Understanding immune phenotypes and their spatial relationships to breast adenocarcinoma in FFPE tissues

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Abstract

The relationship between elements of the immune system and breast tumors in situ requires an approach that leverages multiplexed immunohistochemistry (mIHC) with multispectral imaging to facilitate precise image analyses. To achieve this, we developed a novel 7-color mIHC assay, based on tyramide signal amplification, that allowed us to reliably interrogate PD1, PDL1, CD8, FoxP3, CD68, and cytokeratin, in formalin-fixed, paraffin-embedded (FFPE) samples of human breast cancer. Imaging was performed using the multispectral Vectra system and inForm image analysis software. Using this mIHC panel and the cell segmentations and phenotyping tools in inForm, we were able to reliably identify PD-1+ cytotoxic T cells (PD-1+ CD8+), PD-L1+ regulatory T cells (PD-L1+ FoxP3+), PD-L1+ tumor associated macrophages (PD-L1+ CD68+) and PD-L1+ breast tumor cells (PD-L1+ CK+). With cell phenotypes within the tumor microenvironment determined based on specific co-localized staining combinations, various measures of cell density (per um^2) we employed spatial point pattern analyses to examine spatial relationships between specific phenotypes. With this analysis, we are able to describe distances between PD-1+ cytotoxic T cells and PD-L1+: regulatory T cells; tumor associated macrophages; and breast tumor cells. With this combined mIHC, multispectral imaging and advanced image analysis, we demonstrate a novel method which allows for unique tumor microenvironment assessments within in breast cancer. Through the preservation of tumor architecture available in archival FFPE tissues, these methods can advance our understanding of unique tumor microenvironment interactions, and could provide the ability to stratify responses to immunotherapies.

Methods

To interrogate immune checkpoint expression in breast cancer, *in situ,* a novel soon-to-be released 7-color PD-1 Checkpoint Panel assay was developed to visaulize expression of PD-1, PD-L1, CD8, FoxP3, CD68, and cytokeratin (CK). In brief, tissue samples were de-waxed, rehydrated, and subject to antigen retrieval via microwave. Using a serial immunostaining protocol, called OPAL™, the samples were incubated in anti-PD-L1 for 30’. Samples were then washed, and incubated with a secondary rabbit/mouse HRP polymer for 10’. Immediately post secondray HRP, the samples were incubated with Opal fluorophore for 10’, washed, and subjected to another antigen retrieval. This process was repeated 6 times to incorporate CD8, PD-1, FoxP3, CD68, and CK (Table 1). Finally, samples were rinsed, and incubated with DAPI for 5’, prior to coverslipping. Multispectral images were captured using Vectra™, and image analysis, including phenotyping, was performed using inForm ™. Density measures and spatial point pattern analysis were then performed on all inForm output data.

Opal 7 PD-1 Checkpoint Panel

The Opal 7 PD-1 Checkpoint Panel is a soon-to-be released self-contained assay featuring all primary antibodies for PD-1, PD-L1, CD8, FoxP3, CD68, and cytokeratin, all secondary-HRP reagents, and additional Opal mutliplexing reagents (Figure 1).

Antibody	Series Order	Opal Fluorophore
PD-L1	1	Opal 520
CD8	2	Opal 570
PD-1	3	Opal 540
FoxP3	4	Opal 620
CD68	5	Opal 650
CK	6	Opal 690

Table 1. PD-1 Checkpoint Panel. Fluorophore pairings and order of application for all targets in the PD-1 Checkpoint assay.

Figure 1. PD-1 Checkpoint Panel Kit

Vectra Multispectral imaging

The Vectra 200-slide multispectral system is a highly automated system capable of both 4 and 10x whole slide fluorescent scanning, and 10, 20, and 40x high-power multispectral fluorescent imaging.

Results

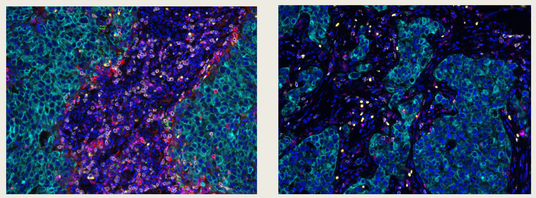


Figure 2. Multiplexed staining for CD8, CD68, FoxP3, PD-1, PD-L1, and Cytokeratin in human breast cancer.
Shown are two examples of the PD-1 Checkpoint Panel on two human breast cancer samples. CD8 is pink, CD68 is magenta, FoxP3 is yellow, PD-1 is red, PD-L1 is green and Cytokeratin is aqua.

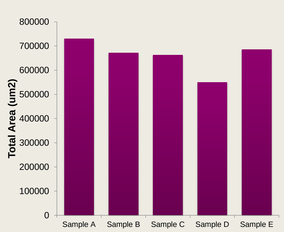
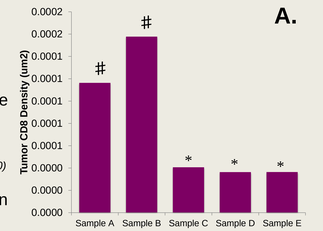
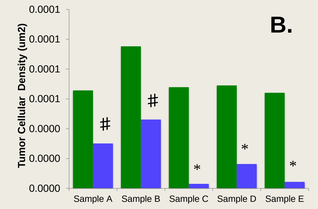


Figure 3. Tumor area analysis in human breast cancer.
Five individual breast cancer samples stained with the PD-1 Checkpoint Panel were subject to multispectral image analysis with Vectra. Tumor area measurements for five 20x fields/case were calculated, and averaged. ANOVA yielded no significant differences between cases. $F_{(4,20)}$ = 0.84109; p=0.551.

Figure 4. Tumor cellular density in human breast cancer.
A. Tumor CD8 densities (magenta) were calculated for the five breast cancer samples. ANOVA indicated significant differences between cases ($F_{(4,20)}$ = 5.562; p<0.05). Similar differences were also observed in the stroma (data not shown). B. Based on significant CD8 density differences, FoxP3 (blue) and CD68 (green) densities were calculated, with significant differences observed for FoxP3 (blue; t = 3.726, df = 3, p<0.05), but not for CD68 (green; t = 1.210, df = 3, p = N.S.). Similarly, these difference were also observed in the tumor-assocaiated stroma (data not shown). For all, * < #





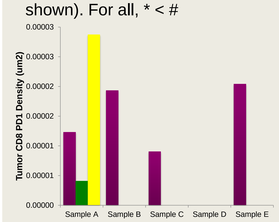
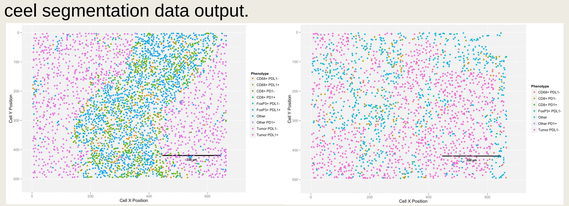


Figure 5. Assessment of PD-1 and PD-L1 on cellular phenotypes in human breast cancer.
Density assessments of CD8+ PD-1+ (magenta), CD68+ PD-L1+ (green), and CK+ PD-L1+ (yellow) within the tumor compartment of all cases, revealed no significant differences, based on CD8 densities.

Figure 6. Phenotype locations in human breast cancer.
Examples of two PD-1 Checkpoint Panel stained human breast cancer samples with phenotype locations determined from inForm ceel segmentation data output.



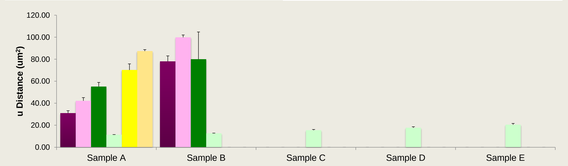
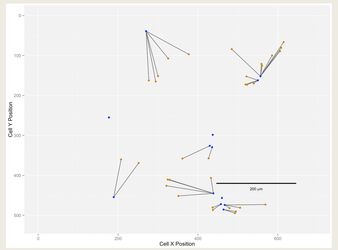
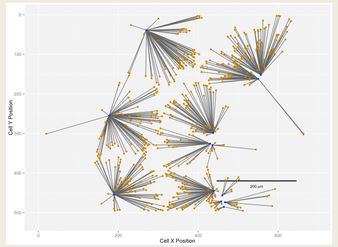


Figure 7. Assessment of cellular phenotype proximities in human breast cancer. Average distances (um^2) for phenotype proximities between: CD8+ PD1+ and CD68+ PDL1+ (magenta); CD8+ PD1- and CD68+ PDL1+ (pink); CD8+ PD1+ and FoxP3+ PDL1+ (green); CD8+ PD1- and FoxP3+ PDL1+ (light green); CD8+ PD1+ and CK+ PD-L1+ (yellow); and CD8+ PD1- and CK+ and PD-L1+ (light yellow).

Figure 8. Spatial point patterning in human breast cancer.
Examination of spatial relationships between CD8+ and CD68+ phenotypes using nearest-neighbor analyses. A. Analysis of nearest CD8+ PD1+ (orange) to each neighbor CD68+ PDL1+ (blue). B. Analysis of nearest CD8+ PD1- (orange) to each neighbor CD68+ PDL1+ (blue). Nearest-neighbors indicated by solid line connection.





Summary and Conclusions

Here we present the results of a novel soon-to-be released 7-color PD-1 Checkpoint Panel we have developed, based on tyramide signal amplification, that allowed us to reliably interrogate PD-1, PD-L1, CD8, CD68, FoxP3, and cytokeratin, in formalin-fixed, paraffin-embedded (FFPE) samples of human breast cancer.

- The PD-1 Checkpoint Panel facilitated an effective 7-color multiplex IF stain.
- Interrogation of the multiplexed samples with the Vectra multispectral imaging system allowed for effective signal unmixing to support accurate phenotyping.
- Tumor density assessment of CD8+ lymphocytes demonstrated significant differences among breast cancers.
- Spatial analyses of multiple phenotype densities and interactions distance, enabled by the PD-1 Checkpoint Panel, revealed differences based on CD8 densities.

This demonstrates a novel multiplex staining approach for checkpoint analysis in the breast cancer tumor microenvironment. In larger cohort analyses of tumor architecture, these multiplex staining and multispectral imaging tools can advance our understanding of unique tumor microenvironment interactions.