A Fully-Automated Multiplex Fluorescence IHC Assay with Whole Slide Multispectral Imaging on Mouse Tissue: Phenoptics™ Quantitative Pathology Solutions Translational Workflow

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1 Background

Phenoptics™ quantitative pathology solutions (QPS) is a comprehensive, end-to-end solution consisting of multiplex fluorescence immunohistochemistry staining along with multispectral imaging and tissue analysis.

Here we describe a robust, fully-automated 7-color Opal staining procedure on Formalin-fixed paraffin-embedded (FFPE) section of mouse breast cancer followed by a multispectral whole slide scan and image analysis.

2 Methods

Mouse Breast Cancer Model

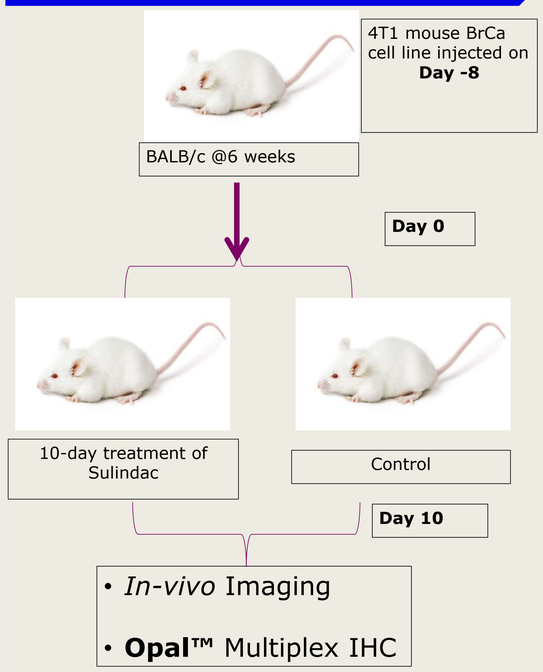


Fig 1. Mouse Breast Cancer Model. BALB/c mice (6 weeks old from Charles River lab) were injected subcutaneously with 1×10^6 4T1 tumor cells in the right flank. Eight days after inoculation, tumor-bearing mice were treated intraperitoneally with or without with 60 mg/kg Sulindac once daily for consecutive 10 days.

Opal Polaris 7-Color Assay & Multispectral imaging on Vectra Polaris

Ten days after Sulindac treatment, tumors from both untreated and treated group were collected then fixed with Formalin for processing to formalin fixed paraffin embedded (FFPE) blocks. Tissue sections from mouse breast cancer FFPE blocks were immunostained using Opal™ Polaris 7-Color Automation IHC kit and Opal Polymer Anti-Rabbit HRP Kit on Leica BOND RX ™ automated stainer. Multispectral fluorescence imagery was acquired on a Vectra Polaris® automated imaging system and analyzed with inForm®, Phenochart®, Image J and R software.

Mouse *in-vivo* Imaging

Non-invasive assessment of Sulindac effects on tumors were assessed on day 2 and 10 post-treatment by fluorescent imaging on the IVIS® SpectrumCT. Treated and untreated 4T1 tumor-bearing mice were anesthetized and injected with either AngioSense 750 EX (AS750, NIR fluorescent vascular probe) or ProSense 750EX (PS750, NIR fluorescent cathepsin activatable probe). Epifluorescence images were captured 24h later, and flank tumor regions were quantified by careful analysis using Living Image® 4.5.

3 Results:

7-Color Whole Slide Multispectral Imaging

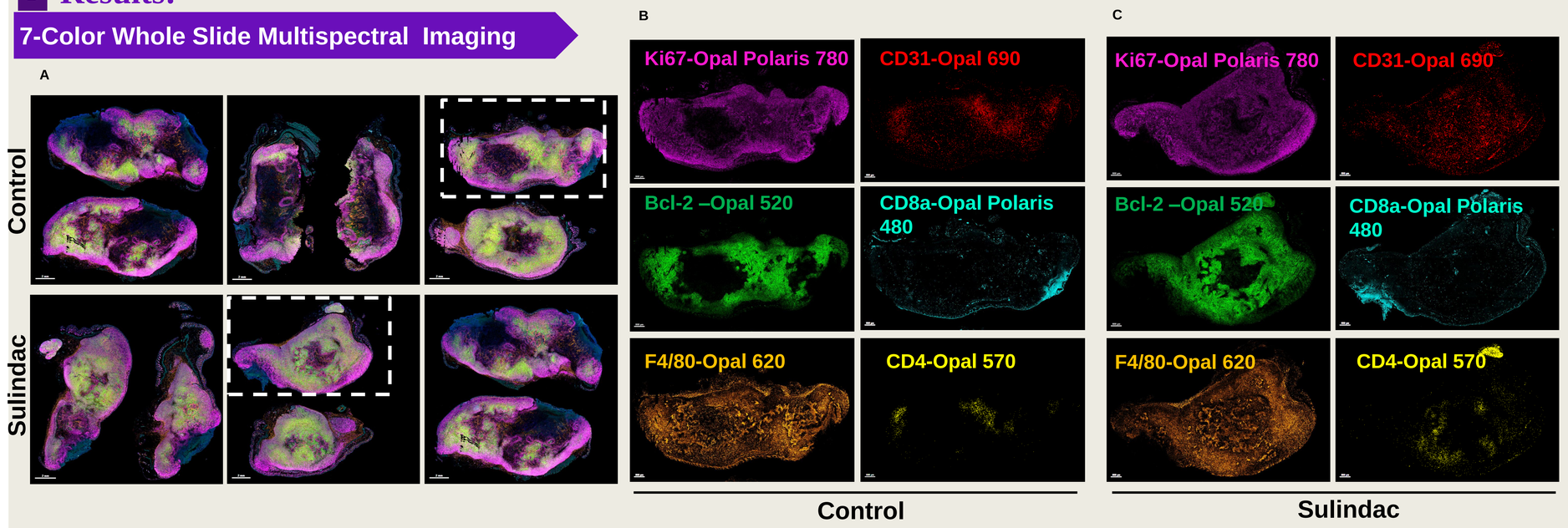
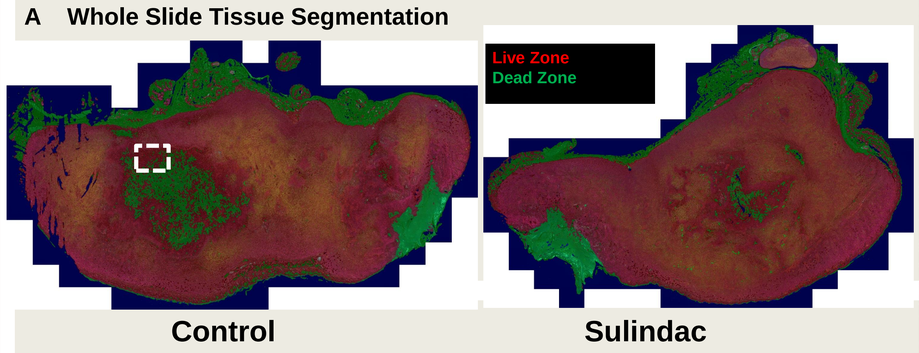


Fig 2. Whole Slide Scan of Opal Polaris 7-Color Fluorescent IHC Assay on Mouse Breast Cancer FFPE Section. A) Whole slide MSI of mouse breast cancer section from control group (top) and 10-day Sulindac treatment group (bottom). Functional classes represented in the multi-marker panel include: Proliferative cells (Ki67), Tumor infiltration lymphocytes (CD4 & CD8), Tumor associated macrophage (F4/80), Angiogenesis (CD31) & Anti-apoptotic cells (Bcl-2). Component planes for each individual marker from the whole slide scan from control group **(B)** and Sulindac treated group **(C)**.

Whole slide Tissue Segmentation and Cell Phenotyping



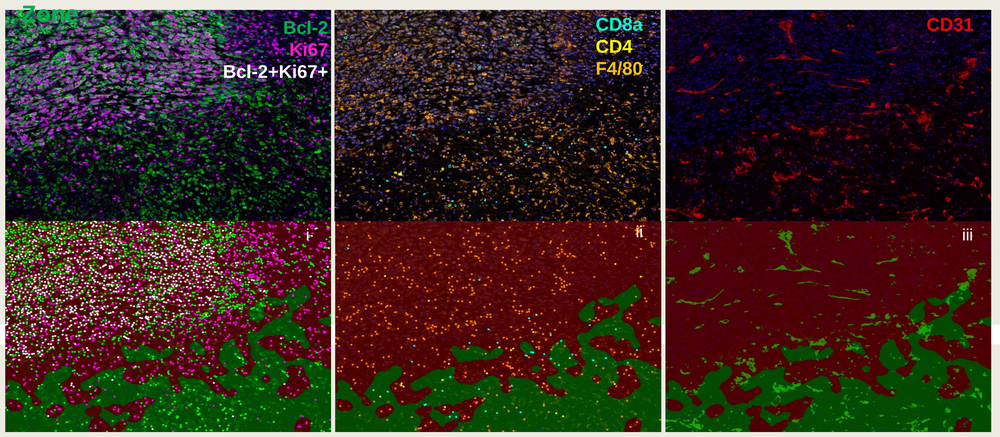
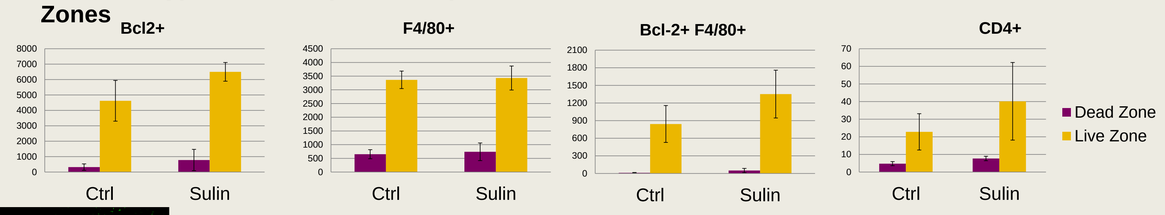
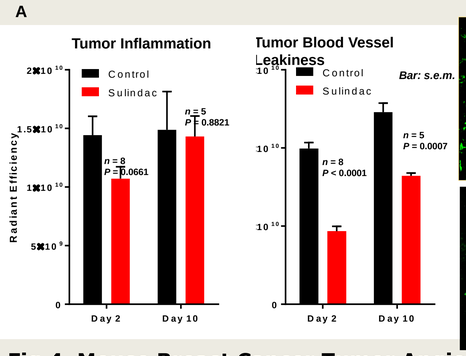
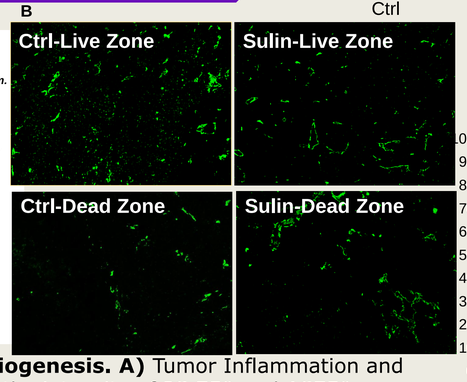


Fig 3. Immune Profiling on Whole Slide View Based on Opal Polaris 7-Color Fluorescent IHC. A) Representative image from whole slide tissue segmentation of mouse breast cancer section based on Ki67 staining (Ki67+: Live Zone & Ki67-: Dead Zone). **B)** Zoomed in views of composite images (top) and tissue segmentation with cell phenotypes(i&ii) or CD31 object segmentation (iii) (bottom). **C)** Cell densities of each phenotype marker within Live Zone and Dead Zone of each tissue section.



Sulindac Reduces Tumor Neovascularization





Tumor Proliferation

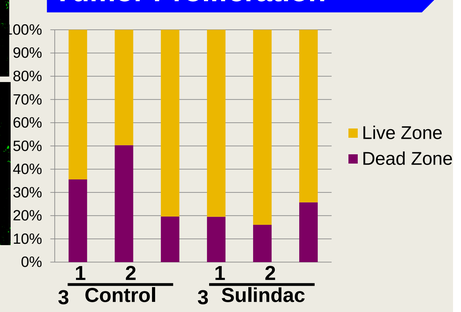


Fig 4. Mouse Breast Cancer Tumor Angiogenesis. A) Tumor Inflammation and Angiogenesis/Leakiness were measured by the intensity of PS 750 and AS750 respectively using *in-vivo imaging.* Data is represented as an average of tumor Radiant Efficiency, a measure of total fluorescence in the tumors. **B)** Representative CD31 staining component planes from Fig. 2

In-vivo imaging results give an overall information that Sulindac effectively reduces tumor angiogenesis. Additionally, CD31 staining from multiplex IHC shows the heterogeneous nature of breast cancer samples and indicates the mechanism of reduced angiogenesis in Sulindac treated group due to reduced micro vessels in the Live Zone of tumor.

Fig 5. Proliferation of Mouse Breast Cancer Tumors. Percentages of Live Zone and Dead Zone of each tumor were calculated by using areas from tissue segmentation based on Ki67 staining.

4 Conclusions

Developing biomarker research strategies for clinically relevant therapies in immuno-oncology (IO) is predicated on the ability to execute fully translational research studies. Here, we demonstrated whole slide analysis utilizing the novel Opal Polaris 7-Color assay plus Opal Polymer anti-Rabbit HRP. The results showed unique tumor vascular signatures and immune context heterogeneity between tumors as well as within tumors. This new application has expanded Phenoptics into whole slide IO tissue research including animal studies, creating a truly translational platform.

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