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# Integrative Methods and Practical Challenges for Single-cell Multi-omics

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## Abstract

Fast-developing single-cell multi-modal omics (scMulti-omics) technologies enable the measurement of multiple modalities, such as DNA methylation, chromatin accessibility, RNA expression, protein abundance, gene perturbation, and spatial information, from the same cell. scMulti-omics can comprehensively explore and identify cell characteristics, while also presenting challenges in developing computational methods and tools for integrative analyses. We review the integrative methods and summarize the existing tools for studying a variety of scMulti-omics data. The various functionalities and practical challenges in using the available tools in the public domain are explored through several case studies. Finally, we identify remaining challenges and future trends in scMulti-omics modeling and analyses.

### Keywords

single-cell sequencing technology; single-cell multi-modality; integrative methods; analysis tools

## Single-cell sequencing technologies and multi-omics data

An individual cell maintains genetic heterogeneity that manifests unique cell functions and states [1]. With rapid developments in cell isolation and high-throughput sequencing technologies, single-cell **omics** (see Glossary) profiling can significantly benefit the study of characteristics and heterogeneity of individual cells, which has historically been confounded by bulk measurements[2]. Opportunities have arisen at the single-cell level to generate

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molecular information about whole-genome gene copies [3, 4], gene expression [5, 6], epigenome [7, 8], **CRISPR perturbation** [9, 10], spatial information [11, 12], and protein abundance [13, 14], among other phenomena. However, different molecules in a cell work synergistically to determine the cell state, and such individual profiling provides only a partial landscape of the entire cell heterogeneity [15, 16].

Single-cell multi-modal omics (scMulti-omics) sequencing technologies have recently emerged and showed advantages of simultaneously measuring multiple modalities from the same individual cell, which enables a more comprehensive exploration of cell behavior and identity [2, 16–19]. These technologies have tremendous potential in precision treatments, drug resistance, and relapse potential for specific tumors since they are particularly useful for studying the cells undergoing rapid differentiation (e.g. cancer and Alzheimer diseases) or evolving highly-diverse sub-populations (e.g. immune cells)[20, 21]. Specifically, scMulti-omics can greatly impact clinical application by identifying novel disease mechanisms. The underlying modalities can help predict drug sensitivities in tumor cells, prior to any *in vivo/vitro* drug doses, in order to exclude low-sensitivity drugs and decrease the diagnostic cost. By 2025, the global scMulti-omics market is anticipated to be $5.32 billion, mainly driven by the increasing need for non-invasive diagnosis and personalized medicine [22].

Intuitively, one omics profile can recover the missing values lost in another. Dropout issues in single-cell RNA-sequencing (**scRNA-Seq**) are prevalent. This problem stems from the inherent issue with sampling, in which samples do not fully represent their target population, especially for low expressed genes. Sampling inefficiencies coupled with the inherent uncertainty that follows sampling procedures lead to loss of data. The gene expression values missing from scRNA-Seq may be recoverable by integrating more omics levels; hence, the integrative analysis of scMulti-omics can theoretically lead to cell state prediction, cell trajectory elucidation, and other functional analyses with high accuracy and low bias [23]. However, considering the intrinsic challenges in sequencing multiple levels of information from the same cell, the sequencing technology accuracy, and the relevant cost, it is not currently feasible to comprehensively profile all aspects of cellular sequence types. Current approaches allow up to four types of omics data to be measured simultaneously, leading to 13 available combinations in the public domain (9 with double-modality sequencing, 3 with triple-modality sequencing, and 1 with quad-modality sequencing). For example, one technique combines measurements of RNA expression, chromatin accessibility, and DNA methylation; another combines RNA expression with spatial information (more details can be found in Figure 1).

It is not surprising that scRNA-Seq serves as a general mediator that is included in most scMulti-omics studies. Not only does RNA’s intermediate position in the central dogma allow for proximity to multiple molecular processes, but also, the application of scRNA-Seq is commercialized and routinely available [24, 25]. At least 43 papers in the past five years published scMulti-omics datasets that were profiled within the same cell (i.e., matched data) (Table 1). By jointly analyzing these matched data, integrative studies may reveal relationships and interactions between various types of molecular information that are otherwise missing in traditional single-omics studies. However, most existing scMulti-omics tools analyze data obtained across single-cell experiments (i.e., unmatched data), which provide opportunities to utilize large amounts of existing single-cell omics data to discover novel insights by properly aligning cross-experimental datasets for integrative analysis. We review integrative methods and their affiliated computational tools designed for matched and unmatched scMulti-omics data. The derived knowledge and insights can provide guidance to choose proper tools regarding supported data types, expected analytical outcomes, functionalities, and performances.

## Integrative methods for scMulti-omics data

Typically, single-omics data is represented by a matrix, with rows representing genes, columns representing cells, and each element of the matrix representing the specific omics information about a feature in the corresponding cell (e.g., gene expression). Joint analyses of scMulti-omics data simultaneously consider both the cross-modality variations and cross-cell correlations. The challenges in developing such integrative methods for scMulti-omics data include unifying different modalities, batch effects between experiments (especially for unmatched data), low sequencing depth, and high-modality interactions. Based on the underlying method design, we summarize the existing integrative methods into the following four broad categories: feature projection, Bayesian modeling, regression modeling, and decomposition (Figure 2).

First, data from different profiling methods can be jointly analyzed through feature projection. Canonical correlation vectorization (**CCV**), modified from **canonical correlation analysis**, investigates the relationship between variables by capturing anchors that are maximally correlated across the datasets [24]. For instance, given matched scRNA-Seq and single-cell ATAC-Sequencing (**scATAC-Seq**) data, CCV identifies the gene features shared between the two matrices by projecting cells. A single matrix can then be generated by projecting expression values and chromatin accessibility of each gene onto a common basis space and normalizing values by penalization and regularization. **Manifold alignment** is another method that has been applied to unmatched scRNA-Seq and epigenomics data to unravel the pseudo-time correlation between, e.g., gene expression and DNA methylation [26, 27]. However, how to legitimately account for batch effects is one of the major difficulties with the projection approach. CCV and Manifold alignment are both feature projection-based dimension reduction techniques. These have the theoretical ability to reduce high-dimensional data down to its critical components in non-linear spaces, ideally emphasizing the features that differentiate cell types. When these methods are applied to multi-omics data, they can denoise each individual dataset and highlight cell-type-specific features, resulting links between multi-omics datasets.

Second, variational Bayes (**VB**) is a stochastic variational inference method based on Bayesian modeling. The underlying method was designed using the hypothesis that the gene copy number (single-cell genome sequencing) is positively correlated with measures corresponding to gene expression (scRNA-Seq) [28]. VB fits the RNA expression matrix to a set of GxC sub-matrices (generated from gene phylogenic analysis, with genes as rows, cells as columns, and gene copy numbers as elements) and integrates the two modalities by finding the lowest variance between the fitted approximate distribution and the true distribution in the original GxC matrix. While the underlying hypothesis for VB is naively intuitive, there are many complex interactions that dictate the level of gene expression. Assuming the hypothesis of a strong positive correlation between gene copy number and gene expression leads to the potential of inconsistent or unreliable results.

The third type of integrative analysis uses various regression models to accurately characterize the high heterogeneity among individual cells and multi-layer data types, simultaneously. Duan and colleagues described a method using a linear regression model to integrate covariates between spatial information and RNA-expression combined with an expectation-maximization approach to fit the two models. The least absolute shrinkage and selection operator (**LASSO**) regression method has been applied to correlate gene expression with chromatin accessible sites [29]. Gradient boosting regression (**GBR**) iteratively fits “weak” models to estimate response variables and align matched gene expression and chromatin accessibility patterns to predict differentially accessible genomics sites for cell type prediction [30]. Hidden Markov random field (**HMRF**) is a graph-based model widely used for pattern recognition in image data analysis. HMRF first builds connections between cells and spatial coordinates and clusters cell groups, integrating gene expression patterns and cell positions [31]. Marked point process (**MPP**) is a nonparametric statistical framework to identify the dependency of spatial distribution and gene expression levels [32]. A more complex multivariate normal model (**MNM**) for spatial transcription analysis defines spatial dependence by combining gene expression profiles with cell locations considering both spatial covariance and nonspatial variance [33]. These regression-based integrative methods vary widely, as does their applicability to integrating multi-omics datasets. The underlying assumptions for regression models are often an issue when applying them to new areas. Specifically, linear regression has strict assumptions that would make it difficult to apply such a parametric approach to genetic data that often does not fit the parametric requirements. However, nonparametric approaches such as MPP do not suffer from this limitation. These types of robust methods have great potential for the integration of multi-omics datasets. In particular, parallels between longitudinal data analysis and spatial omics methods make these types of regression approaches a strong fit.

Lastly, decomposition is a straightforward method for unsupervised data integration. The basic idea is to decompose the original matrices into two low-dimensional submatrices: a coefficient submatrix records signature information (e.g. genes) and a residue submatrix records cell information. One widely used decomposition method in scMulti-omics studies is matrix factorization. The input matrices (e.g. scRNA-Seq and scATAC-Seq data) are decomposed into (*i*) an amplitude matrix (or coefficient matrix) composing genes as rows and factors as columns to reflect gene co-regulation patterns; and (*ii*) a pattern matrix (or residue matrix) composing factors as rows and cells as columns for further cell clusters. Four modified methods have been proposed: integrative nonnegative matrix factorization (**iNMF**) [34], coupled nonnegative matrix factorizations (**coupleNMF**) [35], group factor analysis (**GFA**) [36], and independent component analysis (**ICA**) [37]. **TOPIC** modeling is another decomposition method to discover the shared latent information among input matrices. It has been applied to derive the functional topics of each cell with specific perturbation by integrative analysis of matched scRNA-Seq and single-cell CRISPR screening data [38].

Similar to the feature project-based dimension reduction approaches, the decomposition methods allow for denoising of datasets through dimension reduction in an attempt to emphasize the key features of each cell. When implemented correctly, with the necessary assumptions met, decomposition has the potential to reliably integrate different omics datasets.

Apart from these integrative methods, various advanced methods—such as **network simulated annealing** and deep learning (e.g. **autoencoder**)—have emerged in the last three years for bulk multi-omics studies [39]. However, these approaches have yet to be applied at the single-cell level. Theoretically, all these models can be applied to scMulti-omics analysis with proper optimization, and we believe that the knowledge gained from these methods could be transferred from the bulk level to the single-cell level in the near future.

## Analytical tools for scMulti-omics data

To decipher the secrets hidden in scMulti-omics data, a powerful integrative method is necessary, yet not entirely sufficient. An efficient tool should also be able to properly process the massive and chaotic data before analysis for a more accurate and reliable result. A proper combination of preprocessing steps is necessary to decrease bias and generate meaningful analytical results, including but not limited to data setup, removing low-quality genes and cells, normalizing values across modalities and cells, imputing missing values, and imaging digitalization to generate 2D coordination. In this section, we comprehensively review ten computational integrative tools for scMulti-omics analysis that are equipped with multiple functionalities and various analytical interpretations as mature pipelines (Figure 3, Key Figure).

*Seurat3*—which is based upon a CCV method—is one of the best-developed tools for scRNA-Seq and scMulti-omics data analyses with supreme functionalities and well-written documentation [24, 40]. Released in 2019, the authors demonstrated the use of Seurat3 by (*i*) co-embedding scRNA-Seq and unmatched scATAC-Seq data to reveal cell-type-specific regulatory loci, (*ii*) applying matched RNA expression and cell-surface protein expressions to leverage deeper connections between protein abundance and gene expression, and (*iii*) applying spatial transcriptomic data to predict spatial gene expression patterns and classify subpopulations [24]. Seurat3 inherited all functionalities from its previous version in comprehensive single-cell RNA-Seq analysis [41, 42], cross-experimental single-cell data analysis [43], and integrative bulk and single-cell RNA-Seq data analysis [44], resulting in a wide application range. Its key feature of analyzing multiple modalities at the single-cell level has also been widely applied by other independent labs. More applications of Seurat3 can be found in Table 2.

*MOFA* was developed by Argelaguet and lab members as a statistically rigorous tool that decomposes modality and captures sources of variability between different datasets [36]. MOFA was originally applied on a study with parallel profiled DNA methylation and gene expression revealing the cooperation between the transcriptome and methylation sites. Each factor captured signatures that reflect a specific cell state and can be used to impute missing information in modalities [36]. Catalinas and colleagues applied MOFA on a perturbation-transcription dataset and showed the advantage in capturing relevant gene signatures, of both coding and non-coding transcription compared to the conventional gene differential analysis [45]. MOFA has also been applied to reveal connections between the transcriptome and the epigenetic state in enhancers during germ layer formation and capture the global cell-to-cell variability via factors inferred from triple-modalities [46, 47]. Its new version, MOFA+, claims to have better performance and more functionalities for discovering the association among gene expression, epigenetic variations, and cell fates commitment [48].

Welch's lab recently developed a tool called *LIGER* (linked inference of genomics experimental relationships) that uses the iNFM method to best approximate the original data and identify dataset-specific and shared factors across different datasets. LIGER was originally applied to an unmatched scRNA-Seq and DNA methylation dataset showing the ability in identifying methylation regions that were anticorrelated specific expressions. As with Seurat3, LIGER can also be applied to analyze, e.g. cross-experimental single-cell RNA-Seq data analysis [49].

*MATCHER* aligns the underlying manifolds of diverse single-cell modalities (epigenome and transcriptome) to create an equivalent pseudo-time representation and has been applied to both matched and unmatched single-cell data [26]. MATCHER was initially applied on two publicly available datasets: (*i*) unmatched gene expression, DNA methylation, and chromatin accessibility data from mouse embryonic stem cells, and (*ii*) matched gene expression and DNA methylation data from human-induced pluripotent stem cells [50, 51] (Table 2). As a result, MATCHER identifies sequential changes and reveals the connections of trajectory changes among multiple modalities which provides a great potential for reprogramming and differentiation studies.

*Clonealign* is a tool uniquely designed for the integrative study of scRNA-Seq and gene copy numbers from single-cell DNA sequencing, based on the assumption that the increasing gene copy number will result in an increased expression of the corresponding gene [28]. It was applied to matched scDNA-Seq and 10X scRNA-Seq datasets to identify clone-specific gene expression patterns and the correlation of single-nucleotide variation and expression. However, Clonealign is not suitable for cancers that have quiescent genomes and are devoid of copy number changes, such as sarcomas and karyotypically normal acute myeloid leukemia. As an extension, Clonealign holds the potential to apply to other scMulti-omics studies, such as methylation-transcription and chromatin accessibility-transcription [28].

Pooled CRISPR knockout screening is used to evaluate gene biological functions by comparing gene perturbations with phenotypes (e.g. cell growth). Combining CRISPR screening with scRNA-Seq can elucidate the effects of perturbation on the RNA expression level. *MIMOSCA* is used for perturbation-expression analysis which deciphers the effect of individual perturbations and the marginal contributions of genetic interactions using a maximum likelihood approach and linear model [52]. *MUSIC* is also a tool newly developed for analyzing CRISPR perturbation and RNA expression [38]. It utilizes the TOPIC method to model the perturbation and cell functions from the annotated expression matrix and shows better performance than MIMOSCA [38].

Spatial transcriptomics links differentially expressed genes to the actual cell position in the interactions intra- or inter-subtypes and is critical for understanding cell identity and function in the context of tissue. By including spatial information, researchers can broadly define cell types and subtle alterations without the requirement of direct gene measurement. *Giotto* [53], *Trendscreek* [32], and *SpatialDE* [33] are three well-rounded tools for analyzing spatial transcriptomics data. These pipelines first process the photo image and scRNA-Seq data separately to generate a spatial coordinate figure and cell clusters, respectively. The predicted clusters are then correlated with spatial coordinates to map the spatial-specific cell cluster patterns. A cell spatial network can be built considering both the actual spatial distance between each cell pair and the cell clusters predicted from gene expressions. The spatial variable gene plot intuitively shows the expression patterns at different locations which are intuitively useful to construct the whole tissue regulatory landscape by combining with epigenetic features. Future studies could utilize other omics data to infer relationships between cell positions and causal genetic patterns, and a specific case study for spatial transcriptomics is showcased in our case study.

Despite these ten well-developed tools (Table 2), several other packages were available for specific scMulti-omics data analysis, yet with only scripts and limited functionalities. MOGSA claims to perform gene-set analysis from single-cell transcriptomics and proteomics data to yield insights into the complex molecular machinery of biological systems [54]. Duren and others developed De-Convolution and Coupled-Clustering using the coupleNMF integrative method to intake **scHi-C** and bulk HiChIP data in order to deconvolve the 3D gene contacts into cluster-specific profiles [55]; An R script integrates LASSO to jointly analyze matched RNA expression and chromatin accessibility data from scCAR-Seq [29]; Li and coworkers analyzed matched DNA methylation and chromatin architecture in single cells using an assay, called Methyl-HiC, to reveal coordinated DNA methylation status between distal genomics segments [56]; Clark and teammates developed the scNMT-Seq technique to simultaneously measure matched chromatin accessibility, DNA methylation, and RNA expression, and developed a Bernoulli likelihood-based regression assay to show how parallel profiling of the transcriptome and epigenome could reveal dynamic changes in how chromatin accessibility and DNA methylation interact during differentiation [57]; Adamson and colleagues developed an ICA dependent method named LRICA for Perturb-seq that analyzes gene perturbation and RNA expression in single-cell data [37]. Overall, these tools were only developed as prototypes without executable implementations and user-friendly interfaces. The robustness and usability of these tools need to be further improved for a wider application to other analytical studies.

## Case studies and practical challenges

We applied four existing tools on four publicly available datasets downloaded online to evaluate their compatibilities and functions. We first reproduced the analysis of a spatial transcription dataset from Giotto to showcase the unique outcomes of such an integrative study [53, 58] (Figure 4A). Three signature features were reported: spatially correlated clusters, spatial cell networks, and spatial variable genes. Overall, Giotto is a user-friendly and powerful tool for spatial transcriptome analysis with concise and reproducible tutorials. The unique spatial network can be constructed by associating gene expression levels with cell neighborhoods and intuitively presented on the spatial map. Giotto is one of the few tools that provide web-based interactive visualization and exploration for scMulti-omics analysis.

We further applied three well-cited and widely-used tools (MOFA, LIGER, and Seurat3) on three datasets (scRNA-Seq only, matched scRNA-Seq and scATAC-Seq, unmatched scRNA-Seq and scATAC-Seq) with default parameters (Figure 4B). The performance evaluation and comparison in terms of the cell type prediction led to the following three insights. First, the joint analysis of scRNA-Seq and scATAC-Seq showed better performance in predicting cell clusters than using scRNA-Seq alone, in terms of adjusted rand index, which evaluates the similarity between predicted cell labels and benchmarked cell types (higher score means better clustering performance). Second, the clustering result using matched data was better than that using unmatched data. Third, MOFA was the best-performing tool to analyze matched data and Seurat3 was the best-performing tool for unmatched data. We further evaluated the three tools from four perspectives: CPU running time, enrichment of analytical functionalities, reproducibility of software tutorial, and diversity of result interpretations and visualizations. We conclude that Seurat3 is the most robust and easy-to-use tool among the three (ranked as +++ in Figure 4B). However, the above three tools, as well as most existing integrative tools for scMulti-omics analysis, require different input file formats. For example, Seurat3 requires a gtf file recording gene structure information to create an activity matrix to transfer ATAC peak regions to corresponding genes, while LIGER needs a BED file. MOFA failed to clearly state data preprocessing steps. Such multiple input files and vague tutorials greatly increase the difficulty of the application and reproducibility of tools. More challenges of scMulti-omics analyses can be found in the following section.

## Remaining challenges

While recently developed methods for integrating scMulti-omics data provide new opportunities to jointly analyze different types of single-cell data, there remain several challenges and issues to address for the future of scMulti-omics studies and integrative methods. One challenge is related to the computational issues in dealing with large data. Analyzing massive amounts of data from technologies such as RNA-seq, whole-genome sequencing, and ChIP-seq is an established problem due to their sheer size. Furthermore, single-cell methods have the computational burden of including information from hundreds or thousands of cells. These issues, combined with the multi-omics paradigm of integrating two or more technologies, will exacerbate the problem of dealing with big data. As a result, an important computational challenge to address is to determine how to more efficiently generate, manage, store, and analyze large datasets from a practical and economics standpoint. Since this point is not unique to omics analyses, let alone scMulti-omics methods, there is strong evidence that computational resources will increase. However, technologies allowing for more samples and more levels of information will also be developed, requiring further computational resources. Nevertheless, our capabilities to handle the currently available data will only increase.

Another challenge is related to the analytical capabilities of integrative tools. We have described methods with a variety of functionalities when integrating multi-omics data from both within the same cells and across experiments. However, to our knowledge, existing computational methods cannot perform several important functionalities. For example, from the standpoint of matched data, functionalities including identifying *cis*-regulatory motifs, finding cell-type-specific regulons, and inferring gene regulatory networks are not yet covered by existing integrative methods [59, 60]. While these functionalities may be addressed by methods that integrate data across experiments or even methods that analyze single-omics data [59, 61], there is a lack of integrative methods for matched data. With this type of integrative analysis still in its infancy, it is likely that more integrative methods will be developed to answer a wider range of complex biological questions. Furthermore, while there are options for integrating specific types of single-cell data, drawing inferences from the results is still troubling. Co-inference, the process of simultaneously making inferences from multiple single-cell data types, is quite a difficult challenge. Methods such as co-biclustering, co-imputation, and co-deconvolution [62] are not as straightforward as their singular counterparts. For scMulti-omics to truly provide comprehensive insights, these co-inference methods need to be explored and validated.

The need for a robust benchmarking pipeline is an even bigger concern for scMulti-omics methods than computational challenges. As the number of applicable methods grows, so does the potential benefit of an established benchmarking pipeline. Such a pipeline could retrospectively benchmark established methods or evaluate recently developed methods, allowing for robust scenario-specific validation of each approach. Furthermore, benchmarking pipelines can provide valuable insight into areas in which current methods underperform, highlighting areas of interest for future research and method improvement. Currently, there are benchmarking frameworks and evaluations of various computational methods for single-cell single-omics [63–65], however, no current method is available to scMulti-omics.

## Concluding remarks and future perspectives

Integrative methods for scMulti-omics data provide the opportunity for researchers to jointly analyze different types of molecular information at the single-cell level, producing a more comprehensive view of cellular function. Rather than the traditional single-omics approach of studying biological processes from the genomics or transcriptomics perspective, multi-omics methods allow researchers to explore how two or multiple other realms interact and jointly produce biological observations. We pose several outstanding questions to be answered in the field of scMulti-omics analysis (see Outstanding Questions). Several future trends in scMulti-omics data analyses are listed below.

The application of new methods will inevitably lead to substantial improvements in scMulti-omics. One trend in single-omics single-cell analyses is the use of bulk RNA-seq data to impute scRNA-seq data [66–68]. The sparsity of scRNA-seq data stemming from low coverage and inefficiencies in sequencing methods means that the imputation of gene expression is a consistent hurdle in scRNA-seq analyses. Nevertheless, these issues will persist in scMulti-omics and must be dealt with accordingly.

Another trend throughout much of the computational sciences is the development of machine learning (**ML**) and artificial intelligence (**AI**) methods [69–72]. Within the scope of multi-omics, these approaches most presumably benefit the areas of imputation and matching of data across experiments [73–77]. Using ML/AI methods, researchers can potentially construct tools that will capture intricacies between data across experiments that cannot be manually identified and programmed into methods. This would allow for a more reliable linkage of multi-omics data, which will prove especially beneficial for benchmarking. One example is the autoencoder, which compresses input data and filters it through a bottleneck, followed by the decoding of the same data in an attempt to preserve the key defining features of the data.

Lastly, the nature of all technologies is to advance. From Sanger sequencing in the 1970s to next-generation sequencing of the late 1990s and early 2000s, methods for profiling omics data have continually evolved [78]. More recently, so-called third-generation sequencing provides an even greater opportunity to understand complex systems biology, although there is some difficulty in separating the generations of sequencing due to the rapid advances [79]. The most advanced methods are now considered fourth-generation sequencing, and they allow for *in situ* profiling, preserving the available spatial context of the data [80]. These advancing technologies will inevitably be integrated within multi-omics methods, requiring further development of technologies to integrate the new levels of information gained.

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## GLOSSARY

**Artificial intelligence (AI)**

a kind of machine algorithm processing data analysis with mimic cognitive functions.

**Autoencoder**

is a type of multi-layer artificial neural network for unsupervised and efficient data coding and specifically useful for dimension reduction.

**Canonical correlation analysis**

is a statistical method for investigating relationships between two data sets aiming to identify shared sources of variation in a pair of data sets (e.g. two scRNA-Seq data from different sources).

**Canonical correlation vectorization (CCV)**

is a **dimensional reduction** method based on augmented implicitly restarted Lanczos bidiagonalization algorithm capturing features that are maximally correlated across multiple datasets. It takes datasets $X_1 \ldots X_n$ and finds the projection vectors $W = w_1 \ldots w_n$ that optimize $\underset{w_1 \ldots w_n}{\operatorname{argmax}} \sum_{i\,<\,j} w_i^T X_i^T X_j w_j$ with $w_n^T X_n^T X_n w_n = 1$.

**Coupled nonnegative matrix factorization (coupleNMF)**

finds a subset of genes in unmatched data in which one modality is highly predictable from another. It considers a regression model and estimates parameters by fitting a penalized least-square problem based on the two modalities.

**CRISPR perturbation**

is an artificial technique that induces specific genetic perturbations to the cells. It allows the controllable repression of gene expression by knocking out the corresponding genes or single nucleotides.

**Dimensional reduction**

is the process of reducing the variables by identifying the principle features that explain data variations.

**Gradient boosting regression (GBR)**

uses the idea of iteratively fitting “weak” models in order to build a “stronger” model that can more accurately estimate a response variable.

**Group factor analysis (GFA)**

is an unsupervised dimension reduction approach that decomposes input matrices into a product of matrices. A set of $m$ data matrices can be decomposed as $Y^m = ZW^{mT} + \varepsilon^m$ where $Y^m$ are input matrices and $Z$ denotes latent factors. $W^m$ is amplitude matrix reflecting feature patterns, and $\boldsymbol{\varepsilon}^{\boldsymbol{m}}$ denotes cell clusters.

**Hidden Markov random field (HMRF)**

is a graph-based model for transfer pixel intensities over a 2D image using estimated variables from other modalities to reduce the spatial constraints and build connections between neighbor cells.

**Independent component analysis (ICA)**

is a dimensional reduction method that decomposes observed data before ICA is applied to a low-rank matrix to capture underlying processes. The ICA model can be defined as modeling $\boldsymbol{Y} = \boldsymbol{AS}$ where $Y$ is the input, $A$ is the mixing matrix, and $S$ contains the independent components.

**Integrative non-negative matrix factorization (iNMF)**

can be defined as $E_i \approx H_i\,(W + V_i)$ where $E_i$ is the $i^{th}$ dataset, $H_i$ contains the dataset-specific components, $V_i$ approximates dataset-specific effects, $W$ approximates the shared effects.

**LASSO regression**

is a type of linear regression that shrinks regression coefficients by subjecting the coefficients to a constraint, allowing for feature selection and regularization.

**Machine learning (ML)**

is an application of AI that automatically train parameters from known samples to predicting patterns from unknown samples, without explicit programming.

**Manifold alignment**

infers **pseudotime** patterns by simultaneously measuring shared latent variables from multiple modalities. Each modality fits in a linear or non-linear model embedded to a squared exponential kernel, and the latent time variable can be found by maximizing the posterior distribution of a multivariate Gaussian.

**Multivariate normal modeling (MNM)**

decomposes a data vector into spatial and nonspatial components, and can be defined as: $y = f((x_1, x_2, \ldots)) + \psi$, where $y$ is the spatial covariance, $f$ is the spatial variance component that parametrizes covariance based on pairwise distances of samples of interest, $(x_1, x_2, \ldots)$ are the spatial coordinates, and $\psi$ is an independent observation term that models the nonspatial variance component.

**Network simulated annealing**

is a probability technique for approximating the global optimum to solve the combinatory problems in network construction.

**Omics is**

a collection of comprehensive data reflecting the attribution of a particular molecular type, e.g. genomics, transcriptomics, proteomics, etc.

**Pseudotime**

represents a theoretical timeline that estimated from the progression of input modality patterns.

**scHi-C**

uses specific restriction enzymes to digest genome per individual cell allowing the examination of genome 3D organization [81]. It is very useful for predicting the topologically associated domains.

**Single-cell Assay for Transposase-Accessible Chromatin using sequencing (scATAC-Seq)**

identifies the open regions on chromatins to indicate whether the corresponding gene is accessible to be bound by the transcriptome factors.

**Variational Bayes (VB)**

derives a lower bound estimator (a stochastic objective function) for a variety of directed graphical models with continuous latent variables.

**Topic modeling**

is a probabilistic generative model that used to aim to detect “topics” across a collection of documents. When applying in the bioinformatics area, it identifies a shared topic distribution across each input modality.

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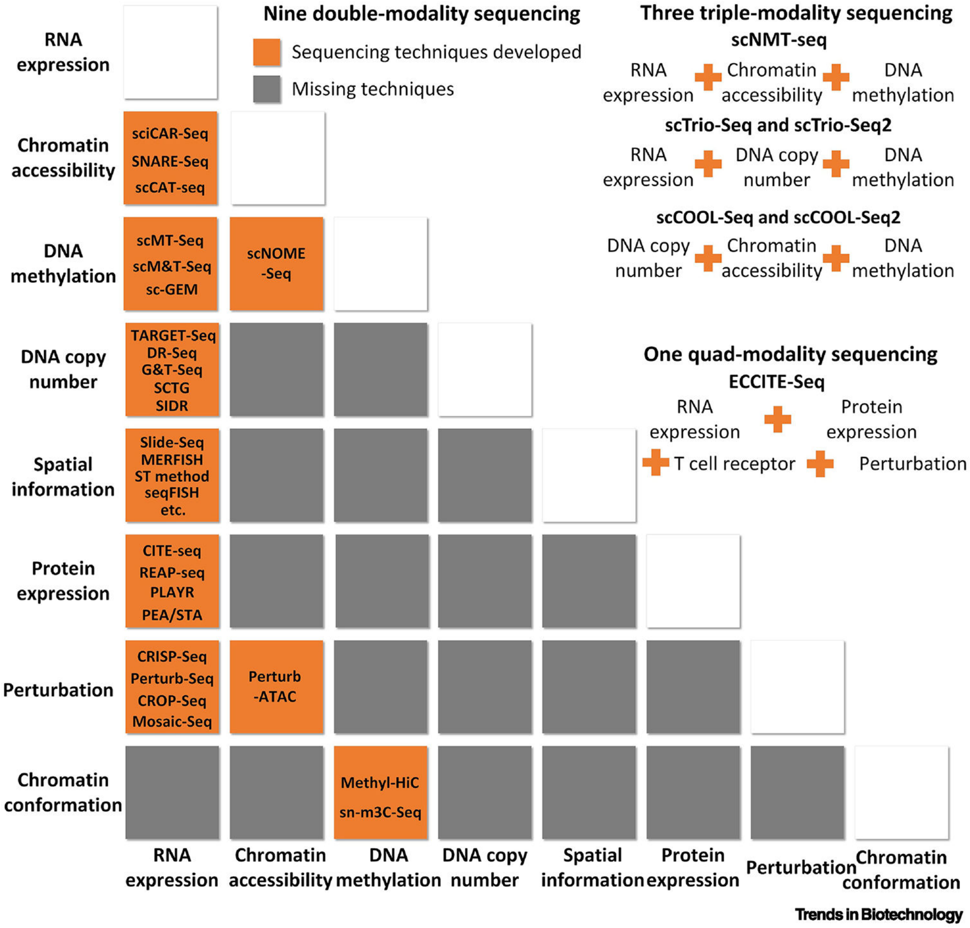
## OUTSTANDING QUESTIONS

- How do the challenges in designing computational methods, from both infrastructure and algorithmic points of view, limit our understanding of single-cell multi-omics processes?
- How reliably do methods that integrate data across experiments replicate the connections between data types?
- What techniques would allow researchers to accurately conclude the reliable performance of integrating methods?
- Would efforts toward a generation of a robust single-cell multi-omics tool that can integrate any type of omics data be more beneficial to the single-cell multi-omics field than explicit work towards detailed improvements and innovations in focused on defined data types?
- To what extent can AI/ML be used to impute or infer the spatial information from single-cell multi-omics data?
- How can AI/ML methods be used to effectively integrate numerous multi-omics data types across numerous experiments?
- What challenges does the “black box” problem in AI/ML pose in determining the effectiveness of linking cross-experiment data?
- Will single-cell multi-omics methods become so widespread that biologists begin to routinely collect data with these types of methods in mind?
- How will single-cell multi-omics drive insights into heterogeneous regulation across the same or different cell states and tissue types?
- How much insight from single-cell multi-omics can be gained in the scope of genomic variation—specifically intronic variation—and its impact on gene expression and regulation?
- How will single-cell multi-omics’ inevitable implementation in the realm of precision medicine aid in the elucidation of specific mechanisms behind cancer cell development and aid in targeted therapies?

## HIGHLIGHTS

- Applying integrative methods to single-cell multi-omics data opens a new window into the understanding of heterogeneous mechanism landscapes and cell-cell interactions.
- Integration of cross-experiment data poses a special challenge.
- A comprehensive understanding of the underlying methods is necessary to determine which pipeline is appropriate for a given single-cell multi-omic dataset.
- We designed and implemented two case studies to demonstrate the application of available single-cell multi-omic tools, where new insights and practical challenges are generated.
- Among the numerous remaining challenges in single-cell multi-omics, establishing a robust benchmarking pipeline is paramount.
- Trends observed in traditional multi-omics, including machine learning, artificial intelligence, and evolving technologies, are paralleled in single-cell multi-omics methods.



Nine double-modality sequencing

🟧 Sequencing techniques developed

⬛ Missing techniques

|  | RNA expression | Chromatin accessibility | DNA methylation | DNA copy number | Spatial information | Protein expression | Perturbation | Chromatin conformation |
|---|---|---|---|---|---|---|---|---|
| RNA expression | □ |  |  |  |  |  |  |  |
| Chromatin accessibility | sciCAR-Seq<br>SNARE-Seq<br>scCAT-seq | □ |  |  |  |  |  |  |
| DNA methylation | scMT-Seq<br>scM&T-Seq<br>sc-GEM | scNOME-Seq | □ |  |  |  |  |  |
| DNA copy number | TARGET-Seq<br>DR-Seq<br>G&T-Seq<br>SCTG<br>SIDR | ⬛ | ⬛ | □ |  |  |  |  |
| Spatial information | Slide-Seq<br>MERFISH<br>ST method<br>seqFISH<br>etc. | ⬛ | ⬛ | ⬛ | □ |  |  |  |
| Protein expression | CITE-seq<br>REAP-seq<br>PLAYR<br>PEA/STA | ⬛ | ⬛ | ⬛ | ⬛ | □ |  |  |
| Perturbation | CRISP-Seq<br>Perturb-Seq<br>CROP-Seq<br>Mosaic-Seq | Perturb-ATAC | ⬛ | ⬛ | ⬛ | ⬛ | □ |  |
| Chromatin conformation | ⬛ | ⬛ | Methyl-HiC<br>sn-m3C-Seq | ⬛ | ⬛ | ⬛ | ⬛ | □ |

Three triple-modality sequencing

scNMT-seq

RNA expression + Chromatin accessibility + DNA methylation

scTrio-Seq and scTrio-Seq2

RNA expression + DNA copy number + DNA methylation

scCOOL-Seq and scCOOL-Seq2

DNA copy number + Chromatin accessibility + DNA methylation

One quad-modality sequencing

ECCITE-Seq

RNA expression + Protein expression + T cell receptor + Perturbation

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**Figure 1.**

Existing scMulti-omics combinations and representative sequencing techniques. By December 2019, multiple sequencing techniques have been developed to acquire nine combinations of two modalities from a single cell, and three for triple-modality and 1 for quad-modality. Names of sequencing methods are listed accordingly, and most of them are not commercialized.

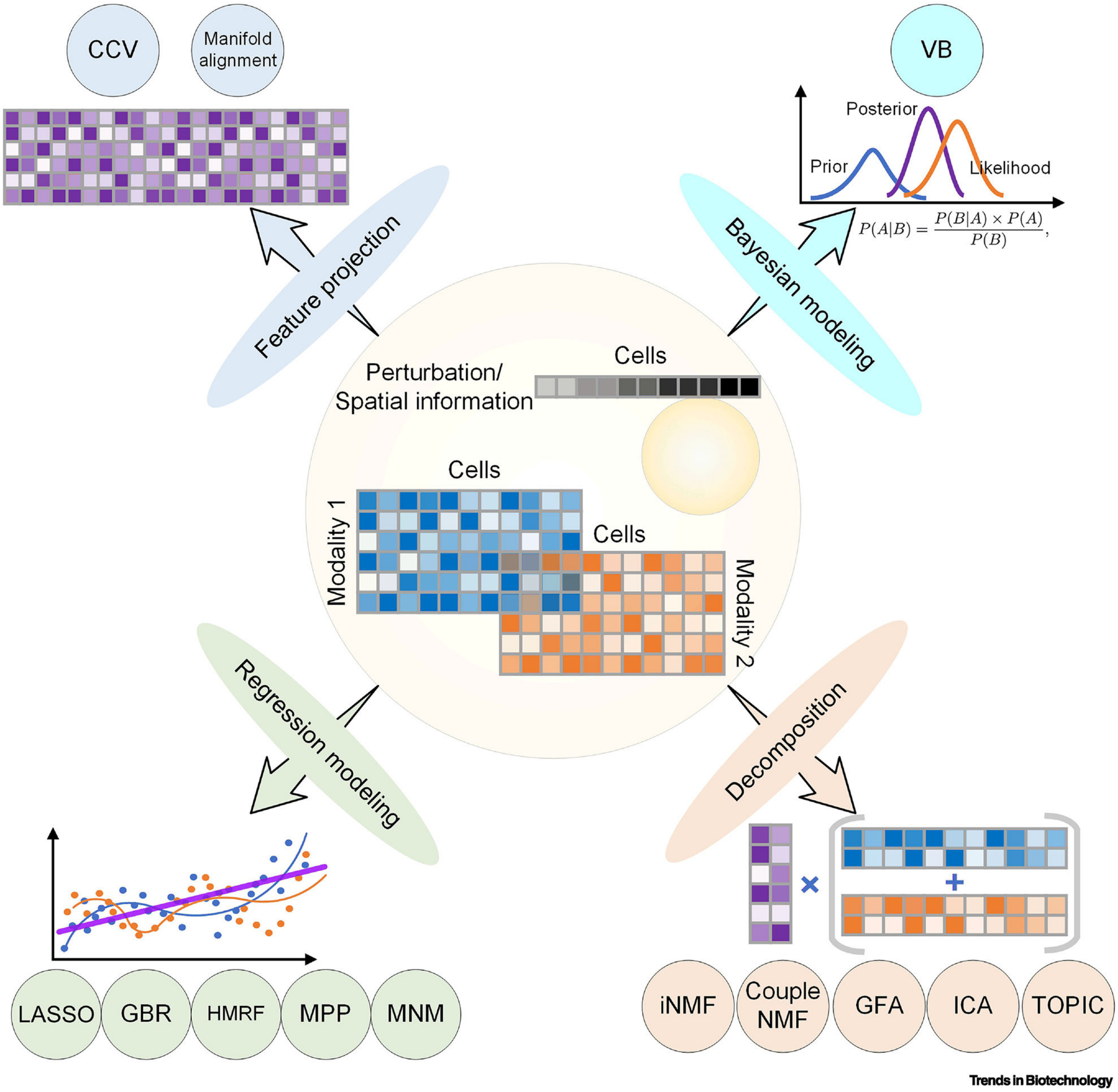
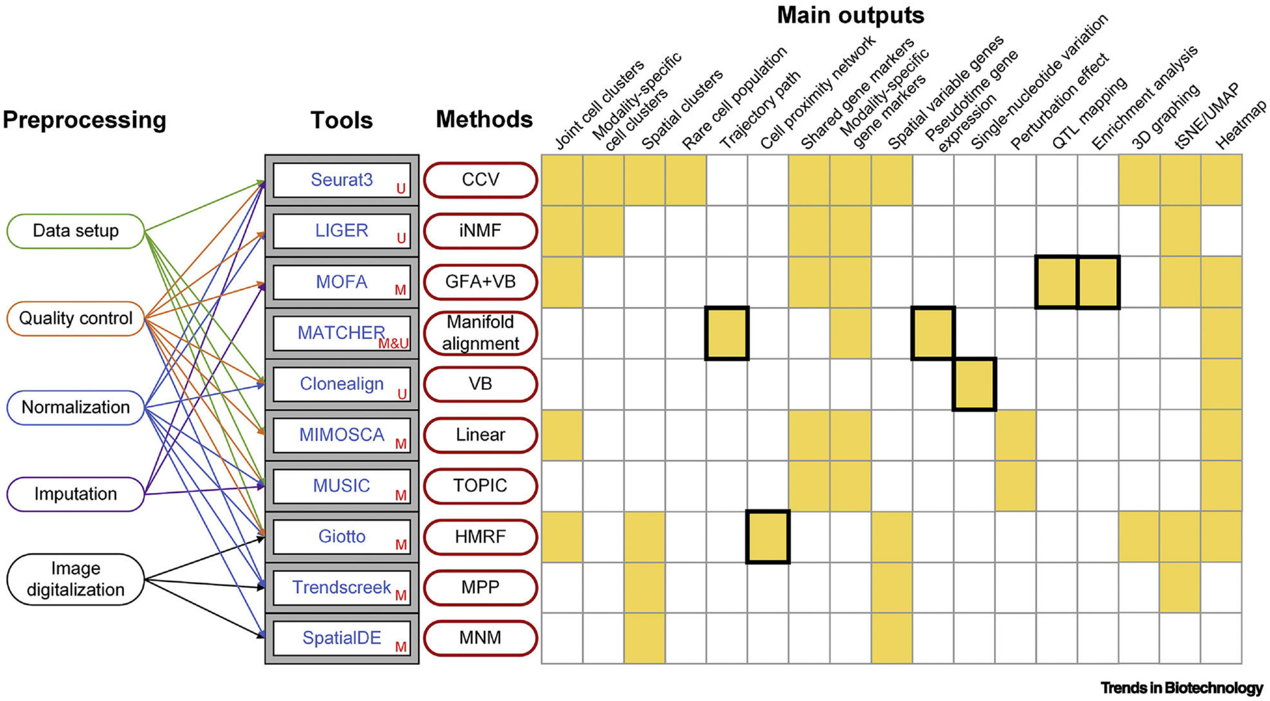


**Figure 2.**

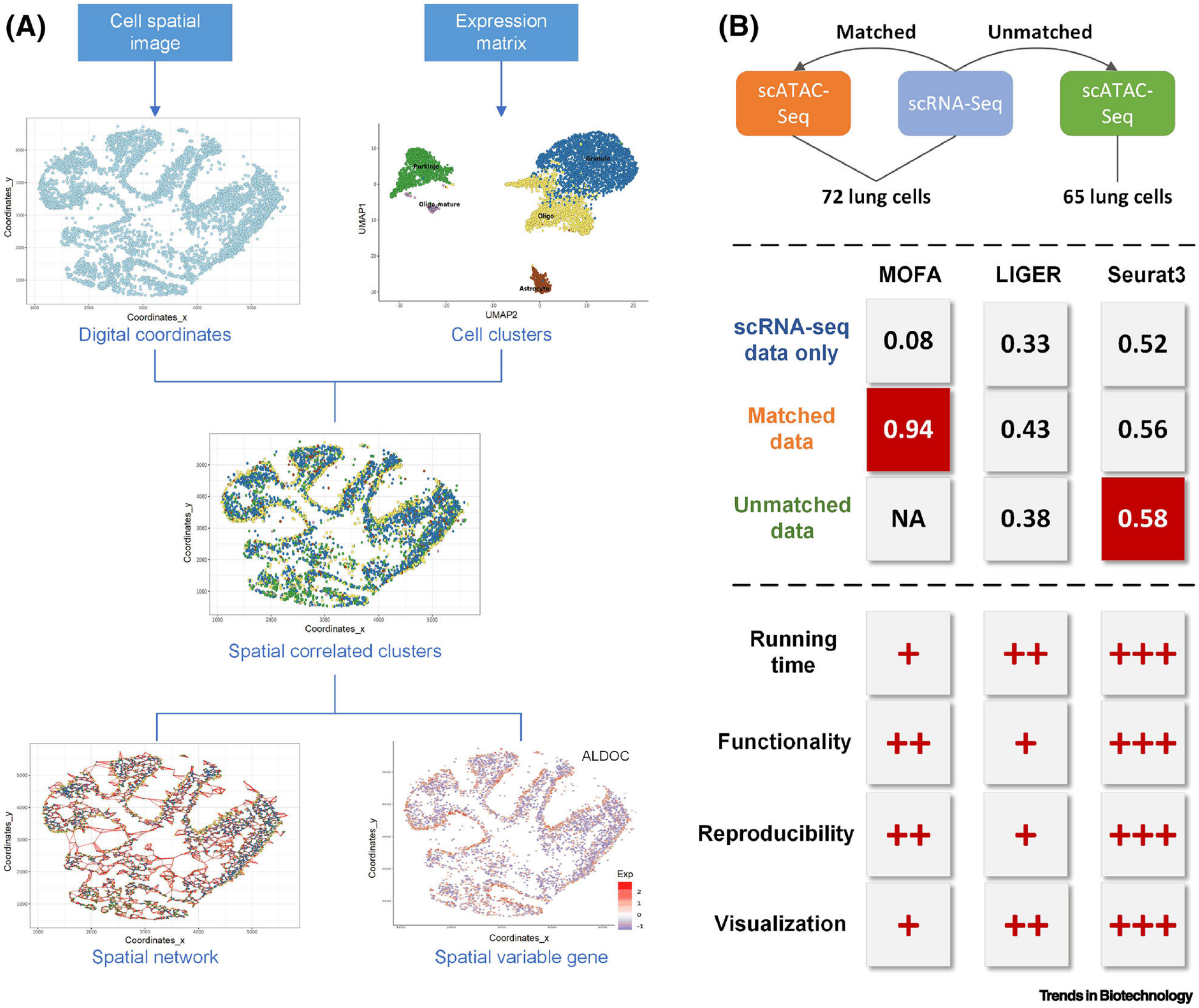
Integrative methods for scMulti-omics. We summarized all 13 integrative algorithms applied on scMulti-omics analysis into four categories, and each of which is followed by a graph for understanding.



| Tools | Methods | Joint cell clusters | Modality-specific cell clusters | Spatial clusters | Rare cell population | Trajectory path | Cell proximity network | Shared gene markers | Modality-specific gene markers | Spatial variable genes | Pseudotime gene expression | Single-nucleotide variation | Perturbation effect | QTL mapping | Enrichment analysis | 3D graphing | tSNE/UMAP | Heatmap |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Seurat3 U | CCV | ■ | ■ | ■ | ■ | | | ■ | ■ | ■ | | | | | | ■ | ■ | ■ |
| LIGER U | iNMF | ■ | ■ | | | | | ■ | ■ | | | | | | | | ■ | |
| MOFA M | GFA+VB | ■ | | | | | | ■ | ■ | | | | | ▣ | ▣ | | ■ | ■ |
| MATCHER M&U | Manifold alignment | | | | | ▣ | | | ■ | | ▣ | | | | | | | ■ |
| Clonealign U | VB | | | | | | | | | | | ▣ | | | | | | ■ |
| MIMOSCA M | Linear | ■ | | | | | | ■ | ■ | | | | ■ | | | | | ■ |
| MUSIC M | TOPIC | | | | | | | ■ | ■ | | | | ■ | | | | | ■ |
| Giotto M | HMRF | ■ | | ■ | | | ▣ | | | ■ | | | | | | ■ | ■ | ■ |
| Trendscreek M | MPP | | | ■ | | | | | | ■ | | | | | | | ■ | |
| SpatialDE M | MNM | | | ■ | | | | | | ■ | | | | | | | | |

**Figure 3, Key Figure.**

Ten tools integrated with proper preprocessing steps, core integrative methods, and adequate results interpretations for integrative analysis of scMulti-omics. U (unmatched), M (matched), and M&U (both matched and unmatched) represent the data type that the original tool’s paper claimed to support. The main outputs are summarized based on original papers and tool tutorials from our investigations. Black frames indicate unique outputs.



| | MOFA | LIGER | Seurat3 |
|---|---|---|---|
| scRNA-seq data only | 0.08 | 0.33 | 0.52 |
| Matched data | 0.94 | 0.43 | 0.56 |
| Unmatched data | NA | 0.38 | 0.58 |

| | MOFA | LIGER | Seurat3 |
|---|---|---|---|
| Running time | + | ++ | +++ |
| Functionality | ++ | + | +++ |
| Reproducibility | ++ | + | +++ |
| Visualization | + | ++ | +++ |

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**Figure 4.**

Two case studies for scMulti-omics analysis. (A) Main outcomes of Giotto. (B) Assessment of cell type prediction using MOFA, LIGER, and Seurat3, in terms of ARI (adjusted rand index) scores on three datasets. The three tools were further systematically evaluated and ranked, where +++ represents the most efficient tool in terms of CPU running time, provides the best practical support in analytical functionalities and visualizations, and has the most robustness in reproducibility of results; and + represents less efficiency, practical support, and robustness.

## Table 1:

Publicly available matched scMulti-omics data.

| Single-cell Profiling | Seq tech | Accession | Ref |
|---|---|---|---|
| **1** RNA expression<br>**2** DNA copy number | G&T-seq | EGAS00001001204 | [82, 83] |
| | | E-ERAD-381 | |
| | SIDR | PRJEB20144 | [84] |
| | TARGET-Seq | GSE105454 | [85] |
| | SCTG | SRP040646 | [86] |
| | DR-seq | GSE62952 | [87] |
| **1** RNA expression<br>**2** DNA methylation | sc-GEM | SRR3748387 | [51] |
| | scM&T-seq | GSE74535 | [50] |
| | scMT-seq | GSE76483 | [88] |
| **1** RNA expression<br>**2** Chromatin accessibility | sci-CAR | GSE117089 | [29] |
| | scCAT-seq | Along with the paper | [89] |
| | SNARE-seq | GSE126074 | [90] |
| | CITE-seq + scATAC-Seq | GSE139369 | [91] |
| **1** DNA methylation<br>**2** Chromatin accessibility | scNOME-seq | GSE83882 | [92] |
| **1** RNA expression<br>**2** Protein expression | CITE-seq | GSE100866 | [13] |
| | | GSE128639 | [24] |
| | REAP-seq | GSE100501 | [14] |
| | PLAYR | Along with the paper | [93] |
| | PEA/STA | Along with the paper | [94] |
| **1** DNA methylation<br>**2** Chromosome conformation | Methyl-HiC | GSE119171 | [56] |
| | sn-m3C-Seq | GSE124391 | [95] |
| **1** RNA expression<br>**2** DNA perturbation | Perturb-seq | GSE90546 | [37] |
| | | GSE90063 | [52] |
| | CRISP-seq | GSE90486 | [96] |
| | CROP-seq | GSE92872 | [97] |
| | | GSE108699 | [98] |
| | Mosaic-Seq | GSE81884 | [99] |
| **1** RNA expression<br>**2** Spatial information | osmFISH | http://linnarssonlab.org/osmFISH | [11] |
| | STARmap | https://www.starmapresources.com/data/ | [12] |
| | seqFISH | Along with the paper | [100] |
| | MERFISH | Along with the paper | [101] |
| | | GSE113576 | [102] |
| | Slide-Seq | https://singlecell.broadinstitute.org/single_cell/study/SCP354/slide-seq-study | [58] |
| | Spatial transcriptomics (10X spatial) | https://github.com/SpatialTranscriptomicsResearch/st_pipeline | [103] |

| Single-cell Profiling | | Seq tech | Accession | Ref |
|---|---|---|---|---|
| | | | https://www.10xgenomics.com/solutions/spatial-gene-expression/ | [104–106] |
| | | MASC-Seq | Along with the paper | [107] |
| | | ST-RNA-Seq | SRP100428 | [108] |
| **1**<br>**2** | Chromatin accessibility<br>RNA perturbation | Perturb-ATAC | GSE116297 | [10] |
| **1**<br>**2**<br>**3** | RNA expression<br>DNA methylation<br>Chromatin accessibility | scNMT-seq | GSE109262 | [57] |
| **1**<br>**2**<br>**3** | RNA expression<br>DNA copy number<br>DNA methylation | scTrio-seq | GSE65364 | [109] |
| | | scTrio-seq2 | GSE97693 | [110] |
| **1**<br>**2**<br>**3** | DNA copy number<br>DNA methylation<br>Chromatin accessibility | scCOOL-seq | GSE78140 | [111] |
| | | | GSE100272 | [112] |
| | | iscCOOL-seq | GSE114822 | [113] |
| **1**<br>**2**<br>**3**<br>**4** | RNA expression<br>Protein expression<br>T cell receptor<br>Perturbation | ECCITE-Seq | GSE126310 | [114] |

## Table 2.

Tools for scMulti-omics analysis and application examples.

| Tools[a] | Implemented in[b] | Data[c] | Number of Cells | Main biological insights | Ref |
|---|---|---|---|---|---|
| MATCHER | Py | RE+DM+CA | 5,151 | Establish a continuum that ranges from pluripotency to a differentiation primed state; while the shared master time was highly correlated in the matched dataset which was useful for determining the overall reprogramming progress of each cell | [26] |
| MIMOSCA | Py | RE+GP | 200,000 | Predict the regulation of anti-parasitic response genes *Gbp2,2b,3,4,5* and 7 by inducing CRISPR/Cas9 knockout, targeting 24 transcriptome factors; suggest *Stat2's* impact on *Gpb* genes may be mediated through *Irf8*. | [52] |
| MOFA | R/Py | RE+DM | 87 | Reveal the cooperation between the transcriptome and methylation sites during the transition from naive to primed pluripotent states. Factor-specific markers were identified, such as *Rex1/Zpf42, Tbx3*, and *Fbxo15*. | [36] |
| | | RE+PE | 200,000 | Identified 44 genes whose activation induces a zygotic genome activation-like transcriptional response, including 40 novel maternal proteins. | [45] |
| Clonealign | R | RE+DC | 1,152 | Build single-cell phylogeny with four distinct clades and eight sub-clades. The intra-clonal clustering identified cell cycle corresponding clusters. | [28] |
| Trendsceek | R | RE+SI | ~10,000 | Identify 35 significant genes with expression primarily in nongranular cells including Ptn, Nr2f2, and Fabp7 in mouse olfactory bulb tissue. | [32] |
| SpatialDE | Py | RE+SI | ~10,000 | Identify 67 spatial variable genes with spatial dependencies of the gene expression variance and showed clear spatial substructure, consistent with matched tissues in mouse olfactory bulb. | [33] |
| Seurat3 | R | RE+CA | 14,249+100,000 | Reveal cell-type-specific regulatory loci whose accessibility profiles were consistent with expected patterns. | [24] |
| | | | 35,882 | Identify 91,601 putative peak-to-gene linkages and inferred the potential oncogene RUNX1. | [91] |
| | | | 7,846 | Reveal cell-state transcriptional regulators and lineage relationships in mammary gland cells. | [115] |
| | | RE+PE | 33,454 | Matched RNA expression and 25 cell-surface protein expressions, leveraging connections between protein abundance and gene expression. | [24] |
| | | RE+SI | 14,249 | Predict spatial gene expression patterns and spatial subpopulations. | [24] |
| LIGER | R | RE+DM | 55,803+3378 | Resulted in 37 neuron clusters and identified methylation regions that were anticorrelated with *Arx* expression, including a validated *Arx* enhancer. | [49] |
| MUSIC | R | RE+GP | 32,777 | Identify a novel knockout effect on cell migration impacted by the perturbation of *Cebpb* on immune cell activation, and gene-gene perturbation associations between *Cebpb* and other gene perturbations. | [38] |
| Giotto | R | RE+SI | 913 | Identify six global and distinctive clusters including excitatory neurons (Icam), GABAergic neurons (Slc32a1), and four smaller groups; Visualize both single-cell resolution heterogeneity in both expression and spatial space representations. | [53] |

[a] Tools that are equipped with multiple functions; developed methods with only scripts are not considered in this table; tools are sorted by publishing year from old to new.

[b] The platform of each tool, either as an R or Python (Py) package.

[c] Data type combination examples using the corresponding tool. RE=RNA expression, DM=DNA methylation, CA=chromatin accessibility, PE=protein expression, SI=Spatial information, DC=DNA copy, GP=Gene perturbation, HM=HiChiP.