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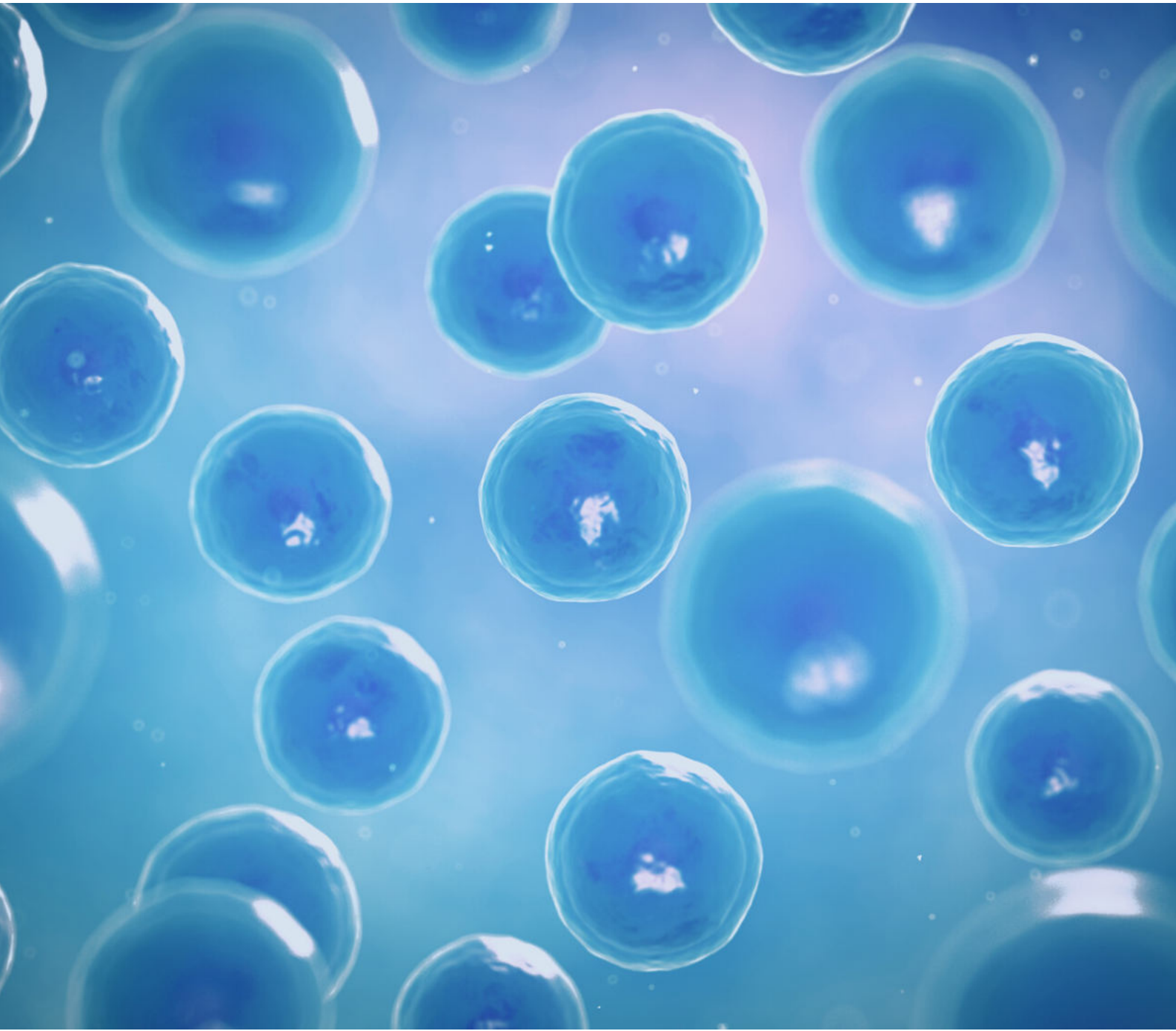
DIFFERENTIATION AND APPLICATION OF SINGLE CELL RNA SEQUENCING DATA ANALYSIS



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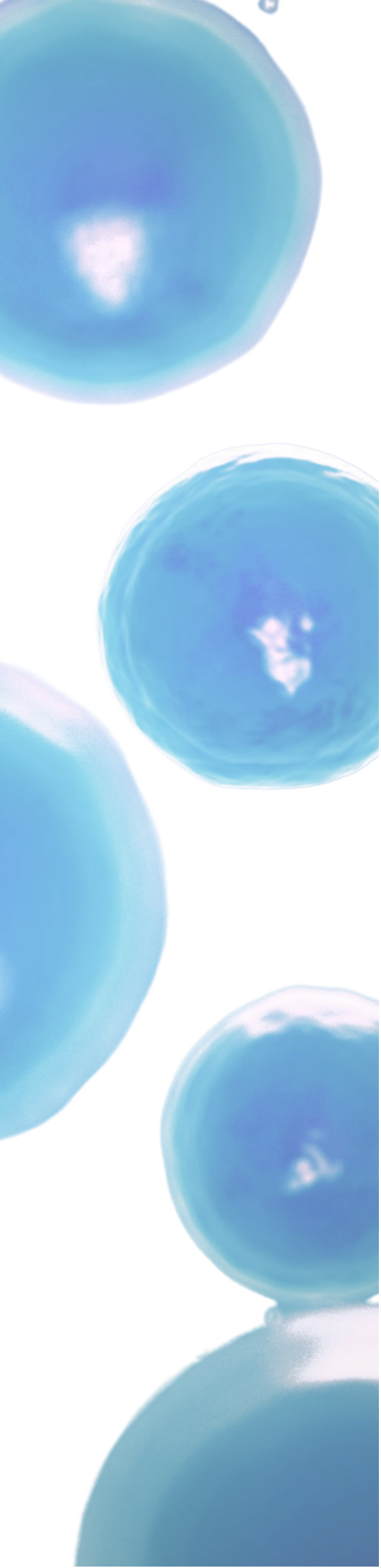


INTRODUCTION

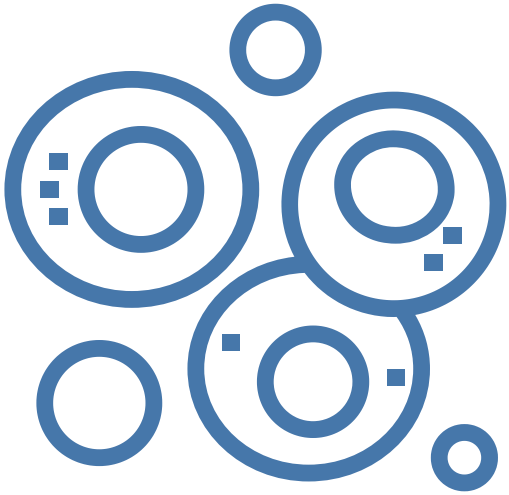
Single-cell sequencing technology is a way to get genomic, transcriptomic, and multi-omics information from a cell to show differences between cell populations and the evolution of cells. Single-cell sequencing techniques turn out to be helpful in many ways: they help draw out cellular maps better than standard sequencing methods, distinguish between a small number of cells, find heterogeneity relationships between different single cells, and show differences and evolutionary links between cell populations. This eBook talks about how single-cell sequencing results can be used and analyzed in different areas of health and medicine for a broad range of applications [1].

EXPLORE THE ADVANTAGES OF SINGLE CELL RNA SEQUENCING (SCRNA-SEQ)

A tissue or cell population is used as the starting material for bulk RNA sequencing (RNA-Seq), which yields a variety of diverse gene expression patterns from the research sample. Since each cell has a unique set of somatic mutations and transcriptional controls, all these changes will most likely be reflected at the RNA level. Individualized cell study is necessary for clinical diagnosis, treatments, and research at the transcriptome level. The development of scRNA-Seq technology was aided by this requirement. The technique has added valuable potential to the genomics toolbox in medical research studies.

While bulk RNA-Seq assesses the average gene expression across the population of cells in a sample and identifies changes across sample conditions, the scRNA-Seq analyses the gene expression of individual cells in a sample and discovers variations between cell types and states.

At the experimental level, the bulk RNA-Seq involves RNA extraction, fragmentation, reverse transcription, ligation of sequencing adapters, and PCR amplification. In the case of scRNA-Seq, the protocol majorly resembles the bulk RNA-Seq. However, RNA is labeled with cell-specific identifiers and reverse transcribed, which turns RNA into cDNA. The major advantage here in scRNA-Seq is the additional use of unique molecular identifiers and spike-ins, which helps to reduce noise levels in the scRNA-Seq protocol and promises to offer a better resolution of cellular distinctions [2].



At the analytical level, the bulk RNA-Seq data is assessed for estimating gene and transcript expression, differential expression analysis, and alternative splicing, while scRNA-Seq data can be further analyzed for dimensionality reduction, cell subpopulation identification, and trajectory analysis.

In terms of general applications for cancer categorization, biomarker and gene fusion findings, illness diagnostics, and therapeutic treatment optimization, single-cell sequencing will undoubtedly play a better role in biological characteristics understanding. Here, we have distilled some of the specificities of single cell RNA sequencing data analysis compared to general RNA sequencing data that may lead to additional applications [Table 1].

Product	Bulk RNA-Seq	Single Cell RNA-Seq
Sample Requirements	Total cell ≥2M	Total cell ≥ 500K Cell size < 50-60μm (the width of the microfluidic channel) Cell viability: ≥ 80%
Principle	Oligo dT magnetic beads to enrich polyA or de-rRNA to enrich mRNA	Use millions of unique Barcodes and UMIs to label cells and intracellular genes, respectively, to achieve cell differentiation and single-cell quantification
Experiment Procedure	Extract the RNA in the sample, enrich the mRNA in the nucleic acid, randomly interrupt it and reverse-transcribe it into cDNA, connect the adapter and carry out PCR enrichment	The GEMs are collected and flowed to the reservoir, where the Gel beads dissolve to release the Barcode sequence, and start labeling the sample. The products containing the Barcode information in each droplet are mixed to construct a standard sequencing library.
Sequencing Data Volume	6-12G/sample	15-30M/cell
Analysis	Gene quantification: obtain the mean value of gene expression within the sample; differential gene screening; differential gene function enrichment analysis	Cell counting and cell typing; intracellular gene quantification; intercellular marker gene screening; gene functional enrichment typing
Scope of Application	Growth and development of animals and plants, shape differences, environmental stress, etc.	The sample size is too small for routine RNA sequencing; research on heterogeneity between cells, such as genetics, development, disease mechanism, etc.

Table 1: Differentiation comparison of bulk RNA-Seq and single cell RNA-Seq

STANDARD DATA ANALYSIS OF SINGLE CELL RNA SEQUENCING

I. Cell Calling and UMI Counting

Cell Ranger is a software package officially provided by 10x genomics for its single-cell transcriptome data analysis. Cell Ranger aligns the fastq sequencing data generated by sequencing to the reference genome for cell and UMI counts to generate a cell-gene expression matrix [3].

A specific, damage-induced quiescent cell type known as the resurrection stem cell was discovered in one study from a nature publication that used cell ranger counts and single-cell RNA sequencing to assess the healing mouse gut. In order to revive the homoeostatic stem cell compartment and rebuild the intestinal epithelium, the study defined a special stem cell that is activated by injury and plays its role in the healing process [4].

Cells	Sample 1	Sample 2	Sample 3
Estimated Number of Cells	3,000	3,000	3,000
Fraction Reads in Cells	81.9%	64.7%	91.7%
Mean Reads per Cell	62,217	55,413	63,737
Total Genes Detected	19,314	19,671	18,691
Median Genes per Cell	1,219	1,060	156
Median UMI Counts per Cell	3,847	3,339	517

Table 2: Expression distribution of cells

II. Clusters of Cells

Many clustering techniques have been developed because of the crucial role played by the clustering step for subsequent analysis of the scRNA-Seq data. Simple k-means clustering, hierarchical clustering, and their variants, and the more advanced approaches such as likelihood-based mixture modeling and spatial clustering based on density are all approaches that are adopted for cluster analysis [5].

In one such study, the single-cell model-based deep embedded clustering algorithm scDeepCluster was created. It concurrently teaches feature representation and clustering through explicit modeling of the production of scRNA-Seq Seq data. In the data scRNA, data analysis scDeepCluster outperformed other methods under various clustering performance metrics and demonstrated better scalability. This technique was based on testing extensive simulated data and real datasets from four representative single-cell sequencing platforms. scDeepCluster is a promising application for massive grouping amounts of scRNA-Seq Seq data due to its accuracy and effectiveness [2].

In another research study, breast cancer diagnoses were assessed using feature-selected cell cluster analysis. Four well-known clustering learning algorithms—K-means, SOM, HC, and PAM—were used to conduct clustering on the chosen features. The study presented valuable elements in feature selection for finding clusters in breast cancers. It provided insights for better data mining and machine learning application in cluster analysis for breast cancer diagnosis in the future [6].

- **UMAP/tSNE cell typing**

Since bulk RNA sequencing counts the expression of all cells, it cannot be used for cell typing. In tissue samples containing complicated cell populations, recent developments in single-cell RNA sequencing (scRNA-SeqSeq) technology have made it possible to identify specific cell types, such as epithelial cells, immune cells, and fibroblasts. The major challenge with the sequence data is, however, the high dimensionality, sometimes referred to as the "curse of dimensionality," which is faced

during cluster analysis for cell typing. It could be challenging to pinpoint the specific clusters of data points based on the measured distances between the data pairs when they are represented in a higher dimensional space. One way to get around the high-dimensionality curse is by dimensionally reducing or projecting data into a lower-dimensional space[7].

Unified manifold approximation and projection (UMAP) and t-distributed stochastic neighbor embedding (t-SNE) are perhaps the most used nonlinear dimensionality reduction methods for displaying high-dimensional. This method is a vitalthe first stage in the analysis process[8]. The algorithm preserves the local geometric structure information of high-dimensional data in the low-dimensional space as much as possible and groups cells with similar expression patterns together to realize cell typing. The t-SNE method was used to visualize data that was analyzed in a research study intended for multiplexed detection of proteins, transcriptomes, clonotypes, and CRISPR alterations in cancer sample assays [9].

III. Quantitative Differential and Functional Analysis of Genes

The procedure of statistically assessing the normalized read count data to find measurable variations in expression levels between experimental groups is known as differential expression analysis.

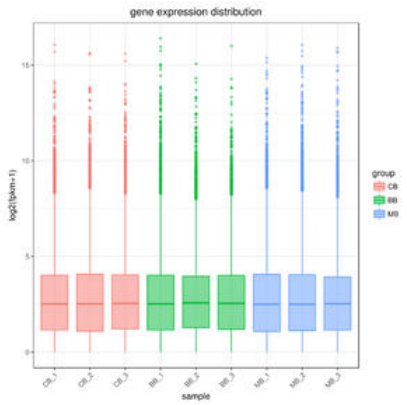


Figure 1*: Bulk RNA-Seq: expression levels of all genes in a sample

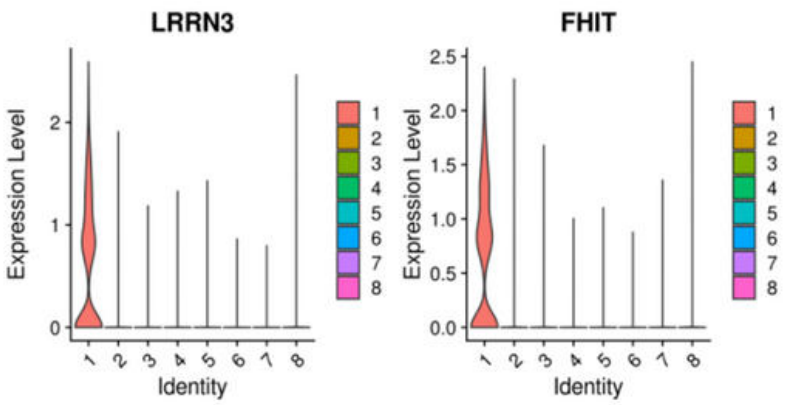


Figure 2*: scRNA-Seq: expression of a single gene in each cell cluster

The process of dissecting and analyzing a set of genes to determine which genes are responsible for regulating biological pathways is known as functional analysis of genes. These procedures entail the use of cell sequencing and are extremely important in biological and medical research. Both quantitative differential gene difference and functional enrichment studies can be carried out using bulk RNA-Seq and scRNA-Seq.

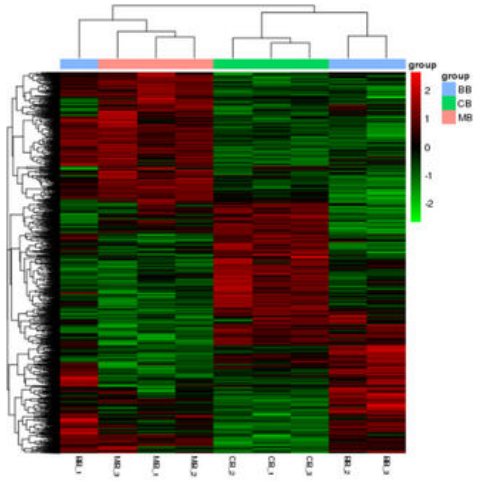


Figure 3*: Bulk RNA-Seq: differential genome clustering heatmap for different combinations

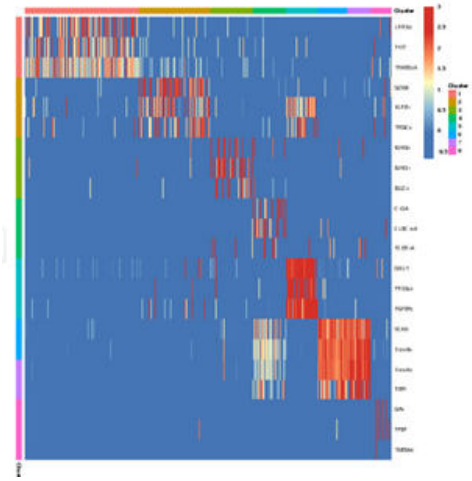


Figure 4*: scRNA-Seq: Clustering Heatmaps of Different Cell Clusters

While bulk RNA-Seq primarily focuses on the quantification of genes in a single sample and the differential enrichment between different samples/combinations, scRNA-Seq analyses the gene expression level of a single sample cell within a sample, identifies mark genes and performs differential analysis between different cell clusters.

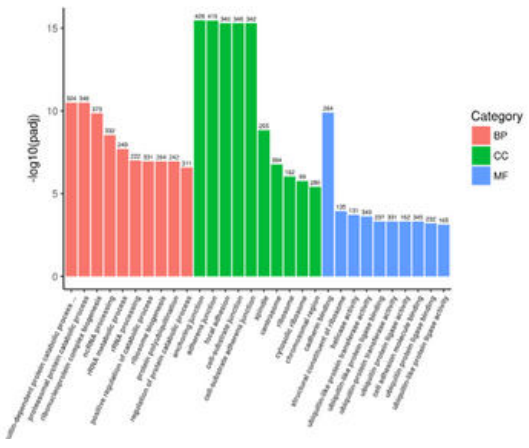


Figure 5*: Bulk RNA-Seq: GO enrichment analysis of differential genes in different combinations

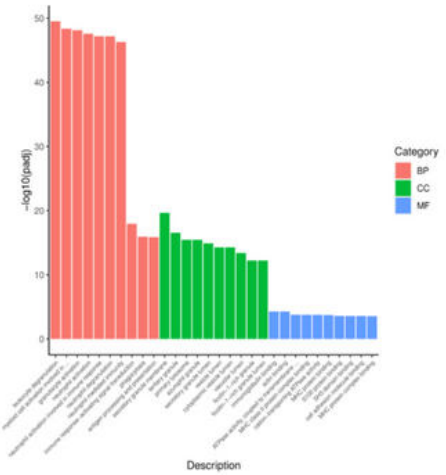


Figure 6*: scRNA-Seq: GO enrichment analysis of differential genes in different cells

ADVANCED DATA ANALYSIS IN MULTIPLE APPROACHES

I. Cell Type Annotation

Gene measurements at individual cells may be used to examine a variety of tissues and organs at various stages, as opposed to bulk RNA-Seq measurements, which average the gene expressions over a population of cells. A typical objective in many single-cell research is to determine and define the different cell types in the sample using the single-cell transcriptome data. To achieve this, several strategies have been created.

	SingleR	Garnett	scMCA	sciBet	NovoCM	NovoGS	Celaref	Similarity
Source	Nature Immunology	Nature Methods	Cell	Preprint	Novogene	Novogene	Github	Novogene
Version	v1.0-2019	v1.0-2019	v1.0-2018	v1.0-2019	v1.0-2019	v1.0-2019	v1.0-2019	v1.0-2019
Principle/ Catalogue	Similarity	Similarity&MarkerGene	Similarity	Similarity&MarkerGene	MarkerGene	MarkerGene	Similarity	Similarity
Method	Similarity to Ref	Learning algorithm	Similarity to Ref	Gamma-Poission; Entropy Test	Hypergeometric test	GSVA	Similarity to Ref	Learning algorithm
Target	Cell-Significant & Cluster-Significant	Cell-Significant & Cluster-Significant	Cell-Significant & Cluster-Significant	Cell-Significant & Cluster-Significant	Cluster-Significant	Cell-Significant & Cluster-Significant	Cluster-Significant	Cell-Significant & Cluster-Significant
Ram/Core	80/1	50/1	20/1	20/1	20/1	80/1	50/1	50/1
Limitation	HCA/MCA Database	incomprehensive Tissues and cells	Only for Mouse	Tendentious enrichment for non-immunity cell	Databases (CellMarker and PanglaoDB)		Incomprehensive cells No_cells <= 3w	HCA/MCA Database and Self-Database
Advantage	Common	Learning	Rapid and accuracy for Mouse	Rapid and accuracy for Immunity cells	Celltyping By Markergene & more accuracy		-	Learning
Accuracy	~72.7%	~63.6%	~80.0%	~60.0%	~83.3%	~66.7%	-	-
Grade	★★★★	★★★	★★★★★	★★★	★★★★★	★★★	★★	★★

Table 3: Summary of cell definition methods. Note: accuray is evaluated by ari, and the percentage is displayed

- **Marker gene:**

Genes expressed significantly higher in one kind of cell but only moderately expressed in other types are referred to as cell-type-specific (CTS) genes or marker genes. The study of RNA transcriptional data relies heavily on these genes, which specify cellular identity [10]. Single-cell sequencing combines the expression level of the marker gene to identify cell type. This has led to boosting our understanding of cell biology and the molecular causes of illnesses.

The identification of diagnostic marker genes from the gene expression profiles is a vital step in the application of microarrays to cancer diagnoses.

For the development of therapies and the diagnosis and prognosis of tumours, these molecular markers provide invaluable supplementary information. One research study reported the curation of a repository of microbial marker genes and predictors and described the development of a computational pipeline termed "Mi2P" for using this repository. The aim of the study was to predict the prediction of host phenotype. The study concentrated on microbial marker genes associated with type II diabetes and liver cirrhosis, as well as the effectiveness of immunotherapy for non-small-cell lung cancer (NSCLC) and renal cell carcinoma (RCC). The study identified the marker genes from the metagenomic data. The researchers demonstrated that predictors constructed from these microbial marker genes could quickly and accurately predict the host phenotype in the presence of microbiome data [11].

- **Expression profile similarity:**

The technique of cell type identification uses the expression profile of an unknown cell type to do a correlation analysis with the expression profile of a known cell type. If the correlation is high, this type of cell is identified. R packages such as SingleR and celaref can be used to analyse the correlation. A cell type identification method may be developed based on the observation that cells of the same type evoke comparable gene expression patterns, but cells of different kinds evoke distinct gene expression patterns. In the past, only cell similarity was assessed, but research published in 2021 included the computation of the dissimilarity index to the analysis and suggested a new approach.

To create the incidence matrix, the approach first calculated the similarity and dissimilarity between cells. Next, it used the K-means algorithm in a low-dimensional space to achieve clustering. On various real single-cell RNA sequencing datasets, the suggested enhanced spectral clustering approach outperformed the traditional spectral clustering method in identifying various kinds of cells [12].

- **Statistical model building classifier:**

The expression profiles of known cell types can be used as a training set to build a classifier and input the expression profiles to classify and identify our target cells. R language package such as “Garnett” can be used for such sequence expression profiles data. A research article in 2019 used a tool, scPred to classify individual cells with high accuracy using machine learning on training data from pancreatic tissue, mononuclear cells, colorectal tumor biopsies, and circulating dendritic cells. To confirm the existence of tumour cells, surgical samples of stage IIA intestinal gastric adenocarcinoma from two individuals were taken together with matched-normal epithelium. It was possible to identify tumour cells from 1905 epithelial cells using their microsatellite stability. A classifier model was developed by the researchers to distinguish between epithelial cells from tumours and normal tissue. The outcomes of the study showed that, as long as the cells are not of highly uncommon kind in the training data, scPred can properly classify them[13]. ScType is another computational structure that considers completely mechanized and super quick cell-type determination dependent simply upon scRNA-Seq information and an extensive cell marker data set as foundation data. ScType conveys unbiased and reliable cell type distinguishing proof by guaranteeing the particularity of positive and negative marker qualities across groups and cell types utilizing six scRNA-Seq datasets from human and animal tissue distinctions [14].

II. Pseudotime Analysis

Pseudotime analysis, also known as cell trajectory analysis, is often used in development-related studies to infer the differentiation trajectory of cells or the evolution of cell subtypes during development.

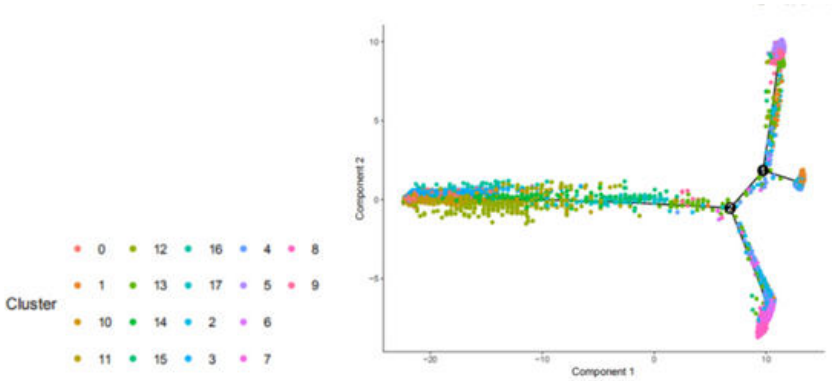


Figure 7*: Dissect cellular decisions with branch analysis

The construction of cell lineages is used to study the changes in gene expression over time in various cell subsets. A group of cells contains cells in a variety of developmental phases, including differentiated, undifferentiated, and intermediate cells. Some cells develop early, others develop late, and some are in a phase transition. Algorithms are used to infer the relative differentiation time of each cell based on gene expression, which results in differentiation trajectories[15].

Pseudotime analysis application in NSCLC (non-small cell lung cancer)

Non-small cell lung cancer (NSCLC) is a highly heterogeneous condition. The microenvironment around cancer cells affects how the disease develops and how well it responds to treatment. Individualized therapy can be influenced by knowledge of the NSCLC's cellular ecological environment.

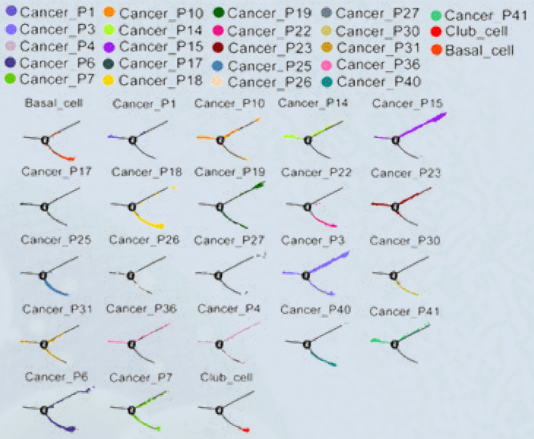


Figure 8: Phenotypes of lung epithelial cells and their evolutionary trajectory into cancer cells[16]

In a study that was published in the journal Nature in 2021, researchers gathered the primary foci of 42 patients with advanced (stage III/IV) NSCLC, examined the transcriptome of 90,406 cells, and built a single-cell map of the disease. This work revealed the tumor heterogeneity and properties related to tumor-associated neutrophils, which will help to clarify their function in non-small cell lung cancer. Rare cell types in cancers including follicular dendritic cells and T helper 17 were found by researchers. The findings showed that there was significant variation in the cellular make-up, chromosomal organization, developmental course, intercellular signaling network, and phenotypic dominance of tumours from various patients. The study discovered a link between tumour heterogeneity and tumour-associated neutrophils, which may assist clarify their role in NSCLC and result in more effective therapy and curing methods[16].

II. Tumour Mutation Analysis

Tumour mutation load (TML), otherwise called tumour mutation burden (TMB), is a strategy for distinguishing and evaluating how much non-equivalent, physical changes that happen in disease cells per mega base of genomic areas of interest. TML has arisen as an indicator of patient order for immunotherapy reaction. For somatic large-scale chromosomal copy number variations (CNVs), such as gains or deletions of whole chromosomes or significant parts of chromosomes, tumour mutation analysis can be performed using tumour single-cell RNA sequencing data.

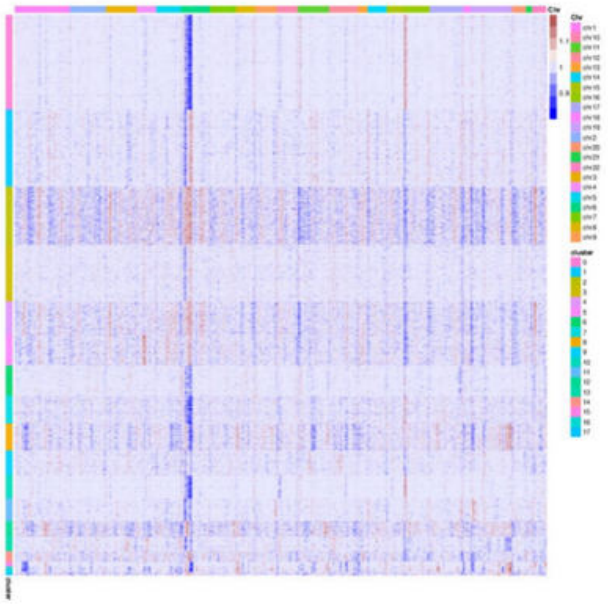


Figure 9*: Distribution of CNVs per cell in the sample

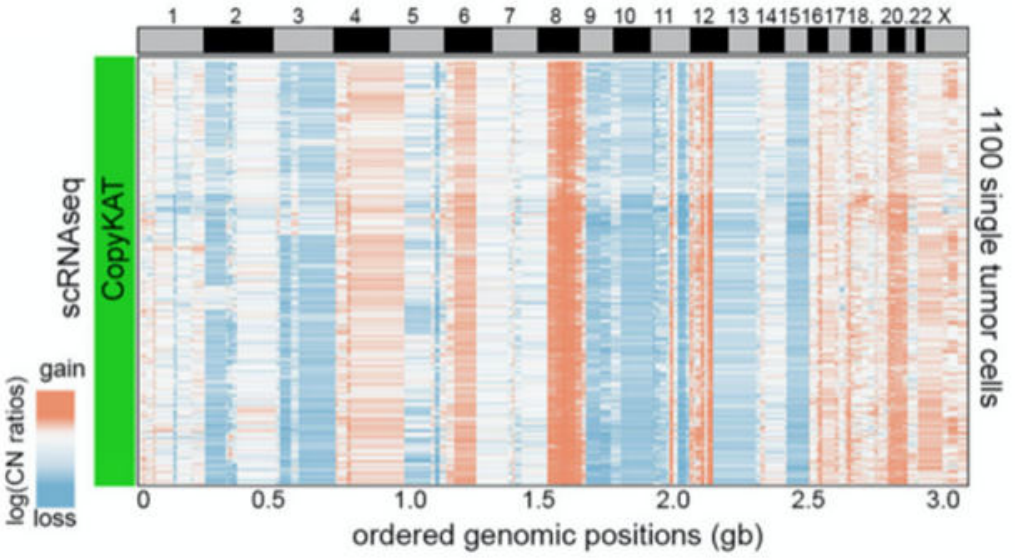


Figure 10*: The distribution of CNVs at each position of the chromosome in each cell

🕮 Intratumoral heterogeneity (ITH) by tumour mutation analysis

Although intratumoral heterogeneity (ITH) is a crucial aspect of cancer, it is still unclear where it originates from. The investigation of cancer heterogeneity at the cell level is perhaps one of the most intense tools. In one of the cancer research studies, researchers carried out a high-throughput single-cell transcriptome sequencing investigation of 20 patients' peritoneal metastases from advanced gastric cancer. The cellular source of intratumor heterogeneity in patients with gastric cancer was shown for the first time with the construction of a single-cell atlas of 45,048 peritoneal tumours. It was shown that the lineage makeup of tumour cells was highly correlated with patient survival by comparing peritoneal tumour cells from patients who had longer survival times and those who had short survival times. These findings offer a means of patient stratification and target identification for potential therapeutic use [17].

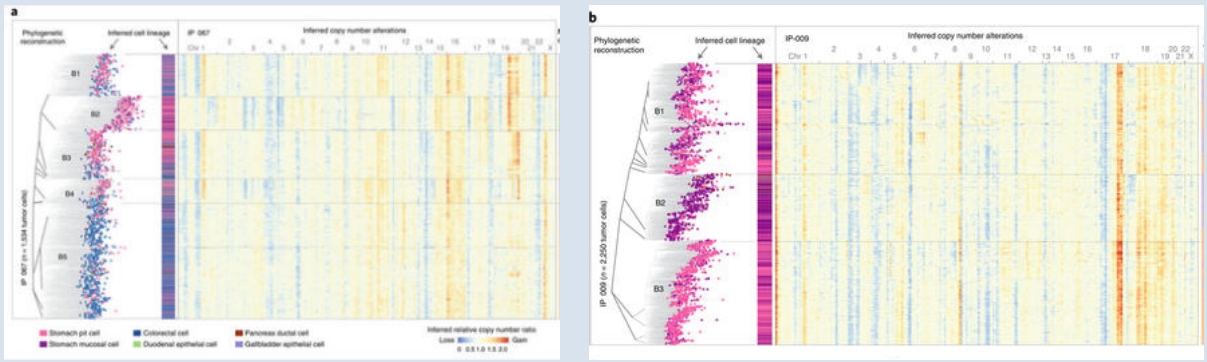


Figure 11: The diversity in tumor cell lineage compositions links to ITH at transcriptomic, genotypic and molecular levels[17]

IV. Cell Communication Analysis and Its Role

Receptor-ligand assays are used to measure cell communication. Development, differentiation, and inflammation are just a few of the bioinformatic processes that are regulated by ligand-receptor-mediated cellular interactions in biological cells. A cellular regulatory network that controls the progression of many physiological processes is made up of the interactions between cells.

Through the interaction of ligands and receptors, cells can play significant roles in tumour formation, drug resistance, immunological infiltration, and inflammation. These ligands can either drive various cell types to approach one another and perform their functions in a membrane-bound form, or they can be secreted and coupled to receptors in a soluble form.

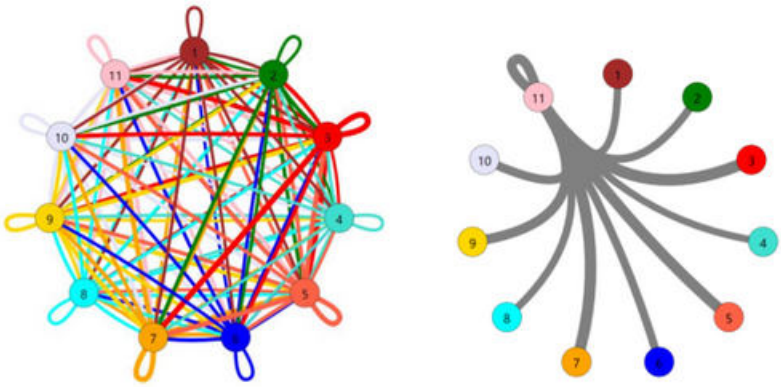


Figure 12*: Intercellular ligand receptor pair distribution map

A case study of ligand-receptor interactions in the tumour microenvironment facilitating cell-to-cell communication

The clear cell renal cell carcinoma (ccRCC) microenvironment is important for both cancer development and medication resistance. However, the cellular makeup and underlying gene regulatory characteristics of ccRCC are still poorly understood.

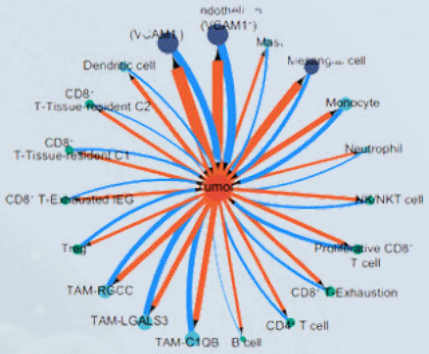


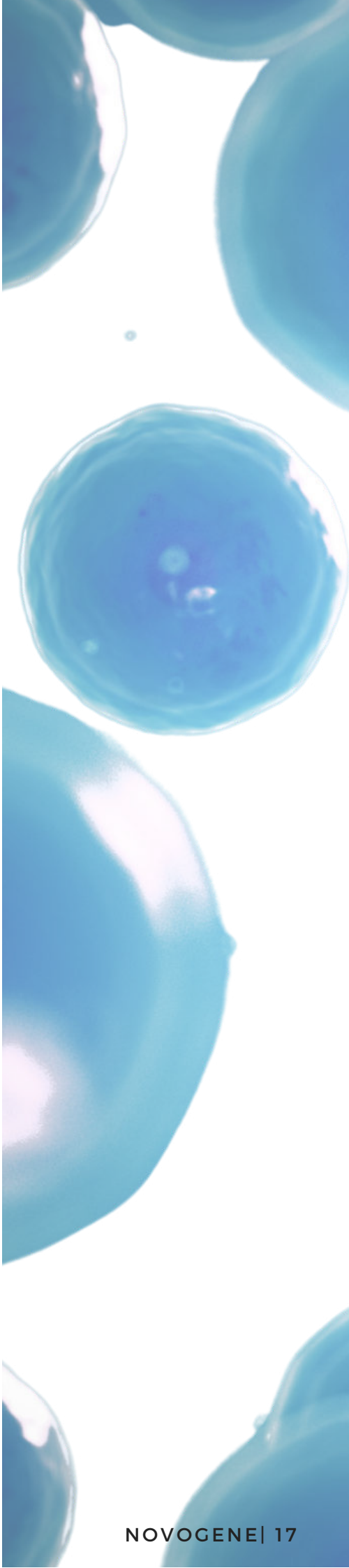
Figure 13: Ligand–receptor-based interaction between tumor and immune cells[18]

In order to create transcriptional and epigenomic maps of ccRCC, scientists used scRNA-Seq and single-cell sequencing assay for transposase-accessible chromatin (scATAC) sequencing in a research study published in 2021. The results of the study discovered tumour cell-specific regulatory programs that are controlled by four important transcription factors (HOXC5, VENTX, ISL1, and OTP), which are involved in cancer. A thorough investigation of chromatin accessibility and gene expression in CD8+ T cells and macrophages was carried out to identify unique regulatory components in respective subpopulations. The study explained how ligand-receptor interactions in the tumour microenvironment facilitate cell-to-cell communication. A multi-omics approach further clarified the cellular heterogeneity of ccRCC and revealed possible treatment targets[18].

CONCLUSION

The discovery of novel biomarkers or therapeutic targets can be sparked by an understanding of physiological cycles and neurotic causes at the single-cell level, as is the case in the case of cancer. This will provide a rational foundation for better disease diagnosis and treatment.

This eBook focused on single-cell sequencing methods and their uses in studying various single cells, including those found in tumours, conceptive medicine, and immunology. It highlighted the significant benefits of single-cell sequencing breakthroughs. However, single-cell sequencing methods continue to face challenges, including laborious activity and high location costs, which limit the use of the invention. It is anticipated that developments in single-cell sequencing technologies will become more available, refined, robust, and reasonable, enabling them to be used in critical analysis and play a key role in clinical discoveries and therapy.



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