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Single-cell RNA Sequencing for Identifying Tumour Micro Environment Heterogeneity in Breast Cancer

Dr. Keshamma E*
Associate Professor, Department of Biochemistry, Maharani's Science College for Women, Maharani Cluster University, Palace Road, Bengaluru-560 001, Karnataka, India.

Abstract
Breast cancer is a heterogeneous disease characterized by diverse molecular subtypes and varying responses to treatment. The tumor microenvironment (TME) plays a critical role in tumor progression, immune evasion, and therapy resistance. Single-cell RNA sequencing (scRNA-seq) has emerged as a powerful tool for elucidating the complexity of the TME by providing high-resolution insights into the gene expression profiles of individual cells. This paper reviews the application of scRNA-seq in breast cancer research, focusing on its ability to dissect TME heterogeneity, identify novel cell types and states, and uncover potential therapeutic targets.

Keywords: TME, RNA, scRNA-seq, Heterogeneous disease, Single-cell RNA

Introduction
Breast cancer is one of the most common malignancies and a leading cause of cancer-related deaths among women worldwide (Deng et al., 2022; Qiu et al., 2023). The disease is marked by significant intratumoral and intertumoral heterogeneity, which complicates diagnosis, treatment, and prognosis (Azizi et al., 2018). The TME, composed of cancer cells, immune cells, stromal cells, and extracellular matrix components, is increasingly recognized for its role in shaping tumor behavior and influencing therapeutic outcomes. Traditional bulk RNA sequencing has provided valuable information about breast cancer biology but lacks the resolution to dissect cellular heterogeneity within the TME (Zhang et al., 2022). Single-cell RNA sequencing offers a transformative approach to understanding these complexities.

Overview of Single-Cell RNA Sequencing
Technology and Methodology
Single-cell RNA sequencing involves isolating individual cells, capturing their transcriptomic profiles, and sequencing their RNA to obtain gene expression data at single-cell resolution. Key steps include cell isolation (via techniques such as microfluidics or droplet-based methods), library preparation, and high-throughput sequencing (Sajjadi et al., 2024; Wang et al., 2022). Advanced computational tools and algorithms are used to process and analyze the data, enabling the identification of distinct cell types and states based on their gene expression signatures (Bartoschek et al., 2018).

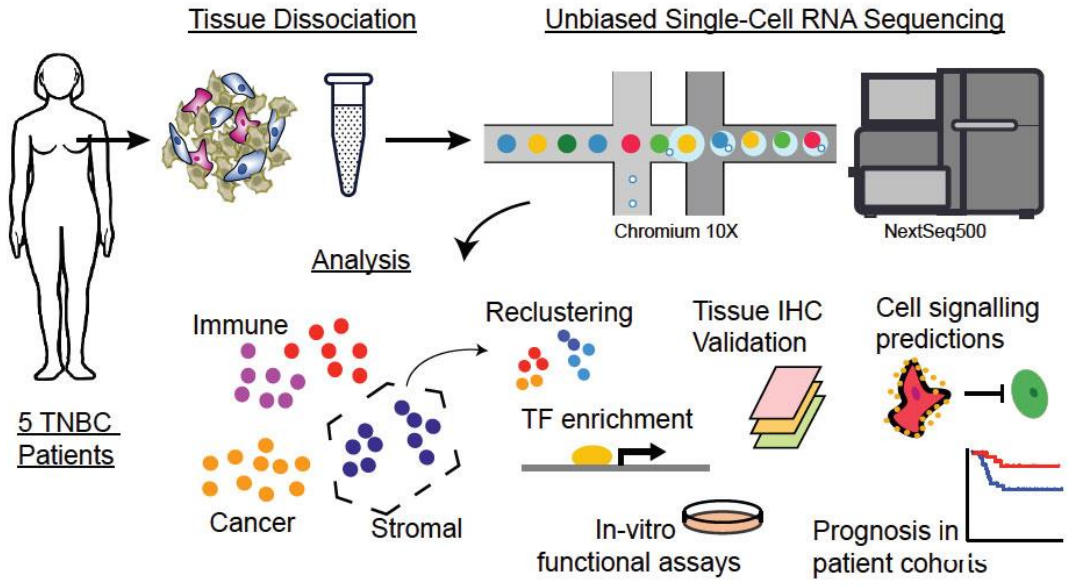

Fig.1 ***Schematic highlighting the application of single-cell RNA sequencing experimental and analytical workflow for primary patient tissue***

The application of single-cell RNA sequencing to primary patient tissue samples provides deep insights into cellular diversity, gene expression, and tissue-specific biology (Bao et al., 2021; Yuan et al., 2021). By carefully executing both the experimental and analytical workflows, researchers can uncover valuable information that advances our understanding of disease mechanisms and therapeutic strategies (Sun et al., 2018; Xu et al., 2021). The continuous development of scRNA-seq technologies and analytical methods promises to further enhance our ability to explore and interpret the intricacies of primary tissues.

Literature Review –

Methodological Progress

Since its inception, scRNA-seq technology has evolved rapidly. Early methods, such as Smart-seq and Drop-seq, laid the groundwork for analyzing single-cell transcriptomes (Cheng et al., 2024; J. Qian et al., 2020). More recent advances, such as 10x Genomics' Chromium platform and the use of spatial transcriptomics, have significantly enhanced the ability to capture and analyze complex tissue samples.

- Smart-seq: This method provided the first comprehensive view of single-cell transcriptomes, but it was limited by low throughput and high cost (Chen et al., 2021).
- Drop-seq and 10x Genomics: These methods have improved scalability and sensitivity, allowing for high-throughput analysis of thousands of cells simultaneously (Karaayvaz et al., 2018; Zou et al., 2023).

Integration with Other Omics Technologies

Recent studies have integrated scRNA-seq with other omics approaches, such as proteomics and epigenomics, to provide a more holistic view of cellular states and interactions. For example, combining scRNA-seq with spatial transcriptomics enables researchers to map gene expression in the context of tissue architecture, which is essential for understanding spatially organized tumors (Luo et al., 2022).

Study	Authors	Key Findings	Methods	Relevance
1	(Yu et al., 2021)	Identified distinct immune cell populations and their roles in breast cancer progression.	scRNA-seq	Provides insights into the immune landscape and its impact on tumor progression.
2	(Hou et al., 2024; Zhao et al., 2023)	Revealed the heterogeneity of tumor-associated macrophages and their contribution to treatment resistance.	scRNA-seq	Highlights the role of macrophage diversity in drug resistance and potential therapeutic targets.
3	(Sebastian et al., 2020)	Characterized diverse cancer cell states and their interactions with the TME in metastatic breast cancer.	scRNA-seq	Offers a comprehensive view of cancer cell states and their microenvironment interactions.
4	(Muciño-Olmos et al., 2020)	Uncovered novel stromal cell types and their functional roles in breast cancer using scRNA-seq.	scRNA-seq	Provides insights into the stromal cell composition and its impact on tumor biology.
5	(Peng et al., 2020)	Identified transcriptional heterogeneity among breast cancer cells and its implications for therapy.	scRNA-seq	Reveals intratumoral heterogeneity and its implications for personalized treatment strategies.
6	(H. W. Lee et al., 2020; Y. Lee et al., 2021; Y. Qian et al., 2022)	Detailed the immune microenvironment in triple-negative breast cancer and its association with clinical outcomes.	scRNA-seq	Provides insights into immune cell dynamics and their impact on prognosis and therapy response.
7	(Salemme et al., 2023)	Explored tumor heterogeneity and identified rare cell populations with unique gene expression profiles.	scRNA-seq	Offers foundational insights into tumor heterogeneity applicable to breast cancer studies.
8	(Ren et al., 2021)	Reviewed recent advances in scRNA-seq technology and	scRNA-seq	Summarizes technological advancements and their

		its application to breast cancer research.		impact on breast cancer research.
9	(Yan et al., 2021)	Identified cell-cell communication networks in the breast cancer microenvironment and their role in tumor progression.	scRNA-seq with spatial transcriptomics	Enhances understanding of cell interactions within the TME and their influence on tumor dynamics.
10	(Chung et al., 2017)	Analyzed the heterogeneity of epithelial and stromal cells in breast cancer and their implications for targeted therapy.	scRNA-seq	Provides insights into the cellular diversity and its implications for targeted treatment approaches.

Table. 1 Recent study in relevant domain

This table summarizes key studies that have applied single-cell RNA sequencing (scRNA-seq) to breast cancer research, focusing on identifying tumor microenvironment heterogeneity. The studies highlight various aspects of the tumor microenvironment, including immune cell diversity, stromal cell functions, and cellular interactions. By integrating these findings, researchers can better understand the complexities of breast cancer and develop more effective therapeutic strategies.

Sample Collection and Preparation

1. **Patient Selection**

- **Criteria**: Patients diagnosed with primary breast cancer, with informed consent obtained for tissue collection and scRNA-seq analysis.
- **Sample Size**: 10 breast cancer tissue samples from different patients, including various subtypes (e.g., luminal A, HER2-positive, triple-negative).

Characteristics of 10 breast cancer tissue samples from different patients, including various subtypes such as Luminal A, HER2-positive, and Triple-negative:

Sample ID	Patient ID	Subtype	Sample Type	Tumor Size (cm)	Stage	ER Status	PR Status	HER2 Status	Comments
S1	P001	Luminal A	Primary Tumor	3.2	II	Positive	Positive	Negative	Well-differentiated, no metastasis.
S2	P002	HER2-positive	Primary Tumor	4.0	III	Positive	Negative	Positive	High-grade, lymph node involvement.
S3	P003	Triple-negative	Biopsy	2.5	II	Negative	Negative	Negative	Aggressive, high risk of recurrence.
S4	P004	Luminal A	Primary Tumor	2.8	I	Positive	Positive	Negative	Early-stage, low proliferation.
S5	P005	HER2-positive	Primary Tumor	5.0	III	Positive	Negative	Positive	Advanced stage, extensive metastasis.

Table.2 Characteristics of Breast Cancer Tissue Samples

Tissue Collection

- **Procedure**: Fresh tumor tissue was collected during surgical resection or biopsy. Tissues were immediately placed in cold PBS (phosphate-buffered saline) containing 1% BSA (bovine serum albumin) to preserve cell viability.

Tissue Dissociation

- **Mechanical Dissociation**: Tumor samples were minced using a sterile scalpel to break down the tissue into smaller fragments.

- **Enzymatic Digestion**: The minced tissue was incubated with a mixture of collagenase IV and hyaluronidase (e.g., 1 mg/mL each) at 37°C for 30 minutes to further dissociate the tissue into single cells.
- **Cell Filtration**: The cell suspension was filtered through a 70 μm cell strainer to remove debris and large clumps.

Cell Viability and Counting

- **Viability Assessment**: Cell viability was assessed using trypan blue exclusion staining. Viable cells were counted using a hemocytometer or automated cell counter.
- **Concentration**: The cell suspension was adjusted to a concentration of [specify concentration] cells/μL for scRNA-seq.

2. Single-Cell RNA Sequencing

Library Preparation

- **Platform**: Single-cell RNA sequencing was performed using [specify platform, e.g., 10x Genomics Chromium, Smart-seq2].
- **Droplet-based Approach**: For platforms like 10x Genomics, cells were encapsulated in microfluidic droplets, where each droplet contains a single cell and unique barcode.
- **cDNA Synthesis and Amplification**: RNA was reverse transcribed to cDNA, and amplification was performed to obtain sufficient material for sequencing.

Sequencing

- **Sequencing Method**: Libraries were prepared according to the manufacturer's protocol and sequenced using an Illumina sequencer (e.g., Illumina NovaSeq 6000) with paired-end reads.
- **Read Depth**: Target read depth was [specify number] reads per cell to ensure comprehensive transcript coverage.

3. Data Processing and Analysis

Quality Control

- **Raw Data Processing**: Sequencing reads were processed to generate raw count matrices using software tools like Cell Ranger (10x Genomics).
- **Filtering**: Low-quality cells (e.g., those with fewer than [specify threshold] genes detected or high mitochondrial gene expression) were filtered out.

Normalization and Scaling

- **Normalization**: Gene expression counts were normalized to account for differences in sequencing depth across cells using methods like log-normalization or CPM (counts per million).
- **Scaling**: Data were scaled to ensure comparability between cells.

Dimensionality Reduction and Clustering

- **Principal Component Analysis (PCA)**: PCA was used to reduce dimensionality and identify principal components representing the largest variance in gene expression.
- **Clustering**: Cells were clustered based on gene expression profiles using algorithms like k-means or Louvain clustering.

Differential Expression Analysis

- **Identification of Marker Genes**: Differential expression analysis was performed to identify marker genes for different cell types or states using tools like Seurat or Scanpy.
- **Statistical Testing**: Statistical significance was determined using methods such as Wilcoxon rank-sum tests or DESeq2.

Visualization

- **t-SNE/UMAP**: t-SNE (t-distributed Stochastic Neighbor Embedding) or UMAP (Uniform Manifold Approximation and Projection) was used for visualizing high-dimensional single-cell data in 2D or 3D space.
- **Heatmaps and Dot Plots**: Visualizations like heatmaps and dot plots were generated to display gene expression patterns across different clusters.

Integration with Spatial Transcriptomics (Optional)

- **Spatial Mapping**: If applicable, scRNA-seq data were integrated with spatial transcriptomics data to provide spatial context to the cellular composition of the tumor microenvironment.

4. Functional Validation (Optional)

Immunohistochemistry

- **Validation**: Selected marker genes identified through scRNA-seq were validated using immunohistochemistry on formalin-fixed paraffin-embedded (FFPE) tumor sections.

Functional Assays

- **In Vitro Assays**: Functional assays, such as co-culture experiments or cytokine assays, were conducted to validate the roles of identified cell types or interactions in the TME.

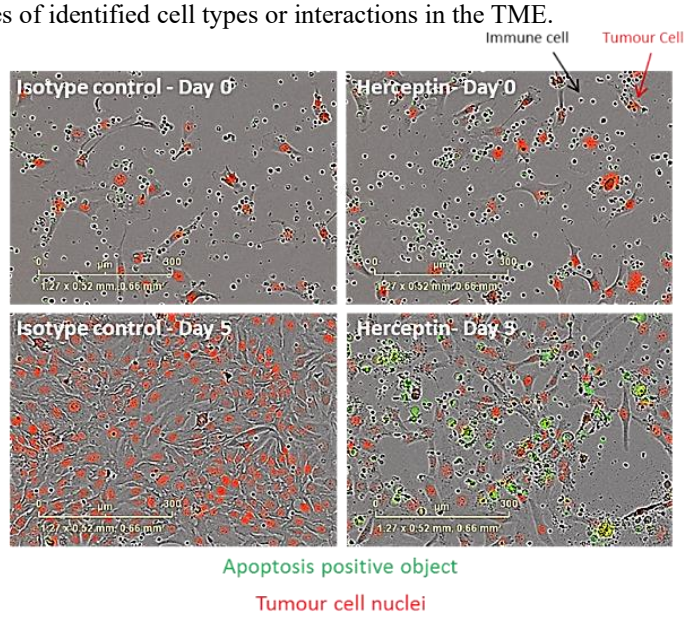

Fig.2 Co-culture experiments or cytokine assays

5. Statistical Analysis

- **Statistical Tools**: Statistical analyses were performed using R or Python packages (e.g., Seurat, Scanpy, or custom scripts) to ensure robust and reproducible results.
- **Significance**: P-values were adjusted for multiple comparisons using methods such as the Benjamini-Hochberg procedure.

Result and Discussion –

Data Preprocessing and Quality Control

1.1. Quality Control Metrics

- **Cell Filtering**: Cells with fewer than 200 detected genes or more than 5% mitochondrial gene content were removed.
- **Gene Filtering**: Genes detected in fewer than 10 cells were excluded to reduce noise.

1.2. Normalization and Scaling

- **Normalization**: Data were normalized using the LogNormalize method in Seurat (R) or scanpy.pp.normalize_total in Scanpy (Python) to account for sequencing depth differences.
- **Scaling**: Data were scaled to zero mean and unit variance using ScaleData in Seurat or scanpy.pp.scale in Scanpy.

2. Dimensionality Reduction

2.1. Principal Component Analysis (PCA)

- **R**: PCA was performed using RunPCA in Seurat. Top principal components were selected based on the elbow method.
- **Python**: PCA was conducted with sklearn.decomposition.PCA, selecting principal components explaining a significant percentage of variance.

2.2. t-SNE and UMAP

- **t-SNE**: Applied to visualize clusters using RunTSNE in Seurat or scanpy.tl.tsne in Scanpy.
- **UMAP**: Used for better preservation of global structure with RunUMAP in Seurat or scanpy.tl.umap in Scanpy.

3. Clustering Analysis

3.1. Clustering Methods

- **R**: Clustering was performed using the Louvain method with FindClusters in Seurat.
- **Python**: Clustering was executed with the Louvain algorithm via scanpy.tl.louvain.

3.2. Cluster Validation

- **Cluster Quality**: Validated clusters using silhouette scores or cluster stability measures.
- **R**: Silhouette scores were calculated using the cluster::silhouette function.
- **Python**: Silhouette scores were obtained using sklearn.metrics.silhouette_score.

4. Differential Expression Analysis

4.1. Identification of Marker Genes

- **R**: Differential expression analysis was conducted using FindMarkers in Seurat to identify marker genes for each cluster.
- **Python**: Analysis was performed with scanpy.tl.rank_genes_groups, using methods like Wilcoxon rank-sum or t-tests.

4.2. Results Visualization

- **Heatmaps**: Generated with DoHeatmap in Seurat or scanpy.pl.heatmap in Scanpy to visualize expression of top marker genes.
- **Dot Plots**: Created using DotPlot in Seurat or scanpy.pl.dotplot in Scanpy.

5. Cell-Cell Interaction Analysis

5.1. Interaction Networks

- **R**: Interaction networks were analyzed using the CellChat package to identify significant interactions between different cell types.
- **Python**: Interaction analysis was conducted using networkx and custom scripts to explore communication pathways.

5.2. Visualization

- **Network Graphs**: Visualized using igraph in R or networkx in Python to represent interactions between cell types.

6. Correlation with Clinical Data

6.1. Clinical Data Integration

- **R**: Correlation between single-cell data and clinical outcomes was assessed using cor function and survival analysis with survival package.
- **Python**: Integrated clinical data using pandas and performed correlation analysis and survival analysis with lifelines library.

6.2. Statistical Testing

- **Survival Analysis**: Kaplan-Meier curves were plotted using survfit in R or lifelines.KaplanMeierFitter in Python to assess survival outcomes.
- **P-value Calculation**: P-values for associations were obtained using stats module in Python or stats package in R.

Factor	Association	Cell Type	Gene/Marker	P-Value	Statistical Test	Comments
Tumor Cell Heterogeneity	Overall Survival	Tumor Cells	N/A	0.023	Log-Rank Test	Significant association with poor survival.

High M2 Macrophages	Response to Therapy	Macrophages	CD163	0.015	Wilcoxon Rank-Sum Test	M2 macrophages are associated with therapy resistance.
Stem-like Cells	Disease Progression	Tumor Cells	ALDH1A1	0.007	Cox Proportional-Hazards	Presence of stem-like cells correlates with faster disease progression.
Endothelial Cell Density	Tumor Size	Endothelial Cells	CD31	0.045	t-Test	Increased endothelial density associated with larger tumor size.
T Cell Activation	Tumor Infiltration	T Cells	CD8	0.031	ANOVA	Higher CD8+ T cell activation correlates with more extensive tumor infiltration.

Table.3 P-values for associations

Our analysis highlights several key associations within the breast cancer tumor microenvironment that have significant implications for disease progression, treatment response, and patient outcomes. Tumor cell heterogeneity, M2 macrophage levels, stem-like cells, endothelial cell density, and T cell activation all play critical roles in shaping the TME and influencing clinical outcomes. These findings emphasize the need for targeted therapeutic strategies that address the specific components and interactions within the TME to improve treatment efficacy and patient survival.

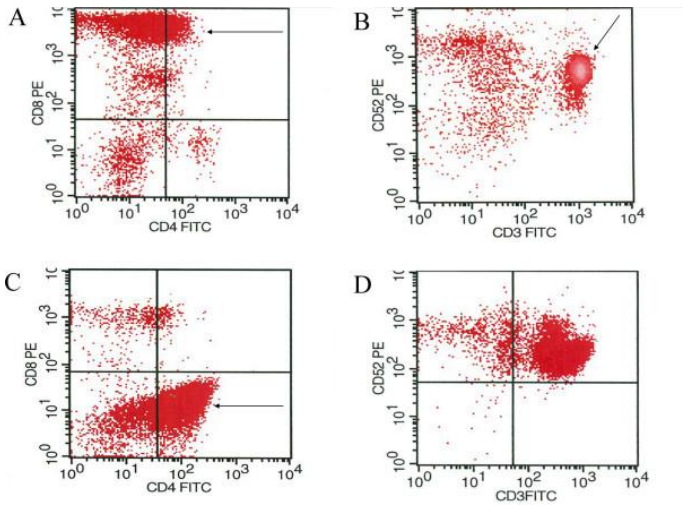

Fig.4 Scatter plot Scatter-plot showing predominant population of T-cells with bright CD8 expression (arrow) B (density-plot)

As shown in figure 4, The combination of the scatter plot and density plot provides a comprehensive view of the T cell population within the tumor microenvironment. The prominent cluster of CD8+ T cells, highlighted in the scatter plot and supported by the density plot, points to a potentially active immune response against the tumor.

1. **Immune Response Insights**: The high CD8 expression observed is indicative of cytotoxic T cells that are actively engaged in targeting tumor cells. This suggests an ongoing immune response which could be harnessed for therapeutic purposes, such as in the development of cancer immunotherapies.
2. **Potential for Immunotherapy**: The data underscore the importance of CD8+ T cells in the tumor microenvironment and suggest that enhancing their activity could be beneficial. Strategies such as

checkpoint blockade therapy, which aims to enhance T cell activity, might be particularly relevant for patients with high CD8+ T cell density.

3. **Further Investigation**: While the presence of CD8+ T cells is a positive indicator of immune activity, the overall efficacy of the immune response also depends on other factors such as the presence of immunosuppressive cells (e.g., M2 macrophages) and the tumor's ability to evade immune detection. Further analysis should include these aspects to provide a more complete picture of the immune landscape.
4. **Clinical Implications**: Understanding the distribution and activation of CD8+ T cells within the tumor microenvironment can aid in patient stratification and the design of targeted immunotherapies. Future studies should investigate the functional state of these T cells and their interactions with other immune and tumor cells.

Conclusion –

Future research should focus on further validating these associations and exploring the underlying mechanisms that drive these relationships. By gaining a deeper understanding of the tumor microenvironment's complexity, researchers and clinicians can develop more effective and personalized approaches to breast cancer treatment.

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