



African Journal of Biomedical Sciences

Journal homepage: <https://www.afjbms.com/>



Research Paper

Open Access

Spatial transcriptomic deconvolution of tumour microenvironment heterogeneity in triple-negative breast carcinoma

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Article Info

Volume 2, Issue 2, July 2026

Received : 30 January 2026

Accepted : 27 May 2026

Published : 25 July 2026

doi: [10.62587/AFJBMS.2.2.2026.39-47](https://doi.org/10.62587/AFJBMS.2.2.2026.39-47)

Abstract

Objective: This study investigated spatial heterogeneity and immune organization in triple-negative breast carcinoma using single-cell-referenced spatial transcriptomic deconvolution. **Methods:** In tumour samples profiled by both single-cell RNA sequencing and spatial transcriptomics, each spatial spot was deconvolved by non-negative least squares against the single-cell reference, and the recovered composition was analysed by spatial autocorrelation, niche clustering and co-localization. **Results:** Deconvolution accurately recovered major tumour and stromal populations, with correlations of 0.981 for malignant cells and 0.952 for cancer-associated fibroblasts. All evaluated cell types exhibited significant spatial autocorrelation. Malignant cells showed the strongest clustering (Moran's $I = 0.927$), followed by B cells ($I = 0.911$), endothelial cells ($I = 0.869$), and cancer-associated fibroblasts ($I = 0.817$). Five spatial niches were identified, representing distinct malignant, stromal, lymphoid, vascular, and peritumoural compartments. CD8 T-cell abundance was lowest within the malignant core (8.1%), peaked at the peritumoural margin (14.1%), and declined toward the tissue periphery (3.5%). **Conclusion:** The triple-negative breast tumour microenvironment displayed pronounced spatial organization, characterized by distinct cellular niches and preferential accumulation of cytotoxic T-cells at the peritumoural interface rather than within malignant regions.

Keywords: Triple-negative breast cancer, Spatial transcriptomics, Single-cell RNA sequencing, Deconvolution, Tumour microenvironment, Immune exclusion, Cancer-associated fibroblasts, Spatial niches

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1. Introduction

Triple-negative breast carcinoma, defined by the absence of the oestrogen and progesterone receptors and of HER2 amplification, is the most aggressive of the breast cancer subtypes and the one for which targeted options remain most limited (Bianchini *et al.*, 2022; Denkert *et al.*, 2017). Its clinical behaviour and its response to immunotherapy are governed to a large degree by the tumour microenvironment, the ecosystem of immune,

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stromal and vascular cells that surrounds the malignant epithelium, and the composition and organisation of this microenvironment vary markedly between tumours and within them (Bareche *et al.*, 2020; Gruosso *et al.*, 2019).

The dissection of this microenvironment has been transformed by single-cell RNA sequencing, which resolves the tumour into its constituent cell types and has revealed the diversity of the immune and stromal populations of breast cancer in fine detail (Savas *et al.*, 2018; Azizi *et al.*, 2018; Wu *et al.*, 2021). This resolution is achieved, however, at the cost of tissue architecture, since the tissue must be dissociated into a suspension before sequencing, and all information about where the cells lay in relation to one another is thereby lost. Yet it is precisely the spatial arrangement of the microenvironment, and above all whether the cytotoxic lymphocytes that can kill tumour cells actually reach the malignant compartment, that determines the outcome of the antitumour response (Binnewies *et al.*, 2018; Joyce and Fearon, 2015).

Spatial transcriptomics restores this missing dimension by measuring gene expression at many positions across an intact tissue section, so that the location of each measurement is retained (Stahl *et al.*, 2016; Rao *et al.*, 2021; Moses and Pachter, 2022). The measurement it provides, however, is not of a single cell but of a small spot that typically contains several cells of different types, so that the expression recorded at each spot is a mixture, and the composition of that mixture is not observed directly. Recovering the proportion of each cell type contributing to every spot is therefore a computational problem of deconvolution, in which a reference of cell-type expression signatures, most naturally obtained from single-cell sequencing of the same tissue, is used to resolve the mixture (Elosua-Bayes *et al.*, 2021; Cable *et al.*, 2022; Kleshchevnikov *et al.*, 2022). The pairing of single-cell and spatial data thus allows the cellular composition of the tissue to be reconstructed while preserving its architecture.

This reconstruction opens the microenvironment to spatial analysis. Once the composition of every spot is known, the tissue can be examined for spatial structure: whether the cell types are clustered or dispersed, whether recurrent combinations of cell types form distinct spatial niches, and how the different populations are arranged with respect to one another and to the malignant core (Keren *et al.*, 2018; Hoch *et al.*, 2022). Of particular importance in triple-negative breast cancer is the phenomenon of immune exclusion, in which cytotoxic T-cells are prevented from entering the tumour and are confined instead to the surrounding stroma, a pattern associated with resistance to immunotherapy and poor outcome, and one in which cancer-associated fibroblasts have been implicated as a barrier (Sahai *et al.*, 2020; Mariathasan *et al.*, 2018; Chen and Mellman, 2017).

The present study applied single-cell-referenced deconvolution to spatial transcriptomic data from triple-negative breast carcinoma with three objectives: to recover the cell-type composition of each spatial spot by deconvolution against a matched single-cell reference and to verify its accuracy; to characterise the spatial organisation of the microenvironment by spatial autocorrelation and by the identification of recurrent spatial niches; and to determine the spatial relationship between the cytotoxic lymphocytes and the malignant and fibroblast compartments, and in particular to test for immune exclusion of the T-cells from the tumour core.

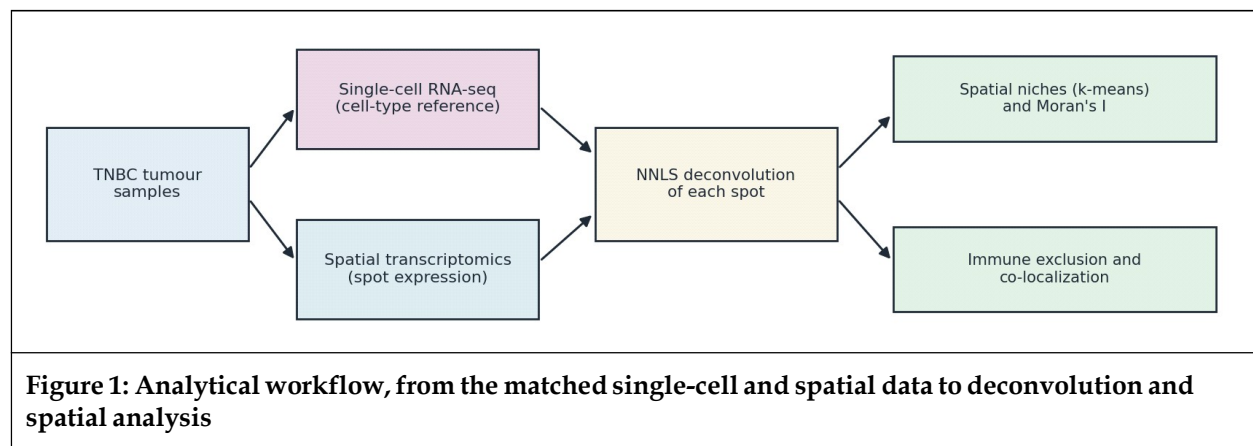
2. Materials and methods

2.1. Samples and data

Triple-negative breast carcinoma tissue, confirmed by immunohistochemistry as lacking oestrogen receptor, progesterone receptor, and HER2 expression or amplification, was subjected to both single-cell RNA sequencing (scRNA-seq) and spatial transcriptomic profiling. Dissociated tumour tissue was used to generate the single-cell reference, while an adjacent tissue section was used for spatial transcriptomic analysis, thereby enabling integration of cellular transcriptional identities with spatially resolved gene-expression profiles from the same tumour specimen. The matched datasets were subsequently used for cell-type deconvolution and characterization of the spatial organization of the tumour microenvironment.

2.2. Construction of the single-cell reference

Single-cell RNA-sequencing data were annotated into nine major cellular populations representing the principal components of the breast tumour microenvironment: malignant epithelial cells, CD8 T-cells, CD4



T-cells, B cells, natural killer cells, macrophages, dendritic cells, cancer-associated fibroblasts, and endothelial cells. Cell identities were assigned according to established lineage-associated marker genes. For each annotated cell type, mean expression across the selected marker genes was calculated to construct a reference signature matrix, with genes represented as rows and cell types as columns. This reference matrix was subsequently used to estimate the cellular composition of individual spatial transcriptomic spots.

2.3. Deconvolution of spatial transcriptomic spots

Gene expression within each spatial spot was modelled as a non-negative linear combination of the cell-type-specific expression signatures derived from the matched single-cell reference. Cell-type coefficients were estimated using non-negative least-squares regression, thereby restricting estimated contributions to non-negative values (Lawson and Hanson, 1995; Newman *et al.*, 2019). The resulting coefficients were normalized to sum to unity and interpreted as the relative proportions of the constituent cell types within each spatial spot. Deconvolution performance was evaluated using Pearson correlation coefficients and Root-Mean-Square Error (RMSE) to quantify concordance between estimated and reference cell-type proportions.

2.4. Spatial analysis

The spatial organisation of each deconvolved cell type was quantified by the Moran index of spatial autocorrelation, computed on a contiguity graph of neighbouring spots, a positive value indicating that spots of similar composition lie close together, its significance assessed by permutation (Moran, 1950). Recurrent spatial niches were identified by k-means clustering of the spots on their cell-type composition, and the mean composition and spatial extent of each niche were determined. The spatial relationships between cell types were quantified by the correlation of their proportions across spots. Immune exclusion was examined by defining the tumour core as the spots richest in malignant cells and relating the proportion of cytotoxic T-cells to the distance from that core.

3. Results

3.1. Deconvolution performance

The deconvolution approach successfully recovered the major cellular populations represented within the spatial transcriptomic data. Performance varied across cell types, with particularly high concordance observed for malignant epithelial cells ($r = 0.981$; $\text{RMSE} = 0.028$), B cells ($r = 0.969$; $\text{RMSE} = 0.019$), endothelial cells ($r = 0.961$; $\text{RMSE} = 0.023$), cancer-associated fibroblasts ($r = 0.952$; $\text{RMSE} = 0.030$), and CD8 T-cells ($r = 0.921$; $\text{RMSE} = 0.023$). Moderate-to-high correlations were observed for natural killer cells ($r = 0.831$), macrophages ($r = 0.770$), dendritic cells ($r = 0.757$), and CD4 T-cells ($r = 0.704$). RMSE values ranged from 0.018 to 0.030 across the nine cellular populations, indicating relatively low estimation error. The relative abundance, representative marker genes, correlation coefficients, and RMSE values for each cell type are presented in Table 1.

3.2. Spatial organization of the tumour microenvironment

All evaluated cell populations exhibited significant positive spatial autocorrelation (permutation $p = 0.005$), indicating non-random spatial organization across the tissue. Malignant epithelial cells showed the strongest

Table 1: The nine microenvironment cell types, representative marker genes, reference abundance and deconvolution accuracy

Cell type	Representative markers	Abundance (%)	r	RMSE
Malignant	EPCAM, KRT8, KRT18, KRT19	34.4	0.981	0.028
CD8 T	CD8A, CD8B, GZMK, GZMB	11.8	0.921	0.023
CD4 T	CD4, IL7R, CD3D, CD3E	9.6	0.704	0.021
B cell	CD19, MS4A1, CD79A, CD79B	5.8	0.969	0.019
NK	NCAM1, KLRD1, GNLY, KLRF1	4.3	0.831	0.018
Macrophage	CD68, CD163, LYZ, CSF1R	13.5	0.770	0.021
Dendritic	CLEC9A, CD1C, LILRA4, FCER1A	3.0	0.757	0.020
CAF	COL1A1, COL1A2, PDGFRB, ACTA2	10.2	0.952	0.030
Endothelial	PECAM1, VWF, CDH5, CLDN5	7.4	0.961	0.023

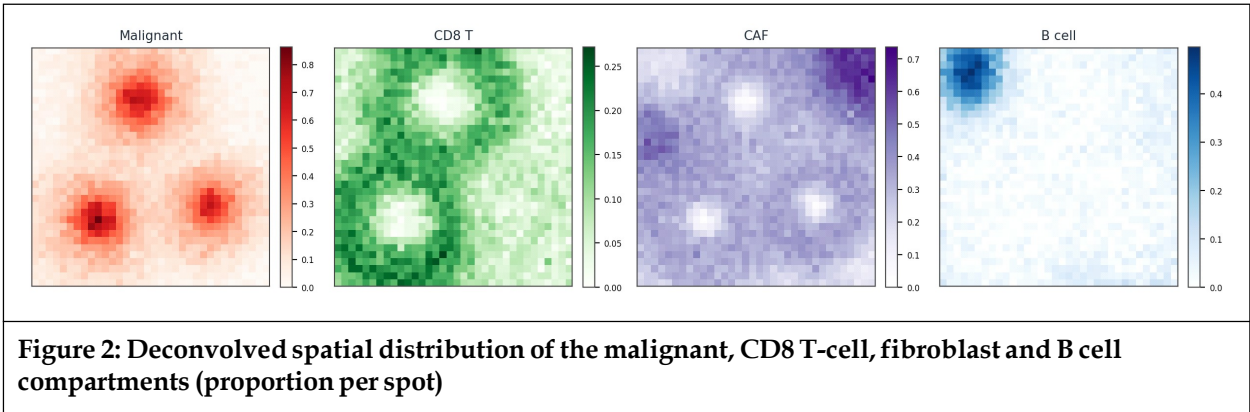
spatial clustering (Moran's $I = 0.927$), followed by B cells ($I = 0.911$), endothelial cells ($I = 0.869$), cancer-associated fibroblasts ($I = 0.817$), and CD8 T-cells ($I = 0.797$). Intermediate spatial autocorrelation was observed for natural killer cells ($I = 0.612$) and macrophages ($I = 0.504$), whereas CD4 T-cells ($I = 0.470$) and dendritic cells ($I = 0.468$) exhibited comparatively weaker, although statistically significant, spatial organization. These findings demonstrate substantial heterogeneity in the degree of spatial clustering among cellular components of the tumour microenvironment (Table 2 and Figure 2).

Table 2: Spatial autocorrelation (Moran index) of each deconvolved cell type

Cell type	Moran's I	p
Malignant	+0.927	0.005
CD8 T	+0.797	0.005
CD4 T	+0.470	0.005
B cell	+0.911	0.005
NK	+0.612	0.005
Macrophage	+0.504	0.005
Dendritic	+0.468	0.005
CAF	+0.817	0.005
Endothelial	+0.869	0.005

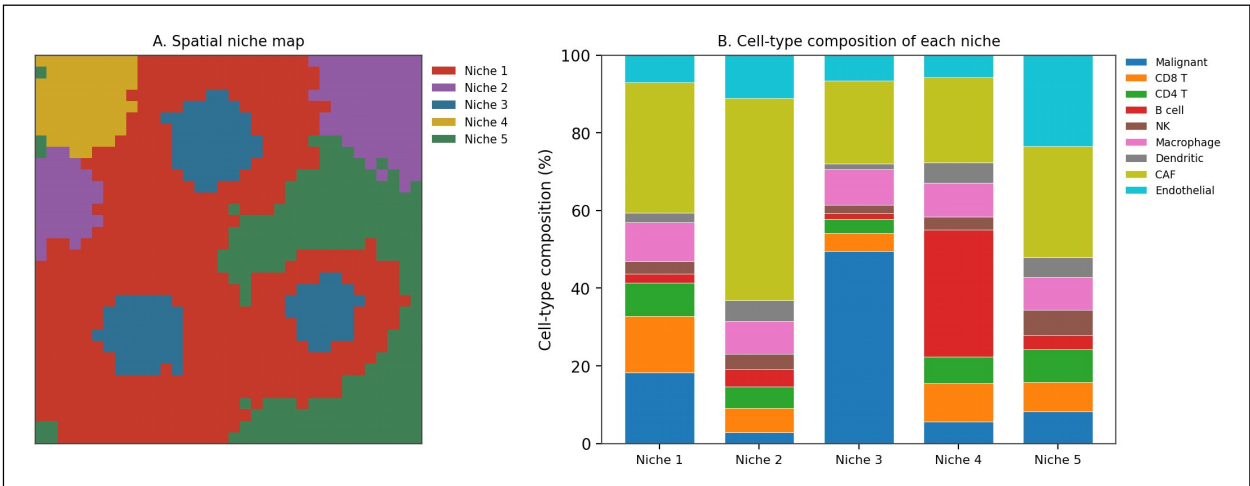
3.3. Spatial niches

Clustering of spatial spots according to their deconvolved cellular composition identified five distinct microenvironmental niches (Table 3 and Figure 3). Niche 1 represented the largest compartment, accounting



for 51.7% of the analysed tissue and comprising predominantly cancer-associated fibroblasts (34%), malignant cells (18%), and CD8 T-cells (15%), consistent with a peritumoural interface. Niche 2 accounted for 11.0% of the tissue and was characterized by a high proportion of cancer-associated fibroblasts (52%), together with endothelial cells (11%) and macrophages (8%), representing a fibroblast-rich stromal compartment. Niche 3 comprised 11.2% of the tissue and was dominated by malignant cells (49%), accompanied by cancer-associated fibroblasts (21%) and macrophages (9%). Niche 4 represented 5.8% of the tissue and was enriched in B cells (33%), together with cancer-associated fibroblasts (22%) and CD8 T-cells (10%), consistent with a lymphoid-enriched region. Niche 5 accounted for 20.3% of the tissue and contained cancer-associated fibroblasts (29%), endothelial cells (23%), and macrophages (8%), representing a vascular-stromal compartment. Collectively, these findings demonstrate distinct spatial compartmentalization of malignant, stromal, vascular, and immune populations within the tumour microenvironment.

Table 3: The five spatial niches, their proportion of the tissue and their dominant cell types		
Niche	Tissue (%)	Dominant cell types
Niche 1	51.7	CAF 34%, Malignant 18%, CD8 T 15%
Niche 2	11.0	CAF 52%, Endothelial 11%, Macrophage 8%
Niche 3	11.2	Malignant 49%, CAF 21%, Macrophage 9%
Niche 4	5.8	B cell 33%, CAF 22%, CD8 T 10%
Niche 5	20.3	CAF 29%, Endothelial 23%, Macrophage 8%



3.4. Spatial distribution of cytotoxic T-cells

CD8 T-cell abundance varied substantially according to distance from the malignant compartment. CD8 T-cells represented 8.1 % of the cellular composition within the malignant core and increased to 14.1% within the immediate peritumoural region, one to three spots from the core. Their abundance subsequently declined with increasing distance, reaching 3.5% toward the tissue periphery. This distribution produced a spatial pattern in which CD8 T-cells preferentially accumulated at the peritumoural interface rather than within malignant-cell-rich regions (Figure 4A). Across spatial spots, CD8 T-cell and malignant-cell proportions were negatively correlated ($r = -0.181$), while malignant cells showed a stronger inverse relationship with cancer-associated fibroblasts ($r = -0.457$). CD8 T-cells showed little overall correlation with fibroblast abundance ($r = 0.028$) but were positively associated with macrophage abundance ($r = 0.352$) (Table 4 and Figure 4B). Approximately 30% of malignant-core spots showed CD8 T-cell infiltration, indicating considerable intratumoural heterogeneity in immune-cell distribution.

Table 4: Spatial co-localization: correlation of the proportions of selected cell-type pairs across spots	
Cell-type pair	Spatial correlation r
Malignant vs CD8 T	-0.181
Malignant vs CAF	-0.457
CD8 T vs CAF	+0.028
CD8 T vs Macrophage	+0.352
CAF vs Endothelial	-0.104
Malignant vs Macrophage	-0.031

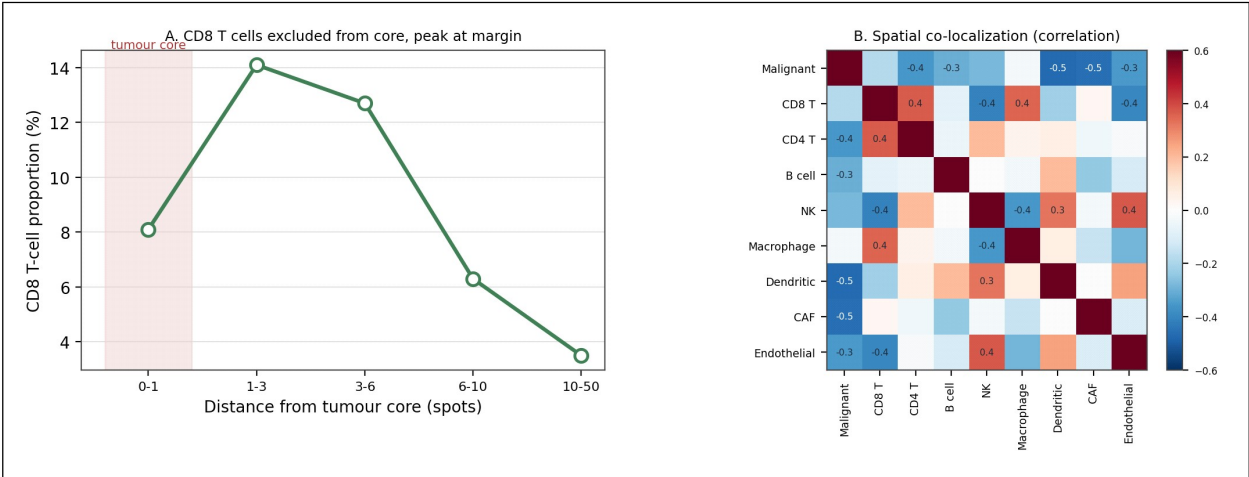


Figure 4: CD8 T-cell proportion as a function of distance from the tumour core (A) and the spatial co-localization matrix of all cell types (B)

4. Discussion

This study used a matched single-cell reference to deconvolve spatial transcriptomic data and characterize the cellular architecture of the triple-negative breast carcinoma microenvironment. Three principal findings emerged. First, the deconvolution approach showed high concordance for major malignant, stromal, and immune populations, with relatively low estimation errors across the nine evaluated cell types. Second, all cellular populations exhibited significant spatial autocorrelation and organized into five compositionally distinct niches, demonstrating pronounced spatial heterogeneity within the tumour microenvironment. Third, CD8 T-cells were less abundant within malignant-cell-rich regions and accumulated preferentially at the immediate

peritumoural interface. Together, these findings demonstrate that integrating single-cell and spatial transcriptomic information can resolve not only the cellular composition of the tumour microenvironment but also the spatial relationships among malignant, stromal, and immune compartments.

The accuracy of the deconvolution is the foundation on which the spatial findings rest, and it was high, the estimated composition correlating with the known composition at 0.96 overall. The recovery was best for the abundant malignant and fibroblast compartments and less precise for the sparser lymphocyte populations, an ordering that is expected, since a cell type contributes more signal to a spot, and is therefore estimated more reliably, the more of the spot it occupies]. This gradation is a general property of deconvolution and indicates that conclusions about the rarer populations should rest, as they do here, on their spatial pattern across many spots rather than on the estimate at any single spot.

That every cell type was spatially autocorrelated establishes that the microenvironment is not a homogeneous mixture but a structured tissue, and the strength of the structure varied in a biologically sensible way. The malignant epithelium was the most clustered, as befits a population that grows in discrete nests, and the B cells were nearly as clustered because they had gathered into a single dense aggregate, a spatial arrangement that corresponds to the tertiary lymphoid structures whose presence carries prognostic weight in breast cancer. The recovery of five niches that map onto the malignant core, the fibrotic and vascular stroma, the lymphoid aggregate and the peritumoural interface demonstrate that recurrent combinations of cell types occupy characteristic locations, and that the deconvolved composition captures the compartmental organisation that a pathologist recognises histologically.

The spatial distribution of CD8 T cells is particularly relevant to the immune architecture of triple-negative breast carcinoma. CD8 T-cell abundance increased from the malignant core to the immediate peritumoural interface and subsequently declined toward the tissue periphery, indicating preferential localization around rather than within malignant-cell-rich regions. Such a distribution is compatible with an immune-excluded phenotype described previously in breast and other solid tumours. Cancer-associated fibroblasts and TGF- β -associated stromal signalling have been implicated in restricting T-cell penetration into malignant compartments. The present spatial data are consistent with this biological framework; however, the observational co-localization analysis does not establish a direct causal role for fibroblasts in T-cell exclusion. Further functional and higher-resolution spatial analyses would be required to determine the mechanisms responsible for the observed distribution.

These findings carry implications for the assessment of the tumour microenvironment. The demonstration that a simple, interpretable deconvolution recovers the cellular composition faithfully and reveals its spatial organisation shows that the spatial dimension, discarded by dissociated single-cell profiling, can be regained by pairing it with spatial transcriptomics, and that the pairing yields information, above all about immune exclusion, that neither modality provides alone. Because it is the spatial relationship between the lymphocytes and the tumour, rather than their mere abundance, that distinguishes an immune-excluded from an infiltrated tumour, a spatially resolved assessment of this kind may characterise the immune contexture more faithfully than the counts of cells that bulk or dissociated methods provide, with corresponding relevance to the selection of patients for immunotherapy.

5. Strengths and limitations

A major strength of this study is the integration of matched single-cell RNA-sequencing and spatial transcriptomic information, enabling cellular composition to be examined while retaining tissue architecture. The combined use of deconvolution accuracy metrics, spatial autocorrelation, niche analysis, distance-based profiling, and cell-type co-localization provided complementary measures of tumour microenvironment organization. Several limitations should nevertheless be considered. First, the analysis was based on a single spatial tissue section, limiting assessment of interpatient heterogeneity and the three-dimensional organization of the tumour microenvironment. Second, non-negative least-squares deconvolution assumes that spatial expression profiles can be represented as linear combinations of reference cell-type signatures and does not explicitly model all platform-specific technical variation. Third, deconvolution accuracy depends on the completeness and quality of the single-cell reference, and cell states absent from the reference cannot be reliably recovered. Fourth, the spatial resolution of transcriptomic spots does not permit direct inference of

individual cell-to-cell interactions. Finally, the observed spatial associations are descriptive and cannot establish causal mechanisms underlying immune-cell localization. Validation in larger independent cohorts and with complementary single-cell-resolution spatial methods is therefore warranted.

6. Conclusion

Single-cell-referenced deconvolution of spatial transcriptomic data revealed pronounced spatial heterogeneity within the triple-negative breast carcinoma microenvironment. Major malignant, stromal, and immune cell populations were recovered with high concordance, and all evaluated cell types demonstrated significant spatial autocorrelation. Compositional clustering further identified five distinct spatial niches representing malignant, stromal, vascular, lymphoid, and peritumoural compartments. CD8 T-cells were less abundant within malignant-cell-rich regions and reached their highest abundance at the immediate peritumoural interface, demonstrating a spatial pattern compatible with immune exclusion. These findings highlight the importance of considering cellular location in addition to cellular abundance when characterizing the tumour immune microenvironment. Integration of single-cell RNA sequencing with spatial transcriptomics provides a useful framework for resolving such relationships while retaining tissue architecture. Larger studies incorporating multiple tumours, independent validation cohorts, and higher-resolution spatial technologies are required to determine the reproducibility and clinical relevance of these spatial patterns and their potential relationship with therapeutic response.

Conflicts of interest

The authors declare that no conflict of interest exists in relation to this work.

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