Precise spatial multiplexing of immune cell diversity in clinical breast tumor specimens with ChipCytometry™

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Abstract

Highly multiplexed spatial biomarker analysis has demonstrated the potential to advance our current understanding of the immune system and its role in cancer – from tumor initiation to metastatic progression. Previously, a trade-off between plex and spatial context meant that our understanding of immune cell involvement in cancer was limited by either single-plex technologies with spatial context (e.g., immunohistochemistry) or highly multiplexed technologies without spatial context (e.g., flow cytometry and single-cell RNA sequencing). ChipCytometry™ is a novel, highly multiplexed technology that preserves both plex and spatial context to deeply profile immune cell diversity at single-cell resolution. ChipCytometry uses commercially available antibodies and combines iterative immuno-fluorescent staining with high-dynamic range imaging to profile dozens of protein biomarkers in a single tissue specimen. Cellular phenotypes are identified with via flow cytometry-like hierarchical gating from standard multichannel OME-TIFF images, compatible with a variety of computational tools being developed for multiplexed analysis and visualization. Here, we use ChipCytometry to identify and quantify key immune cell subtypes in a fresh frozen tissue sample from a patient with breast cancer. The results show precise expression levels for each biomarker in the assay in each individual cell in the sample, while maintaining spatial positioning of each cell. Spatial analysis reveals quantifiable heterogeneity of immune cell infiltration within the tumor samples, demonstrating the utility of the ChipCytometry platform for the in-depth immune profiling of clinical tissue samples.

Methods

Sample Preparation: Five µm FF tissue sections were mounted onto glass coverslips. Sections were loaded onto ZellSafe™ microfluidic chips to preserve sample integrity during serial delivery of reagents.
Data Collection: ROIs were selected based on an initial scan of autofluorescence in a single fluorescent channel. Sections were stained with fluorescent antibodies and incubated for 15 min at RT. Sample was imaged in up to 5 channels, then fluorescence was photobleached. Sections were stained in cycles until all targets were imaged (Table 1).
Image Analysis: Custom software was used to align and overlay scanned ROIs. Cell segmentation was performed using an internal AI-algorithm. Cell phenotypes were identified using hierarchical gating strategy.

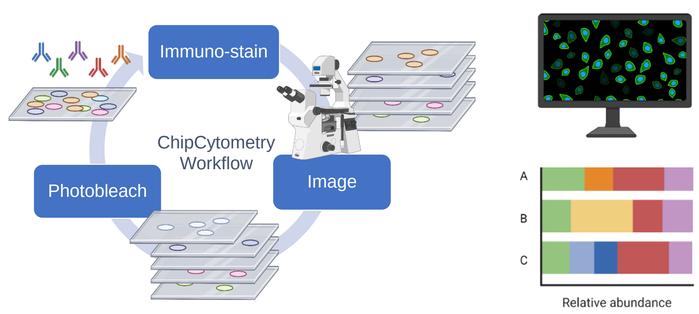

Figure 1. ChipCytometry workflow. The ChipCytometry workflow is based on successive rounds of staining with up to 5 fluorescently labeled antibodies, imaging, and photobleaching. Multi-channel OME-TIFF images are registered and stitched together to produce a single composite image. Custom sample-specific AI-based algorithms enable cell segmentation and phenotyping prior to quantitative analysis. (Made with BioRender)

Cycle	Target
1	CD8
	CD11c
	CD14
	CD123
2	CD38
	CD20
	CD56
	CD4
3	CD3
	FoxP3
	CD45RO
4	CD45
	CD68
	PD-1
	CD45RA
5	CD31
	HER-2
6	DNA
	Pan-CK
	EpCAM
	HLA-DR

Table 1. Antibody staining plan. A 21-plex antibody panel was applied to the specimens in a 6-cycle assay. Each target corresponds to a monoclonal fluorescently labeled antibody (with the exception of DNA dye).

Results

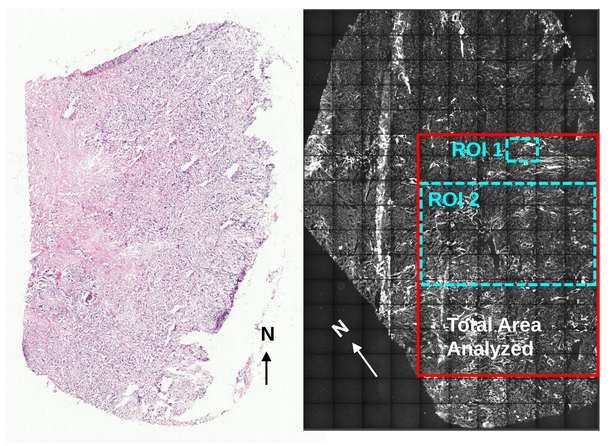

Figure 1A. Overview H&E image of breast tumor specimen. H&E staining revealed a large region of invasive carcinoma.

Figure 1B. Autofluorescence image of breast tumor specimen. A subset of the area was selected for further profiling with ChipCytometry (red rectangle).

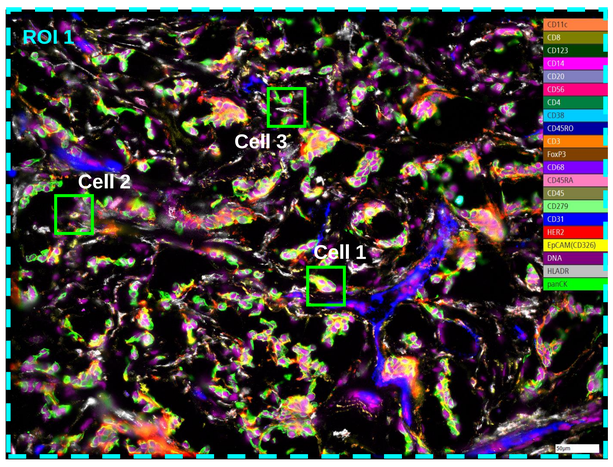

Figure 2A. Whole slide staining of breast carcinoma tissue. A 21-plex antibody staining plan was used to image the entire tissue specimen. Here, we show a single image tile from a subset of the total area analyzed (0.36 mm² of 23.76 mm²) highlighting the tissue architecture including tumor and immune cells.

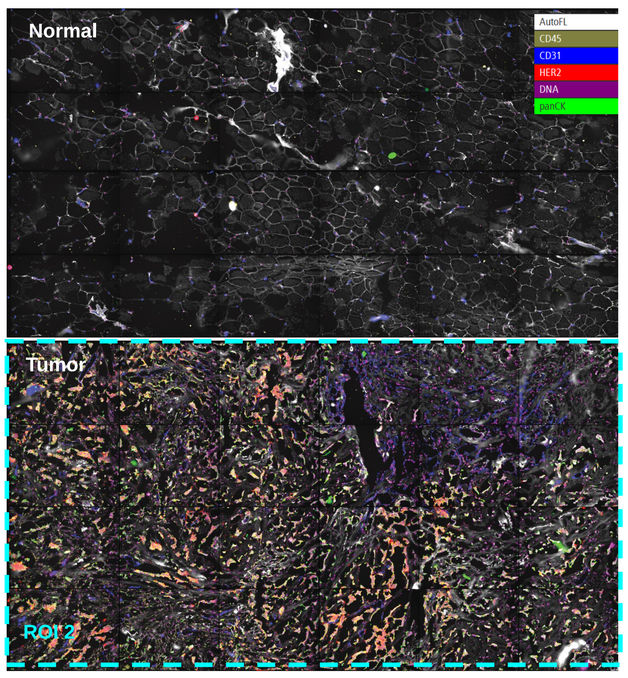

Figure 3. Staining of normal (top) and tumor (bottom) breast tissue. Overview images (8.64 mm² each) of the tumor sample with higher immune (CD45+) and epithelial (Pan-CK+) cell density than the normal tissue. AF and DNA stain reveal adipocyte-rich regions of normal tissue.

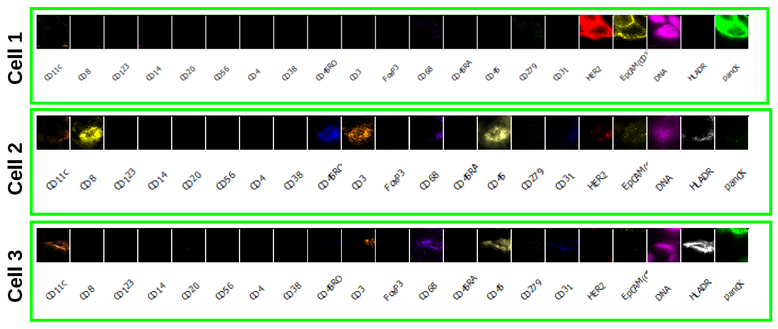

Figure 2B. Highly multiplexed single cell imaging. All 21 markers were used for cell phenotyping. Here, we show marker strips for 3 different cells: a tumor cell (cell 1) expressing HER2, EpCAM, and panCK; a cytotoxic T cell (cell 2) expressing CD45, CD3, CD8, and CD45RO; and a macrophage (cell 3) expressing CD45, CD68, HLA-DR, and CD11c.

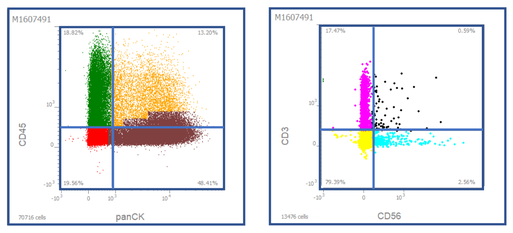

Figure 4. Representative dot plots showing hierarchical gating strategy. From image data, cells were segmented and interrogated for quantitative expression of each marker to enable the identification of cell phenotypes via flow cytometry-like hierarchical gating. This series represents a single gating strategy to identify some key immune cells including CD3+ T cells and CD56+ NK cells.

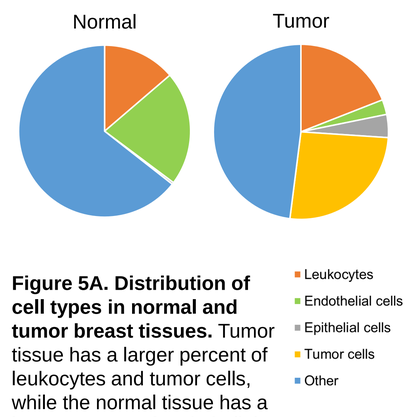

Figure 5A. Distribution of cell types in normal and tumor breast tissues. Tumor tissue has a larger percent of leukocytes and tumor cells, while the normal tissue has a larger percent of endothelial cells.

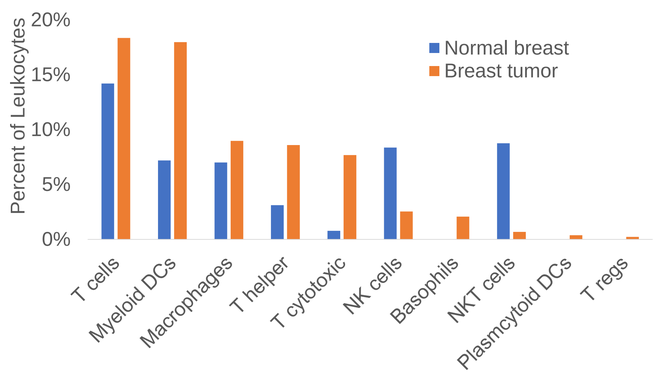

Figure 5B. Abundance of leukocyte subtypes in normal and tumor breast tissues. Breast tumor tissue contains a larger percent of leukocyte subtypes than normal tissue.

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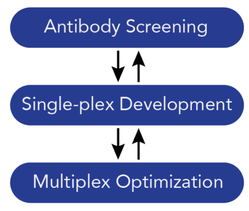

Figure 6. Validation of commercial antibodies for ChipCytometry. Each antibody undergoes clone screening, single-plex development to optimize dilution and evaluate target-specificity, and multiplex optimization to evaluate antibody cross-reactivity in a multiplexed assay.

Conclusions

- We demonstrate the utility of ChipCytometry to generate highly-multiplexed, spatially-resolved protein expression data from a clinical sample. We show quantitative measurement of 21 clinically relevant biomarkers at the single-cell level for every cell in this tissue specimen from a patient with HER2+ breast cancer.
- ChipCytometry is a multiplexed imaging method that uses commercial antibodies from any vendor to spatially resolve protein targets *in situ*. ChipCytometry does not require any additional abstractions (e.g., oligo barcoding), which enables a simpler validation workflow and greater target versatility.
- We quantify relevant populations of tumor and immune subpopulations, revealing high relative abundance of key immune cell subtypes in this breast tumor tissue. Quantification of cell populations expressing very high or low levels of a single marker is more challenging and made possible through high-dynamic range (HDR) imaging.

Selected Publications

1. Carstensen, S., Holz, O., Hohlfeld, J. M., & Müller, M. (2021). Quantitative analysis of endotoxin-induced inflammation in human lung cells by Chipcytometry. Cytometry Part A, 99(10), 967–976. https://doi.org/10.1002/cyto.a.24352
2. FitzPatrick, M. E. B., Provine, N. M., Garner, L. C., Powell, K., Amini, A., Irwin, S. L., Ferry, H., Ambrose, T., Friend, P., Vrakas, G., Reddy, S., Soilleux, E., Klenerman, P., & Allan, P. J. (2021). Human intestinal tissue-resident memory T cells comprise transcriptionally and functionally distinct subsets. Cell Reports, 34(3), 108661. https://doi.org/10.1016/j.celrep.2020.108661
3. Hagel, J. P., Bennett, K., Buffa, F., Klenerman, P., Willberg, C. B., & Powell, K. (2021). Defining T Cell Subsets in Human Tonsils Using ChipCytometry. The Journal of Immunology, 206(12), 3073–3082. https://doi.org/10.4049/jimmunol.2100063
4. Jarosch, S., Köhlen, J., Sarker, R. S. J., Steiger, K., Janssen, K.-P., Christians, A., Hennig, C., Holler, E., D'Ippolito, E., & Busch, D. H. (2021). Multiplexed imaging and automated signal quantification in formalin-fixed paraffin-embedded tissues by ChipCytometry. Cell Reports Methods, 1(7), 100104. https://doi.org/10.1016/j.crmeth.2021.100104
5. Jarosch, S., Köhlen, J., Wagner, S., D'Ippolito, E., & Busch, D. H. (2022). ChipCytometry for multiplexed detection of protein and mRNA markers on human FFPE tissue samples. *STAR Protocols*, *3*(2), 101374. https://doi.org/10.1016/j.xpro.2022.101374
6. Schupp, J., Christians, A., Zimmer, N., Gleue, L., Jonuleit, H., Helm, M., & Tuettenberg, A. (2021). In-Depth Immune-Oncology Studies of the Tumor Microenvironment in a Humanized Melanoma Mouse Model. International Journal of Molecular Sciences, 22(3), 1011. https://doi.org/10.3390/ijms22031011

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