Visualizing the immune response

An optimized fluorescent 9-plex spatial proteomics workflow enables single cell quantification within human invasive ductal carcinoma (IDC) breast cancer research

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Abstract

New spatial proteomic workflows with innovations in enzyme activated spatial amplification reagents and fluorescent spectral unmixing enable simultaneous detection of 8 biomarkers within a single tissue section and allow the deep examination of specific cellular interactions and greater characterization of cellular phenotypes. Traditional immunohistochemistry (IHC) approaches are limited to resolving spectrally distinct signals for up to four targets. Increasing the number of unique targets that can be detected in a single sample relies on the unmixing of overlapping spectral signatures and drives multidimensional analysis of complex cell-tissue systems.

Using a spatial imaging system and staining 8 immunologically relevant targets with spectrally unique fluorophores, we were able to produce high-resolution images of whole tissue sections with spectrally unmixed channels necessary for downstream analysis. Quantitative image analysis measured the spatial distribution of distinct cell populations based on the identified biomarkers within the tumor microenvironment. The analysis software enabled single cell analysis and provided insight to specific localization of myeloid (CD68+ cells) and lymphoid (CD3+, CD4+, CD8+ and CD20+ cells) subpopulations along with proliferating cells in the context of extracellular matrix neighborhoods within a single tissue slice. The results point to the benefit of a spectrally unmixed 9-plex sample analysis (8 biomarkers and a nuclear counterstain) when reviewing the spatial relationships between immune cell types within the tumor microenvironment. Optimizing the spatial amplification reagents, purposely paired to a spatial imager, and combining downstream single cell analysis enables comprehensive immuno-characterization of complex tissues in a single sample.

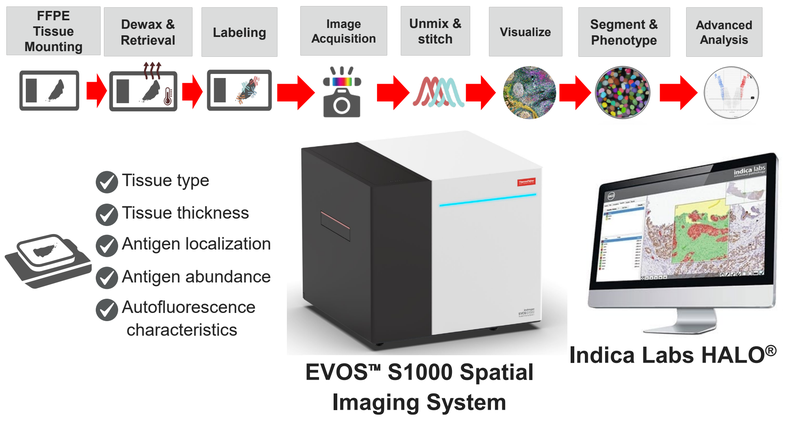
Introduction

Spatial Biology Provides Deep Insights into FFPE Tissue Samples

Ability to analyze single cells in spatial context provides a variety of information
- Cellular phenotyping
- Cell types, cell states and cell functions
- Cell-cell interactions
- Cellular neighborhoods
- Tissue microenvironments and architecture

Workflow

Immunology targets labeled with antibody-based detection, to characterize how cells interact across complex tissues imaged on a high-resolution spatial slide scanner.



Multiplex Labeling for Spatial Biology

Aluora™ Spatial Amplification Kits

Aluora kits utilize tyramide-like signal amplification labeling technique for enhanced sensitivity and intensity. Combining the brightness of Invitrogen™ fluorescent dyes with polyHRP-mediated signal amplification labeling permits high-fidelity multiplexing of a variety of validated antibody clones.

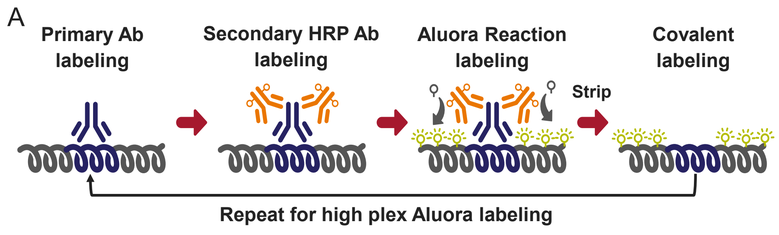
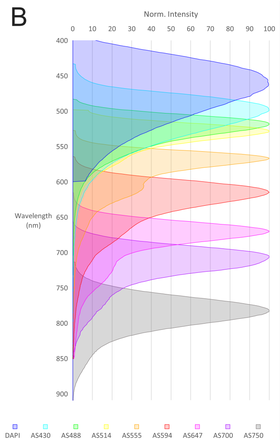


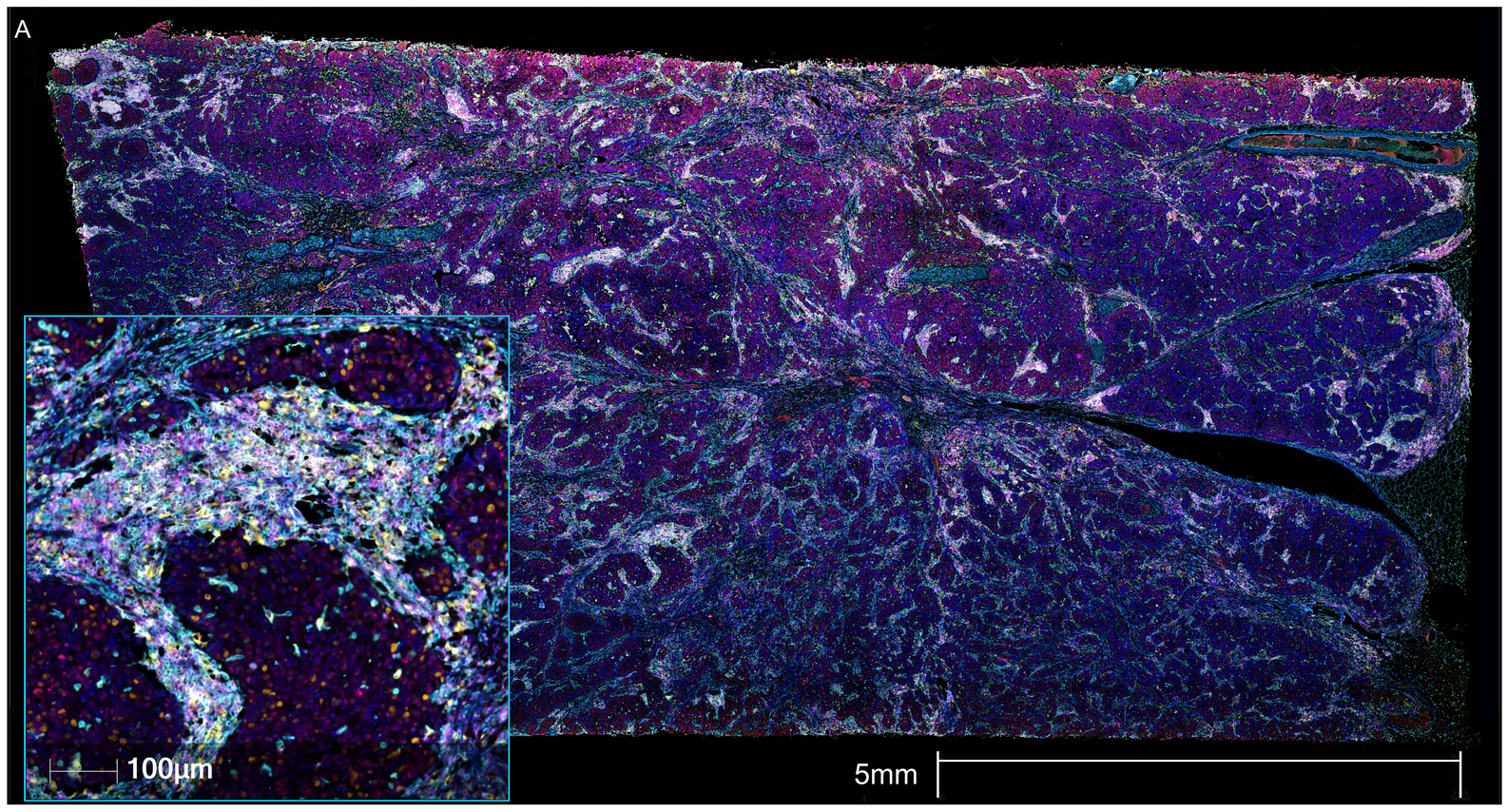
Figure 1. Multiplex labeling workflow with the Aluora kits uses horse radish peroxidase (HRP) for enzymatic conversion of the Aluora dyes to covalently bind tissue on and surrounding the protein epitope targeted primary antibody. A) Diagram of workflow. B) Emission spectra and table of Aluora products.

B



Channel		Ex/Em	PolyHRP-Goat Anti-Mouse IgG Kit	PolyHRP-Goat Anti-Rabbit IgG Kit	HRP-Streptavidin Kit
430	Aluora 430	427/499	A40001329	A40001337	A40001345
488	Aluora 488	493/518	A40001330	A40001338	A40001346
514	Aluora 514	512/529	A40001331	A40001339	A40001347
555	Aluora 555	553/567	A40001332	A40001340	A40001348
594	Aluora 594	589/615	A40001333	A40001341	A40001349
647	Aluora 647	652/670	A40001334	A40001342	A40001350
700	Aluora 700	687/706	A40001335	A40001343	A40001351
750	Aluora 750	757/783	A40001336	A40001344	A40001352

Results



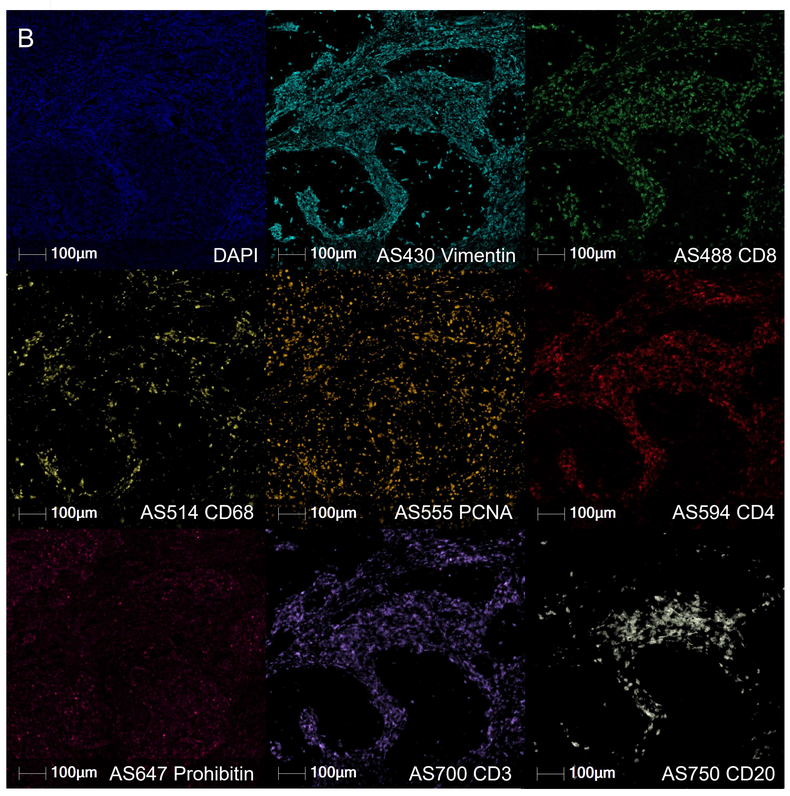


Figure 2. Invasive ductal carcinoma tissue stained with the 8-plex Aluora spatial amplification assay and DAPI. Human invasive ductal carcinoma of breast tissue processed and stained with the Aluora spatial amplification 8-plex assay. A) Composite image with zoomed in inset and B) individual unmixed channels. Images and spectral unmixing were performed on the EVOS S1000 Spatial Imaging System.

Spectral unmixing enables high-plex spatial biology imaging

Our study utilized the Aluora Spatial Amplification 8-plex assay to process and stain human invasive ductal carcinoma of breast tissue. By employing the EVOS S1000 spatial imaging system, we successfully generated high-resolution images of the entire tissue section using a 20x objective (figure 2A). We were able to distinguish the unique staining patterns of the 8 different biomarkers and DAPI (figure 2B). These images were crucial for downstream analysis, as they allowed for precise examination of 8 immunologically relevant targets in context of their specific colocalizations and interactions.

Materials and methods

Sample Preparation

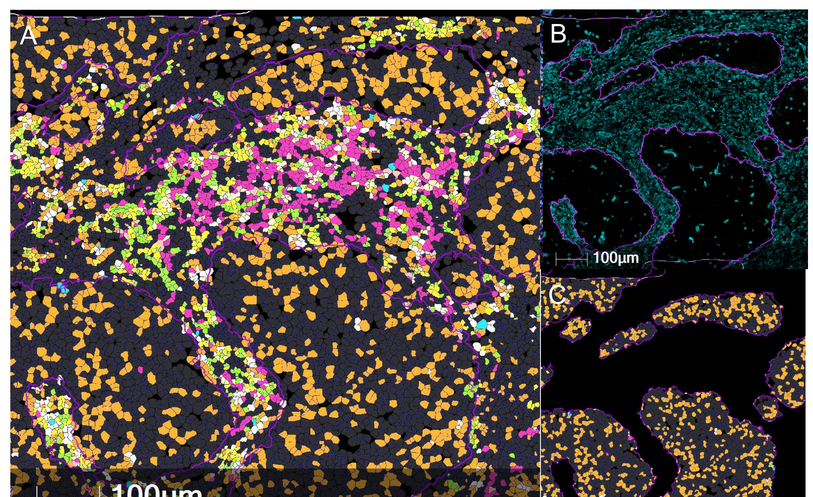
Formalin-fixed, paraffin-embedded human breast invasive ductal carcinoma tissue samples were obtained from Biochain Institute Inc. These slides were then processed using a Leica Bond RXm and stained with primary antibodies (table below) and the Aluora Spatial Amplification kits, as described in figure 1. Images were acquired and spectrally unmixed on the EVOS S1000.

Invitrogen™ primary antibody	Catalog no.	Staining concentration
CD3d Recombinant Rabbit Monoclonal Antibody	MA5-32462	1:1,000
CD4 Monoclonal Antibody (N1UG0)	14-2444-82	1:5,000
CD20 Monoclonal Antibody (L26)	14-0202-82	1:1,000
CD68 Monoclonal Antibody (KP1)	14-0688-82	1:5,000
CD8a Monoclonal Antibody (C8/144B)	14-0085-82	1:1,000
Prohibitin Monoclonal Antibody (II-14-10)	MA5-12858	1:1,000
PCNA Monoclonal Antibody (PC10)	13-3900	1:10,000
Vimentin Monoclonal Antibody (V9)	MA5-11883	1:10,000

Data Analysis

Analysis of the multiplex immunofluorescence stitched image was performed on the Indica Labs HALO (version 4.0.5107.318) software. Manual annotation using the Magnetic Pen identified vimentin positive and vimentin negative regions. Cell detection and phenotyping was performed using the Indica Labs-HighPlex FL version 4.2.14, utilizing the Halo AI Nuclei Seg V2-FL classifier. Phenotypes were identified by positive expression of specified markers in the following table.

Phenotype	Target(s)
B cells	CD20+
Macrophages	CD68+
Cytotoxic T cells	CD3+/CD8+
Helper T cells	CD3+/CD4+
Proliferating cells	PCNA+



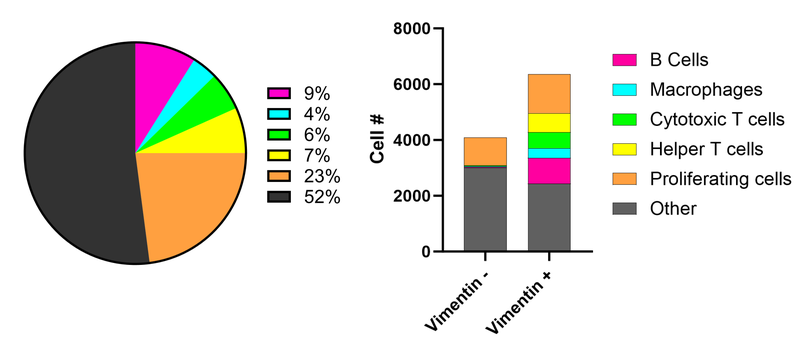


Figure 3. Single cell segmentation and phenotyping reveal spatial distribution of immune cell subpopulations. Human invasive ductal carcinoma of breast tissue analyzed to identify single cell target expression and specific localization of immune cell subpopulations. A) 0.7 mm^2 area analyzed containing 10454 cells, with white cell segments indicating dual positive proliferating & immune cell phenotypes. B) Tissue segmentation identifying vimentin positive and negative regions. C) Identification of region-specific cell localization.

Immune cell populations colocalize with extracellular matrix vimentin

Cellular phenotyping enabled characterization of the 1.06 million cells identified in the 81mm^2 tissue section (figure 3) assigned 25% of the cells as immune cells. 23% of the nonimmune cells were proliferating. We were able to calculate the percentage of both myeloid (CD68+ cells) and lymphoid (CD3+, CD4+, CD8+ and CD20+ cells) subpopulations in specific region of the tissue (figure 3B), specifically within the extracellular matrix vimentin positive area (figure 3C). While proliferating PCNA+ cells are found in both regions, the immune cells are dominantly in the extracellular matrix vimentin neighborhoods of the cancer tissue. This provided crucial insights into the peritumoral restriction of immune cell subpopulations within this section, and information that would not have been evident in a non-spatial, bulk phenotyping assay (flow cytometry/single cell RNA seq).

Conclusions

- With the new spatial proteomic workflows highlighting the synergy between Aluora dyes and EVOS S1000, we were able to produce high-resolution images of whole tissue sections with 9 spectrally unmixed channels necessary for downstream analysis.
- HALO analysis software enabled single cell phenotyping of the tissue revealing immune cell colocalization with vimentin positive areas.

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