Spatially Resolved Heterogeneity of Triple Negative Breast Cancer

Tissue-Level Insights with Visium Spatial Gene Expression

Triple negative breast cancer (TNBC) is unresponsive to traditional hormone or targeted therapies and highly heterogeneous. Developing novel treatment options, therefore, requires a deeper understanding of its biological underpinnings and cell-type composition. In combination with single nuclei RNA-seq data and pathologist annotation, Visium Spatial Gene Expression provided a fuller understanding of the biology within a TNBC section by enabling improved resolution of intratumoral heterogeneity, localization and characterization of putative cancer stem cells, and cell subtype identification of infiltrating immune cells.

Graphical abstract

Highlights

- Characterization of intratumoral heterogeneity within pathologist-annotated invasive carcinoma
- Integration of spatial and single cell gene expression data to visually localize enrichment of specific cell subtypes and states
- Refinement of immune cell boundaries and immune cell-type composition
- Identification and localization of putative cancer stem cells
Introduction

Extensive heterogeneity exists in the breast cancer tumor microenvironment. However, clinical characterization has been primarily limited to pathologist annotation and tissue staining for three key genes: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Triple negative breast cancer (TNBC) expresses none of the usual breast cancer therapeutic targets, including ER, PR, and HER2, making it unresponsive to either hormone therapy or targeted anti-HER2 drugs like trastuzumab.

Improving diagnoses and therapeutic outcomes requires further investigation of the underlying biology of TNBC to understand its complexity and develop effective treatments. The spatial transcriptomics technology of Visium Spatial Gene Expression combines the benefits of histology and immunofluorescence with tissue-wide whole transcriptome analysis, enabling simultaneous molecular and pathological characterization of an entire tissue section (Figure 1). These capabilities, coupled with single cell or single nuclei gene expression profiling, make it possible to create a tissue-level map of distinct cell types and states.

Armed with a more complete view of disease, including cell-type identification and localization across an entire tissue section, spatial transcriptomics can augment pathology to accelerate clinical advances. Here, we illustrate how the integration of spatial and single nuclei gene expression data can better characterize triple negative breast cancer by resolving intratumoral heterogeneity, defining the extent of immune infiltration, and localizing specific cell types and states.

Figure 1. Visium Spatial Gene Expression provides a ready-to-use, robust workflow for whole tissue section analysis of gene expression that integrates easily with current histological laboratory methods and tools for tissue analysis. Fresh-frozen tissue was sectioned, placed onto a library preparation slide, fixed, stained with H&E before imaging, and permeabilized to release mRNA that binds to spatially barcoded capture probes, allowing for the capture of gene expression information. cDNA was synthesized from captured mRNA and washed off the slide before sequencing library construction. Libraries were sequenced and data analyzed and visualized using Space Ranger and Loupe Browser.
Methods

Human Tissue Specimens from triple negative breast cancer biopsy were obtained from BioIVT Asterand. Fresh-frozen tissue was sectioned using a cryostat, placed onto a Visium Gene Expression slide, fixed, and stained with hematoxylin and eosin according to the Demonstrated Protocol: Methanol Fixation, H&E Staining & Imaging for Visium Spatial Protocols (Document CG000160). Stained tissue sections were imaged at 20X magnification using brightfield settings and manually annotated by a pathologist. After imaging, the tissue was permeabilized using optimal conditions developed according to the Visium Spatial Gene Expression Reagent Kits - Tissue Optimization User Guide (Document CG000238), releasing mRNA that bound to spatially barcoded capture probes on the slide. Following permeabilization, cDNA was synthesized from captured mRNA and sequencing libraries were prepared according to the Visium Spatial Gene Expression Reagent Kits - Library Construction User Guide (Document CG000239). A Spatial Gene Expression library from an adjacent section was prepared the same way, before enriching for genes of interest using the 10x Genomics Human Pan-Cancer Panel according to the Targeted Gene Expression Reagent Kits User Guide (Document CG000293). The resulting libraries were sequenced and analyzed using Space Ranger pipeline v1.1 and visualized on top of the tissue image in Loupe Browser v4.1. Pathologist annotations were imported into Loupe Browser as an additional classification category.

Single Cell Gene Expression processing of two additional fresh-frozen TNBC samples was performed according to the Chromium Next GEM Single Cell 3’ Reagent Kits v3.1 User Guide (Document CG000204), and the resulting data for 86,249 single nuclei were aggregated before clustering and annotated manually using a marker gene approach. Since each Visium gene expression spot can overlay multiple cells, cell-type anchors determined by single nuclei RNA-seq were used to inform spatial identification of dominant cell subtypes on the Visium samples using Seurat v3.2 (1). Visium Spatial Gene Expression spots in the TNBC section were genotyped using souporcell variant identification pipeline4 (2). Variants were called in bulk and genotyped using VarTrix with clustering parameters set at k = 2 in order to restrict classification to unambiguous cancer or not-cancer cells, with the assumption that the majority of cells would be either tumor or normal.

Results

Intratumoral heterogeneity

Pathologist annotation segregated the TNBC section into five regions (Figure 2B and 2C). The bulk of the section was annotated as Invasive Carcinoma (green), meaning the cancer cells have spread beyond the milk duct and invaded the surrounding breast tissue, and have the potential to spread to other parts of the body. Surrounding the Invasive Carcinoma are Immune Cells (blue), which are interspersed with discrete regions of Ductal Carcinoma In Situ (DCIS, light green), areas of non-invasive cancer where atypical cells line the milk ducts. Along the upper left periphery lies Fibrous Tissue (red), predominantly made up of extracellular matrix. Normal gland (orange), is also present.

Arrayed across the Visium Spatial Gene Expression slide are approximately 5,000 gene expression spots. Each spot contains spatially barcoded probes that bind mRNA from the cells in the tissue section above it. Space Ranger performs graph-based clustering of each spot to segregate them based on gene expression. Clusters can be visualized spatially, with reference to the tissue section (Figure 2D), or as UMAP projections showing the distance between gene expression–based clusters (Figure 2E). While a traditional single cell UMAP is made up of dots representing single cells, with Visium Spatial Gene Expression each dot represents a tissue-covered spot that may include mRNA from one to ten cells.

In general, there is high correlation between pathologist annotations and graph-based clusters determined by gene expression, particularly at region boundaries. Graph-based Cluster 3 (green) primarily overlaps with the pathologist-annotated region labeled as fibrous tissue, and Clusters 6 (brown) and 7 (pink) overlap largely with immune cells. Cluster 8 (grey) shows consistent localization with DCIS. Using Spatial Gene Expression, additional heterogeneity can be identified within regions, particularly for invasive carcinoma, which is made up of four distinct gene expression–based clusters, including Cluster 1 (blue), Cluster 2 (orange), Cluster 4 (red), and Cluster 5 (purple). A list of select differentially expressed genes for all eight graph-based clusters is provided in Table 1.
While whole transcriptome analysis is ideal for discovery and full characterization of graph-based clusters, targeted transcriptome analysis can provide exceptional value in the disambiguation of spatial heterogeneity and validation of preliminary findings. Using the Human Pan-Cancer Panel with Targeted Gene Expression from 10x Genomics, similar cluster patterns and transcriptional profiles were resolved on a second section of the same TNBC sample (Figure 2F). 

Spatial resolution of unique cell types and cell states
Visium spots have a diameter of 55 microns and typically capture 1–10 cells per spot, depending on tissue thickness and cell density. To increase resolution of spatially resolved gene expression to the single cell level, single nuclei RNA-sequencing (snRNA-seq) of two TNBC samples with Chromium Single Cell Gene Expression was used to identify cellular subtypes within the tissue (Figure 3A). Gene expression profiles for both tissues were processed in Seurat and batch corrected using Harmony (3).
<table>
<thead>
<tr>
<th>Cluster</th>
<th>Gene</th>
<th>Name</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MMP7</td>
<td>Matrix metallopeptidase 7</td>
<td>Exhibits elevated expression in several types of human cancer</td>
</tr>
<tr>
<td>1</td>
<td>FYB1</td>
<td>FYN binding protein 1</td>
<td>Involved in platelet activation and IL-2 expression</td>
</tr>
<tr>
<td>2</td>
<td>FDCSP</td>
<td>Follicular dendritic cell secreted protein</td>
<td>Secreted protein that binds to activated B cells to regulate antibody responses may promote cancer cell invasion and migration</td>
</tr>
<tr>
<td>2</td>
<td>BCL2A1</td>
<td>BCL2 related protein A1</td>
<td>Reduces release of pro-apoptotic cytochrome c, and serves as a direct transcription factor of NK-κB in response to inflammatory mediators</td>
</tr>
<tr>
<td>3</td>
<td>TNXB</td>
<td>Tenascin XB</td>
<td>Extracellular matrix glycoprotein with anti-adhesive effects</td>
</tr>
<tr>
<td>3</td>
<td>CLDN5</td>
<td>Claudin 5</td>
<td>Integral membrane protein and component of tight junctions</td>
</tr>
<tr>
<td>4</td>
<td>KLK6</td>
<td>Kallikrein related peptidase 6</td>
<td>Implicated in carcinogenesis and may serve as a biomarker for multiple cancer types</td>
</tr>
<tr>
<td>4</td>
<td>CENPW</td>
<td>Centromere protein W</td>
<td>Putative oncogene</td>
</tr>
<tr>
<td>5</td>
<td>CST1</td>
<td>Cystatin SN</td>
<td>Favorable prognostic marker in breast cancer</td>
</tr>
<tr>
<td>5</td>
<td>POSTN</td>
<td>Periostin</td>
<td>Secreted extracellular matrix protein that plays a role in cancer stem cell maintenance and metastasis</td>
</tr>
<tr>
<td>6</td>
<td>CXCL14</td>
<td>C-X-C motif chemokine ligand 14</td>
<td>Chemoattractant for neutrophils and expressed by myeloid dendritic cells</td>
</tr>
<tr>
<td>6</td>
<td>CD79B</td>
<td>CD79b molecule</td>
<td>Part of B lymphocyte antigen receptor</td>
</tr>
<tr>
<td>7</td>
<td>IGLC3</td>
<td>Immunoglobulin lambda constant 3 (Kern-Oz+ marker)</td>
<td>Part of the constant region of B-cell antibodies</td>
</tr>
<tr>
<td>7</td>
<td>MZB1</td>
<td>Marginal zone B and B1 cell specific protein</td>
<td>Marker of plasma B cells</td>
</tr>
<tr>
<td>8</td>
<td>PIGR</td>
<td>Polymeric immunoglobulin receptor</td>
<td>Favorable prognostic marker in breast cancer</td>
</tr>
<tr>
<td>8</td>
<td>CLU</td>
<td>Clusterin</td>
<td>Secreted chaperone protein that can protect against apoptosis</td>
</tr>
</tbody>
</table>

*Table 1. Select differentially expressed genes for spatially defined clusters.*
Cell-type anchors determined by snRNA-seq were matched to Visium Gene Expression spots where these cell types were abundant. For additional detail on how to generate and match cell-type anchors, consult the “Analysis, visualization, and integration of spatial datasets with Seurat” vignette (4).

The extent and composition of immune cell infiltration in a tumor informs prognosis. Visium Spatial Gene Expression provides a unique view into both the identity of infiltrating cells as well as the extent of penetration into the cancerous region. By leveraging paired snRNA-seq data, spots containing specific subtypes of T cells could be identified. In this tissue section, T cells could be found throughout the immune cell–annotated region, as well as within the invasive carcinoma region, well beyond the exterior histological boundary (Figure 3B). This cluster, T cells-1, expressed PD-1, perforin, granzyme, and other checkpoint inhibitors, suggesting an exhausted T-cell phenotype and limited anti-tumor efficacy.

In the absence of snRNA-seq data, the predominant cell type in graph-based clusters can also be inferred based on gene expression. Cluster 7 (pink), along the periphery of the pathologist-annotated invasive carcinoma region (Figure 2D), expressed numerous immunoglobulin genes, along with MZB1 and JCHAIN, both markers of plasma cells, suggesting that in this patient, a novel B-cell response may have been mounted against the tumor. Analysis of paired, full-length Ig receptors in B cells from this sample could provide additional prognostic value and potential tumor-specific antibody sequences.

Figure 3. Complementary single cell and spatial analyses reveal the localization of unique cell types and states. A. UMAP projection of 86,249 single nuclei isolated from two TNBC tumors and annotated by cell subtype based on marker gene expression. B. Regions of specific T-cell subtype infiltration were visualized by combining Spatial Gene Expression and single nuclei RNA-seq data. Here, exhausted T cells can be seen surrounding the tumorigenic region. C. Localization of mammary stem cells, identified by CD49f expression in snRNA-seq data and visualized over H&E image. CD49f cells are putative breast cancer stem cells, though genetic analysis (D) suggests these cells have not yet transformed into tumorigenic cells. D. Using gene expression data, the genotype of each Visium spot was inferred as either tumor or normal, aiding in the identification of putative cancer stem cells. Genotypic designation is based on variant calling with the analysis package souporcell (2). Blank spots denote where a genotypic assignment could not be made.
By coupling snRNA-seq and spatial analysis, CD49f<sup>hi</sup> mammary stem cells (MaSCs), a potential tumor stem cell population, were identified and localized predominantly within the fibrous tissue (Figure 3C). Transformation of MaSCs into breast cancer stem cells may result in CD49f<sup>hi</sup> cells within a breast tumor, the presence of which have been associated with increased chance of cancer recurrence (5). Given the identification of CD49f<sup>hi</sup> cells in this TNBC section, knowing whether the cells are tumorigenic could help inform prognosis.

To discriminate between tumor and normal cells, spots were individually genotyped using the souporcell variant identification pipeline (Figure 3D). Souporcell is intended to be a genetic demultiplexer, but by treating tumor and non-tumor cells as genetically distinct samples, it was possible to differentiate between putative tumor spots and non-transformed normal spots as long as the divergent alleles were expressed. In this biopsy, the regions containing CD49f<sup>hi</sup> MaSCs appear genetically normal, suggesting these cells have not yet transformed into breast cancer stem cells, though they may be driven towards a cancer phenotype, given their gene expression profiles. Particularly for cancer analysis, a cell’s transcriptional phenotype is only part of the equation. Gene expression changes can be driven by mutational burden, and knowing what genetic aberrations underlie transcriptional differences can give a better sense of why a tumor is progressing and how it may respond to a range of therapeutic options.

**Conclusion**

In cases of triple negative breast cancer, where hormone therapies and targeted anti-HER2 treatment are ineffective, surgical resection of cancerous tissue often becomes the first line of defense. Improved understanding of the biological underpinnings and cell-type composition of TNBC is critical to developing novel targeted treatment options. The characterization of cancer stem cells is particularly important due to their heterogeneity and plasticity.

In a complete tissue section of TNBC, Visium Spatial Gene Expression allowed for detailed resolution of tumor heterogeneity, localization of putative cancer stem-cell subtypes, and delineation of the extent and cellular identity of immune infiltration. By providing spatial orientation alongside transcriptional profiles, Visium Spatial Gene Expression complements and can be combined with traditional histological techniques, including H&E staining, pathological annotation, and immunofluorescence. Coupled with snRNA-seq, this analysis provided spatial resolution of specific cell subtypes, including CD49f<sup>hi</sup> mammary stem cells and infiltrating exhausted T cells, creating a powerful and visual cell atlas. Complementary single cell and spatial approaches can reveal the cellular and spatial organization of complex tissues like TNBC, offering a systematic method for dissecting the intricate organization of the tumor and its microenvironment.

**References**

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