of emerging high-dimensional technologies. In particular, single-cell methods offer considerable opportunities to analyse organoids at unprecedented scale and depth, enabling comprehensive characterisation of cellular processes and spatial organisation underpinning organoid heterogeneity. This review evaluates state-of-the-art analytical methods applied to organoids, discusses the latest advances in single-cell technologies, and speculates on the integration of these two rapidly developing fields.

Main Text

Organoids at the Interface of Basic Biology and Translational Research

Organoids are stem cell-based self-organising three-dimensional (3D) tissue models that are widely adopted as an intermediate culture system between traditional tissue culture and animal models. As a versatile experimental medium, organoids have been used to address diverse biological and clinical questions, ranging from basic stem cell and developmental biology [1,2] to disease modelling [3], drug screening [4,5], and rational design of personalised medicine [6].

Organoids can be generated from a variety of tissue origins, including adult stem cells, embryonic stem cells, induced pluripotent stem cells (iPSCs), and tumour biopsies [7]. In contrast to homogenous 2D cell lines and cell line-derived spheroids, organoids are self-organising **heterocellular** (see Glossary) systems capable of recapitulating physiologically relevant phenotypes of their tissue of origin [8]. When compared to animal models and clinical biopsies, the relative simplicity of organoids renders them more amenable to genetic modification, more compatible

with high-throughput applications, and in general offers greater experimental flexibility [9] (Figure 1).

Organoids are powerful **biomimetic** models able to recapitulate tissue architecture and functionality [7], but their utility has been limited by the availability of analytical tools that can generate representative and reproducible data from them. Traditional bulk technologies (e.g. western blotting, confocal microscopy) have been successfully applied to organoid research, but their low-dimensional readouts are unable to reveal finer details of organoid heterogeneity. To fully leverage the biological complexity of organoids, high-dimensional methodologies that enable the inspection of organoids at single-cell resolution are required. Here, we review state-of-the-art analytical methods that have been – or could be – applied to organoid research, evaluating their strengths and weaknesses in light of the biological questions being addressed. In particular, we will discuss the latest development in high-dimensional single-cell technologies and speculate on their integration into future organoid studies.

Dimensionality of Data

The concept of '**data dimensionality**' can be ambiguous and warrants definition. Within the scope of this review, the distinction between low- and high-dimensional biological data concerns three aspects: 1) the number of *cells* analysed, 2) the number of *parameters* measured, and 3) the number of experimental *conditions* evaluated per assay (Figure 2). The dimensionality of a dataset is limited by its smallest dimension along these three axes. For example, bulk **-omic** analyses of organoid populations are considered low-intermediate dimensional due to the lack of single-cell resolution despite the richness of parameters being measured. Similarly, the dimensionality of florescent imaging approaches is limited by the number of parameters that can be measured per cell (typically <5). This definition also distinguishes high-dimensional and high-throughput applications — the latter not necessarily high-dimensional as the increased throughput may only be applied to one dimension. For example, high-throughput viability-based drug screens can measure a vast number of conditions but only with one parameter, live vs. dead. Finally, we do not consider **multimodal** analyses adding extra dimensions to our

framework as extra **modalities** can be considered an extension of the parameter axis.

There is a dichotomy of methods applied to organoid research: namely the 'population' approach that considers organoids as constituents of a larger culture, and the 'reductionist' approach that treats organoids as self-organised cell assemblies. While the former is largely compatible with traditional bulk analysis (e.g. organoid viability drug screening [4,5]), the latter requires single-cell methods to capture organoid heterogeneity (e.g. cell-type and cell-state specific signalling analysis of organoids [10]) (Figure 2). No single tool is optimal for all applications — the selection of experimental methods should always be determined by the biological question being addressed, and multiple tools may be needed to tackle the same problem from different angles.

Low-intermediate Dimensional Organoid Analysis

During the last decade, organoid technology has undergone unprecedented technical advances to occupy a unique niche at the interface of basic science and

translational research (Figure 1). Despite its increasing complexity, the core idea of the technology is that organoids are miniature organs that can be used as proxies of their tissues of origin, recapitulating their histological and pathological characteristics. Given their role as tissue models, low-dimensional methods routinely used for analysing tissues have been applied to organoid characterisation and led to significant biological insights.

Organoid Imaging

In its simplest and most widely adopted form, organoids are cultured in an **extracellular matrix (ECM)** such as Matrigel, collagen, or polyethylene glycol (PEG) hydrogel [11]. The semi-transparency of the matrices allows direct inspection of organoids in a non-intrusive way, enabling direct interpretation of the state of an organoid culture. Since organoids self-organise, their morphology can be indicative of their interaction with the ECM [12] as well as key developmental processes such as proliferation, differentiation, and morphogenesis [1,13]. In addition, the internal structure of organoids can be reconstructed via tissue clearing followed by high-resolution 3D imaging [14]. When applied to cerebral organoids, Renner and

colleagues demonstrated the presence and interconnectivity of distinct ventral and dorsal neuroepithelia as well as timed generation of differentiated neurons, confirming that the spatial-temporal patterning events that govern human brain development can be recapitulated in organoids [15]. Recent developments in deep **convolutional neural networks (CNNs)** have been applied to automated recognition and quantification of human intestinal organoids from brightfield images [16]. Future iterations of such imaging methods will parse organoids with more complicated structural features or even track the development of organoids over time, ideally at single-cell resolution [17]. This will lead to more comprehensive and standardised morphological characterisation of heterogenous organoid populations.

Morphological analysis reveals that organoids structurally resemble their tissue of origin [15]. In order to demonstrate that the cellular composition is also preserved, histological profiling (e.g., H&E) and immunostaining (e.g., immunohistochemical (IHC), immunofluorescence (IF)) are the most routinely used tools. Whole-mount staining represents the structural complexity of organoids but can be obscured by

the thickness of the sample [14], while imaging of organoid-derived monolayers solves this problem and enables luminal access to the otherwise enclosed structure [18,19]. Extensive efforts have been made to validate the histological fidelity of organoids regarding cell-type and cell-state compared to the tissue being modelled (e.g., brain [20], stomach [21], lung [22], and various cancers [5,23–25]). Despite that such analysis can only describe 2D cross-sections of organoids irrespective of their 3D complexity, histology remains the cornerstone of organoid characterisation and underlies the paradigm of using organoids as *ex vivo* tissue models.

Advances in high-resolution microscopy enable 3D imaging of entire organoids with single-cell resolution, opening opportunities for the inspection of organoid morphology and composition in its native spatial context [1,14]. **High-content imaging (HCI)** empowered by florescent light-sheet microscopy has been applied to time-course characterisation of intestinal organoids, enabling accurate reconstruction of their developmental trajectory [1,26]. Although florescent read-outs limit the number of parameters measured by IF, a major advantage of HCI is the large number of conditions that can be assessed (>1000s). When incorporated

into existing high-throughput organoid imaging platforms [4], HCI will further improve the consistency and accuracy of phenotypic organoid characterisation.

Organoid Bulk Analysis

Imaging technologies are useful for the examination of organoids with spatial resolution, but biological insights obtained from low-parameter florescent imaging alone can be limited. The ease of retrieving organoids from ECM ensures that established methods in molecular biology, biochemistry, and bulk **next-generation sequencing (NGS)** technologies can be applied to the analysis of organoid populations with minimal requirement for protocol adaptation, offering easily accessible avenues for mechanistic investigation (Figure 2).

Bulk analysis technologies are useful when the biological question is not confounded by sample heterogeneity. In the case of organoids, this could include mutational profiles of clonal cancer patient-derived organoids demonstrated by whole-genome sequencing (WGS) [5,27] and whole-exome sequencing (WES) [3,25], **CRISPR/Cas** mediated genome editing confirmed by real-time PCR [28] and

western blotting [29], drug screening based on the measurement of organoid population viability [4,5], or directed organoid differentiation analysed by bulk -omic technologies [30]. In each case, experimental variables alter the majority of cells in an organoid population and the parameter metric can therefore be detected by bulk technologies.

Bulk analysis can generate a high-level overview of organoid cultures and can be used as an intermediate step to identify key biological questions that may require higher-dimensional follow-up. However, a major caveat of applying bulk analytical methods to organoids is that they flatten a high-dimensional biological system into a single-layered readout, leading to the loss of cell-type and cell-state specific information underpinning organoid heterogeneity. In order to fully understand the complexity of organoids and to unleash their potential as biomimetic tissue models, robust and cost-effective high-dimensional technologies are required.

Organoid Research in the Era of Single-cell Technology

Imaging and bulk analysis have given rise to considerable understanding of organoid biology, justifying their utility as *ex vivo* tissue surrogates. In many cases, however, information obtained with low-dimensional technologies can be inadequate, as they fail to address one of the most fundamental features of organoid cultures – their heterogeneity – at a sufficient level to generate informative biological insight (Figure 3a). Recent advances in high-dimensional single-cell technologies have enriched the toolbox for organoid analysis, enabling systematic characterisation of their cellular composition [31], developmental trajectory [1], -omic landscapes [2], and cell signalling profiles [10] (Figure 3b). Here we review the latest developments in single-cell technologies, reflect on the perspective of their application to organoid studies, and discuss ongoing efforts aimed at integrating these two complementary fields.

Single-cell Technologies Coming of Age

Complex biological processes performed by multicellular organisms are regulated by -omic level molecular networks across multiple modalities [32]. A cell's genome dictates its genetic composition, whereas the proteome describes executors of its

biological functions. The epigenome determines the cell's identity and function, which is also reflected in its transcriptome. Single-cell technologies aim to generate -omic scale profiles, describe cross-omic regulatory relationships, and, when combined with genetic engineering, deduce the causality between genotype and phenotype – all at the resolution of individual cells [33]. As complex heterocellular systems comprising multiple cell-types and cell-states simultaneously, organoids are uniquely placed to benefit from advances in single-cell technologies.

Over the past decade, single-cell technologies have undergone substantial development, leading to a variety of methods able to profile cellular phenotypes across different modalities (Figure 4). Among all technologies, single-cell RNA-sequencing (scRNA-seq) is the most established and widely used. While early versions of scRNA-seq protocols required manual isolation of individual cells [34], the introduction of plate-based protocols greatly increased sample throughput from the order of 10s to 100s of cells (e.g., Smart-seq2 / Smart-seq3 [35,36], CEL-Seq2 [37]), and such methods have been successfully applied to organoid cells [2]. Advances in microfluidics and nanotechnology led to the development and

commercialisation of droplet-based scRNA-seq platforms such as Drop-seq [38] and inDrop [39], where 1000s of single cells are partitioned into discrete nanolitre droplets, their mRNA being released, barcoded, and used for pooled cDNA library construction. One of the key limitations of droplet-based methods is that they are designed for viable cells to achieve best data quality, meaning that they may not be suitable for applications where prompt processing of fresh samples is not always possible. However, it has been recently demonstrated that droplet-based methods can generate robust data from single nuclei [40,41] or methanol-fixed cells [42], making them applicable to challenging samples such as frozen tissues and organoids [43]. In addition, the development of the subnanolitre well-based method Seq-Well enables efficient single cell partitioning at lower sample input compared to droplet-based methods [44], making it especially suitable for organoid applications where the amount of starting material can be limited.

Taking an alternative approach, combinatorial single-cell indexing platforms based on split-and-pool DNA-barcoding strategies (e.g., SPLiT-seq [45], sci-RNA-seq [46], and sci-RNA-seq3 [47]) were invented to circumvent the process of physical

encapsulation of single cells, offering a highly scalable workflow to characterise transcriptomes in complex tissues. For example, using sci-RNA-seq3, Cao and colleagues profiled the transcriptome of more than 2 million single cells and constructed a comprehensive developmental trajectory of mouse embryos staged between 9.5 and 13.5 days of gestation [47]. It is worth noting that both SPLiT-seq and sci-RNA-seq are compatible with fixed materials by design [45–47], meaning that they should be compatible with intracellular protein assays that require fully permeabilised cells as input. All of these features make split-pool barcoding scRNA-seq strategies particularly well suited to organoids. Moreover, the integration of combinatorial single-cell indexing with oligo-tag sample multiplexing (e.g., MULTI-seq [48], ClickTag [49]) will essentially eliminate the upper limit of the number of cells and conditions that can be analysed in a single scRNA-seq assay.

In addition to the transcriptome, single-cell technologies have enabled deep profiling of the genome [50], the proteome [51], the methylome [52,53], histone modification profiles [54,55], chromatin accessibility landscapes [56,57], and chromosome conformation [58] at varying coverage and throughput (Figure 4a).

The maturation of these methods provides unique opportunities for integrative single-cell analysis, giving rise to holistic representations of cell-type and cell-state at the resolution of individual cells (Figure 4b). For example, by integrating scRNA-seq, scATAC-seq and the split-pool barcoding strategy, Zhu and colleagues performed high-throughput single-cell profiling of the transcriptome and chromatin accessibility of fetal mouse forebrains. The multimodal approach allowed developmental trajectories to be inferred from regulatory relationships between ***cis*-regulatory elements (CRE)** and their putative target genes, which is not feasible when scRNA-seq or scATAC-seq is used alone or at lower throughput [59]. Although extremely powerful and high-dimensional, data generated from multi-omic single-cell assays are usually sparse with limited coverage of the modals of interest [60] – this presents unique challenges and opportunities for computational approaches (refs [33] and [61] provide comprehensive reviews of this topic). To our knowledge, no multi-omic studies have been reported from organoids, suggesting the existence of an uncharted territory and exciting possibilities for new biological discoveries (Figure 4a).

Although spatial-organisation and CRISPR-mediated genome editing are not classically considered as cellular modalities, recent efforts have pioneered integrative methods that can bring contextual and functional insight into high-dimensional single-cell analysis [62–65] (Figure 4b). Spatial -omic technologies have been successfully applied to cerebral organoids [66], showing promise for high-dimensional organoid characterisation in their native context. Of note, scRNA-seq occupies the central position in existing multimodal -omic technologies (Figure 4b), due to the ease of efficient RNA capture and cDNA amplification. We expect future multimodal technologies employing direct or indirect incorporation of oligo tags to label additional cellular modalities, making them capturable, amplifiable, and ultimately sequenceable [64,65]. Finally, from the perspective of data analysis, cell-type information acquired from scRNA-seq can be used as a reference to interpret the other modalities, further highlighting its pivotal role in multi-omic technologies [33].

Data Analysis for Single-cell -Omic Technologies

Single-cell technologies generate high-dimensional data that necessitates the development of novel data analysis pipelines (Figure 5a). As guidelines for single-cell -omic data analysis have been extensively covered elsewhere (e.g., scRNA-seq [67,68], scDNA-seq [69], and scATAC-seq [70]), we will focus our discussion on approaches particularly relevant to organoid studies.

Given the sparsity and increased dimensionality of single-cell datasets, one of the key steps of single-cell data analysis is feature selection based on data variance, aiming at increasing signal-to-noise ratio and reducing computational burdens [33,68]. The selection of meaningful biological features can be further aided by **principal component analysis (PCA)**, which, together with ***t*-SNE (*t*-distributed stochastic neighbour embedding)** and **UMAP (Uniform Manifold Approximation and Projection)**, make the most widely used dimensionality reduction tools to facilitate data visualisation and summarisation [68]. Different dimensionality reduction methods have different strengths and weaknesses. For example, *t*-SNE resolves distinct cell populations but fails to preserve global structure of the data, which can be readily represented by UMAP [71]. Given that

organoids often contain differentiation trajectories, dimensional reduction techniques that maintain global structure are often preferable. Additional algorithms have been developed to preserve both local and global distances (e.g., SPRING [72], PHATE [73]), with the application of SPRING to human cerebral organoids successfully revealing development trajectories from pluripotent stem cells to cortical neurons [2]. The typical data analysis step following dimensionality reduction is to organise cells into clusters based on similarities of their measured profiles, where graph-based community detection methods such as the **Louvain algorithm** (and the improved Leiden algorithm [74]) are predominantly used. Using prior-knowledge databases as a reference, clusters can be annotated with specific cell-types and cell-states determined by differential gene expression, and then be used as anchor points for downstream data interpretation. Collectively, these computational methods can be used to clearly resolve discrete cell-types and cell-states in single-cell organoid data.

One of the most established applications of scRNA-seq analysis is lineage reconstruction via **pseudotime analysis** (e.g., PAGA [75], Slingshot [76]) and **RNA**

315 **velocity** estimation [77]. The key assumption of these algorithms is that the
316 biological system of interest contains cells encompassing a continuous
317 developmental trajectory, which is also a definitive character of organoids. More
318 specifically, the selection of pseudotime analysis methods depends on the structure
319 of the expected trajectories (e.g. linear, bifurcating, or tree-shaped) [78]. For
320 example, in order to identify the branch point between budding organoids and
321 enterocysts, Serra and colleagues analysed the development of intestinal organoids
322 using Wishbone [79], an algorithm designed to infer bifurcating trajectories that is
323 well suited to their biological process of interest [1]. Finally, deep profiling of
324 ligand-receptor expression and interaction enabled by algorithms such as
325 CellPhone DB [80], CellChat [81], and NicheNet [82] has enabled inference of cell-
326 cell communication in complex tissues [83,84]. Such methods will also be highly
327 applicable to organoid co-cultures, where the key considerations will involve the
328 selection of physiologically relevant cell-types to be modelled, the determination
329 of the number of cells to be sequenced and the sequencing depth, and the
330 inclusion of meaningful and comparable monoculture controls.
331

For multi-omic technologies, the integration of different modalities (e.g. RNA and protein) can be performed at the data acquisition and/or data analysis stage(s) (Figure 5b). As each modality emphasises a particular aspect of the underlying biology, independent analysis of multiple modalities may produce conflicting identifications of cell clusters that will confound downstream analysis [33]. Various methods have been developed to enable joint analysis of multimodal datasets (e.g., LIGER [85], MOFA+ [86]), primarily based on the assumption that data obtained from multiple molecular layers of the same cells or biological replicates should share common manifold structures [33,87]. Additional measures have been taken to address the sparsity of data generated from single-cell experiments [67]. Taken together, the interweaving development of experimental procedures and data analysis approaches will lead to comprehensive single-cell representations of complex biological systems including organoids in the coming years.

High-dimensional Single-cell Organoid Analysis

The heterogenous composition of organoids makes them a natural use case of single-cell technologies. When the biological process of interest is only present in

subpopulations of an organoid culture, the ability to obtain single-cell readout is not only informative but essential. For example, Grün and colleagues reported the first single-cell transcriptomic profiles of murine small intestinal organoids and identified a rare type of *Reg4*-expressing enteroendocrine cells that are undetectable if the organoids are homogenised and analysed as a bulk sample [31]. Using a series of CRISPR-engineered colorectal cancer (CRC) organoid models, Han and colleagues performed scRNA-seq and demonstrated that microenvironmental TGF-β signalling can induce YAP/TAZ-dependent transcriptional reprogramming and lineage reversion, ultimately leading to Wnt independence – a mechanistic insight not easily obtainable from bulk analysis [29]. Moreover, by implementing iTracer, an inducible lineage recording system coupling CRISPR/Cas9 genomic scarring and spatial scRNA-seq, He and colleagues analysed the dynamics of cell fate commitment during cerebral organoid regionalisation with spatial-temporal resolution [66].

In addition to intra-organoid heterogeneity, the complexity of organoid cultures can be further increased by co-culture with heterotypic cell-types. Single-cell

366 technologies are particularly useful for analysing organoid co-cultures, where the
367 resolution of cell-type-specific information is essential for meaningful downstream
368 analysis. While traditional flow cytometry is usually sufficient to retrieve cell-type
369 and cell-state information from organoids and organoid co-cultures [88,89], recent
370 developments in **mass cytometry** have enabled high-dimensional analysis of cell-
371 type, cell-state, and post-translational modifications (PTMs) in one multiplexed
372 experiment. This has led to the discovery of novel connections between epithelial
373 cell-intrinsic and extrinsic signalling in a CRC tumour microenvironment organoid
374 co-culture model [10].
375
376 Beyond the characterisation of heterogeneity, comprehensive profiling of organoids
377 enabled by single-cell technologies can also be used as a benchmark tool to
378 evaluate their physiological relevance and, where the phenotypic similarity can be
379 faithfully established, generate extrapolatable understanding of healthy and
380 diseased tissues. For example, Velasco and colleagues performed scRNA-seq
381 analysis of 21 human dorsal forebrain organoids derived from four independent
382 iPSC lines, demonstrating cross-organoid reproducibility in cell-type composition

and developmental trajectory, as well as their striking similarity to endogenous fetal brains [20]. Transcriptomic comparison of organoids with their tissue of origin has also been applied to the intestine [31], the lung [22], the kidney [90], and the gastrula [91]. scRNA-Seq will likely become a routine quality control procedure for newly established organoid models. In the context of disease modelling, single-cell analysis has been used to demonstrate the physiological relevance of organoids [5,92] and to justify their use as avatars of personalised medicine [5,93]. However, it is important to note that while organoids generally display remarkable resemblance to their tissue of origin, discrepancies do exist due to suboptimal modelling of the native microenvironment and/or long-term *in vitro* propagation, which can provide insight for the refinement of organoid culture protocols [94–96] but also hold implications for the development and evaluation of organoid-based personalised therapies [93,84]. Thus, while single-cell technologies are an excellent way to analyse organoids, they can also be used to improve the biomimetic accuracy of organoid cultures in the future [96].

Concluding Remarks and Future Perspectives

Over the past decade, organoid technology has undergone considerable growth and revolutionised basic science and translational research. Traditional low-dimensional techniques provide useful tools to demonstrate the physiological relevance of organoids and to justify their utility, while advances in single-cell technologies hold promise to enable high-dimensional organoid characterisation at unprecedented scale and depth. Although extensive integration of single-cell technologies to organoid research is yet to be accomplished (Figure 4a), there is little reason why the frontiers of the two fields cannot be jointly extended as both technical and biological challenges are being actively addressed (see Outstanding Questions).

Substantial efforts have been made to improve the reproducibility of organoid cultures [97] and to leverage their experimental flexibility [10,98]. The fidelity of organoids can be increased via refinements of the culture condition [95] and culture format (e.g., **organ-on-a-chip** [99,100]). As a result, organoids are becoming increasingly accurate and versatile biomimetic models, providing unique

opportunities for single-cell technologies where a highly amenable experimental system is key to generating biological insights.

Single-cell technologies are often pioneered using solution-phase model systems such as **peripheral blood mononuclear cells (PBMCs)**. Application of single-cell technologies to organoids requires that high-quality single-cell suspensions can be generated from the cultures without compromising the underlying biology. This can be challenging for solid-phase 3D organoids but remediable if fixed samples can be used as the input of downstream single-cell experiments [42]. Meanwhile, the latest developments in single-cell technologies highlight increased sample throughput [47,59], increased data modality [33,67], and improved compatibility with challenging samples [45–47]. Unfortunately, most single-cell technologies fail to couple the high-dimensional -omic analysis with robust functional assays, and we anticipate the development of such methods to be a primary challenge for the field going forward.

Organoids combine the complexity of tissues with the flexibility of cell lines and are therefore uniquely positioned to leverage emerging high-dimensional technologies. Through the future application of multiplexed multimodal single-cell technologies, we expect high-dimensional analysis of biomimetic cultures to revolutionise the study of healthy and diseased tissues.

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Glossary

ATAC-seq (assay for transposase-accessible chromatin using sequencing): a method to assess genome-wide chromatin accessibility by inserting sequencing adapters into open chromatin with the hyperactive mutant Tn5 transposase.

Biomimetic: synthetic systems or methods that mimic biological processes.

BS-seq (bisulfite-sequencing): a method to determine the pattern of DNA methylation by treating DNA with bisulfite prior to sequencing. Bisulfite converts cytosine residues to uracil but leaves 5-methylcytosine residues unaffected.

***cis*-regulatory elements (CRE):** non-coding DNA sequences that regulate the transcription of neighbouring genes, such as promoters, enhancers, and silencers.

Convolutional neural networks (CNNs): a branch of machine learning algorithms inspired by the organisation of the animal visual cortex. A convolutional neural network consists of an input and an output layer, as well as multiple hidden layers.

CRISPR / Cas (clustered regularly interspaced short palindromic repeats / CRISPR associated protein): a genetic engineering technology derived from the adaptive immune system of bacteria.

CUT&RUN (cleavage under targets and release using nuclease): a method that uses a target-specific primary antibody and a protein A—protein G-micrococcal nuclease (pAG-MNase) to profile protein-DNA interactions.

Data dimensionality: a metric of experimental methods determined by the number of cells, parameters, and experimental conditions being evaluated per assay.

Extracellular matrix (ECM): a 3D macromolecular network providing structural support to cells.

Heterocellular: a culture / population comprising different cell-types.

High-content imaging (HCI): high-throughput automated image acquisition and analysis workflows that enable extraction of quantitative multi-parametric data at the single-cell resolution.

Louvain algorithm: a graph-based unsupervised method to detect communities from large networks via modularity maximisation.

Mass cytometry: a single-cell cytometric method that utilises rare-earth metal coupled antibodies along with the high-mass accuracy of mass spectrometry.

Modality: refers to cellular molecules such as DNA, RNA, and proteins in the context of single-cell -omic technologies.

Multimodal: data of multiple modalities, e.g., of DNA and RNA.

Next-generation sequencing (NGS): high-throughput DNA sequencing technologies applying the concept of massively parallel processing (e.g., Illumina sequencing).

Omic: collective description of large numbers of cellular molecules such as genes, proteins, and RNAs.

Organ-on-a-chip: three-dimensional cell culture platforms empowered by microfluidics and nanotechnologies that simulate mechanics and physiological activities of entire organs and organ systems.

Peripheral blood mononuclear cells (PBMCs): heterocellular leukocyte samples mainly comprising lymphocytes and monocytes.

Principal component analysis (PCA): an unsupervised linear transformation method used for dimensionality reduction and data visualisation.

Pseudotime analysis: methods to extract latent temporal information from high-dimensional datasets followed by mapping cells onto the reconstructed trajectories.

RNA velocity: a high-dimensional vector that predicts future states of single cells based on profiles of unspliced and spliced mRNA.

***t*-SNE (*t*-distributed stochastic neighbour embedding):** a non-linear dimensionality reduction algorithm used for data visualisation that resolves distinct clusters from high-dimensional datasets.

UMAP (uniform manifold approximation and projection): a non-linear dimensionality reduction technique for data visualisation that preserves the global structure of high-dimensional datasets.

Figure Legends

Figure 1 – Organoid Culture Overview.

Organoids occupy a unique position in existing experimental biological systems. When compared to traditional cell lines and cell line-derived spheroids, organoids are 3-dimensional culture systems able to self-organise and therefore ensure higher physiological relevance. Organoids can be generated from primary tissues but offer greater experimental flexibility, as they are more compatible with high-throughput applications and more amenable to genetic modification. The strengths of the organoid technology enable its application in diverse fields such as drug screening, disease modelling, developmental biology, and personalised medicine.

Figure 2 – Low-dimensional versus High-dimensional Organoid Analysis.

Organoid analysis can be performed at the population or the single-cell level, generating data with increasing dimensionality. Data dimensionality is determined by three independent factors: 1) the number of cells, 2) the number of parameters, and 3) the number of conditions being analysed per assay. In general, analyses of organoid populations (e.g., microscopy, bulk molecular analysis, bulk -omic analysis,

and viability screen) are considered low-intermediate dimensional as they cannot generate single-cell readout. In contrast, cytometry, high-content imaging, and single-cell -omic technologies can provide high-dimensional biological insight on a cell-by-cell basis. IHC, immunohistochemistry; IF, immunofluorescence; ChIP-seq, chromatin immunoprecipitation-sequencing [101]; **CUT&RUN**, cleavage under targets and release using nuclease [98]; **ATAC-seq**, assay for transposase-accessible chromatin using sequencing [98,102]; **BS-seq**, bisulfite-sequencing [103,104].

Figure 3 – Comparison of Organoid Analytical Methods.

(a) Organoid cultures exhibit heterogeneity at various levels. While the inter-culture variability introduced by technical (e.g., protocol, experimental batch) or biological (e.g., tissue of origin) differences can be captured by low-dimensional methods such as microscopy and bulk analysis, single-cell analysis is required to generate high-dimensional insight into intra-organoid heterogeneity including information of cell-type and cell-state. (b) Different analytical methods are capable of profiling the phenotypes of organoids at different levels of detail. When compared to microscopy and low-dimensional bulk analysis, single-cell methods can generate

holistic representations of organoid phenotypes including viability, cellular composition, spatial patterning, -omic profile, developmental trajectory, and cell-cell communication.

Figure 4 – Overview of Single-cell Technologies.

(a) Overview of existing single-cell technologies demonstrating the throughput and the number of parameters being measured, as well as the modality / modalities being analysed. Notably, among all the techniques available, only scRNA-seq, scATAC-seq, smFISH, and CyTOF have been reported with organoid studies, highlighting gaps in the application of single-cell technologies to organoid research. (b) Overview of existing multi-omic single-cell technologies and their integration with spatial resolution and CRISPR-mediated genetic modification. The nodes represent the modality of interest with the multimodal technologies labelling the connecting edges. CEL-seq, cell expression by linear amplification and sequencing [37]; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing [105]; CyTOF, cytometry time-of-flight [106]; dsciATAC-seq, droplet single-cell assay for transposase-accessible chromatin using sequencing [57]; ECCITE-seq, expanded

CRISPR-compatible cellular indexing of transcriptomes and epitopes by sequencing [64]; G&T-seq, genome and transcriptome sequencing [107]; IMC, imaging mass cytometry [106]; MERFISH, multiplexed error-robust fluorescence *in situ* hybridization [62]; REAP-seq, RNA expression and protein sequencing assay [108]; scATAC-seq, single-cell assay for transposase-accessible chromatin using sequencing [56]; scBS-seq, single-cell bisulfite sequencing [109]; scChIP–seq, single-cell chromatin immunoprecipitation followed by sequencing [54]; sciATAC-seq, single-cell combinatorial indexing assay for transposase-accessible chromatin using sequencing [110]; sci-CAR , single-cell combinatorial indexing chromatin accessibility and mRNA sequencing [111]; sci-MET, single-cell combinatorial indexing for methylation analysis [53]; sci-RNA-seq, single-cell combinatorial indexing RNA sequencing [46,47]; SCI-seq, single-cell combinatorial indexed sequencing [50]; sciMAP-ATAC, single-cell combinatorial indexing on microbiopsies assigned to positions for the assay for transposase accessible chromatin [112]; scM&T-seq, single-cell methylome and transcriptome sequencing [113]; scNMT-seq, single-cell nucleosome, methylation and transcription sequencing [114]; scNOMe-seq, single-cell nucleosome occupancy and methylome sequencing [115];

SCoPE-MS, single cell proteomics by mass spectrometry [51]; scTrio-seq, single-cell triple omics sequencing [116]; seqFISH+, sequential fluorescence *in situ* hybridization+ [63]; Slide-seq [117,118]; smFISH, single-molecule fluorescence *in situ* hybridization [31,119]; SNARE-seq, single-nucleus chromatin accessibility and mRNA expression sequencing [120]; snmC-seq, single methylcytosine sequencing [52]; SPLiT-seq, split-pool ligation-based transcriptome sequencing [45]; uliCUT&RUN, ultra-low input CUT&RUN [55].

Figure 5 – Single-cell Data Analysis.

(a) Overview of the unimodal single-cell data analysis workflow. Raw data from single-cell experiments undergo data pre-processing to generate normalised count matrices, followed by feature selection and dimensionality reduction to enable data visualisation. Cells can be clustered and annotated to facilitate downstream data interpretation. Depending on the modality being analysed, the data can be used to perform genomic analysis (e.g., mutational analysis, copy number variation (CNV) identification, and lineage tracing), epigenomic analysis (e.g., chromatin accessibility profiling, cis-regulatory element (CRE) identification, and pseudotime estimation),

as well as transcriptomic analysis (e.g., cell-type identification, developmental trajectory reconstruction, and inference of cell-cell communication). (b) Multimodal datasets can be generated from separate or joint experiments and analysed independently or integratively. Challenges in both experimental procedures and data analysis workflows are being actively addressed to ultimately enable holistic multi-omic characterisation of single cells.

Outstanding Questions

- What are the bottlenecks that limit the physiological relevance of organoids to be further improved?
- Can high-dimensional technologies be used to improve the biomimetic accuracy of organoids as tissue models?
- Can multimodal single-cell technologies reveal novel phenotypes in organoids?
- Can single-cell analysis of organoid co-cultures be used to model cell-cell communication in tissues?
- Is it possible to combine -omic technologies with robust functional assays?
- Is there an upper limit of the number of modalities that can be measured simultaneously in organoids?
- Can single-cell technologies be used to generate spatial-temporal -omic profiles of live organoids?

Highlights

- Organoids are heterocellular biomimetic tissue models transforming basic science and translational research.
- Traditional low-dimensional methods have provided remarkable biological insights when applied to organoid research. However, to fully unleash the potential of organoids as *ex vivo* tissue models, high-dimensional technologies are needed.
- Single-cell technologies enable the study of cellular processes across multiple modalities at considerable scale and depth, leading to significant progress in organoid biology.
- Advances in experimental procedures as well as data analysis approaches open opportunities for the integration of single-cell technologies and organoid research.

Figure 1

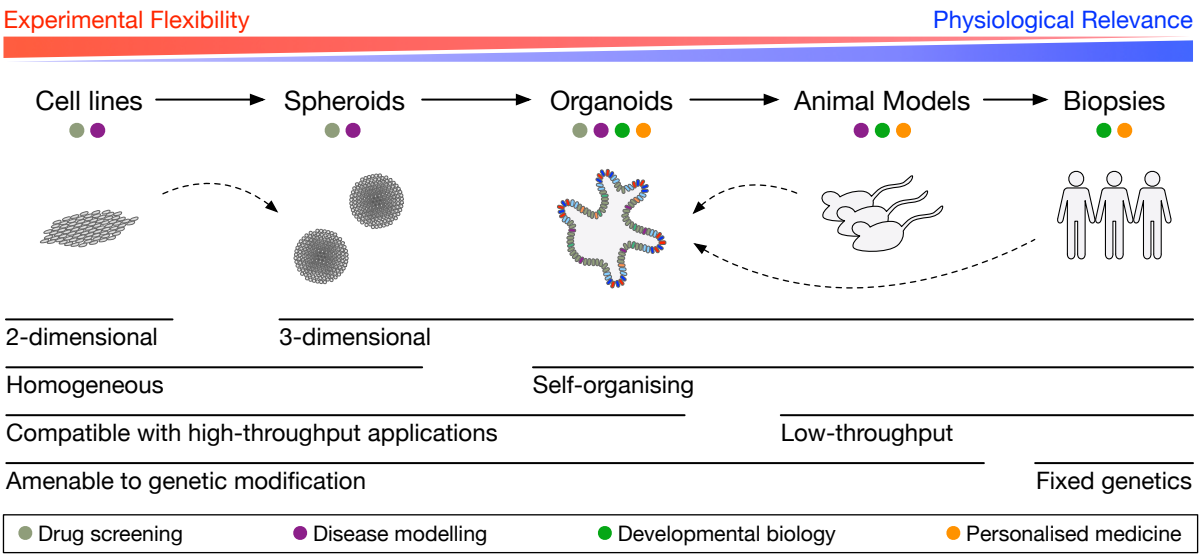

Experimental Flexibility
Physiological Relevance
Cell lines
Spheroids
Organoids
Animal Models
Biopsies
2-dimensional
3-dimensional
Homogeneous
Self-organising
Compatible with high-throughput applications
Low-throughput
Amenable to genetic modification
Fixed genetics
Drug screening
Disease modelling
Developmental biology
Personalised medicine

Figure 2

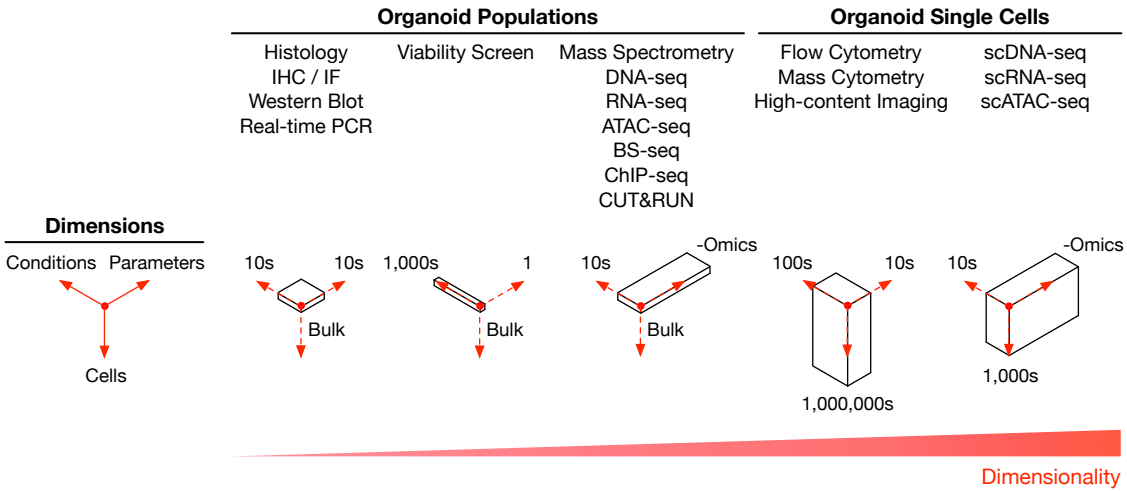

Organoid Populations
Organoid Single Cells
Histology
IHC / IF
Western Blot
Real-time PCR
Viability Screen
Mass Spectrometry
DNA-seq
RNA-seq
ATAC-seq
BS-seq
ChIP-seq
CUT&RUN
Flow Cytometry
Mass Cytometry
High-content Imaging
scDNA-seq
scRNA-seq
scATAC-seq
Dimensions
Conditions
Parameters
Cells
10s
10s
Bulk
1,000s
1
Bulk
10s
-Omics
Bulk
100s
10s
1,000,000s
10s
-Omics
1,000s
Dimensionality

Figure 3

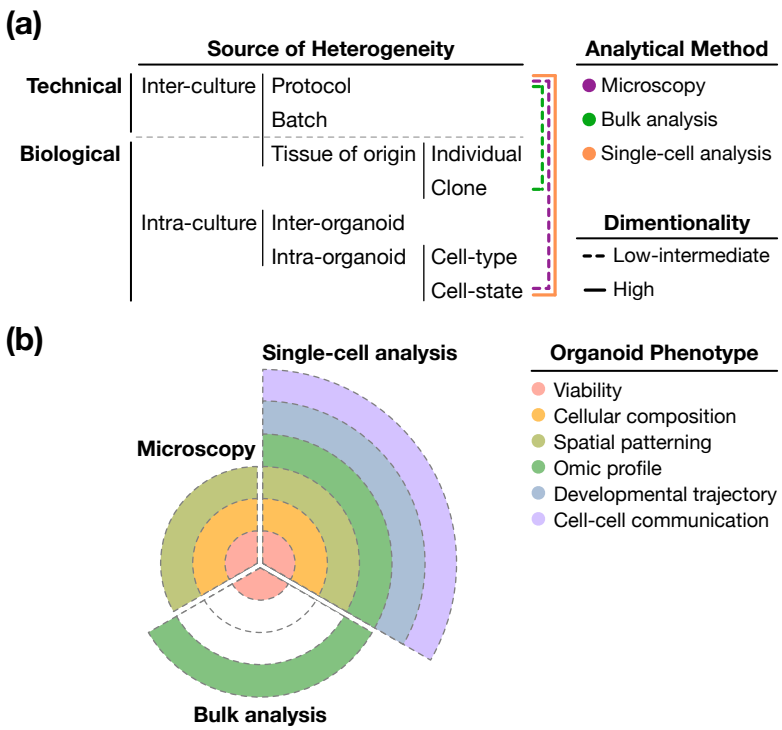
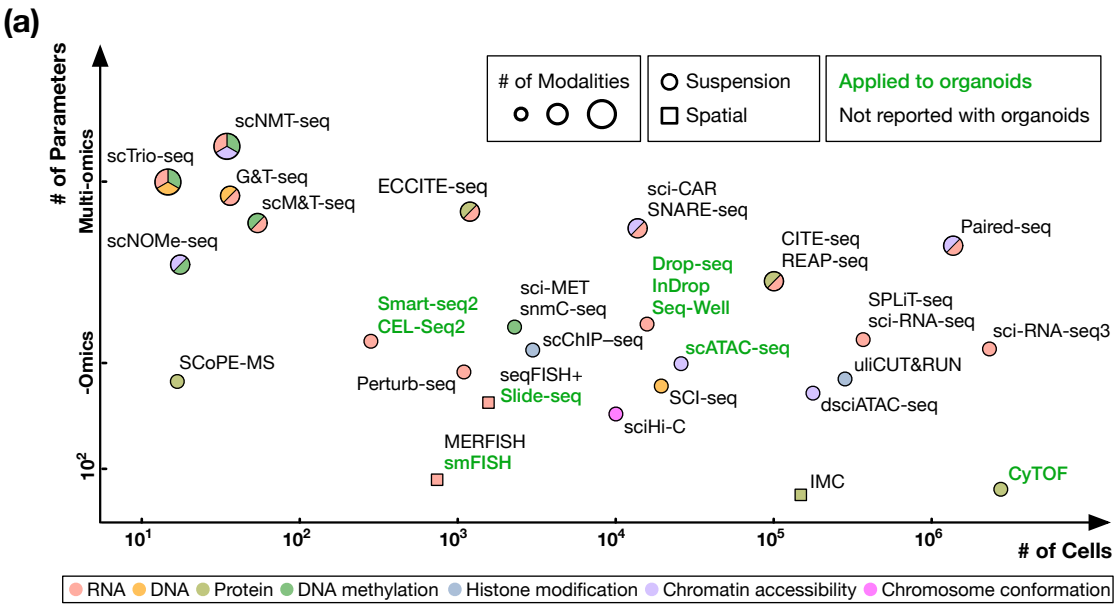

(a)
Source of Heterogeneity
Technical
Inter-culture
Protocol
Batch
Biological
Tissue of origin
Individual
Clone
Intra-culture
Inter-organoid
Intra-organoid
Cell-type
Cell-state
Analytical Method
Microscopy
Bulk analysis
Single-cell analysis
Dimentionality
Low-intermediate
High
(b)
Single-cell analysis
Microscopy
Bulk analysis
Organoid Phenotype
Viability
Cellular composition
Spatial patterning
Omic profile
Developmental trajectory
Cell-cell communication

Figure 4


(a)
# of Parameters
Multi-omics
-Omics
10^2
# of Modalities
Suspension
Spatial
Applied to organoids
Not reported with organoids
scNMT-seq
scTrio-seq
G&T-seq
scM&T-seq
scNOMe-seq
ECCITE-seq
sci-CAR
SNARE-seq
CITE-seq
REAP-seq
Paired-seq
Drop-seq
InDrop
Seq-Well
sci-MET
snmC-seq
Smart-seq2
CEL-Seq2
scChIP–seq
SPLiT-seq
sci-RNA-seq
sci-RNA-seq3
scATAC-seq
SCoPE-MS
uliCUT&RUN
Perturb-seq
seqFISH+
Slide-seq
SCI-seq
dsciATAC-seq
sciHi-C
MERFISH
smFISH
IMC
CyTOF
10^1
10^2
10^3
10^4
10^5
10^6
# of Cells
RNA
DNA
Protein
DNA methylation
Histone modification
Chromatin accessibility
Chromosome conformation

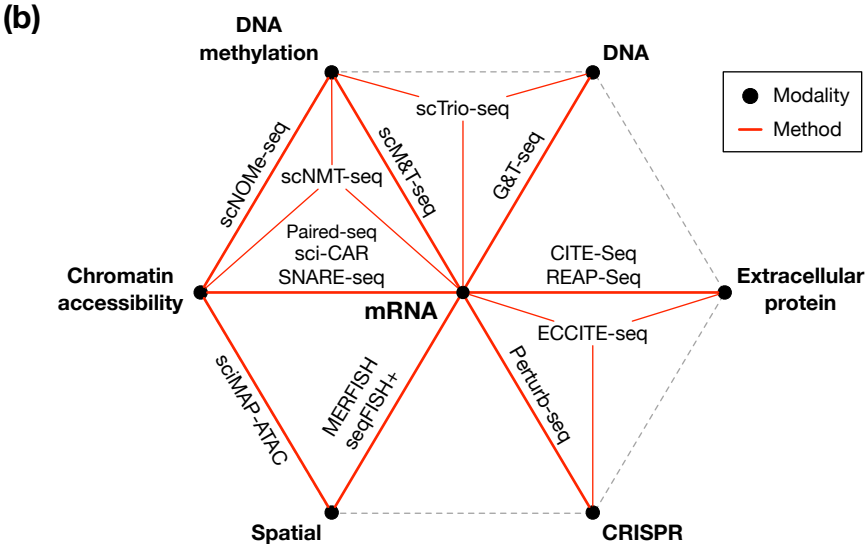
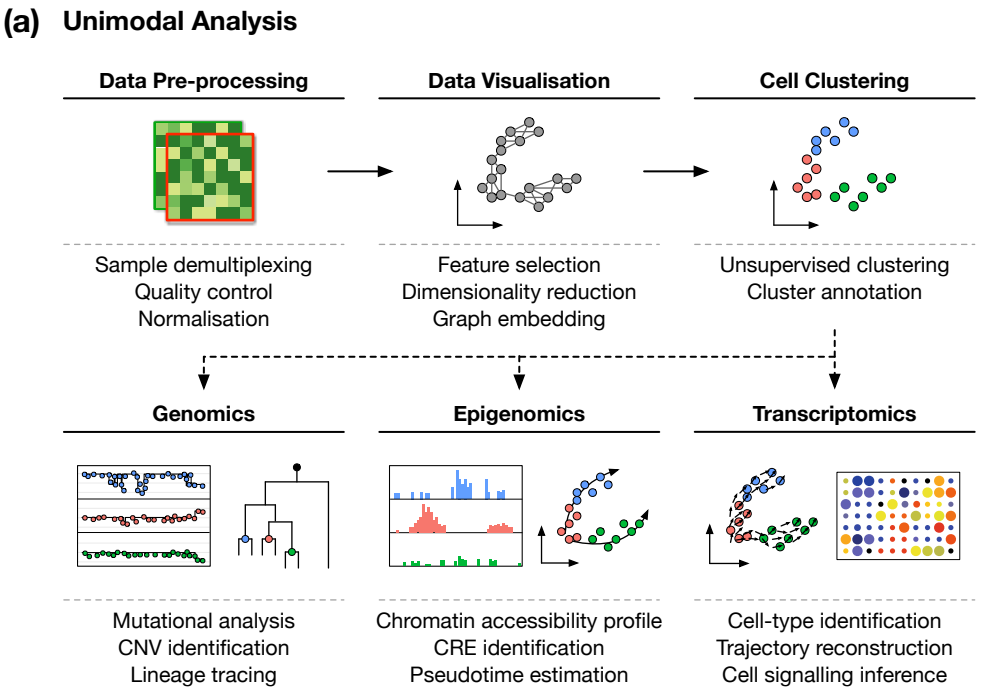
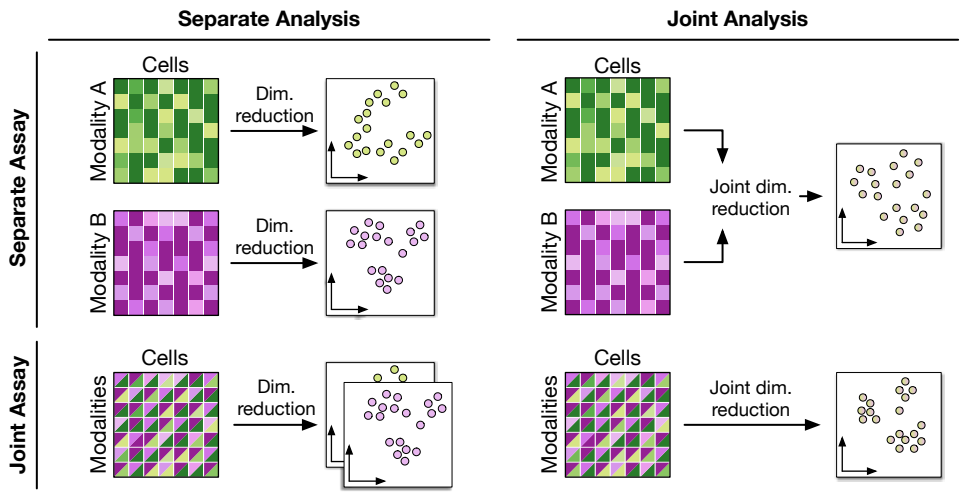

(b)
DNA methylation
DNA
Modality
Method
scTrio-seq
scNOMe-seq
scM&T-seq
G&T-seq
scNMT-seq
Paired-seq
sci-CAR
SNARE-seq
CITE-Seq
REAP-Seq
Chromatin accessibility
mRNA
Extracellular protein
ECCITE-seq
sciMAP-ATAC
MERFISH
seqFISH+
Perturb-seq
Spatial
CRISPR

Figure 5


(a) Unimodal Analysis
Data Pre-processing
Data Visualisation
Cell Clustering
Sample demultiplexing
Quality control
Normalisation
Feature selection
Dimensionality reduction
Graph embedding
Unsupervised clustering
Cluster annotation
Genomics
Epigenomics
Transcriptomics
Mutational analysis
CNV identification
Lineage tracing
Chromatin accessibility profile
CRE identification
Pseudotime estimation
Cell-type identification
Trajectory reconstruction
Cell signalling inference


(b) Multimodal Analysis
Separate Analysis
Joint Analysis
Separate Assay
Joint Assay
Cells
Modality A
Modality B
Modalities
Dim. reduction
Joint dim. reduction