Precise spatial multiplexing of immune cell diversity in clinical tumor samples 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 HER2+ 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

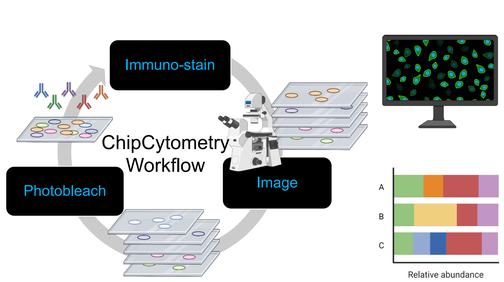

Figure 1. ChipCytometry workflow. The ChipCytometry workflow is based on successive rounds of staining with up to 5 fluorescently labeled antibodies, imaging, and photobleaching. Multi-channel OME-TIFF images are registered and stitched together to produce a single composite image. Custom sample-specific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

Target	Dilution
CD3	1:100
CD4	1:100
CD8	1:200
CD11c	1:200
CD14	1:100
CD20	1:200
CD31	1:400
CD38	1:100
CD45	1:500
CD45RA	1:1800
CD45RO	1:900
CD56	1:100
CD68	1:300
CD123	1:100
PD1	1:100
EpCAM	1:1800
CD340	1:600
DNA	1:100000
HLA-DR	1:600
FoxP3	1:100
Pan-CK	1:1800

Table 1. Antibody staining plan. A 21-plex antibody staining plan was applied to this breast cancer specimen.

ChipCytometry Protocol:

Sample Preparation

1. 5 µm FF tissue sections were mounted onto glass coverslips
2. Sections were loaded onto ZellSafe™ microfluidic chips to preserve sample integrity during serial delivery of reagents

Data Collection

1. ROIs were selected based on an initial scan of autofluorescence in a single channel
2. Sections were stained with fluorescent antibodies and incubated for 15 min at RT
3. Sample was imaged in up to 5 channels, then fluorescence was photobleached
4. Step 4 and 5 were repeated in rounds until all targets were imaged (Table 1)

Image Analysis

1. Custom software was used to align and overlay scanned ROIs
2. Cell segmentation was performed using an internal AI-algorithm
3. Cell phenotypes were identified using hierarchical gating strategy

Results

Here we present the analysis of a roughly 8 mm² of sample area (Fig. 2A) of 15 mm² scanned area. Most tumor cells (Pan-CK+) are also HER2+ (Fig. 2B), which suggests this is a carcinoma – a malignancy of epithelial origin. Yet, we found a single region of normal epithelium (Pan-CK+/HER2-) with relatively normal tissue architecture (Fig. 2C). Each antibody was subjected to rigorous testing during antibody validation (Fig. 4). All 21 markers in this assay were used for cell phenotyping using a hierarchical gating strategy based on expression values (Fig. 3 and 5). The resulting cell populations were quantified (Table 2) and represented in terms of percent of all cells segmented (Fig. 6A) and percent of all leukocytes (Fig. 6B).

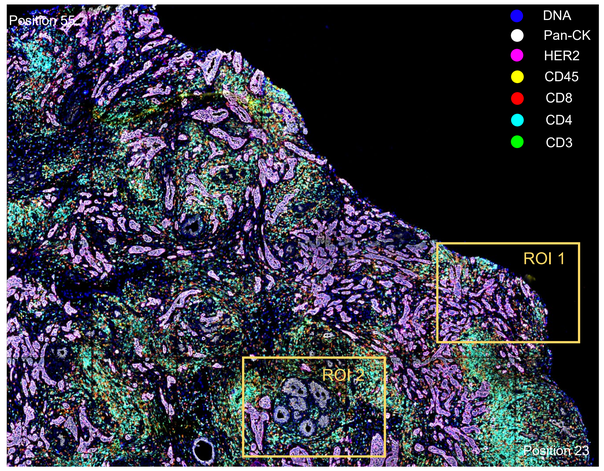

Figure 2A. Whole sample staining of HER2+ breast carcinoma tissue. A 21-plex antibody staining plan was used to image the entire tissue specimen. Here, we show a subset (7) of those markers and a subset of the area analyzed (8 mm² of 15 mm²) using key markers to highlight tissue architecture including tumor and immune cells.

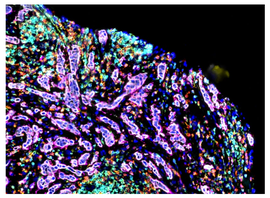

Figure 2B. Zoomed in view of ROI 1, HER2+ region of epithelial tissue.

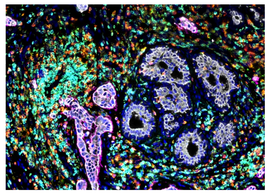

Figure 2C. Zoomed in view of ROI 2, HER2- region of epithelial tissue (Pan-CK+).

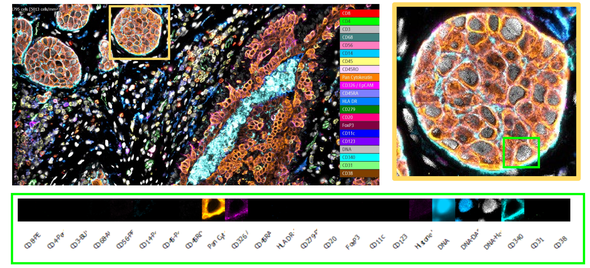

Figure 3. Highly multiplexed single cell imaging. All 21 markers (note different colors) were used for cell phenotyping. A region of relative low cell density is highlighted to distinguish individual markers. Here, a single tumor cell expresses pan-cytokeratin, EpCAM, HER2, and DNA markers.

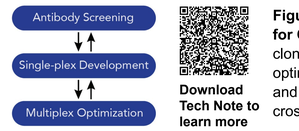

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Figure 4. Validation of commercial antibodies for ChipCytometry. Each antibody undergoes clone screening, single-plex development to optimize dilution and evaluate target-specificity, and multiplex optimization to evaluate antibody cross-reactivity in a multiplexed assay.

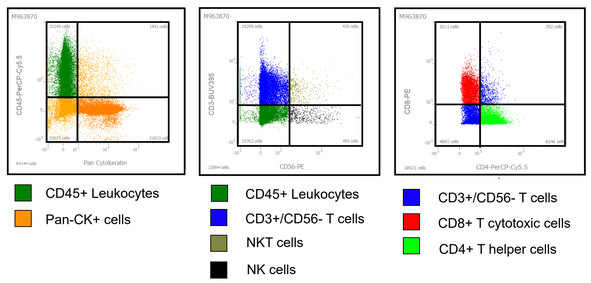
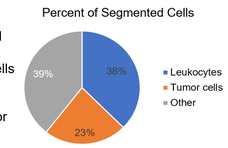


Figure 5. Representative dot plots showing hierarchical gating strategy. From image data, cells were segmented, and interrogated for quantitative expression of each marker to enable the identification of cell phenotypes via flow cytometry-like hierarchical gating. This series represents a single gating strategy to identify some key immune cells including T helper and T cytotoxic cells.

Cell Population	Parent Gate	Gating Strategy	% of Total Leukocytes	Absolute Count
All cells	Hoechst Stain for DNA	N/A	-	**94184**
Leukocytes	All	CD45+ Pan CK-	**100.00%**	**35245**
T cells	CD45+ Leukocytes	CD3+ CD56-	**54.75%**	**19298**
NK cells	CD45+ Leukocytes	CD3- CD56+	**1.40%**	**495**
NKT cells	CD45+ Leukocytes	CD3+ CD56+	**1.24%**	**439**
T cytotoxic	T cells	CD4-CD8+	**14.22%**	**5011**
T helper	T cells	CD4+CD8-	**23.40%**	**8246**
T regulatory	T helper	FoxP3+	**1.33%**	**468**
B cells	CD45+ Leukocytes	CD3-CD20+	**1.57%**	**554**
Macrophages	CD45+ Leukocytes	CD68+	**9.11%**	**3210**
Dendritic Cells	CD45+ CD3- CD20- CD14-	HLA-DR+ CD56-	**18.44%**	**6499**
Myeloid DCs	Dendritic Cells	CD11c+ CD123-	**13.24%**	**4666**
Plasmacytoid DCs	Dendritic Cells	CD11c- CD123+	**0.21%**	**74**
Tumor cells	All	CD45- Pan-CK+	-	**21833**

Table 2. Quantification of cell populations. Cell populations were determined via a hierarchical gating strategy, and each population was quantified. Absolute cell counts and percent of total leukocytes are represented here.

Figure 6A. Percent of all cells. Most segmented cells were either leukocytes (38%) or tumor cells (23%).



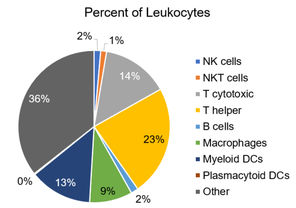


Figure 6B. Percent of leukocytes. Most identified leukocytes were T helper or T cytotoxic cells.

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 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 T helper and T cytotoxic cells in this HER2+ breast cancer 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.

Scan to learn more about ChipCytometry

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. 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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