |
| | BRIF-seq | [154] | N/A |
| | scAba-seq (5hmC) | [45] | N/A |
| | CLEVER-seq (5fC) | [46] | N/A |
| | scMAB-seq (5fC/5caC) | [47] | N/A |
| | RSMA | [48] | CRC cell line [48] |
| | scCGI-seq | [49] | Chronic myeloid leukemia cell line [49] |
| | epi-gSCAR | [50] | Acute myeloid leukemia cell lines [50] |
| | scAEBS | [44] | Circulating tumor cells [44] |
| | scTEM-seq | [155] | Acute myeloid leukemia cell line [155] |
| | scTAM-seq | [51] | N/A |
| Chromatin Accessibility | scATAC-seq (microfluidics based) | [58,59] | Basal cell carcinoma [59], multiple myeloma [61], prostate cancer [62], triple-negative breast cancer [63,64], drug-resistant lung cancer [65], gynecological cancers [66], glioblastoma [156], renal cancer [157], breast metastasis [158], chronic lymphocytic leukemia [71], CRISPR perturbation [68–70], Barret metaplasia [159], bladder cancer [160], drug-resistant leukemic T cells [161], lung adenocarcinoma metastasis [162] |
| | scATAC-seq (plate based) | [60] | N/A |
| | sciATAC-seq | [163] | Mouse lung adenocarcinoma [164] |
| | SNuBar-ATAC | [165] | Lung cancer [165] |
| | HyDrop-ATAC | [166] | N/A |
| | scMNase-seq | [73,74] | N/A |
| | scGET-seq | [167] | Cervical cancer cell line [167] |
| | sciMAP-ATAC | [109] | N/A |
| | Spatial ATAC-seq | [110] | N/A |
| Histone modifications and DNA-protein interactions | scChIP-seq | [75] | Breast cancer [77,78] |
| | scChIC-seq | [76] | N/A |
| | iscChIC-seq | [168] | N/A |

*(continued on next page)*

Table 1. (continued)

| Epigenetic mechanism | Technique | Designed by | Current applications in cancer |
|---|---|---|---|
| | scChIL-seq | [169] | Breast cancer cell line [169], chronic myeloid leukemia cell line [169] |
| | ACT-seq | [170] | N/A |
| | CoBATCH | [171] | N/A |
| | uliCUT&RUN | [172] | N/A |
| | scCUT&Tag | [173,174] | Glioblastoma [175], various cancer cell lines [176] |
| | AutoCUT&Tag | [79] | Mixed-lineage leukemia [79], chronic myeloid leukemia cell line [79] |
| | sciTIP-seq | [177] | CRC cell line [177] |
| | REpi-seq | [178] | N/A |
| | Spatial-CUT&Tag | [107] | N/A |
| | Epigenomic MERFISH | [108] | N/A |
| Chromatin 3D interactions | scHiC | [82,83] | Cervical cancer cell line [83], chronic myeloid leukemia cell line [83] |
| | scDAM-ID | [84] | Chronic myeloid leukemia cell line [84] |

[a]N/A indicates methodologies that have not yet been applied in cancer research.

Although single cell DNA methylation-sequencing technologies are still nascent, they have already provided new crucial insights into important questions about tumor heterogeneity, such as intratumoral epigenetic diversity, TME, and metastasis. Using scRRBS on patient-derived chronic lymphocytic leukemia (CLL) [52] and glioblastoma samples [53], we can observe the significant intratumoral epigenetic diversity of cancer cells, showing that important epigenetic routes significantly contribute to tumor heterogeneity. scBS-seq in CRC tumors showed that some partial methylation domains (PMDs) exhibit extensive epigenetic heterogeneity between cells of the same tumor, which can be explained by TME cues [54]. scBS-seq has even been applied to the study of patients with common variable immune deficiency (CVID), in which B cell differentiation is impaired and increases the risk of malignancy by up to 12-fold [55]. Lastly, scWGBS showed that circulating tumor cells (CTCs), which form clusters to increase their metastatic potential, exhibit

### Box 2. Current limitations of single cell epigenetic-sequencing technologies

Enormous efforts have been made to develop epigenomic tools capable of profiling, individually and simultaneously, the different epigenetic layers at a single cell resolution. However, the application of these technologies is still nascent and not well established. This is partly due to the current challenges these approaches pose, such as low throughput, limited coverage per cell, and elevated costs [137]. Many experimental issues need to be tackled, such as amplification bias, differences of library sizes across samples, and DNA damage upon processing (e.g., bisulfite treatment), among others. Additionally, the numerous techniques and methodologies used for the same purpose (e.g., there are more than ten epigenetic techniques for only studying single cell DNA methylation, see Table 1 in the main text) emphasizes the lack of standardization and the need to perform benchmarking across these technologies.

Conversely, computational analysis represents a major challenge due to the nature of single cell data, characterized by large-scale dimensions and high intrinsic noise and sparsity. Since the resulting data matrixes are often sparse (due to low coverage), most approaches cannot be loci specific (e.g., in single cell DNA methylation), but instead clusters are generated (t-SNEs/UMAPs) after high-dimensional data reduction. To compensate for missing values, imputation (e.g., KNN), pseudo-bulking, and genomic binning are often used. It is also necessary to evaluate the integration of multi-omics data, including paired multimodal data. New multi-omic integrative pipelines were recently designed to facilitate the analysis of multiple layers of epigenetic data simultaneously, such as MOFA+ [138,139], scMVP [140], Babel [141], Maestro [142], or EpiScanpy [116]. In addition, new user-friendly interfaces, such as ShinyArchR for scATAC-seq [143] or ChromSCape for scHi-C [144], will make analysis more accessible for nonbioinformaticians.

significant promoter hypomethylation of key stemness-/proliferation-associated genes, mimicking embryonic stem cell biology; treatment with Na+/K+ ATPase inhibitors leads to DNA methylation of these regions, resulting in CTC dissociation and metastasis suppression [56].

### Chromatin accessibility

Genome-wide DNA accessibility assays facilitate the study of epigenomic alterations by identifying DNA regulatory elements (REs), such as gene promoters and enhancers, involved in the repression or activation of gene expression. These noncoding regions are relevant to understand cancer biology and identify pathways controlling tumorigenesis [57].

The most used technique to investigate this at the single cell resolution is single-cell ATAC-seq (scATAC-seq), or an alternative modality based on combinatorial cellular indexing (sciATAC-seq). Both methods are based on inserting sequencing adapters into accessible genome areas to facilitate Tn5 transposition as a measure of open chromatin [58]. There are two main protocols: microfluidics techniques encompassing either droplet-based [59] or plate-based techniques [60]. Several studies have used scATAC-seq, often in combination with scRNA-seq, to dissect the intrinsic properties of different cancer types and associate chromatin accessibility variation with gene expression.

Following this approach, a study investigated the mechanisms behind cellular plasticity in multiple myeloma, highlighting the acquisition of aberrant transcriptional states within cancer cells promoted by epigenetic reprogramming affecting cell chromatin landscape and enhancer usage. Owing to this unprecedented resolution, it was observed that these different transcriptional states coexist within individual myeloma cells, conferring them a plasticity that leads to dedifferentiation and that promotes the expression of surface markers that are not specific to the lineage and that could represent new immunotherapeutic targets. Moreover, this study showed that myeloma cells dedifferentiate independently of their genotype, revealing the possibility to target epigenomic rather than genomic states. These insights show the potential of scATAC-seq to unveil tumoral epigenomic heterogeneity and to discover new actionable targets [61]. With the same methodology, a study conducted in prostate cancer identified pre-existing and treatment-persistent cell subsets with regenerative properties after exposure to therapy [62]. Additionally, scATAC-seq profiling of the TME in basal cell carcinoma facilitated the characterization of regulatory networks in immune, stroma, and malignant cells. This technique enabled the identification of regulatory mechanisms related to T cell exhaustion in tumor-infiltrating lymphocytes [59].

scATAC-seq is also useful to understand variations in the response to cancer treatment. For example, profiling of chromatin accessibility and the transcriptome of immune cells in patients with TNBC treated with chemotherapy alone or in combination with checkpoint inhibitors revealed that high levels of baseline CXCL13+ T cells had a pivotal role in effective responses to the combination treatment [63]. scATAC-seq also revealed synthetic lethalities and epigenetic resistance mechanisms after treatment in TNBC and lung cancer [64,65]. Additionally, the *de novo* acquisition of RE, analyzed by scATAC-seq, has been reported to drive oncogenic pathways in gynecological malignancies [66].

Chromatin accessibility is also relevant to differentiate between mature and stem-like populations, being the key determinants of the latter in many cancers. A recent study in primary glioblastoma using scATAC-seq revealed that tumor-initiating cells are heterogeneous, exhibiting three different states associated with survival, which are controlled by unique transcription factors (TFs) [67].

Single cell chromatin accessibility profiling can be used in combination with CRISPR/Cas9 technology to detect changes in the epigenome preceded by CRISPR perturbation and explore TF-binding dynamics in cancer [68–70]. More recently, a new protocol involving the modification

of scATAC-seq to enrich transposable-accessible mitochondrial DNA and to infer tumoral clonal dynamics has been developed [71,72].

In addition to these approaches, another technique, called single cell micrococcal nuclease sequencing (scMNase-seq), allows the measurement of chromatin compaction as well as nucleosome positioning in single cells [73,74]. To do so, the linker DNA between the nucleosome cores is digested by MNase, enabling subsequent sequencing of the protected DNA regions.

### Histone modifications and DNA–protein interactions

Another layer of epigenetic regulation is orchestrated by a range of post-translational modifications in histones, which lead to transcriptionally permissive or repressive chromatin states. The characterization of these histone marks at a single cell resolution can help to properly dissect regulatory heterogeneity within cancerous cells, enabling the detection of rare chromatin states that might be associated with drug resistance and relapse. This is achievable owing to the development of several techniques that facilitate the profiling of histone modifications in single cells. One such technique is single-cell ChIP-seq (scChIP-seq), which can map histone marks, TFs, and other DNA-interacting proteins within the genome [75]. Similarly, single-cell chromatin immunocleavage sequencing (scChIC-seq) is a method to analyze histone modifications at a single cell resolution. By the binding of MNase to a specific antibody that targets a histone modification of interest, the surrounding DNA is cleaved into small fractions followed by sequencing [76]. Other novel techniques are detailed in Table 1.

These methodologies can facilitate the segmentation of tumor cells based on their histone modification profiles, fostering the identification of rare populations with distinct properties. By applying scCHIP-seq in patient-derived xenograft models of TNBC, a repressive H3K27me3 mark was found to be depleted in several genes associated with chemotherapy resistance in a subset of resistant cells. Interestingly, these chromatin features were also found in a fraction of cells from the untreated drug-sensitive tumor, suggesting that cells with epigenetic characteristics of drug resistance already exist before therapy [77]. Another recent study in TNBC, also using scCHIP-seq, showed that cancer cells exhibit bivalent chromatin states with permissive H3K4me3 and repressive H3K27me3 modifications, indicating that those cells that manage to deplete H3K27me3 marks are prone to becoming chemotolerant. The authors also showed that treating cells with a combination of a H3K27me3 demethylase inhibitor and chemotherapy reduced the number of persistent cells, suggesting an enhanced therapeutic approach for TNBC [78]. Finally, a similar study was performed in samples from patients with mixed-lineage leukemia using an automatized variant of scCUT&Tag (AutoCUT&Tag) for studying the leukemogenic histone methyltransferase KMT2A, uncovering the epigenetic heterogeneity of patient samples and predicting sensitivity to DOT1L pharmacological inhibition [79]. These promising discoveries reinforce the potential of the aforementioned methodologies to study tumor cell epigenomes from a single cell perspective.

### Chromatin 3D architecture

The 3D structure of the genome is governed by chromosome conformation and folding within the nucleus and has been reported to have a significant role in the regulation of gene expression by, for instance, promoting the interaction of enhancers and promoters that regulates the expression of specific genes [80]. The nuclear architecture comprises chromosomal compartments that facilitate the emergence of *trans*-regulatory elements, long-range loops, topologically associating domains (TADs), and lamina-associated domains (LADs). In some cancers, this genome architecture is disrupted due to genomic rearrangement or structural variations, affecting the regulatory landscape of the cancer cell [81].

Single cell Hi-C (scHi-C) enables in-depth interaction profiling by relying on proximity ligation followed by massively parallel sequencing. It helps to decipher millions of loci concomitantly and assess cell–cell variability in terms of chromosome structure within the nucleus [82,83]. Although there are many studies that have used Hi-C to explore the genome architecture of different types of cancer, they have been performed at the bulk level. Additionally, a technology called single cell DNA adenine methyltransferase identification (scDamID), enables genome-wide mapping of LADs at a single cell resolution [84]. Currently, neither single cell 3D approaches have been applied in cancer research.

## Single cell epigenetic multi-omic approaches

Complex biological systems, such as cancer, cannot be fully understood by either exploration at the bulk level or by inspection of individual layers of information. Therefore, it is crucial to integrate different 'omics at a single cell resolution to decipher the mechanisms and regulatory heterogeneity that drive cancer development and progression (Figure 2) [85].

The recent development of tools has permitted more comprehensive epigenomic analysis by simultaneously exploring several layers of the epigenome in individual cells. Single-cell Nucleosome

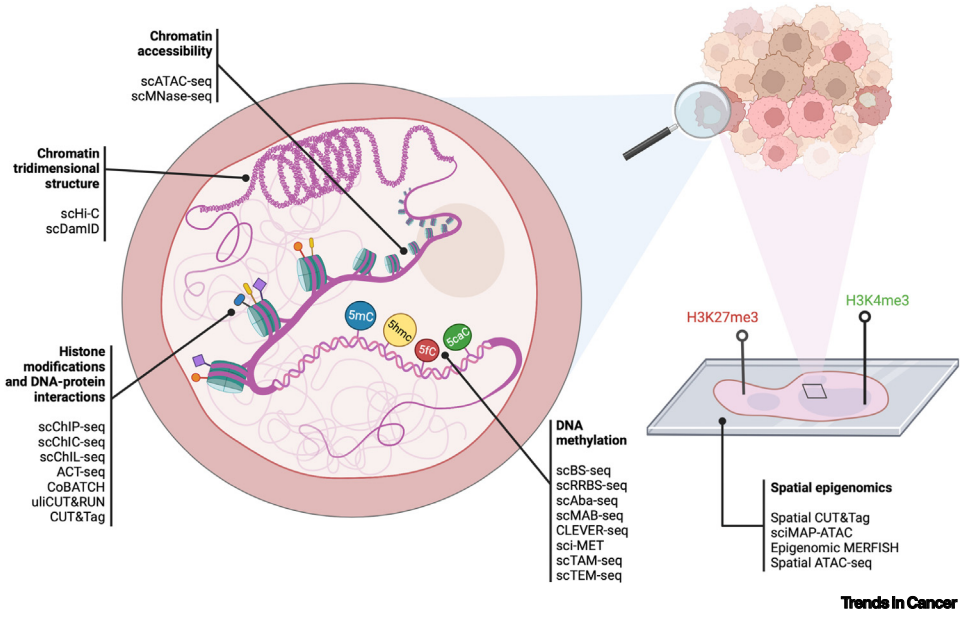


Figure 2. Single cell-sequencing and spatial technologies to study the different epigenetic mechanisms in cancer. Abbreviations: ACT-seq, antibody-guided chromatin tagmentation sequencing [170]; CLEVER-seq, chemical-labeling-enabled C-to-T conversion sequencing [46]; CoBATCH, combinatorial barcoding and targeted chromatin release [171]; epigenomic MERFISH, epigenomic multiplexed error robust fluorescence *in situ* hybridization [108], scABA-seq, single cell restriction endonuclease AbaSI sequencing [45]; scATAC-seq, single cell sequencing assay for transposase-accessible chromatin [59]; scBS-seq, single cell bisulfite sequencing [41]; scChIC-seq, single cell chromatin immunocleavage sequencing [76]; scChIL-seq, single cell chromatin integration labeling [169]; scChIP-seq, single cell chromatin immunoprecipitation followed by sequencing [75]; scCUT&Tag, single cell cleavage under targets and tagmentation [174]; scDamID, single cell DNA adenine methyltransferase identification [84]; scHi-C, single cell Hi-C [82,83]; sciMAP-ATAC, single cell combinatorial indexing on microbiopsies assigned to positions for the assay for transposase accessible chromatin [109]; sci-MET, single cell combinatorial indexing for methylation analysis [152]; scMAB-seq, single cell methylase-assisted bisulfite sequencing [47]; scMNase-seq, single cell micrococcal nuclease sequencing [74]; scRRBS-seq, single cell reduced representation bisulfite sequencing [39,40]; scTAM-seq, single cell targeted analysis of the methylome [51]; scTEM-seq, single cell transposable element methylation sequencing [155]; spatial ATAC-seq, spatially resolved chromatin accessibility profiling [110]; spatial CUT&Tag, spatial histone modification profiling with cleavage under targets and tagmentation [107]; uliCUT&RUN, ultra-low-input cleavage under targets and release using nuclease [172].

Occupancy and Methylome sequencing (NOME)-seq enables the profiling of chromatin accessibility and DNA methylation status of each cell [86]. Furthermore, it is also possible to simultaneously analyze the chromatin 3D structure and the methylome in individual cells with tools such as scMethyl-HiC and sn-m3C-seq [87,88]. Additionally, single-cell Chromatin Overall Omic-scale Landscape Sequencing (scCOOL-seq) can provide a more in-depth characterization of the epigenome, by exploring the nucleosome positioning, chromatin accessibility, DNA methylation, copy number alterations (CNAs), and ploidy in each single cell [89]. A variation of scCOOL-seq that also combines scRNA-seq (scCOOL-seq + scRNA-seq) allowed the identification of two novel prognosis pancreatic ductal adenocarcinoma biomarkers (ZNF667 and ZNF667-AS1) [90]. As a side note, in a similar fashion to some bulk DNA methylation array-based methods [91], CNAs can also be inferred from mono-omic single cell DNA methylation-sequencing technologies, by splitting the genome in windows/bins of equal size, calculating the number of CpGs detected per window, and normalizing by the total number of detected CpGs per cell, using non-malignant cells as a baseline reference [35].

It is relevant to combine epigenomic approaches with the simultaneous interrogation of the transcriptome to understand how the epigenome landscape of a cell is reflected in its transcriptional state. In most cases, expression heterogeneity is not fully explained by genetic alterations, but rather by cell–cell epigenetic cues. An example of this is the joint multiplexed-scRRBS/scRNA-seq dual-protocol (Smart-RRBS), optimized by the Landau lab, which allows the simultaneous interrogation of DNA methylation, transcriptomics, and CNAs in a single cell and has been successfully used to unravel the epigenetic clonal heterogeneity found in CLL and glioblastoma cells [35,92]; both of these studies show how important the intratumoral epigenetic heterogeneity in cancer progression is: in the first study, Gaiti *et al.* demonstrated that epigenetic information enables the mapping of CLL lineage history and the prediction of its evolution after therapy [92]. In the second study, Chaligne *et al.* demonstrated the epigenetic heritability in glioma cells, with key differences in cell plasticity states between IDH-mutant cells and IDH-wild-type glioblastoma [35].

Another multi-omic protocol is scTrio-seq, which also interrogates single cell DNA methylome, transcriptome, and CNAs, and has enabled study of tumoral heterogeneity by identifying epigenetically diverse cancer subpopulations in samples from patients with either CRC or hepatocellular cancer that may reflect the different lineages in the tumor [93,94]. There are many additional tools that combine single cell transcriptomics with DNA methylation (scM&T-seq [95], scTrio-seq [94], and snmCT-seq [96]), chromatin accessibility (scCAT-seq [97] and joint scATAC+RNA-seq [98]), DNA–protein interactions (scDam&T-seq [99]) and multiple epigenomic layers (scNMT-seq [100] and scNOMeRe-seq [101]).

Every additional piece of information that we could gain in a single cell will allow a better understanding of the co-occurrence of certain genetic/transcriptomic/epigenomic states in a tumor. Currently, scCOOL-seq coupled with scRNA-seq [90] is the one technique that simultaneously analyzes the most layers. There are more multi-omic approaches available, catalogued in Table 2.

## Spatial epigenomics

Although all these technologies provide relevant information on the epigenome of each individual cell, its spatial localization within the tumor is lost upon dissociation. Spatial technologies based on RNA, DNA, multiplexed fluorescence, and isotope labeling now enable exploration of the cell type composition of a tissue preserving the spatial information [102–104]. Incorporation of the spatial context has helped investigation of the architecture of several tumors, as well as understanding how both malignant and non-malignant cells, such as stromal and immune

Table 2. Currently available multi-omic methodologies for epigenetic single cell sequencing

| Technique | Basis | Designed by | Current applications in cancer |
|---|---|---|---|
| scM&T-seq | Methylome + transcriptome + genotype | [95] | N/A[a] |
| scMT-seq | Methylome + transcriptome | [179] | N/A |
| Smart-RRBS | Methylome + transcriptome + CNA | [92,180] | Chronic lymphocytic leukemia [91], Glioblastoma [35] |
| scmCT-seq | Methylome + transcriptome | [96] | N/A |
| scGEM | Methylome + transcriptome + genotype | [181] | Lung cancer [181] |
| scTrio-seq | Methylome + transcriptome + CNA | [94] | CRC [93], hepatocellular carcinoma [94] |
| scNMT-seq | Methylome + transcriptome + accessibility | [100] | N/A |
| scNOME-seq | Methylome + accessibility | [86] | Chronic myeloid leukemia cell line [86] |
| scNOMeRe-seq | Methylome + transcriptome + accessibility | [101] | N/A |
| scCOOL-seq | Methylome + accessibility + CNA + ploidy | [89] | N/A |
| iscCOOL-seq | Methylome + accessibility + CNA + ploidy | [182] | N/A |
| scCOOL-seq + scRNA-seq | Methylome + accessibility + CNA + transcriptome | [90] | Pancreatic ductal carcinoma [90] |
| scCAT-seq | Accessibility + transcriptome | [97] | Lung cancer [97], various cancer cell lines [97] |
| Joint scATAC-Seq/scRNA-seq | Accessibility + transcriptome | [98] | Chronic myeloid leukemia cell line [98] |
| scDAM&T-seq | Protein-DNA interactome + transcriptome | [99] | Chronic myeloid leukemia cell line [99] |
| T-ATAC-seq | Accessibility + TCR sequencing | [183] | Acute T cell leukemia cell line [183] |
| scMethyl-HiC | Methylome + chromatin conformation | [86] | N/A |
| sn-m3C-seq | Methylome + chromatin conformation | [87] | N/A |
| SNARE-seq | Accessibility + transcriptome | [184] | Chronic myeloid leukemia cell line [184] |
| Paired-seq | Accessibility + transcriptome | [185] | Hepatocellular carcinoma cell line [185] |
| Paired-Tag | Histone modifications + transcriptome | [186] | N/A |
| scChaRM-seq | Methylome + transcriptome + accessibility | [187] | N/A |
| NEAT-seq | Accessibility + transcriptome + nuclear proteomics | [188] | Chronic myeloid leukemia cell line [188] |
| ASAP-seq | Accessibility + proteomics + mitochondrial DNA capture | [189] | N/A |
| Spear-ATAC | Accessibility + short guide RNA integration detection | [70] | N/A |
| Pi-ATAC | Accessibility + proteomics | [190] | Chronic myeloid leukemia cell line [190] |

*(continued on next page)*

Table 2. (continued)

| Technique | Basis | Designed by | Current applications in cancer |
|---|---|---|---|
| scCUT&TAG-pro | DNA–protein interactions + surface proteins | [191] | N/A |
| SHARE-seq | Accessibility + transcriptome | [192] | N/A |
| Multi-scale multiplexed FISH imaging technology for simultaneous imaging of genomic loci alone or with nascent RNA transcripts | Transcriptome + chromatin conformation | [193] | N/A |
| Multimodal approach (DNA seqFISH+, multiplexed immunofluorescence + RNA seqFISH) | Histone modifications + transcriptome + chromatin conformation | [194] | N/A |

[a]N/A indicates methodologies that have not yet been applied in cancer research.

cells, are displayed within the TME [105,106]. The opportunity to leverage this methodology by also spatially profiling epigenomic states within the tissue would provide exceptional resolution of the mechanisms driving cancer evolution. Recently, a study presented a spatial histone modification-profiling approach based on a combination of in-tissue deterministic barcoding and cleavage under targets and tagmentation (CUT&Tag) chemistry, called spatial-CUT&Tag. By genome-wide mapping of different histone modifications across a tissue section of mouse embryos, the authors were able to recapitulate the spatial position of the different cell types and characterize how epigenetic regulation is displayed throughout the tissue [107]. Epigenomic multiplexed error robust fluorescence *in situ* hybridization (Epigenomic MERFISH) is another recent tool for spatially histone modification profiling, which involves *in situ* tagmentation and transcription followed by highly multiplexed imaging. This high-resolution targeted approach allows mapping of single cell epigenomes in a tissue region [108]. In addition, there are spatially resolved epigenomics approaches that rely on measuring chromatin accessibility. Single cell combinatorial indexing on Microbiopsies Assigned to Positions for the Assay for Transposase Accessible Chromatin (sciMAP-ATAC) is a method that profiles the chromatin states of individual cells in a cubic region defined by a microbiopsy punch [109]. Finally, spatial ATAC-seq is another recent method for chromatin accessibility mapping that can reveal the epigenetic landscape at cellular level over a tissue section [110].

Although these developing methodologies are recent and have not yet been applied in cancer research, they hold the potential to explore how regulatory programs occur throughout the tumor tissue. By incorporating the spatial context, we will be able to visualize how cancer subclones with distinct epigenetic properties, such as stem-like subclones, are localized and investigate whether their position compromises their future clonal evolution and expansion. Apart from spatial tumoral heterogeneity, they will also contribute to understanding how cancer cells interact with each other and with the TME. This crucial information can shed light on epigenetic mechanisms behind the variability in patient outcomes and resistance to treatments, such as the aberrant regulatory programs that facilitate cancer cell evasion of the immune system. Another potential benefit of these technological breakthroughs is the possibility to improve or design new therapeutic approaches by leveraging the localization of the subclones with distinct regulatory mechanisms.

Nevertheless, these spatial epigenomics technologies work only on fresh tissue sections. Currently, it is possible to analyze formalin-fixed paraffin-embedded (FFPE) samples for spatial transcriptomics [111]. Given that FFPE is the standard format to archive and preserve clinical

samples, FFPE-based spatial epigenomics techniques would represent a breakthrough in this field. [112].

## Concluding remarks and future perspectives

The emergence of single cell-sequencing technologies over the past few years has opened a new dimension for how to study cancer biology. Genetic and transcriptomic high-throughput single cell-sequencing technologies have already proven to be extraordinary tools to ultimately dissect tumors cell by cell, 'atomizing' the complex tumoral heterogeneity that otherwise would be impossible to decipher [8]. Nevertheless, cancer is, by nature, an epigenetic disease, the progression of which is largely dependent on non-genetic determinants [5]. Indeed, epigenetics is a crucial mechanistic layer without which essential information about tumor heterogeneity would be lost. Scientists across the globe have developed dozens of new technologies that allow the analysis epigenetic mechanisms at the single cell level, including the development of multi-omic approaches to simultaneously unravel multiple layers of information (Tables 1 and 2). Unfortunately, due to their novelty and complexity, most of these technologies have not yet been applied in cancer research (see Outstanding questions). Nonetheless, there are extraordinary examples showing how useful epigenetic single cell technologies are at unraveling tumoral heterogeneity, encouraging the development of new, more fine-tuned methodologies that may overcome current limitations (Box 2). The creation of automatized, high-throughput platforms for single-cell epigenetics, similar to those already applied for microfluidics-based scATAC-seq [59], would help to standardize this process, making it more 'user-friendly' and more available to any type of research laboratory. Another possible alternative would be the use of novel long-read, third-generation sequencing technologies, such as Oxford Nanopore, especially at detecting single cell DNA methylation, which would not require any bisulfite conversion or PCR amplification steps (using PCR-free based barcoding), thus avoiding DNA degradation and elongation biases [113]. This technology is already being used to detect DNA 5mC methylation at the bulk level (PromethION™ Nanopore platform) [114]. In addition, a new method for sequencing the transcriptome at the single cell level, named scCOLOR-seq, was recently developed using Nanopore technology [115]. Despite this progress, no epigenetic long-read single cell sequencing has yet been developed. Furthermore, there is a need for benchmarking and standardization of bioinformatic pipelines when analyzing single cell epigenetic-sequencing data, making this process more comprehensible for all bioinformaticians [116].

Despite these limitations, single cell epigenetic technologies have a bright future ahead, especially when we consider all the potential benefits that they could offer from a clinical viewpoint [117]. At present, several bulk epigenetic-based tests are used as diagnostic/prognostic tools in cancer. For example, MGMT and MLH1 promoter methylation analysis by methylation-sensitive PCR (MSP) or pyrosequencing is commonly used to prognosticate and diagnose patients with glioblastoma and sporadic CRC, respectively [118,119]. Continuing with glioblastoma, nearly all IDH-mutant gliomas manifest a positive CpG island methylator phenotype (G-CIMP+), linking this DNA methylation status to a favorable prognosis [120]. In addition, and similar to the well-known ColoPrint and MammaPrint gene expression-based CRC and breast cancer classifiers [121,122], several epigenetic DNA methylation-based signatures show promising clinical results, as in the classification of cancers of unknown primary using bulk-based DNA methylation arrays [123] or in the early detection of blood-circulating tumor cells from patients with CRC, breast or prostate cancer [44,124,125]. Even though these bulk-based epigenetic clinical approaches are useful, their major drawback lies in the unsolved tumor heterogeneity. Under the bulk average may lie important low-abundant, undetected subclones that are key for the prognosis of certain cancers, such as glioblastoma, one of the most heterogeneous cancers [126]. Additionally, the different types of non-malignant cell in the TME also have a significant clinical impact on patient

### Outstanding questions

How can we overcome and improve experimental limitations, such as limited throughput, low coverage, and high costs, of single cell epigenomics technologies?

Will robust computational tools capable of analyzing and integrating all single cell epigenomic layers of information, also combined with other single cell 'omics data, be developed in the near future?

Will single cell epigenomics approaches provide new insights that promote and accelerate the development of novel therapies in cancer?

How is epigenomic heterogeneity displayed across the cellular distribution of a tumor and how is this related to the cellular crosstalk between cancerous cells and with the TME?

outcome, such as in melanoma, where determining whether the tumor is 'hot' or 'cold' (tumors with high or low content of immune cells, respectively) is crucial for predicting responses to immunotherapy [127].

Thus, applying single cell epigenetic technologies will enable the discovery of new predictive/diagnostic biomarkers to detect these subclones, to develop highly specific and personalized therapeutic strategies that will avoid potential resistance to therapy, and even detecting early metastatic events. In addition, since FFPE-archived samples are the most common way to preserve patient biopsies, it is necessary to develop suitable tools to study FFPE samples at the single cell level in a retrospective manner. Of course, many obstacles lie along this long road to clinics: first, these technologies are economically expensive and, thus, unaffordable by hospital services; second, the lack of commercially standardized and automatized protocols; and lastly, the enormous complexity of the generated data calls for simplified, targeted-based analyses.

It is difficult to say whether this technology will be applied in clinics in the immediate future, but what is clear is that all the knowledge that we will gain from these single cell epigenetic approaches over the next few years will be groundbreaking, in terms of both basic and clinical scenarios.

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## Declaration of interests

M.E. is a consultant for Ferrer International and Quimatryx. The other authors declare no conflict of interest.

## References

1. Hanahan, D. (2022) Hallmarks of cancer: new dimensions. *Cancer Discov.* 12, 31–46
2. Turajlic, S. *et al.* (2019) Resolving genetic heterogeneity in cancer. *Nat. Rev. Genet.* 20, 404–416
3. Shaffer, S.M. *et al.* (2017) Rare cell variability and drug-induced reprogramming as a mode of cancer drug resistance. *Nature* 546, 431–435
4. Chen, J.F. and Yan, Q. (2021) The roles of epigenetics in cancer progression and metastasis. *Biochem. J.* 478, 3373–3393
5. Nam, A.S. *et al.* (2021) Integrating genetic and non-genetic determinants of cancer evolution by single-cell multi-omics. *Nat. Rev. Genet.* 22, 3–18
6. Allis, C.D. and Jenuwein, T. (2016) The molecular hallmarks of epigenetic control. *Nat. Rev. Genet.* 17, 487–500
7. Darwiche, N. (2020) Epigenetic mechanisms and the hallmarks of cancer: an intimate affair. *Am. J. Cancer Res.* 10, 1954–1978
8. Yalan, L. *et al.* (2021) Applications of single-cell sequencing in cancer research: progress and perspectives. *J. Hematol. Oncol.* 14, 91
9. Dagogo-Jack, I. and Shaw, A.T. (2018) Tumour heterogeneity and resistance to cancer therapies. *Nat. Rev. Clin. Oncol.* 15, 81–94
10. McGranahan, N. and Swanton, C. (2017) Clonal heterogeneity and tumor evolution: past, present, and the future. *Cell* 168, 613–628
11. Oakes, C.C. *et al.* (2014) Evolution of DNA methylation is linked to genetic aberrations in chronic lymphocytic leukemia. *Cancer Discov.* 4, 348–361
12. Tay, R.E. *et al.* (2021) Revisiting the role of CD4 + T cells in cancer immunotherapy-new insights into old paradigms. *Cancer Gene Ther.* 28, 5–17
13. Baghban, R. *et al.* (2020) Tumor microenvironment complexity and therapeutic implications at a glance. *Cell Commun. Signal* 18, 59
14. Anderson, N.M. and Simon, M.C. (2020) The tumor microenvironment. *Curr. Biol.* 30, R921–R925
15. Marks, D.L. (2016) Epigenetic control of the tumor microenvironment. *Epigenomics* 8, 1671–1687
16. Noble, R. *et al.* (2022) Spatial structure governs the mode of tumour evolution. *Nat. Ecol. Evol.* 6, 207–217
17. Trujillo, J.A. *et al.* (2018) T cell-inflamed versus non-T cell-inflamed tumors: a conceptual framework for cancer immunotherapy drug development and combination therapy selection. *Cancer Immunol. Res.* 6, 990–1000
18. Martínez-Cardús, A. *et al.* (2016) Epigenetic homogeneity within colorectal tumors predicts shorter relapse-free and overall survival times for patients with locoregional cancer. *Gastroenterology* 151, 961–972
19. Lim, J.R. *et al.* (2021) Cancer stem cell characteristics and their potential as therapeutic targets. *Med. Oncol.* 38, 76
20. Neftel, C. *et al.* (2019) An integrative model of cellular states, plasticity, and genetics for glioblastoma. *Cell* 178, 835–849
21. Lee, H.J. (2014) Reprogramming the methylome: erasing memory and creating diversity. *Cell Stem Cell* 14, 710–719
22. Fares, J. *et al.* (2020) Molecular principles of metastasis: a hallmark of cancer revisited. *Signal Transduct. Target. Ther.* 5, 28
23. Patel, S.A. and Vanharanta, S. (2017) Epigenetic determinants of metastasis. *Mol. Oncol.* 11, 79–96
24. Wang, X. *et al.* (2019) Drug resistance and combating drug resistance in cancer. *Cancer Drug Resist* 2, 141–160

25. Hayashi, T. and Konishi, I. (2021) Correlation of anti-tumour drug resistance with epigenetic regulation. *Br. J. Cancer* 124, 681–682
26. Hinohara, K. *et al.* (2018) KDM5 histone demethylase activity links cellular transcriptomic heterogeneity to therapeutic resistance. *Cancer Cell* 34, 939–953
27. Deblois, G. *et al.* (2020) Epigenetic switch-induced viral mimicry evasion in chemotherapy-resistant breast cancer. *Cancer Discov.* 10, 1312–1329
28. Marine, J.-C. *et al.* (2020) Non-genetic mechanisms of therapeutic resistance in cancer. *Nat. Rev. Cancer* 20, 743–756
29. Schuurhuis, G.J. *et al.* (2018) Minimal/measurable residual disease in AML: a consensus document from the European Leukemia Net MRD Working Party. *Blood* 131, 1275–1291
30. van Galen, P. *et al.* (2019) Single-cell RNA-seq reveals AML hierarchies relevant to disease progression and immunity. *Cell* 176, 1265–1281
31. Costa, A. *et al.* (2018) Fibroblast heterogeneity and immunosuppressive environment in human breast cancer. *Cancer Cell* 33, 463–479
32. Aoki, T. *et al.* (2020) Single-cell transcriptome analysis reveals disease-defining t-cell subsets in the tumor microenvironment of classic hodgkin lymphoma. *Cancer Discov.* 10, 406–421
33. Campillo-Marcos, I. *et al.* (2021) Single-cell technologies and analyses in hematopoiesis and hematological malignancies. *Exp. Hematol.* 98, 1–13
34. Miles, L.A. *et al.* (2020) Single-cell mutation analysis of clonal evolution in myeloid malignancies. *Nature* 587, 477–482
35. Chaligne, R. *et al.* (2021) Epigenetic encoding, heritability and plasticity of glioma transcriptional cell states. *Nat. Genet.* 53, 1469–1479
36. Deaton, A.M. and Bird, A. (2011) CpG islands and the regulation of transcription. *Genes Dev.* 25, 1010–1022
37. Berdasco, M. and Esteller, M. (2019) Clinical epigenetics: seizing opportunities for translation. *Nat. Rev. Genet.* 20, 109–127
38. Bell, R.E. *et al.* (2016) Enhancer methylation dynamics contribute to cancer plasticity and patient mortality. *Genome Res.* 26, 601–611
39. Guo, H. *et al.* (2013) Single-cell methylome landscapes of mouse embryonic stem cells and early embryos analyzed using reduced representation bisulfite sequencing. *Genome Res.* 23, 2126–2135
40. Guo, H. *et al.* (2015) Profiling DNA methylome landscapes of mammalian cells with single-cell reduced-representation bisulfite sequencing. *Nat. Protoc.* 10, 645–659
41. Smallwood, S.A. *et al.* (2014) Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. *Nat. Methods* 11, 817–820
42. Farlik, M. *et al.* (2015) Single-cell DNA methylome sequencing and bioinformatic inference of epigenomic cell-state dynamics. *Cell Rep.* 10, 1386–1397
43. Kobayashi, H. *et al.* (2016) Repetitive DNA methylome analysis by small-scale and single-cell shotgun bisulfite sequencing. *Genes Cells* 21, 1209–1222
44. Pixberg, C.F. (2017) Analysis of DNA methylation in single circulating tumor cells. *Oncogene* 36, 3223–3231
45. Mooijman, D. *et al.* (2016) Single-cell 5hmC sequencing reveals chromosome-wide cell-to-cell variability and enables lineage reconstruction. *Nat. Biotechnol.* 34, 852–856
46. Zhu, C. *et al.* (2017) Single-cell 5-formylcytosine landscapes of mammalian early embryos and ESCs at single-base resolution. *Cell Stem Cell* 20, 720–731
47. Wu, X. *et al.* (2017) Simultaneous mapping of active DNA demethylation and sister chromatid exchange in single cells. *Genes Dev.* 31, 511–523
48. Kantlehner, M. *et al.* (2011) A high-throughput DNA methylation analysis of a single cell. *Nucleic Acids Res.* 39, e44
49. Han, L. *et al.* (2017) Bisulfite-independent analysis of CpG island methylation enables genome-scale stratification of single cells. *Nucleic Acids Res.* 45, e77
50. Niemöller, C. *et al.* (2021) Bisulfite-free epigenomics and genomics of single cells through methylation-sensitive restriction. *Commun. Biol.* 4, 153
51. Bianchi, A. *et al.* (2022) scTAM-seq enables targeted high-confidence analysis of DNA methylation in single cells. *bioRxiv* Published online April 12. 2022. https://doi.org/10.1101/2022.04.11.487648
52. Pastore, A. *et al.* (2019) Corrupted coordination of epigenetic modifications leads to diverging chromatin states and transcriptional heterogeneity in CLL. *Nat. Commun.* 10, 1874
53. Johnson, K.C. *et al.* (2021) Single-cell multimodal glioma analyses identify epigenetic regulators of cellular plasticity and environmental stress response. *Nat. Genet.* 53, 1456–1468
54. Huang, Y. *et al.* (2021) Comprehensive analysis of partial methylation domains in colorectal cancer based on single-cell methylation profiles. *Brief. Bioinform.* 22, bbab267
55. Rodríguez-Ubreva, J. *et al.* (2022) Single-cell atlas of common variable immunodeficiency shows germinal center-associated epigenetic dysregulation in B-cell responses. *Nat. Commun.* 13, 1779
56. Gkountela, S. *et al.* (2019) Circulating tumor cell clustering shapes DNA methylation to enable metastasis seeding. *Cell.* 176, 98–112
57. Sur, I. and Taipale, J. (2016) The role of enhancers in cancer. *Nat. Rev. Cancer* 16, 483–493
58. Buenrostro, J.D. *et al.* (2015) Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature* 523, 486–490
59. Satpathy, A.T. *et al.* (2019) Massively parallel single-cell chromatin landscapes of human immune cell development and intratumoral T cell exhaustion. *Nat. Biotechnol.* 37, 925–936
60. Chen, X. *et al.* (2018) A rapid and robust method for single cell chromatin accessibility profiling. *Nat. Commun.* 9, 5345
61. Frede, J. *et al.* (2021) Dynamic transcriptional reprogramming leads to immunotherapeutic vulnerabilities in myeloma. *Nat. Cell Biol.* 23, 1199–1211
62. Taavitsainen, S. *et al.* (2021) Single-cell ATAC and RNA sequencing reveal pre-existing and persistent cells associated with prostate cancer relapse. *Nat. Commun.* 12, 5307
63. Zhang, Y. *et al.* (2021) Single-cell analyses reveal key immune cell subsets associated with response to PD-L1 blockade in triple-negative breast cancer. *Cancer Cell* 39, 1578–1593.e8
64. Shu, S. *et al.* (2020) Synthetic lethal and resistance interactions with BET bromodomain inhibitors in triple-negative breast cancer. *Mol. Cell* 78, 1096–1113
65. Kashima, Y. *et al.* (2021) Single-cell analyses reveal diverse mechanisms of resistance to EGFR tyrosine kinase inhibitors in lung cancer. *Cancer Res.* 81, 4835–4848
66. Regner, M.J. *et al.* (2021) A multi-omic single-cell landscape of human gynecologic malignancies. *Mol. Cell* 81, 4924–4941
67. Guilhamon, P. *et al.* (2021) Single-cell chromatin accessibility profiling of glioblastoma identifies an invasive cancer stem cell population associated with lower survival. *eLife* 10, 1–20
68. Liscovitch-Brauer, N. *et al.* (2021) Profiling the genetic determinants of chromatin accessibility with scalable single-cell CRISPR screens. *Nat. Biotechnol.* 39, 1270–1277
69. Rubin, A.J. *et al.* (2019) Coupled single-cell CRISPR screening and epigenomic profiling reveals causal gene regulatory networks. *Cell* 176, 361–376
70. Pierce, S.E. *et al.* (2021) High-throughput single-cell chromatin accessibility CRISPR screens enable unbiased identification of regulatory networks in cancer. *Nat. Commun.* 12, 2969
71. Lareau, C.A. *et al.* (2021) Massively parallel single-cell mitochondrial DNA genotyping and chromatin profiling. *Nat. Biotechnol.* 39, 451–461
72. Penter, L. *et al.* (2021) Longitudinal single-cell dynamics of chromatin accessibility and mitochondrial mutations in chronic lymphocytic leukemia mirror disease history. *Cancer Discov.* 11, 3048–3063
73. Lai, B. *et al.* (2018) Principles of nucleosome organization revealed by single-cell micrococcal nuclease sequencing. *Nature* 562, 281–285
74. Gao, W. *et al.* (2020) Genome-wide profiling of nucleosome position and chromatin accessibility in single cells using scMNase-seq. *Nat. Protoc.* 15, 68–85
75. Rotem, A. *et al.* (2015) Single-cell ChIP-seq reveals cell subpopulations defined by chromatin state. *Nat. Biotechnol.* 33, 1165–1172
76. Ku, W.L. *et al.* (2019) Single-cell chromatin immunocleavage sequencing (scChIC-seq) to profile histone modification. *Nat. Methods* 16, 323–325

77. Grosselin, K. *et al.* (2019) High-throughput single-cell ChIP-seq identifies heterogeneity of chromatin states in breast cancer. *Nat. Genet.* 51, 1060–1066
78. Marsolier, J. *et al.* (2022) H3K27me3 conditions chemotolerance in triple-negative breast cancer. *Nat. Genet.* 54, 459–468
79. Janssens, D.H. *et al.* (2021) Automated CUT&Tag profiling of chromatin heterogeneity in mixed-lineage leukemia. *Nat. Genet.* 53, 1586–1596
80. Azagra, A. *et al.* (2020) From loops to looks: transcription factors and chromatin organization shaping terminal B cell differentiation. *Trends Immunol.* 41, 46–60
81. Akdemir, K.C. *et al.* (2020) Disruption of chromatin folding domains by somatic genomic rearrangements in human cancer. *Nat. Genet.* 52, 294–305
82. Nagano, T. *et al.* (2013) Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. *Nature* 502, 59–64
83. Ramani, V. *et al.* (2017) Massively multiplex single-cell Hi-C. *Nat. Methods* 14, 263–266
84. Kind, J. *et al.* (2015) Genome-wide maps of nuclear lamina interactions in single human cells. *Cell* 163, 134–147
85. Zhu, C. *et al.* (2020) Single-cell multimodal omics: the power of many. *Nat. Methods* 17, 11–14
86. Pott, S. (2017) Simultaneous measurement of chromatin accessibility, DNA methylation, and nucleosome phasing in single cells. *eLife* 6, e23203
87. Li, G. *et al.* (2019) Joint profiling of DNA methylation and chromatin architecture in single cells. *Nat. Methods* 16, 991–993
88. Lee, D.S. *et al.* (2019) Simultaneous profiling of 3D genome structure and DNA methylation in single human cells. *Nat. Methods* 16, 999–1006
89. Guo, F. *et al.* (2017) Single-cell multi-omics sequencing of mouse early embryos and embryonic stem cells. *Cell Res.* 27, 967–988
90. Fan, X. *et al.* (2022) Integrated single-cell multiomics analysis reveals novel candidate markers for prognosis in human pancreatic ductal adenocarcinoma. *Cell Discov.* 8, 13
91. Blecua, P. *et al.* (2022) Refinement of computational identification of somatic copy number alterations using DNA methylation microarrays illustrated in cancers of unknown primary. *Brief. Bioinform.* 6, bbac161
92. Gaiti, F. *et al.* (2019) Epigenetic evolution and lineage histories of chronic lymphocytic leukaemia. *Nature* 569, 576–580
93. Bian, S. (2018) Single-cell multiomics sequencing and analyses of human colorectal cancer. *Science* 362, 1060–1063
94. Hou, Y. *et al.* (2016) Single-cell triple omics sequencing reveals genetic, epigenetic, and transcriptomic heterogeneity in hepatocellular carcinomas. *Cell Res.* 26, 304–319
95. Angermueller, C. *et al.* (2016) Parallel single-cell sequencing links transcriptional and epigenetic heterogeneity. *Nat. Methods* 13, 229–232
96. Luo, C. *et al.* (2018) Multi-omic profiling of transcriptome and DNA methylome in single nuclei with molecular partitioning. *bioRxiv* Published online October 4, 2018. https://doi.org/10.1101/434845
97. Liu, L. *et al.* (2019) Deconvolution of single-cell multi-omics layers reveals regulatory heterogeneity. *Nat. Commun.* 10, 470
98. Reyes, M. *et al.* (2019) Simultaneous profiling of gene expression and chromatin accessibility in single cells. *Adv. Biosyst.* 3, 1900065
99. Rooijers, K. *et al.* (2019) Simultaneous quantification of protein–DNA contacts and transcriptomes in single cells. *Nat. Biotechnol.* 37, 766–772
100. Clark, S.J. *et al.* (2018) ScNMT-seq enables joint profiling of chromatin accessibility DNA methylation and transcription in single cells. *Nat. Commun.* 9, 781
101. Wang, Y. *et al.* (2021) Single-cell multiomics sequencing reveals the functional regulatory landscape of early embryos. *Nat. Commun.* 12, 1247
102. Ståhl, P.L. *et al.* (2016) Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. *Science* 353, 78–82
103. Rodriques, S.G. *et al.* (2019) Slide-seq: a scalable technology for measuring genome-wide expression at high spatial resolution. *Science* 363, 1463–1467
104. Lewis, S.M. *et al.* (2021) Spatial omics and multiplexed imaging to explore cancer biology. *Nat. Methods* 18, 997–1012
105. Wu, Y. *et al.* (2022) Spatiotemporal immune landscape of colorectal cancer liver metastasis at single-cell level. *Cancer Discov.* 12, 134–153
106. Nieto, P. *et al.* (2021) A single-cell tumor immune atlas for precision oncology. *Genome Res.* 31, 1913–1926
107. Deng, Y. (2022) Spatial-CUT&Tag: spatially resolved chromatin modification profiling at the cellular level. *Science* 375, 681–686
108. Lu, T. *et al.* (2022) Spatially resolved epigenomic profiling of single cells in complex tissues. *bioRxiv* Published online February 19, 2022. https://doi.org/10.1101/2022.02.17.480825
109. Thornton, C.A. (2021) Spatially mapped single-cell chromatin accessibility. *Nat. Commun.* 12, 1274
110. Deng, Y. *et al.* (2021) Spatial-ATAC-seq: spatially resolved chromatin accessibility profiling of tissues at genome scale and cellular level. *bioRxiv* Published online June 7, 2021. https://doi.org/10.1101/2021.06.06.447244
111. Gracia Villacampa, E. *et al.* (2021) Genome-wide spatial expression profiling in formalin-fixed tissues. *Cell Genom.* 1, 100065
112. Zhang, H. *et al.* (2022) Profiling chromatin accessibility in formalin-fixed paraffin-embedded samples. *Genome Res.* 32, 150–161
113. Batista, F.M. *et al.* (2020) Whole genome sequencing of hepatitis A virus using a PCR-free single-molecule nanopore sequencing approach. *Front. Microbiol.* 11, 874
114. Liu, Y. *et al.* (2021) DNA methylation-calling tools for Oxford Nanopore sequencing: a survey and human epigenome-wide evaluation. *Genome Biol.* 22, 295
115. Philpott, N. (2021) Nanopore sequencing of single-cell transcriptomes with scCOLOR-seq. *Nat. Biotechnol.* 39, 1517–1520
116. Danese, A. *et al.* (2021) EpiScanpy: integrated single-cell epigenomic analysis. *Nat. Commun.* 12, 5228
117. Bond, D.R. *et al.* (2020) Single-cell epigenomics in cancer: charting a course to clinical impact. *Epigenomics* 12, 1139–1151
118. Johannessen, L.E. *et al.* (2018) MGMT gene promoter methylation status - assessment of two pyrosequencing kits and three methylation-specific PCR methods for their predictive capacity in glioblastomas. *Cancer Genomics Proteomics* 15, 437–446
119. Locke, W.J. *et al.* (2019) DNA methylation cancer biomarkers: translation to the clinic. *Front. Genet.* 10, 1150
120. Malta, T.M. *et al.* (2018) Glioma CpG island methylator phenotype (G-CIMP): biological and clinical implications. *Neuro-Oncology* 20, 608–620
121. Kopetz, S. *et al.* (2015) Genomic classifier ColoPrint predicts recurrence in stage II colorectal cancer patients more accurately than clinical factors. *Oncologist* 20, 127–133
122. Brandão, M. *et al.* (2019) Mammaprint™: a comprehensive review. *Future Oncol.* 15, 207–224
123. Moran, S. *et al.* (2016) Epigenetic profiling to classify cancer of unknown primary: a multicentre, retrospective analysis. *Lancet Oncol.* 17, 1386–1395
124. Nassar, F.J. *et al.* (2021) Methylated circulating tumor DNA as a biomarker for colorectal cancer diagnosis, prognosis, and prediction. *Clin. Epigenetics* 13, 111
125. Pixberg, C.F. *et al.* (2015) Characterization of DNA methylation in circulating tumor cells. *Genes (Basel)* 6, 1053–1075
126. Friedmann-Morvinski, D. (2014) Glioblastoma heterogeneity and cancer cell plasticity. *Crit. Rev. Oncol.* 19, 327–336
127. Olbryt, M. *et al.* (2020) Biological factors behind melanoma response to immune checkpoint inhibitors. *Int. J. Mol. Sci.* 21, 4071
128. Ortiz-Barahona, V. *et al.* (2020) Use of DNA methylation profiling in translational oncology. *Semin. Cancer Biol.* 83, 523–535
129. Marinov, G.K. and Shipony, Z. (2021) Interrogating the accessible chromatin landscape of eukaryote genomes using ATAC-seq. *Methods Mol. Biol.* 2243, 183–226
130. Nakato, R. and Sakata, T. (2021) Methods for ChIP-seq analysis: a practical workflow and advanced applications. *Methods* 187, 44–53
131. Sati, S. and Cavalli, G. (2016) Chromosome conformation capture technologies and their impact in understanding genome function. *Chromosoma* 126, 33–44

132. Javierre, B.M. *et al.* (2016) Lineage-specific genome architecture links enhancers and non-coding disease variants to target gene promoters. *Cell* 167, 1369–1384
133. Carter, B. and Zhao, K. (2021) The epigenetic basis of cellular heterogeneity. *Nat. Rev. Genet.* 22, 235–250
134. Chakravarthy, A. *et al.* (2018) Pan-cancer deconvolution of tumour composition using DNA methylation. *Nat. Commun.* 9, 3220
135. Ceccarelli, M. *et al.* (2016) Molecular profiling reveals biologically discrete subsets and pathways of progression in diffuse glioma. *Cell* 164, 550–563
136. Völker-Albert, M. *et al.* (2020) Histone modifications in stem cell development and their clinical implications. *Stem Cell Rep.* 15, 1196–1205
137. Mehrmohamadi, M. *et al.* (2021) A comparative overview of epigenomic profiling methods. *Front. Cell Dev. Biol.* 9, 714687
138. Argelaguet, R. *et al.* (2018) Multi-Omics Factor Analysis—a framework for unsupervised integration of multi-omics data sets. *Mol. Syst. Biol.* 14, e8124
139. Argelaguet, R. *et al.* (2020) MOFA+: a statistical framework for comprehensive integration of multi-modal single-cell data. *Genome Biol.* 21, 111
140. Li, G. *et al.* (2022) A deep generative model for multi-view profiling of single-cell RNA-seq and ATAC-seq data. *Genome Biol.* 23, 20
141. Wu, K.E. *et al.* (2021) BABEL enables cross-modality translation between multiomic profiles at single-cell resolution. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2023070118
142. Wang, C. *et al.* (2020) Integrative analyses of single-cell transcriptome and regulome using MAESTRO. *Genome Biol.* 21, 198
143. Sharma, A. *et al.* (2021) ShinyArchR.UiO: user-friendly, integrative and open-source tool for visualisation of single-cell ATAC-seq data using ArchR. *Bioinformatics* 38, 834–836
144. Prompsy, P. *et al.* (2020) Interactive analysis of single-cell epigenomic landscapes with ChromSCape. *Nat. Commun.* 11, 5702
145. Shareef, S.J. *et al.* (2021) Extended-representation bisulfite sequencing of gene regulatory elements in multiplexed samples and single cells. *Nat. Biotechnol.* 39, 1086–1094
146. Chen, H. *et al.* (2021) Single-cell DNA methylome analysis of circulating tumor cells. *Chin. J. Cancer Res.* 33, 391–404
147. Hui, T. *et al.* (2018) High-resolution single-cell DNA methylation measurements reveal epigenetically distinct hematopoietic stem cell subpopulations. *Stem Cell Rep.* 11, 578–592
148. Grants, J.M. (2020) Altered microRNA expression links IL6 and TNF-induced inflammaging with myeloid malignancy in humans and mice. *Blood* 135, 2235–2251
149. Luo, C. *et al.* (2017) Single-cell methylomes identify neuronal subtypes and regulatory elements in mammalian cortex. *Science* 357, 600–604
150. Meir, Z. (2020) Single-cell analysis of clonal maintenance of transcriptional and epigenetic states in cancer cells. *Nat. Genet.* 52, 709–718
151. Luo, C. *et al.* (2018) Robust single-cell DNA methylome profiling with snmC-seq2. *Nat. Commun.* 9, 3824
152. Mulqueen, R.M. *et al.* (2018) Highly scalable generation of DNA methylation profiles in single cells. *Nat. Biotechnol.* 36, 428–431
153. Ma, S. *et al.* (2018) Cell-type-specific brain methylomes profiled via ultralow-input microfluidics. *Nat. Biomed. Eng.* 2, 183–194
154. Li, X. *et al.* (2019) BRIF-seq: bisulfite-converted randomly integrated fragments sequencing at the single-cell level. *Mol. Plant* 12, 438–446
155. Hunt, K.V. *et al.* (2022) scTEM-seq: single-cell analysis of transposable element methylation to link global epigenetic heterogeneity with transcriptional programs. *Sci. Rep.* 12, 5776
156. Wang, L. *et al.* (2019) The phenotypes of proliferating glioblastoma cells reside on a single axis of variation. *Cancer Discov.* 9, 1708–1719
157. Wang, Q. *et al.* (2022) Single-cell chromatin accessibility landscape in kidney identifies additional cell-of-origin in heterogenous papillary renal cell carcinoma. *Nat. Commun.* 13, 31
158. Xu, K. *et al.* (2021) Integrative analyses of scRNA-seq and scATAC-seq reveal CXCL14 as a key regulator of lymph node metastasis in breast cancer. *Hum. Mol. Genet.* 30, 370–380
159. Singh, H. *et al.* (2021) Hybrid stomach-intestinal chromatin states underlie human Barrett's metaplasia. *Gastroenterology* 161, 924–939.e11
160. Wang, H. *et al.* (2021) Single-cell analyses reveal mechanisms of cancer stem cell maintenance and epithelial-mesenchymal transition in recurrent bladder cancer. *Clin. Cancer Res.* 27, 6265–6278
161. Schwartz, G.W. *et al.* (2021) TooManyPeaks identifies drug-resistant-specific regulatory elements from single-cell leukemic epigenomes. *Cell Rep.* 36, 109575
162. Pierce, S.E. *et al.* (2021) LKB1 inactivation modulates chromatin accessibility to drive metastatic progression. *Nat. Cell Biol.* 23, 915–924
163. Cusanovich, D.A. *et al.* (2015) Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science* 348, 910–914
164. Del Priore, I. *et al.* (2021) Protocol for single-cell ATAC sequencing using combinatorial indexing in mouse lung adenocarcinoma. *STAR Protoc.* 2, 100583
165. Wang, K. *et al.* (2021) Simple oligonucleotide-based multiplexing of single-cell chromatin accessibility. *Mol. Cell* 81, 4319–4332
166. De Rop, F.V. *et al.* (2022) Hydrop enables droplet-based single-cell ATAC-seq and single-cell RNA-seq using dissolvable hydrogel beads. *Elife* 11, e73971
167. Tedesco, M. *et al.* (2022) Chromatin Velocity reveals epigenetic dynamics by single-cell profiling of heterochromatin and euchromatin. *Nat. Biotechnol.* 40, 235–244
168. Ku, W.L. *et al.* (2021) Profiling single-cell histone modifications using indexing chromatin immunocleavage sequencing. *Genome Res.* 31, 1831–1842
169. Harada, A. *et al.* (2019) A chromatin integration labelling method enables epigenomic profiling with lower input. *Nat. Cell Biol.* 21, 287–296
170. Carter, B. *et al.* (2019) Mapping histone modifications in low cell number and single cells using antibody-guided chromatin tagmentation (ACT-seq). *Nat. Commun.* 10, 3747
171. Wang, Q. *et al.* (2019) CoBATCH for high-throughput single-cell epigenomic profiling. *Mol. Cell* 76, 206–216
172. Hainer, S.J. *et al.* (2019) Profiling of pluripotency factors in single cells and early embryos. *Cell* 177, 1319–1329
173. Kaya-Okur, H.S. *et al.* (2019) CUT&Tag for efficient epigenomic profiling of small samples and single cells. *Nat. Commun.* 10, 1930
174. Bartosovic, M. *et al.* (2021) Single-cell CUT&Tag profiles histone modifications and transcription factors in complex tissues. *Nat. Biotechnol.* 39, 825–835
175. Wu, S.J. (2021) Single-cell CUT&Tag analysis of chromatin modifications in differentiation and tumor progression. *Nat. Biotechnol.* 39, 819–824
176. Hui, W.W.I. *et al.* (2021) Single-cell mapping of DNA G-quadruplex structures in human cancer cells. *Sci. Rep.* 11, 23641
177. Bartlett, D.A. *et al.* (2021) High-throughput single-cell epigenomic profiling by targeted insertion of promoters (TIP-seq). *J. Cell Biol.* 220, e202103078
178. Ohnuki, H. *et al.* (2021) Iterative epigenomic analyses in the same single cell. *Genome Res.* 31, 1819–1830
179. Hu, Y. *et al.* (2019) Simultaneous profiling of mRNA transcriptome and DNA methylome from a single cell. *Methods Mol. Biol.* 1979, 363–377
180. Gu, H. *et al.* (2021) Smart-RRBS for single-cell methylome and transcriptome analysis. *Nat. Protoc.* 16, 4004–4030
181. Cheow, L.F. *et al.* (2016) Single-cell multimodal profiling reveals cellular epigenetic heterogeneity. *Nat. Methods* 13, 833–836
182. Gu, C. *et al.* (2019) Integrative single-cell analysis of transcriptome, DNA methylome and chromatin accessibility in mouse oocytes. *Cell Res.* 29, 110–123
183. Satpathy, A.T. *et al.* (2018) Transcript-indexed ATAC-seq for precision immune profiling. *Nat. Med.* 24, 580–590
184. Chen, S. *et al.* (2019) High-throughput sequencing of the transcriptome and chromatin accessibility in the same cell. *Nat. Biotechnol.* 37, 1452–1457
185. Zhu, C. *et al.* (2019) An ultra high-throughput method for single-cell joint analysis of open chromatin and transcriptome. *Nat. Struct. Mol. Biol.* 26, 1063–1070

186. Zhu, C. *et al.* (2021) Joint profiling of histone modifications and transcriptome in single cells from mouse brain. *Nat. Methods* 18, 283–292
187. Yan, R. *et al.* (2021) Decoding dynamic epigenetic landscapes in human oocytes using single-cell multi-omics sequencing. *Cell Stem Cell* 28, 1641–1656
188. Chen, A.F. *et al.* (2022) NEAT-seq: simultaneous profiling of intra-nuclear proteins, chromatin accessibility, and gene expression in single cells. *Nat. Methods* 19, 547–553
189. Mimitou, E.P. *et al.* (2021) Scalable, multimodal profiling of chromatin accessibility, gene expression and protein levels in single cells. *Nat. Biotechnol.* 39, 1246–1258
190. Chen, X. *et al.* (2018) Joint single-cell DNA accessibility and protein epitope profiling reveals environmental regulation of epigenomic heterogeneity. *Nat. Commun.* 9, 4590
191. Zhang, B. *et al.* (2022) Characterizing cellular heterogeneity in chromatin state with scCUT&Tag-pro. *Nat. Biotechnol.* Published online March 24, 2022. https://doi.org/10.1038/s41587-022-01250-0
192. Ma, S. *et al.* (2020) Chromatin potential identified by shared single-cell profiling of RNA and chromatin. *Cell* 183, 1103–1116
193. Su, J.-H. *et al.* (2020) Genome-scale imaging of the 3D organization and transcriptional activity of chromatin. *Cell* 182, 1641–1659
194. Takei, Y. *et al.* (2021) Integrated spatial genomics reveals global architecture of single nuclei. *Nature* 590, 344–350