Precise Spatial Multiplexing for Immune Profiling in FFPE Tissues with ChipCytometry™

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Abstract

Highly multiplexed spatial biomarker analysis has the potential to revolutionize the development of targeted drug development and personalized medicine. Pathologists often require the information about spatial context and cellular morphology that tests like immunohistochemistry (IHC) provide, to make diagnoses and treatment decisions. Yet, these methods are limited to measuring a single biomarker at a time. Newer technologies, such as single-cell RNA sequencing, measure many biomarkers at once, but do not provide information on the spatial context of cells *in situ*. Here, we present the analysis of FFPE tissues using ChipCytometry™, a novel method for high-plex biomarker analysis *in situ*. ChipCytometry combines an iterative immuno-fluorescent staining workflow with high-dynamic range (HDR) imaging to facilitate precise spatial phenotyping with single-cell resolution. Standard FCS files are generated from multi-channel OME-TIFF images, enabling identification of cellular phenotypes via flow cytometry-like hierarchical gating. In this study, we identify and quantify tumor and immune cells in melanoma and lung cancer clinical FFPE tissues and present data from appendix FFPE tissue. The results show precise quantification of protein expression in individual cells and quantification of cell populations. Spatial analysis of the samples reveals heterogeneity of immune-rich regions, demonstrating the utility of the ChipCytometry platform for the in-depth immune profiling in clinical FFPE samples.

Methods

1. Sample Preparation: 5 µm FFPE tissue sections were mounted onto glass coverslips. Sections were deparaffinized and rehydrated by serial immersion in citrisolv and graded ethanol. Heat-mediated antigen retrieval (HIER) was performed for 20 min at 95°C. Sections were then loaded onto microfluidic chips to preserve sample integrity during serial delivery of reagents.

2. Data Collection: Regions of interest were selected based on an initial scan in a single fluorescent channel. Sections were subsequently stained, imaged, and quenched in rounds, according to the *staining plan (Table 1)*. Antibodies were incubated for 1 hour at room temperature before washing and imaging.

3. Automated Image Analysis: Image analysis was performed using custom analysis software. Fluorescence intensity images for markers were aligned and overlayed. Image data was used to segment individual cells and cell phenotypes were identified using hierarchical gating strategy and subsequently quantified.

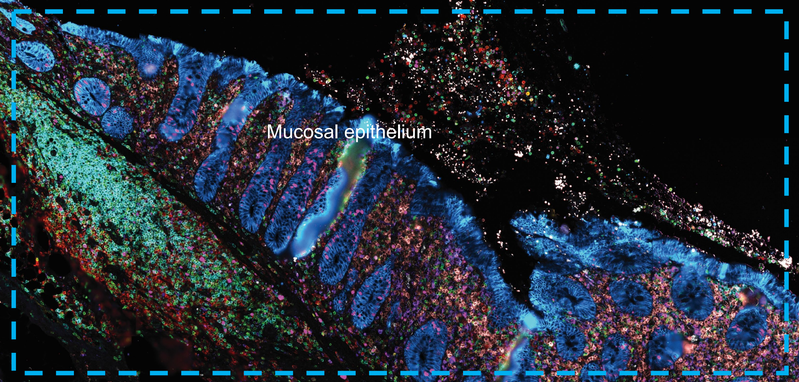


Figure 2. Multiplex immunofluorescence image of mucosal epithelium of appendix tissue (ROI1). ChipCytometry HDR imaging facilitates precise spatial phenotyping with single-cell resolution.

Results

Color	Antibody
	Ki-67
	CD27
	CD3
	CD4
	CD45
	CD68
	FoxP3
	CD45RA
	CD8
	PD-1
	EpCAM
	Granzyme B
	PD-L1

Table 1. Staining plan. The ChipCytometry Spatial Immune Profiling Kit contains 14 pre-validated antibodies for staining FFPE tissues.

The Spatial Immune Cell Profiling Kit is a 14-plex antibody kit targeting key immune and epithelial cells (Table 1) that has been validated in triplicate in three types of FFPE tissue (Table 2). Here, we present the results of precise immune profiling of appendix tissue with the ChipCytometry workflow (Figure 1).

Tested Tissues	Sample Type
Appendix	FFPE Tissue
Lung cancer	FFPE Tissue
Melanoma	FFPE Tissue

Table 2. Tested tissues. The ChipCytometry Spatial Immune Profiling Kit was validated in appendix, lung cancer, and melanoma FFPE tissue sections.

Learn more about
Antibody Validation for ChipCytometry

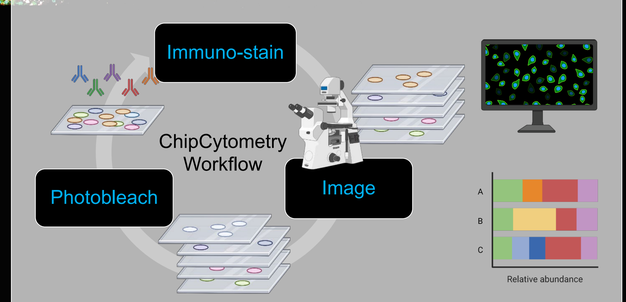


Figure 1. ChipCytometry workflow. The ChipCytometry workflow is based on successive rounds of staining with up to 5 fluorescently labeled antibodies, imaging, and photobleaching. Multi-channel OME-TIFF images are registered and stitched together to produce a single composite image. Custom sample-specific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

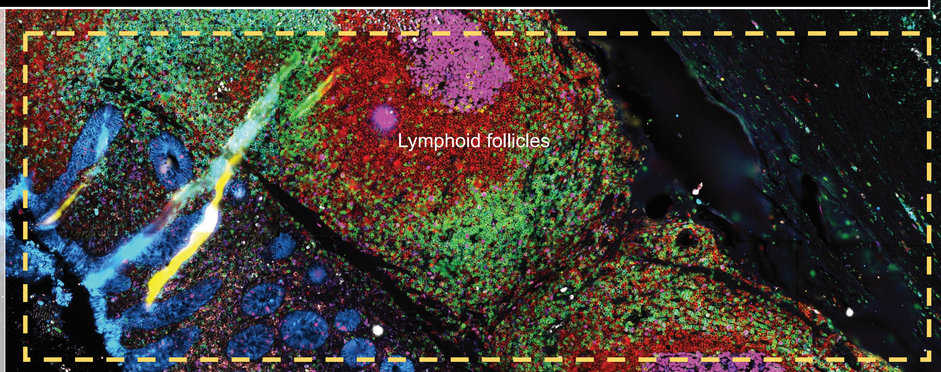
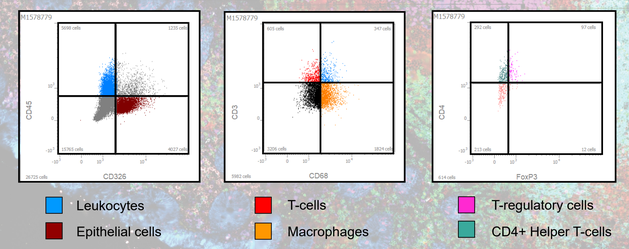


Figure 3. Multiplex immunofluorescence image of lymphoid follicles (ROI2) in appendix tissue. Single-cell resolution enables visualization of individual cells. This region demonstrates various degrees of expression for various immune cell markers.

Figure 4. Hierarchical gating strategy defines cell phenotypes. From image data, cells were segmented, and standard FCS files were generated to enable the identification of cell phenotypes via flow cytometry-like hierarchical gating. These are representative dot plots demonstrating a single gating strategy to identify immune cell subtypes.



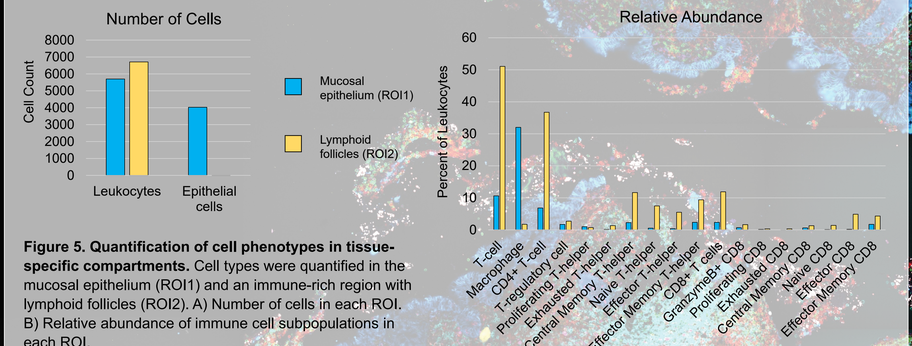


Figure 5. Quantification of cell phenotypes in tissue-specific compartments. Cell types were quantified in the mucosal epithelium (ROI1) and an immune-rich region with lymphoid follicles (ROI2). A) Number of cells in each ROI. B) Relative abundance of immune cell subpopulations in each ROI.

Conclusions

- ChipCytometry is a multiplexed imaging platform that uses open-source reagents for iterative cycles of immuno-staining, imaging, and photobleaching
- ChipCytometry uses HDR imaging and custom analysis software to quantify high- and low-expressing proteins in the same sample
- The Spatial Immune Profiling Kit was used for immuno-phenotyping of human FFPE specimens and was tested in lung cancer, melanoma, and appendix tissue
- Analysis of appendix FFPE tissue revealed quantifiable heterogeneity of protein expression and cell phenotypes

Selected ChipCytometry 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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