Trends in Genetics

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Review

Into the multiverse: advances in single-cell multiomic profiling

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Single-cell transcriptomic approaches have revolutionised the study of complex biological systems, with the routine measurement of gene expression in thousands of cells enabling construction of whole-organism cell atlases. However, the transcriptome is just one layer amongst many that coordinate to define cell type and state and, ultimately, function. In parallel with the widespread uptake of single-cell RNA-seq (scRNA-seq), there has been a rapid emergence of methods that enable multiomic profiling of individual cells, enabling parallel measurement of intercellular heterogeneity in the genome, epigenome, transcriptome, and proteomes. Linking measurements from each of these layers has the potential to reveal regulatory and functional mechanisms underlying cell behaviour in healthy development and disease.

Highlights

To understand intercellular heterogeneity within an organism, it is essential to make coordinated measurements linking the genome and its epigenetic regulation to gene and protein expression at the single-cell level.

Rapid advances in single-cell multiomics approaches have enabled analysis of multiple molecular modalities from the same single cell.

Methods incorporating several modalities now exist, although several challenges remain with regard to resolution, data integration, and scale.

Further developments in multiomics approaches will provide unique insights into the regulatory processes governing how individual cells function collectively to produce whole-organism phenotypes in development, health, and disease.

The many sources of cellular heterogeneity

As fundamental biological units, cells within a multicellular organism are capable of remarkable diversity in form and function throughout development and disease. Individual cells have typically been classified to particular 'types' or 'states' by phenotypic measurement, such as marker gene expression, morphology, or function. scRNA-seq has been instrumental in revealing a broader scheme for cell type classification through simultaneous measurement of the expression of thousands of genes in thousands – even millions – of cells, and therefore more detailed classification of cell types, subtypes, and states in dynamic and complex developmental systems [1–7]. These rapid advances in scRNA-seq technologies have made whole-organism single-cell profiling a reality, underpinning the efforts of major consortia aiming to produce a comprehensive map of cell types in the human body [8].

However, a cell's transcriptome is just one aspect of its phenotype – an incomplete representation of cellular identity, reflecting both the regulatory status of the genome and implied protein production. Cell-type-specific mRNA expression is governed by epigenetic mechanisms and, in general, only has functional potential when translated into protein. Thus, **molecular cellular identity** (see Glossary) is a product of the interplay between many different modalities within the cell (Figure 1, Key figure), all of which can vary as a result of intrinsic and extrinsic factors. To truly understand how individual cells within a multicellular organism can demonstrate such remarkable heterogeneity, it is essential to be able to make coordinated measurements linking the genome and its epigenetic regulation to gene products (transcripts and proteins).

In parallel with the rapid and widespread adoption of scRNA-seq, there has been an adaptive radiation of single-cell multiomics approaches for the simultaneous analysis of multiple molecular modalities from the same single cell (Figure 2). These powerful approaches enable associations to be made between genome sequence, structure, and regulatory state and the transcriptional and proteomic phenotype of the cell. While a cell can be classified on the basis of any one of these measurements, a cell's identity can only be understood through the integration of these different

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layers. This kind of analysis can not only enhance our ability to classify cell identity but brings us closer to being able to perform mechanistic, functional genomic studies of individual cells within a population. This has a particular impact on the study of development, ageing, and disease, where heterogeneity at multiple levels can contribute to cellular phenotypes which have profound impact on the organismal phenotype.

Linking somatic variation and gene expression

Within the lifetime of an organism, genomic diversification between cells – known as **somatic variation** – can occur as a result of programmed and spontaneous mechanisms. Thus, the genomes of individual cells within a multicellular organism can have substantial and significant deviations from the 'prime' genome – that of the fertilised zygote. For example, programmed somatic variation occurs in B and T lymphocytes to produce diversity in specificity of antibody and T cell receptors. Spontaneous somatic variation, where individual cells acquire genomic diversity – from **single-nucleotide variants (SNVs)** to whole-chromosome **copy-number variants (CNVs)** – is common in normal mammalian development and ageing [9,10]. This phenomenon can become pathogenic when a particular variant (or set of variants) acquired in a single cell confers a competitive advantage to the cell and its subsequent progeny. This cellular evolutionary process, where genotypic changes create competitive phenotypic heterogeneity, can lead to clonal expansion and the formation of malignant or cancerous clones through the acquisition of further mutations and genomic rearrangements [11].

Changes in the genome itself have limited impact unless they modify the sequence of genes or their regulatory elements, thereby modifying gene expression and the overall phenotype of the cell. Therefore, linking somatic variation to gene expression in the same cell is critical to understand the functional consequences of acquired mutations and how these can introduce functional cellular heterogeneity. Early single-cell multiomics methods, such as DR-seq (gDNA and mRNA-sequencing) [12] and G&T-seq (genome and transcriptome-sequencing) [13], performed parallel analysis of genomes and transcriptomes of individual cells, typically isolated manually or by **FACS**. In DR-seq, combined amplification of a single cell's genome and transcriptome is performed in a single reaction, while in G&T-seq, mRNA is physically separated from genomic DNA before parallel amplification of both (Figure 3A). Both **plate-based** methods enabled links to be made between genomic variation – from chromosomal copy number down to single-nucleotide resolution – and gene expression. They also demonstrated, for the first time, the direct impact of chromosomal copy number on gene expression in the same cell, with a clear correlation between copy number and gene expression. In the case of G&T-seq it was possible to demonstrate this correlation immediately after the cell cycle in which reciprocal chromosomal gains or losses occurred. Additionally, the combination of full-length RNA-seq and whole-genome sequencing in G&T-seq enabled parallel detection of a fusion transcript and the causative genomic rearrangement in the same cell of a breast cancer cell line. Both of these early methods demonstrated potential for single-cell multiomic studies in cancer (Box 1) in which the transcriptional phenotype of the cell can be associated with evolutionary events recorded in the genome.

These approaches were not without limitations, suffering from sequence errors introduced in the **whole-genome amplification** processes, as well as allelic and locus dropout that is inherent in single-cell genome amplification. Gaining high coverage data from the entire genomes of single cells, in parallel with rich transcriptomic data, is also expensive, which limits reasonable throughput to 100s or 1000s of cells.

More recently, Target-seq [14] was developed to enable parallel mRNA-seq and targeted genotyping, rather than whole-genome sequencing, of the same single cell. The plate-based

Glossary

ATAC-seq: an 'assay for transposase-accessible chromatin with high-throughput sequencing' in which DNA from accessible chromatin is selectively sequenced. This gives an overview of the 'openness' of the chromatin across the genome, probed by hyperactive Tn5 transposase.
Chromatin velocity: a trajectory of cell lineage commitment based on the measurement of changes in euchromatin and heterochromatin in thousands of cells, as measured by the GET-seq assay.
Combinatorial indexing: methods which use serial barcoding of pools of nuclei or cells to generate highly complex combinations of barcodes attached to individual molecules (DNA or RNA), and thus increasing throughput without the need for dedicated microfluidics platforms. These methods are often appropriate for experiments where large number of cells (>1000s) undergo parallel analysis and classification.
Copy-number variant (CNV): an increase or decrease in the number of copies of a region of the genome, ranging from increased numbers of short tandem repeats through to whole chromosome gains and losses.
CUT&Tag: 'cleavage under targets and tagmentation', a method which uses antibody-tethered transposases to target specific DNA–protein interactions for sequencing, including histone modifications and transcription factors.
DNA methylation: in mammals, this is an epigenetic mechanism involving the transfer of a methyl group onto cytosine bases in the genome which can have a regulatory impact on gene expression. This is typically measured using bisulfite sequencing, in which unmethylated cytosines are converted to uracil – which will appear as a thymine base in sequencing data – while methylated cytosines remain unchanged.
Epigenetic plasticity: variability in epigenetic regulation that permits cells to undergo cell fate transitions due to stochastic activation of gene expression.
Euchromatin: loosely packed or 'open' chromatin, which is often the site of active gene expression.
FACS: fluorescence-activated cell sorting, a method for the sorting of single cells based on phenotypic measurements, including size, granularity, and protein/antigen expression.

protocol features an optimised version of the Smart-seq2 mRNA amplification, after which the sample is split and primers targeting regions of interest within the transcriptome and/or genome are used to generate targeted amplicon sequencing libraries. By focussing on known mutations, this approach significantly increases the sensitivity and reduces the cost of mutation detection. A related, microfluidic targeted genome sequencing approach has been commercialised by Mission Bio, enabling high-throughput genotyping of single cells, but linking with protein expression information rather than transcriptomic data. These targeted methods are highly relevant for studies where a known repertoire of mutations is prevalent, such as studies of intratumoural heterogeneity and cancer evolution, where recurrent mutations are common. However, in complex mutational backgrounds, or where mutation discovery is important, bulk or single-cell whole-genome sequencing may still be more applicable.

Methods involving physical separation of the nucleus and cytoplasm of a cell have also been demonstrated (Figure 3B). 'Simultaneous isolation of genomic DNA and total RNA' (SIDR) [15] was the first such method. This approach has seen massive increases in throughput in direct nuclear tagmentation and RNA sequencing (DNTR-seq) [16], which relies on nuclear/cytoplasmic separation, followed by full-length mRNA amplification from the cytoplasmic fraction, and direct tagmentation-based genomic library preparation from the nuclear DNA, obviating the need for a traditional whole-genome amplification step. This represents a significant cost reduction and contributes to the increased scale at which the method can operate. However, like other methods which require physical separation of nucleus and cytoplasm, it is unclear how they are affected by disassembly of the nuclear envelope during the mitotic cell cycle.

Linking the epigenome and gene expression

Although intercellular diversity in genome sequence and structure is common, the phenotypic heterogeneity of cells is a hallmark of multicellular organisms and emerges from the regulation of gene expression through epigenetic modification of the genome. Starting from the same genetic background, cells can acquire highly specialised functions during development and are able to dynamically modify their phenotype in response to environmental stimuli. Many epigenomic approaches have been adapted to make measurements in single cells, but only assays for **DNA methylation** and chromatin accessibility have been incorporated into multiomic assays. These assays, by linking genome regulation and gene expression in the same cell, can shed light on lineage determination, developmental dynamics (Box 2), and mechanisms of disease development.

The first methods that attempted to link epigenetic diversity with transcriptional heterogeneity in single cells focussed on the association between DNA methylation at CpG sites and gene expression. To achieve this, single-cell bisulfite sequencing methods – either post-bisulfite adaptor tagging (PBAT) [17], which measures DNA methylation across the genome, or reduced representation bisulfite sequencing (RRBS) [18], which enriches for regions with high CpG content – have been combined with transcriptomic analysis of individual cells. scM&T-seq (single-cell methylome and transcriptome sequencing) built upon the G&T-seq method (Figure 3A), but instead uses the purified genomic DNA for a modified PBAT protocol, generating genome-wide methylation data, while the transcriptome is again sequenced using a modified Smart-seq2 protocol [19]. The method was first applied to mouse embryonic stem cells to discover novel correlations between heterogeneity at DNA methylation of distal regulatory elements and expression of hundreds of genes, including key pluripotency genes. The G&T-seq approach was also adapted for Smart-RRBS, which enables joint profiling of DNA methylation (by RRBS) and transcriptome analysis [20]. Other approaches involving physical separation of the nucleus and the cytoplasm have been used to obtain gene expression and

Heterochromatin: tightly packed or 'closed' chromatin, which is less accessible for transcription.
Hi-C: a chromosome conformation capture assay which enables the genome-wide measurement of long-range interactions between genomic loci.
Microfluidic assays: in this case referring to assays which isolate individual cells in microfluidic droplets in the presence of barcoded oligonucleotide-coated beads to enable the capture and barcoding of molecules of multiple classes (DNA, RNA, and protein) from single cells. These methods are often appropriate for experiments in which a large number of cells (>1000s) undergo parallel analysis and classification.
Molecular cellular identity: the amalgamation of molecular events that make a cell belong to a particular type or state.
Plate-based assay: in this case refers to a single-cell multiomic approach for which cells are isolated into 96- or 384-well plates for processing (typically) using liquid handling robotics. These methods are often appropriate for experiments where small numbers (100s–1000s) of cells undergo a detailed analysis.
Single-nucleotide variant (SNV): a single base change in the genome.
Somatic variation: genetic diversity occurring between cells within the same organism, arising from mutations occurring after conception.
Whole-genome amplification: describes several possible methods for genome-wide amplification of cellular DNA, in this case to enable single-cell genome sequencing.

Key figure

Multiomic exploration of molecular cell identity

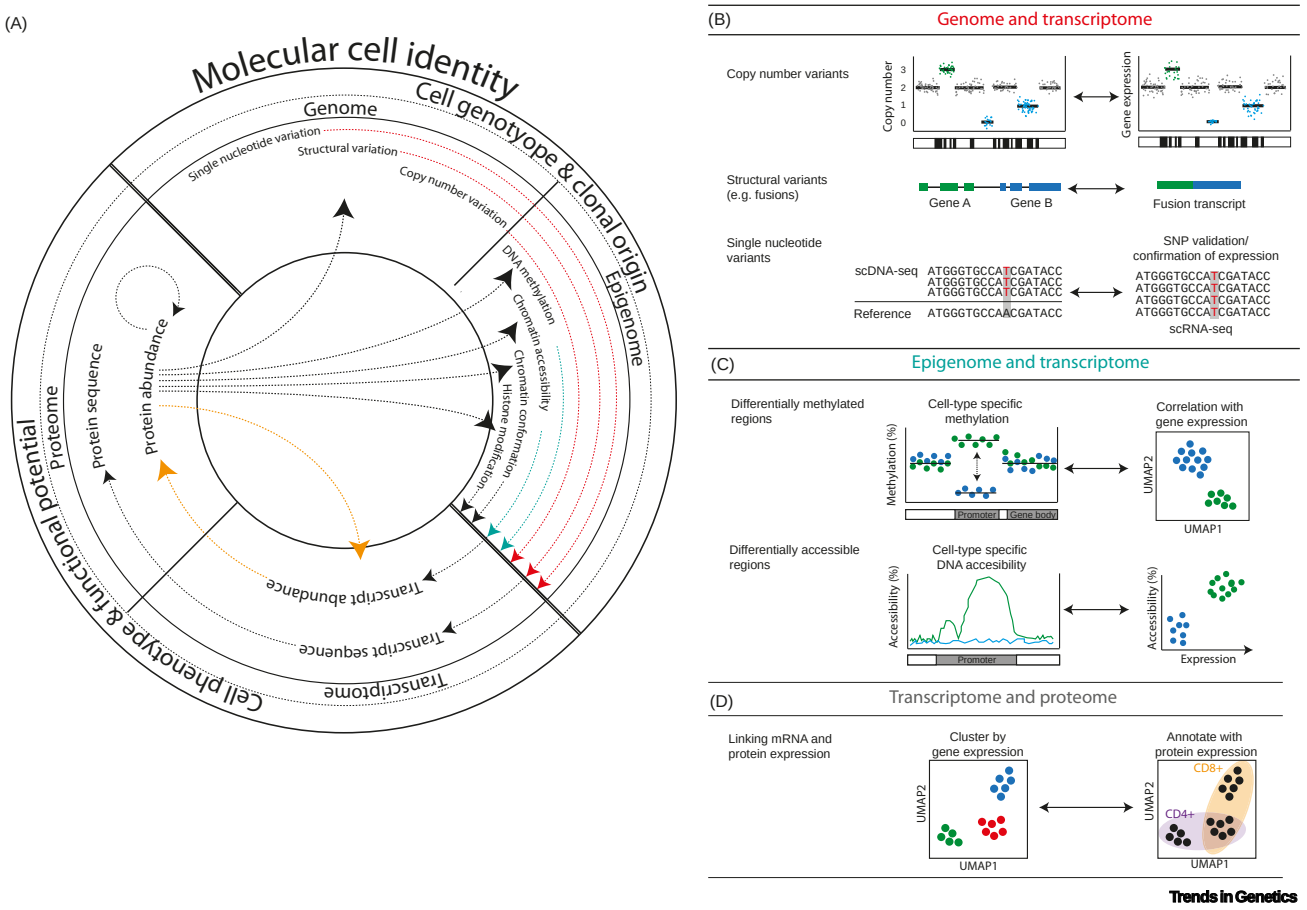

Figure 1. (A) Molecular cell identity comprises the interaction of many different molecular layers within the cell. Genomic and epigenomic variation influence the sequence and abundance of transcripts and proteins, which in turn can influence each molecular layer within the cell. Areas in which single-cell multiomic analysis has made significant advances are highlighted in (B–D).

DNA methylation data from the same single cell, including MT-seq [21] and scTRIO-seq [22] (Figure 3B).

There are still major limitations to the detection of DNA methylation in single cells – bisulfite treatment is destructive to the DNA, resulting in a high level of allelic and locus dropout. Similarly, the sequencing libraries generated using these approaches are typically rich in PCR duplicates, which, combined with dropouts and the expense of pursuing high genomic coverage from single cells, make the measurement of DNA methylation at single-base resolution challenging. Furthermore, the C > T substitution inherent in the approach makes the calling of genomic variants difficult, making existing approaches unsuitable for parallel methylation and SNV calling. Recently, the epi-gSCAR approach (epigenomics and genomics of single cells analysed by restriction) demonstrated the feasibility of bisulfite-free single-cell library preparation – using the methylation-sensitive restriction

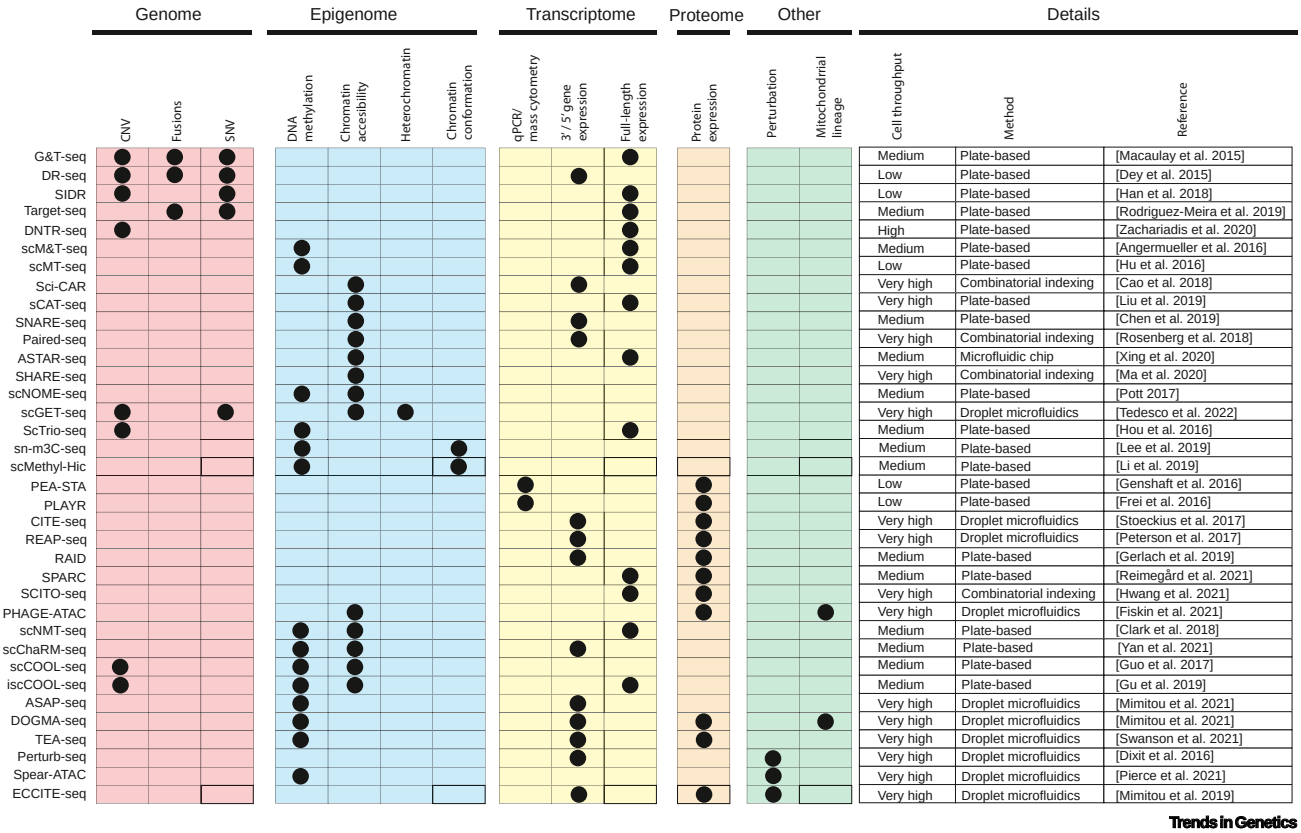

Figure 2. An overview of current single-cell multiomic approaches. See [12,13,15,16,19,21,22,24–26,28,29,36,41,45–50,53–56,58,59,61,63–65,102].

enzyme *HhaI* and quasilinear amplification – to study genome-wide methylation and genomic variation at single-nucleotide resolution in cancer cell lines [23].

The accessibility of sequences within the genome is considered to be a mark of genomic activity, representing the expression of particular genes or the openness of particular sequences, including enhancers or transcription factor binding sites. Chromatin accessibility in single cells is now routinely measured by the 'assay for transposase-accessible chromatin' using sequencing (**ATAC-seq**), in which the Tn5 transposase is used to fragment and insert sequencing adaptors into open regions of the genome (**euchromatin**). Due to the nature of the ATAC-seq method, it is compatible with considerably higher throughput than the analysis of DNA methylation. In general, these high-throughput methods rely on the tagmentation of accessible chromatin in a bulk preparation of nuclei before paired barcoding of the tagmented DNA and RNA from the same cell, either through **combinatorial indexing** or in droplet-based approaches (Figure 3C,D). For example, sciCAR-seq [24], used combinatorial indexing to process over 11 000 nuclei per experiment. Medium-throughput methods, working with intact cells rather than nuclei, have also been described (scCAT-seq, [25] and ASTAR-seq [26]) and may potentially be more applicable to experiments in which rare cells are to be profiled.

Throughput was dramatically increased in Paired-seq [27] by implementation of a ligation-based combinatorial indexing strategy which enabled processing of one million nuclei per experiment. Building on Paired-seq and a similar approach, SPLiT-seq [28] and SHARE-seq [29], further increased the sensitivity of the combinatorial indexing approach to measure the 'chromatin

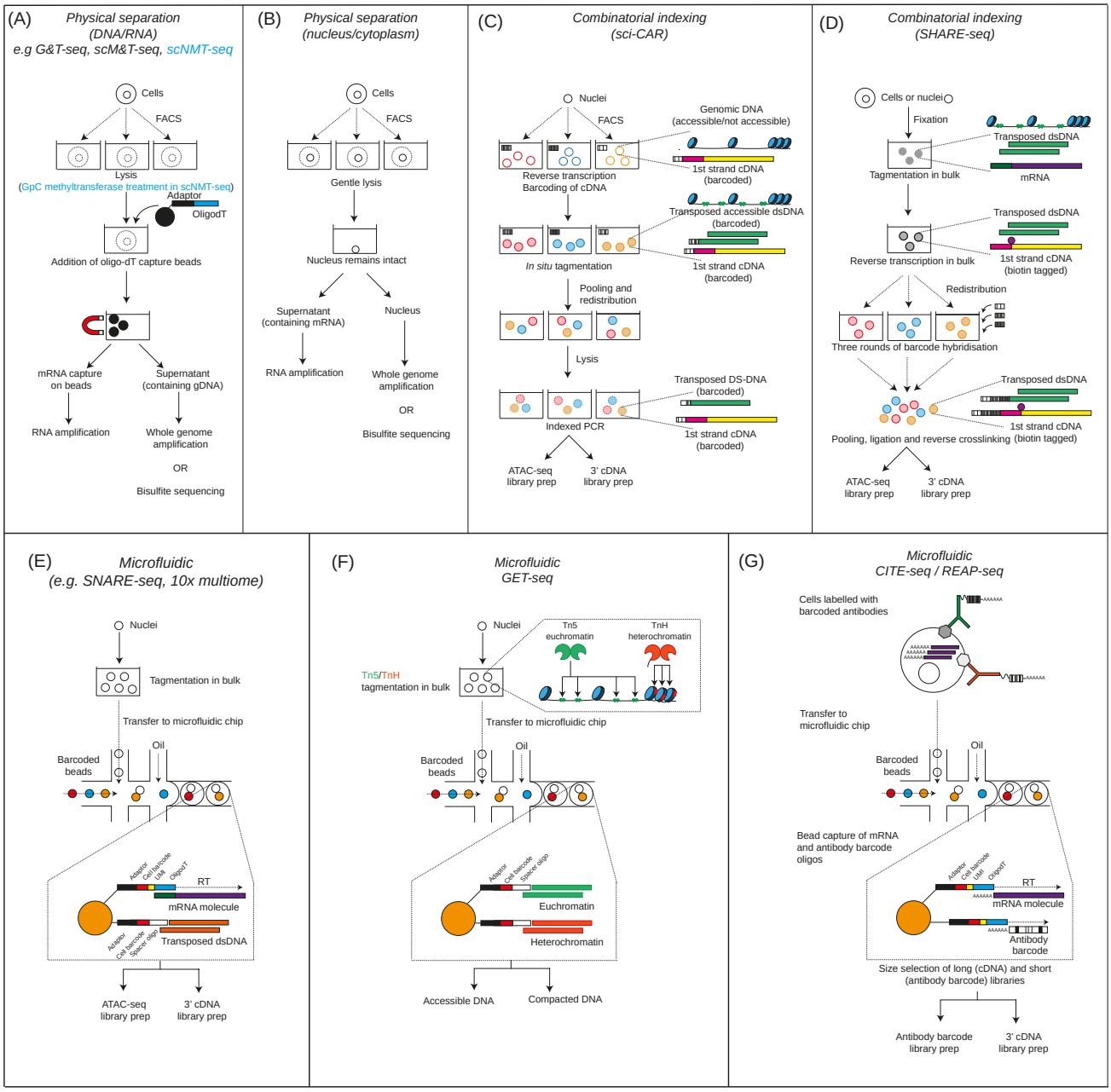

Figure 3. Capturing multiple layers of information from the same single cell. Various approaches have emerged to extract distinct layers of omic information from the same single cell. (A) G&T-seq, and those methods based on it, perform physical separation of genomic DNA and mRNA following capture on magnetic beads. (B) An alternative approach involves physical separation of the nucleus and cytoplasm of the cell. These methods allow both genome sequencing and methylation sequencing to be performed on the isolated DNA. High-throughput combinatorial indexing has been applied in (C) sci-CAR and (D) SHARE-seq to obtain linked transcriptome and chromatin accessibility from the same cell, while droplet-based microfluidic approaches (E) have enabled parallel capture of these modalities using SNARE-seq and the 10X Genomics Chromium platform. (F) Droplet microfluidics has also been used to sequence DNA from accessible and compacted chromatin using GET-seq. (G) CITE-seq and REAP-seq take advantage of polyadenylated oligonucleotide tags attached to antigen-specific antibodies to capture protein expression information in parallel with mRNA expression. Abbreviations: ATAC-seq, 'assay for transposase-accessible chromatin with high-throughput sequencing'; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; FACS, fluorescence-activated cell sorting; G&T-seq, genome and transcriptomesequencing; GET-seq, genome and epigenome by transposases sequencing; sci-CAR, single-cell combinatorial indexing-chromatin accessibility and RNA sequencing; SNARE-seq, single-nucleus chromatin accessibility and mRNA expression sequencing.

Box 1. Single-cell multiomics in cancer evolution

Cancer development within an individual is an evolutionary process in which cells evolve by mutation and subsequent selection for clones with increased proliferative capability, fitness, and resistance to therapeutic intervention. While mutational profiling is informative in understanding tumour evolution, it is critical to link these mutations or genotypes with cellular phenotype or functional data. Single-cell multiomic approaches enable the integration of genotypic information – from single nucleotide to whole chromosome resolution – with epigenomic, transcriptomic, and increasingly, proteomic information, and several landmark studies have demonstrated the application of these approaches in cancer.

The scTrio-seq approach [22] was optimised to profile human colorectal cancer cells from paired primary tumours and lymphatic or liver metastases [72]. In one patient, this permitted the identification of 12 sublineages that originated from two different progenitors, one of which was maintained throughout tumour progression and was still present in both the final neoplasm and distant metastases. Overlaying DNA methylation data along these lineages revealed that methylation levels were homogeneous among cells within the same genetic lineage but varied among different lineages.

Based on the physical separation of genomic DNA and transcriptomes, Smart-RRBS has been applied in the study of epigenetic evolution in chronic lymphocytic leukaemia (CLL) [73]. Here, the parallel measurement of epigenetic and transcriptional changes enabled the linkage of epimutations in *SF3B1*-mutant CLL cells to a 3′ splicing phenotype and subclones of cells with epigenetic and transcriptional phenotypes that expanded following chemotherapeutic treatment. Subsequently, the same approach demonstrated that a decrease in epigenetic–transcriptional coordination in CLL could partially be explained by intercellular epigenetic diversification [74]. The Smart-RRBS approach was also recently applied in the study of primary diffuse glioma, where it enabled joint capture of transcriptional, genetic, and epigenetic data from the same single cell [75]. The scRRBS enabled CNV analysis at 20 Mb resolution for genome-wide analysis, but also 0.1 Mb resolution to reveal CNVs at the *EGFR* locus. Furthermore, it could be used to generate lineage trees from individual glioblastoma samples, with individual branches annotated with transcriptomic cell types and states.

Recently G&T-seq [13] was coupled with laser capture microdissection (LCM) to generate spatially resolved genomic and transcriptional profiles of cancer cells with the potential for lymphovascular invasion in a patient with triple-negative breast cancer [76].

While these studies are still relatively small in scale, continued development of these methods, including increases in throughput and resolution, reductions in cost, and incorporation of additional layers of data, will undoubtedly transform single-cell multiomic profiling into a mainstream tool in the study of cancer evolution.

Box 2. Single-cell multiomics analysis in developmental systems

In multicellular organisms, cells can adapt an immense array of phenotypes and states, despite having the same or highly similar genomes. During development and a healthy lifespan, as cells commit first to specific germ layers then cell types, the regulation of genome function through epigenetic modification is fundamental to the emergence of this complexity. Cell fate decisions are made by individual cells responding to intrinsic and extrinsic factors resulting in changes to epigenomic, transcriptomic, and proteomic aspects of cell identity. The integration of different omic layers of the same single cell through multiomic analysis can provide a unique perspective on the dynamics of these processes.

During early embryogenesis, global demethylation erases the epigenetic signatures of the highly specialised gametes to enable the embryonic cells to become totipotent. scCOOL-seq, which measures DNA methylation, chromatin accessibility, and copy number variation has been applied to study this epigenetic reprogramming in mouse [57] and human embryos [77], revealing the dynamics of parental genome activity in the first cell divisions after fertilisation. The iscCOOL-seq method was subsequently applied to the study of mouse oocytes, identifying dynamic associations between chromatin accessibility, methylation, and expression during oocyte maturation. Similarly, scM&T-seq was also used to explore the heterogeneity in DNA methylation of oocytes from young and aged mice, with those from aged mice showing increased molecular heterogeneity indicative of epigenetic dysregulation [78].

Combined gene expression and whole-genome methylation profiling was used to characterise the post-implantation DNA methylation landscapes in mouse embryos (from eight-cell stage to E6.5 epiblast and extraembryonic ectoderm), revealing divergent methylation patterns in the extraembryonic tissue, with methylation in these lineages mirroring the aberrant methylation of the promoters of developmental genes observed in tumorigenesis [79].

The emergence of high-throughput approaches, combining ATAC-seq and RNA-seq from the same cell, with the potential to integrate protein expression and cell-lineage tracing (e.g., DOGMA-seq), has enormous potential in unravelling the dynamic interactions underpinning molecular cell identity during early development and organogenesis as well as in cellular systems undergoing constant replenishment (e.g., blood).

potential' of individual cells, which explores the predictive power of chromatin accessibility on future gene expression changes and lineage commitment within the cell. Single-nucleus chromatin accessibility and mRNA expression sequencing (SNARE-seq) leverages the **microfluidic** Drop-seq [30] method to perform parallel chromatin accessibility and gene expression measurements on the same nuclei [31]. This approach has been adapted for the 10X Genomics Chromium platform using hydrogel beads carrying separate capture oligonucleotides which capture both the tagmented genome and mRNA. A recent alternative method for single-nucleus multiomic profiling, ISSAAC-seq [32], based on the Sequencing HEteRo RNA-DNA-hYbrid (SHERRY) approach [33], exploits a first tagmentation reaction on accessible chromatin followed by reverse transcription and then a second tagmentation round on DNA–RNA hybrids. Nuclei are loaded on microfluidic or FACS apparatus for single-cell analysis, and separate DNA and RNA libraries are produced, exploiting the different adaptor configuration for the two tagmentation steps.

Linking different aspects of the epigenome

ATAC-seq will only provide sequence information from accessible chromatin and does not capture genetic alterations and chromatin remodelling events associated with **heterochromatin**. Compacted chromatin is crucial for lineage specification [34] and genome stability [35]. Recently, chromatin accessibility profiling has been combined with heterochromatin sampling in the single-cell genome and epigenome by transposases sequencing (scGET-seq) assay [36]. This assay builds on scATAC-seq with droplet microfluidic exploiting engineered transposases to simultaneously probe H3K9me3-enriched compacted chromatin alongside accessible chromatin. This combined epigenomic and genetic characterization allowed for increased resolution in CNV calling and it was used to compute a new metric called **chromatin velocity** – based on the differential enrichment between closed and open chromatin – to reveal patterns of **epigenetic plasticity** during stem cell reprogramming and key transcription factors correlated to developmental commitment. The introduction of engineered transposases in single-cell genomics unlocks immense potential for targeted analysis of other domains within the epigenome. A similar method named scCUT&Tag2for1, a modification of standard **CUT&Tag** [37], uses antibody-guided tagmentation to simultaneously characterise accessible and silenced regulome by targeting the initiation form of RNA polymerase II (Pol2 Serine-5 phosphate) and repressive Polycomb domains (H3K27me3) [38]. A further CUT&Tag development, scCUT&Tag-pro, allows simultaneous profiling of histone modifications with protein abundances on whole cells [39].

Chromatin conformation assays, such as **Hi-C**, have revealed the extent to which three-dimensional conformation of the genome regulates gene expression in health, disease, and senescence. Two multiomic approaches, single-nucleus methyl-3C [40] and scMethyl-HiC [41], have described methods to obtain linked chromatin conformation and methylation data from the same single cell, using bisulfite conversion of crosslinked genomic DNA. These approaches reveal that chromatin conformation alone can identify cell types within heterogeneous populations and differential methylation signatures associated with cell-type-specific chromatin interactions in human brain cells.

Linking transcript and protein expression

Much of cell behaviour is determined by the functions of proteins, and it is generally accepted that mRNA expression levels offer only a weak proxy for direct measurement of protein expression [42]. The obvious biochemical differences between nucleic acids and protein constitute a challenge for developing single-cell approaches – there is no method for protein sequence amplification and so measurements are dependent on antibody-based protein detection or mass spectrometry for peptide identification.

Proximity extension assays (PEAs) have been exploited to detect protein expression using antibodies recognising different epitopes on the same protein. PEA is based on proximity ligation assay (PLA) [43] in which antibodies conjugated with single-stranded DNA oligonucleotides colocalise on the target protein, enabling ligation and generation of a sequence that is detectable, in parallel with mRNA molecules, by qPCR [44,45]. Proximity ligation assay for RNA (PLAYR) expanded the throughput of the PLA approach by detecting transcripts and proteins using mass cytometry, enabling parallel measurement of over 40 different transcripts and protein epitopes in thousands of cells [46]. More recently, the 'single-cell protein and RNA coprofiling' (SPARC) method, in which mRNA and protein lysate are physically separated, enables parallel whole transcriptome mRNA-seq and detection of extracellular and intracellular proteins using PEA [47].

Increases in throughput have been enabled by the combination of oligonucleotide-conjugated antibodies with droplet-based microfluidic (e.g., 10X Genomics) and micro-well platforms (e.g., BD Rhapsody). This approach was pioneered in RNA expression and protein sequencing (REAP-seq) [48], Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) [49]. In these methods, cells are labelled with panels of antibodies, each tagged with a specific polyadenylated barcode which can be captured in parallel with the mRNA from the same cell following lysis (Figure 3G). SCITO-seq demonstrated a combinatorial indexing approach for antibody barcoding, enabling extreme multiplexing of cells as well as multimodal profiling of more than 150 surface proteins in parallel with mRNA expression from the same cells [50].

Antibody-based methods are severely limited by the availability of antigen-specific reagents – detection requires a reliable epitope-specific antibody (or pair of antibodies for PEA-based assays) which dramatically reduces the number of proteins or epitopes that can be surveyed. To obtain a more complete overview of the cellular proteome, antibody-independent methods are essential. Single-cell mass spectrometry-based approaches, such as single cell proteomics by mass spectrometry (SCoPE-MS) [51] and SCoPE2 [52], can analyse thousands of proteins and post-translational modifications in individual cells; however, they have yet to be directly incorporated into a combined multiomics approach. Recently, the PHAGE-ATAC assay [53] demonstrated an alternative approach where antibodies are replaced with nanobody phage-display libraries. This may offer a potential route towards protein detection without the need for antibodies.

Triple and higher-order single-cell multiomics

To fully explore the causes and consequences of intercellular heterogeneity, it is important to simultaneously capture data from as many aspects of the cell as possible. As an early example, scTRIO-seq could simultaneously measure genomic copy number changes at ~10 Mb resolution, DNA methylation, and gene expression from the same cell [22]. The single-cell nucleosome, methylation and transcription sequencing (scNMT-seq) approach [54] combines 'single-cell nucleosome occupancy' and methylome sequencing (scNOMe-Seq [55]) with a modification of G&T-seq [13] in a plate-based assay. In this approach, the genomic DNA is methylated, using a GpC methyltransferase, at GpC sites that are not bound by nucleosomes. Following physical separation of DNA and mRNA, the DNA undergoes bisulfite conversion which allows parallel measurement of nucleosome positioning, DNA methylation, and the cell's transcriptome [54]. A similar approach, scChaRM-seq, was also recently described [56]. NOMe-seq approaches were further adapted for scCOOL-seq [57], which can measure various genomic aspects of the cell in parallel, including chromatin state, nucleosome positioning, DNA methylation, CNV, and ploidy. This method has been modified to incorporate transcriptomic measurements in iscCOOL-seq [58].

Building on microfluidic workflows for parallel ATAC- and CITE-seq from single cells, ASAP-seq [59] demonstrated parallel chromatin accessibility, cell-surface and intracellular protein

measurements. Furthermore, the method enabled mutational profiling of the mitochondrial genome, allowing simultaneous lineage inference from mitochondria mutations, as previously demonstrated with mtscATAC-seq [60]. This was further expanded in the same manuscript to incorporate RNA-seq measurements – thus reading four layers of information from the same cell – in a method referred to as DOGMA-seq [59]. A similar approach, TEA-seq, was also recently described [61]. In these studies, whole cells were analysed instead of nuclei, which has the advantage of allowing more comprehensive phenotypic characterization, surface-marker enrichment prior to analysis, and retention of cytoplasmic RNA in multimodal assays.

By capturing these multiple layers, the epigenetic determinants of differentiation, and their dynamics, can be dissected with unprecedented detail – variations in accessibility and methylation can be directly correlated with variation in gene and protein expression levels. This will enable the construction of genome-wide regulatory models which incorporate the cell as the unit in which genomes are regulated and genes are expressed.

Concluding remarks

The emergence of methods enabling multiomic profiling of single cells continues at a staggering pace. It is now possible to profile multiple molecular layers of thousands of individual cells, with newer methods approaching 'Omni-seq' – where multiple omic measurements can be combined with spatial and lineage-based information to determine a cell's molecular state, microenvironment, and life-history in a single readout [62]. This has significant implications for current and future studies of developmental and cancer biology, where changes in individual cells are fundamental to the progression of healthy development or disease. These methods, especially when coupled with perturbations using the CRISPR/Cas system [63–65], will have immense potential to unravel cellular (epi)genotype/phenotype associations and the mechanisms that govern the emergence of cellular heterogeneity.

However, several challenges remain. At present, the analysis of each molecular level is imperfect – single-cell measurements of any kind are prone to noise and, in particular, drop-out, where critical signals of mutation, modification, or expression may be lost. As methods scale to incorporate thousands, even millions, of cells, there is a concomitant loss of detail per cell (see Outstanding questions). While the future development of methods will undoubtedly see the incorporation of further omics measurements – including expanded proteomic and metabolomic profiling [66] – there is still a need to refine many of the existing methods to obtain high resolution, accurate measurements of base-level events in the genome, and sensitive, quantitative, measurements of both gene and isoform expression from individual cells.

Aside from the macromolecular components of the cell, there are also many metabolites that can be instrumental in the regulation of cell function, and new approaches for their measurement are emerging [67]. No cell lives in isolation – beyond molecular profiling, the life history of the cell and its spatial relationship to other cells are critical determinants of cell identity. Undoubtedly, the considerable advances in cell lineage tracing [68] and spatial transcriptomics [69] will converge with multiomic profiling to enable comprehensive analysis of cellular identity in the context of where it is (Box 3), and where it has come from, but this will come with additional – and complex – computational and data science challenges. While each layer of information added to a multiomic analysis can bring new opportunities to classify cells and their biological context, it will also bring opportunities to study the mechanistic relationships between these different modalities in individual cells. While this an extremely exciting prospect, it requires the development of robust methods for the integration of diverse data types, each with their own idiosyncrasies. Packages such as Seurat [70] and MOFA+ [71] enable data integration from single-cell multiomic experiments, with the latter designed to

Outstanding questions

What is the optimal trade-off between resolution (number of measurements per cell) and throughput (number of cells analysed)?

What are the upper and lower limits of detection required to make meaningful, comprehensive investigations of cell type and state?

By operating at ultra-high-throughput, do we risk missing key details of cellular phenotypes – for example, lowly expressed genes, base-level (epi)genomic variation?

How can key measurements, such as histone modifications, DNA protein interactions, and isoform level gene expression, be integrated into multiomic approaches?

How can the nonmacromolecular components of the cell be integrated into multiomic studies – for example, the metabolome and lipidome?

What are the optimal computational approaches for data integration in multiomics approaches which take into account the various errors and sources of noise in parallel but distinct types of measurement made from the same single cell?

What level of detail is required to build accurate predictive models linking (epi)genomic variation and the function of protein–protein interaction networks?

How can antibody-independent proteomics be integrated with existing multiomic workflows?

Can single-cell multiomics methods be combined with spatial measurements, perhaps even in real time?

Can single-cell whole-genome sequencing – to base level resolution – be enabled at high throughput in a multiomic approach?

What are the applications for wider scientific questions – for example, can single-cell multiomics be applied to assemble and annotate genomes of nonmodel single-cell organisms?

Box 3. Spatial multiomics

The organisation of cellular structures and corresponding cell–cell interactions are fundamental to the operation of any multicellular system. Understanding the spatial organisation of cells within tissues is therefore essential to link molecular cell identities with organ- or organism-level functional biology. However, single-cell methods are not able to capture the spatial context of cells as the analysed tissue must be dissociated in order to be analysed. To address this need, there have been considerable advances in spatial transcriptomics, with transcriptome-wide or targeted approaches revealing gene expression patterns with regional, cellular, and even subcellular resolution [80].

Conventional *in situ* hybridisation [81] allows transcript detection at subcellular resolution, and recent developments of this approach have increased the multiplexing capacity for this approach from tens [82–85] to hundreds and even thousands of transcripts [85–88]. Untargeted methods have also expanded imaging-based *in situ* methodology to genome-wide profiling of gene expression [89,90]. Substrate-based approaches use positionally barcoded oligo-dT microarray features to locally capture mRNA molecules from tissue sections [91], with resolutions ranging from 50 μm (e.g., the 10X Visium platform), spanning multiple cells, through to methods approaching single-cell [92–94] and subcellular (<1 μm) resolution [95]. Spatial epigenomics approaches are also emerging, firstly with sciMAP-ATAC [96], where chromatin accessibility profiles obtained from tissue micropunches were matched with tissue spatial coordinates using combinatorial indexed transposition and sci-ATAC-seq workflow.

Spatial multiomic approaches are emerging – fluorophore- and oligonucleotide-conjugated antibodies can be incorporated into both *in situ* and array-based methods to enable parallel mRNA and protein detection, which has been demonstrated for several of the spatial transcriptomics methods mentioned previously [87,97], using the Nanostring GeoMX DSP instrument [98] and also very recently demonstrated in SPOTS [99], which combines the 10X Genomics Visium Platform with CITE-seq antibody-based protein detection. Novel approaches, such as the recently described DBiT-seq, can perform spatial profiling of mRNA and protein with 10 μm resolution [100]. DBiT-seq is based on two-step microfluidic-delivery of DNA barcodes directly to the surface of a tissue slide, and this approach has also enabled the spatially resolved profiling of accessible chromatin at approximately 20 μM resolution using *in situ* Tn5 transposition combined with microfluidic spatial barcoding [101].

Although only now emerging, these methods are likely to evolve rapidly, and far beyond transcriptomic and proteomic integration. Bringing multiomic methods with single-cell resolution together with imaging approaches will eventually enable comprehensive, three-dimensional molecular profiling of the dynamics of multicellular systems in development and disease.

identify cell classification factors and regulatory dependencies in scNMT-seq data. The continued development of computational tools that go beyond cell type classification, and can infer regulatory networks across multiple layers, is essential for future single-cell multiomic studies.

The ongoing convergence of methods enabling multiomic profiling of cellular molecular identity, localisation, and life history will dramatically change how we study multicellular living systems, offering unique insights into the regulatory processes governing how individual cells function collectively to produce whole-organism phenotypes in development, health, and disease.

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Declaration of interests

F.G. is an inventor of a pending patent application relating to the GET-seq method.

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