Advances in multiplex fluorescence immunohistochemistry: 9-color imaging; whole slide multispectral

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1 Background

We describe two advances in multispectral fluorescence immunohistochemistry (fIHC), a powerful tool for quantifying interactions within the tumor microenvironment.

1. A **fully-automated 8-plex, 9-color assay plus DAPI counterstain** on the same tissue section.
2. A novel scanning method that produces **a multispectral whole slide scan of 6 markers plus DAPI counterstain in ~6 minutes** (1x1.5 cm tissue section).

2 Methods

FFPE samples of primary tumors were immuno-stained using Opal™ reagents manually or on a Leica BOND RX™. Imagery was acquired on a Vectra Polaris® automated imaging system and analyzed with inForm®, MATLAB®, and R software.

Multiplex Staining with Opal Reagents

Opal™ reagents allow multiplex fIHC staining with signal amplification and any combination of mouse and/or rabbit primary antibodies.

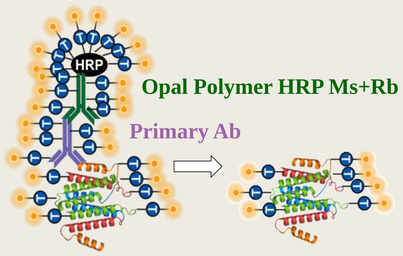
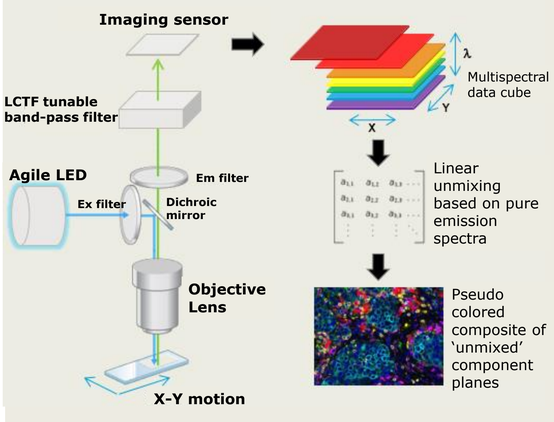


Fig 1. Opal™ Detection. The Opal Polymer HRP amplifies IHC detection by covalently depositing multiple Opal fluorophores near the detected antigen. Then, antibodies are stripped to allow for sequential labeling of multiple markers.

Multispectral Imaging on Vectra Polaris

Fig. 2. Multispectral imaging on the Vectra Polaris is built upon an epifluorescence light path (below, left). Different combinations of agile LED bands, bandpass excitation filters, bandpass emission filters, and a liquid crystal tunable filter (LCTF) are used to select narrow spectral bands that reach the imaging sensor.



For each spectral band, an image is acquired and added to a 'data cube' that contains up to 40 spectral layers (above, right). The data from all spectral layers is then linearly unmixed using previously-determined pure emission spectra for each fluorophore using inForm® software. Intensity values in the resulting 'unmixed' image are directly related to the amount of each dye present.

3 Results: 9-color Multispectral Imaging

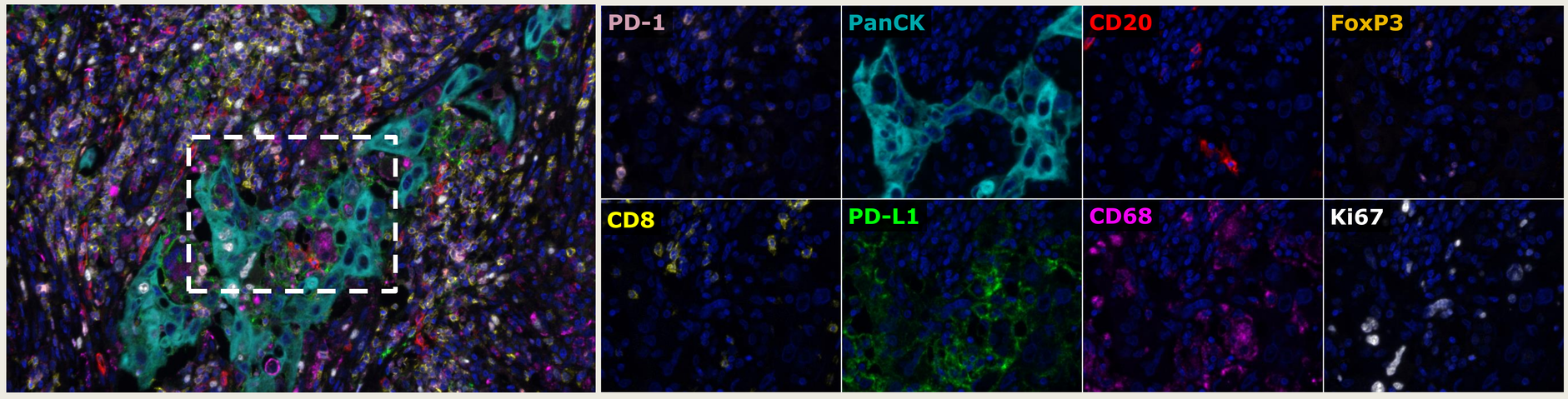
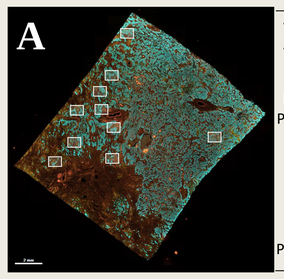


Fig. 3. 8-plex, 9-color panel on human lung cancer section. Two new Opal™ reagents (Opal Polaris 480 and Opal Polaris 780) were combined with the currently available Opal 7-Color Automation IHC Kit to stain and distinguish 8 markers plus DAPI when imaged on the Vectra Polaris®: CD20 (Opal Polaris 480), PD-L1 (Opal 520), CD8 (Opal 540), FoxP3 (Opal 570), CD68 (Opal 620), PD-1 (Opal 650), Ki67 (Opal 690), and PanCK (Opal Polaris 780). Colors assigned to each marker, and associated component planes, are shown on the right.



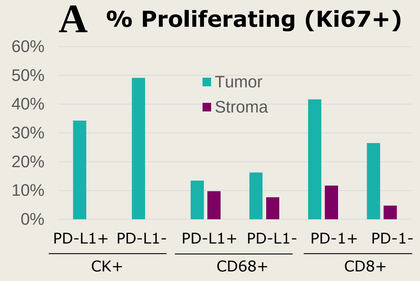
B Phenotype Densities (cells/mm²)

		PD-L1+	CD20+	CD68+	CD8+	FoxP3+	Ki67+	PD-1+	Total Cells
Individual Phenotypes	Stroma	1161	511	813	1169	128	804	690	9779
	Tumor	888	5	239	136	32	1817	116	5239

		CD68+ PD-L1+	CD68+ PD-L1-	CD8+ PD1+	CD8+ PD1-	CD8+ FoxP3+	CD8+ FoxP3-
Double Phenotypes	Stroma	289	523	101	1068	5	1163
	Tumor	91	148	22	115	1	135

Fig 4. Phenotype densities from 9-color panel. A) Whole slide view of lung cancer section shown above in Fig. 3. Multispectral fields were acquired at the locations indicated by the white boxes. **B)** Average densities of each phenotype marker within the acquired fields are shown for each section. Tissue was categorized into tumor and stroma regions using CK positivity by a trained segmentation algorithm in inForm® software. PD-L1 positivity was determined by a fixed intensity threshold; all other phenotypes were determined by a trained inForm® phenotyping algorithm.

A % Proliferating (Ki67+)



B PD-1/PD-L1 proximity

PD-1+ proximity to PD-L1+	
% PD-1+ cells that are within 30 µm of a PD-L1+ cell	57%
Average # PD-L1+ cells within 30 µm of each PD-1+ cell	4.1

PD-L1+ proximity to PD-1+	
% PD-L1+ cells that are within 30 µm of a PD-1+ cell	59%
Average # PD-1+ cells within 30 µm of each PD-L1+ cell	1.9

Fig 5. Cell proliferation (Ki67+) and PD-L1/PD-1 proximity assessment. The 8 immune markers combine to generate more than 20 phenotypes relevant to immuno-oncology. A subset of these markers were studied in relation to proliferation state **(A)** and local PD-L1 proximity **(B)** to characterize the tumor microenvironment**.** For example, **(A)** shows that CD8+ and CD68+ cells were more likely to be proliferating (Ki67+) in tumor vs. stroma, with differences in proliferation observed in the presence or absence of PD-1 or PD-L1. **(B)** shows the high proximity between PD-1+ and PD-L1+ cells in this sample.

4 Results: 7-color Multispectral Whole Slide Scans

Field-based multispectral imaging workflows can accommodate a wide range of fluorophores and up to 9 colors, but can be time consuming as they require up to 50 spectral layers to unmix 9 fluorophores, and often require exposure times in the hundreds of milliseconds.

We have developed complementary high-throughput multispectral scanning approach by optimizing a multispectral workflow for a specific set of 7 fluorophores.

High-throughput multispectral scanning and unmixing performed comparably to field-based multispectral imaging, and outperformed conventional scanning by:

- **Reducing autofluorescence contributions** for all immune markers, **lowering the limit of detection and extending the dynamic range** of some channels by more than 30-fold.
- **Reducing crosstalk** from more than 8% to under 3% (typically <0.5%), thereby **increasing signal accuracy** and reducing false colocalization between non-colocalized markers.

For more details on 7-color whole slide MSI, see related posters via QR code at top right.

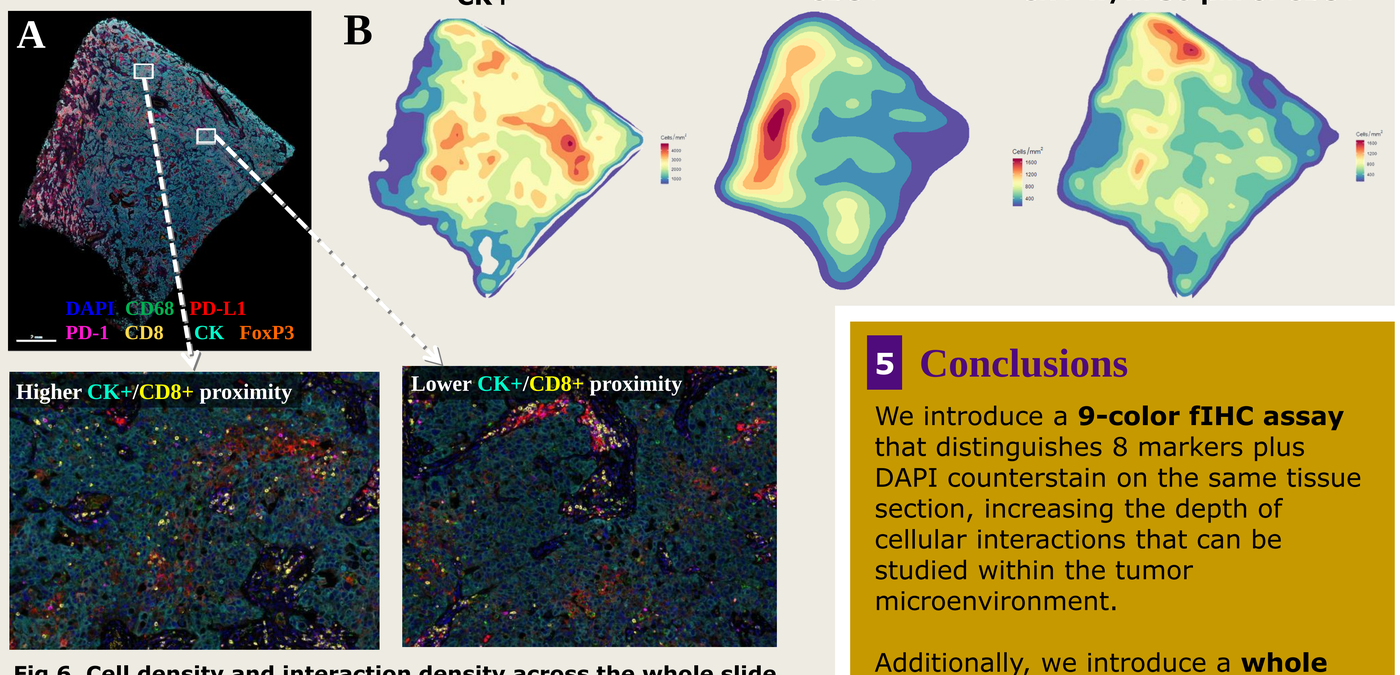


Fig 6. Cell density and interaction density across the whole slide. A) Whole slide MSI of human lung cancer section captured in 6 minutes, shown as composite image with marker colors indicated in key. Cells were phenotyped in inForm®, and interactions assessed with R and phenoptr. **B)** Density contours of CK+ (left), CD8+ (middle), and CK+ within 30 µm of a CD8+ cell (right). **Bottom)** Zoomed in views of A) illustrate differences in CD8+ T-cell (yellow) infiltration within the tissue.

5 Conclusions

We introduce a **9-color fIHC assay** that distinguishes 8 markers plus DAPI counterstain on the same tissue section, increasing the depth of cellular interactions that can be studied within the tumor microenvironment.

Additionally, we introduce a **whole slide multispectral imaging** method that provides rich quantitation of interactions among 6 markers at length scales spanning from cell biology to tumor physiology.

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