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*We've rebranded some of our products.* ***Phenoptics™*** *is now* ***PhenoImager™.***
*and* ***Vectra®*** ***Polaris™*** *is now* ***PhenoImager™*** ***HT****.*

# 8-plex, 9-color Multispectral Imaging

## PUSHING THE BOUNDARIES: OPAL FLUOROPHORES 480 AND 780 OPEN UP A NEW WORLD OF POSSIBILITIES FOR MULTISPECTRAL IMAGING

### Introduction

In the arena of cancer research, new advancements in tissue analysis and biomarker detection are critical to the development of more precise, targeted therapies. Now, more than ever, there is significant emphasis on understanding the underlying interaction between the immune system and the tumor microenvironment and its role in disease progression. To this end, immunofluorescence has greatly increased our understanding of solid tumor biology and immunology, including tumor-infiltrating lymphocytes and cancer-induced architectural alterations, and aided in novel immunology discoveries.

Previously released Opal™ Multiplex 4- and 7-Color Immunohistochemistry (IHC) detection kits from Akoya Biosciences have paved the way for the establishment of multiplexed IHC methods as a preferred method for analysis of cellular composition and interactions in FFPE tissue sections. Open and flexible for use with any primary antibody, researchers can be confident that there will be no cross-reactivity within their samples. Additionally, more information can be gathered from limited samples, including multiple cell phenotypes, and spatial and morphological information that is often neglected with other data collection methods.

We are excited to introduce two new Opal fluorophores, Opal Polaris 480 and Opal Polaris 780. These fluorophores can be acquired either as part of our 7-color Opal Multiplex IHC (Figure 1) or individually, to use in conjunction with the existing 7-Color Opal Detection kits (Table 1). In addition, we will offer the necessary imaging platforms and filters (Table 2) to use with the new fluorophores, which will allow researchers to conduct rapid 7-color whole slide multispectral imaging, as well as the capability of capturing up to eight IHC targets of interest, along with DAPI nuclear stain.

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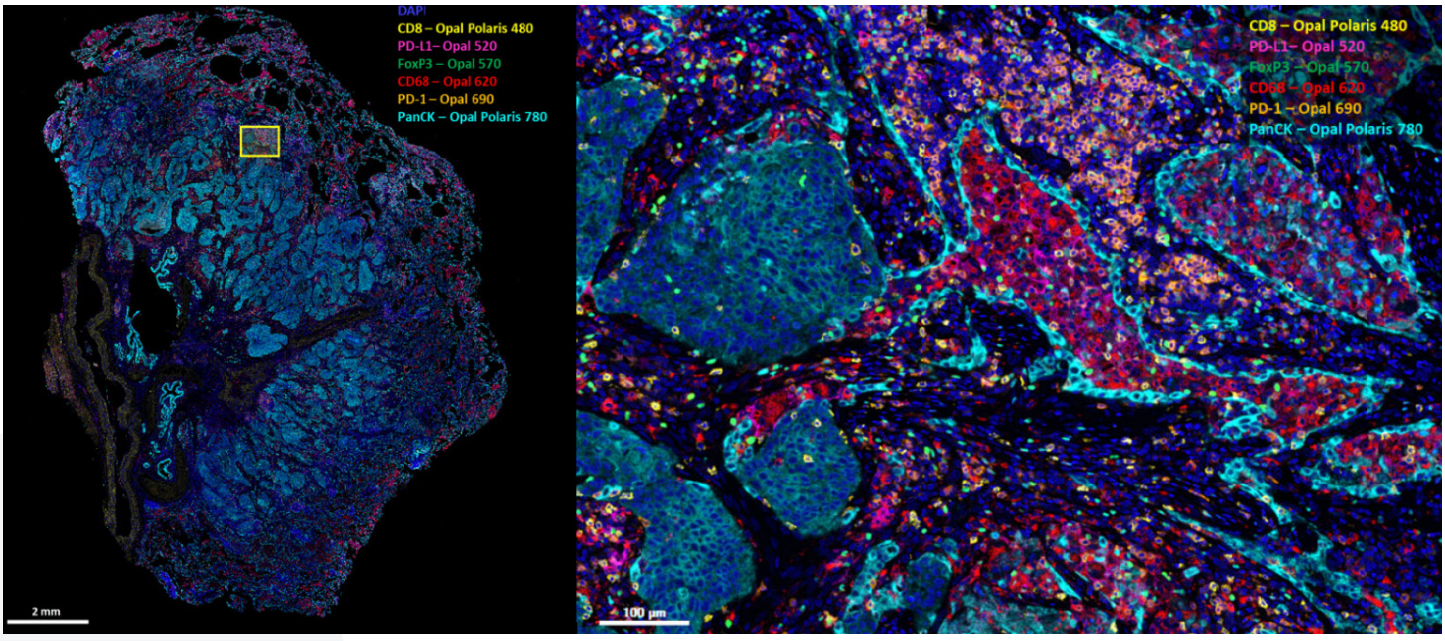


**FIGURE 1**. (Left) Whole slide imaging using 7-color Opal IHC Multiplex staining with new fluorophores, Opal Polaris 480 and Opal Polaris 780, in lung cancer. Image displays cytotoxic T-cells in yellow (CD8), programmed death ligand 1 on cytotoxic T-cell suppressors in magenta (PD-L1), regulatory T-cells in green (FoxP3), macrophages in red (CD68), programmed death receptor 1 on activated/ exhausted T-cells in orange (PD-1), pan cytokeratin stained epithelial tumor cells in cyan (PanCK), and DAPI nuclear counterstain in blue. (Right) 10x magnification of a selected field of view of the tissue sample.

## OPAL FLUOROPHORE EXCITATION AND EMISSION SPECTRA

**TABLE 1.** Opal fluorophores included with each of the three IHC kits, as well as their excitation peak, emission peak, and corresponding cap color.

| FLUOROPHORE | OPAL MULTICOLOR IHC KITS | | | EXCITATION | EMISSION | CAP COLOR |
|---|---|---|---|---|---|---|
| | 4-COLOR | 7-COLOR | 7-COLOR | | | |
| **Spectral DAPI** | √ | √ | √ | 368 nm | 461 nm | Blue |
| **Opal Polaris 480** | | | √ | 450 nm | 500 nm | Violet |
| **Violet Opal 520** | √ | √ | √ | 494 nm | 525 nm | Green |
| **Opal 540** | | √ | | 523 nm | 536 nm | Yellow |
| **Opal 570** | √ | √ | √ | 550 nm | 570 nm | Red |
| **Opal 620** | | √ | √ | 588 nm | 616 nm | Amber |
| **Opal 650** | | √ | | 627 nm | 650 nm | Orange |
| **Opal 690** | √ | √ | √ | 676 nm | 694 nm | Clear |
| **Opal Polaris 780** | | | √ | 750 nm | 770 nm | Pink |

## OPAL FLUOROPHORE AND IMAGING FILTERS

**TABLE 2.** Opal fluorophores and the necessary filters to conduct MSI. The Mantra and PhenoImager™ HT (formerly Vectra® Polaris™) require the Liquid Crystal Tunable Filter (LCTF) to conduct 8-plex, 9-color MSI. The upgraded filter cube for the PhenoImager HT is required for 7-color whole slide unmixing in combination with the Opal™ 7-Color IHC kit. Whole slide MSI cannot be conducted without the filter cube upgrade or and Opal Polaris 780 dyes.

| FLUOROPHORE | FILTERS | | |
|---|---|---|---|
| | MANTRA | PHENOIMAGER HT | |
| | 9-Color Unmixing with LCTF | 7-Color Whole Slide Unmixing | 9-Color Unmixing with LCTF |
| **Spectral DAPI** | DAPI | DAPI + Opal 570/690 | DAPI + Opal Polaris 780 |
| **Opal Polaris 480** | TBD | Opal Polaris 480/620/780 | Opal Polaris 480 + Cy5 |
| **Opal 520** | FITC | Opal 520 | FITC |
| **Opal 540** | FITC, Cy3 | N/A | FITC, Cy3 |
| **Opal 570** | FITC, Cy3, Texas Red | DAPI + Opal 570/690 | FITC, Cy3, Texas Red |
| **Opal 620** | Cy3, Texas Red | Opal Polaris 480/620/780 | Cy3, Texas Red |
| **Opal 650** | Texas Red, Cy5 | N/A | Texas Red, Opal Polaris 480 |
| **Opal 690** | Cy5 | DAPI + Opal 570/690 | Opal Polaris 480 + Cy5 |
| **Opal Polaris 780** | Cy7 | Opal Polaris 480/620/780 | DAPI + Opal Polaris 780 |

**The Benefits of 8-Plex, 9-Color MSI**

One of the biggest limitations in traditional immunofluorescence imaging is the number of IHC targets of interest that can be investigated. Typically, only two to three markers of interest can be studied, not including DAPI nuclear stain. This significantly limits the cell-level biological detail that can be gathered from tissue sections. Furthermore, images may contain significant background autofluorescence, ultimately reducing the range of expression that can be measured, sometimes obscuring expression or leading to false signal.

Multispectral imaging removes these confounding factors by spectral unmixing, which using reference spectra (libraries) to completely remove cross talk between fluorophores, ensuring that the fluorophore signals are accurately captured, while simultaneously removing autofluorescence from the images. You can be confident in the data you collect and analyze.

The addition of the Opal Polaris 480 and 780 fluorophores now greatly expands upon the capabilities that Akoya Biosciences offers. With the addition of the PhenoImager HT platform, 8-plex, 9-color Multiplex IHC becomes a practical reality, enabling deeper understanding of the underlying biology driving disease progression and response, allowing users to discover new biomarkers and to test hypotheses. This new technology makes it possible to phenotype more than 500 cell types defined by different co-expressions, and to observe more cell-to-cell interactions and in-depth architectural details. The complexity of assay validation is somewhat greater for nine colors compared to seven colors, and one can capture twenty 20x multispectral fields for analysis plus a non-spectral whole-slide 20x reference image in as little as 20 minutes (Figure 2).

Together, the addition of these fluorophores to the Opal lineup pushes the boundaries of immuno-oncology research and provides greater visualization and insight into the interplay between tumor and immune cells within the tumor microenvironment.

**TABLE 3.** Opal 7-Color IHC Kits contain the following fluorophores: 520, 540, 570, 620, 650, 690, and spectral DAPI.

| DESCRIPTION | PRODUCT # |
|---|---|
| **Opal 7-Color Manual IHC Kit** | NEL811001KT |
| **Opal 7-Color Automation IHC Kit** | NEL821001KT |
| **Opal 7 Immunology Discovery Kit** | OP7DS2001KT |
| **Opal 7 Tumor Infiltrating Lymphocyte Kit** | OP7TL3001KT |
| **Opal 7 Solid Tumor Immunology Kit** | OP7TL4001KT |

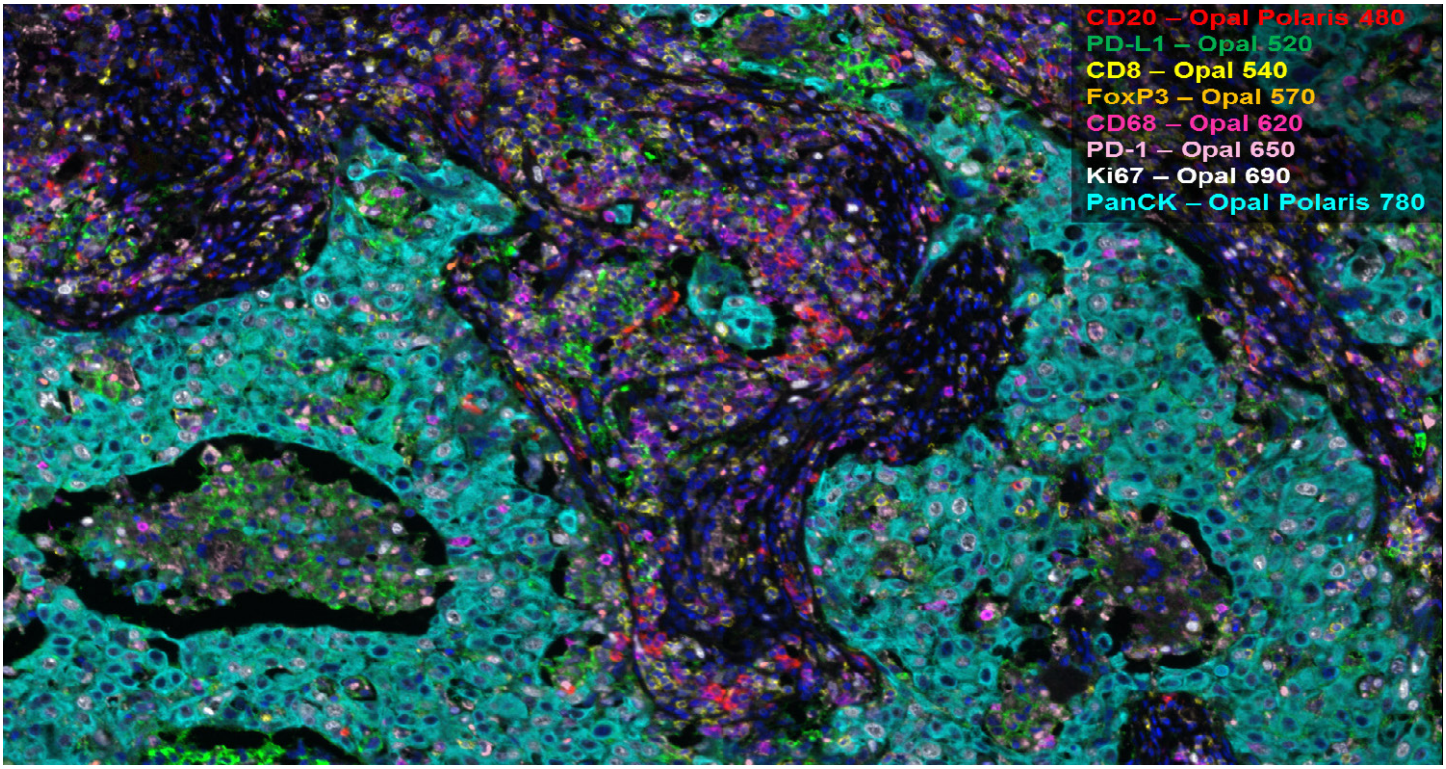


**FIGURE 2**. 9-color Opal IHC staining of lung cancer. Image displays B-cell lymphocytes in red (CD20), programmed cell death ligand 1 on cytotoxic T-cell suppressors in green (PD-L1), cytotoxic T-cells in yellow (CD8), regulatory T-cells in orange (FoxP3), macrophages in magenta (CD68), programmed cell death receptor 1 on activated/ exhausted T-cells (PD-1), nuclear marker for cellular proliferation (Ki67), pan cytokeratin stained epithelial tumor cells in cyan (PanCK), and DAPI nuclear counterstain in blue.

### How to Order for 8-plex, 9-color Multispectral Imaging

To unlock this new technology, individual Opal Reagent Packs for fluorophores 480 (FP1500001KT) and 780 (FP1501001KT) can be purchased separately to work in conjunction with one of the 7-color IHC kits listed below (Table 2).

### Staining Protocol for Opal Polaris 480 and Opal Polaris 780

Opal users can easily assimilate the Opal Polaris 480 fluorophore into their already established protocols for formalin fixed paraffin embedded tissue. Staining with Opal Polaris 480 follows Steps 3-7 in the workflow schematic, similar to Opal fluorophores 520, 540, 570,

620, 650, and 690 (Figure 3), as the Opal signal will not be detrimentally affected by heat treatment for antibody removal. Integration of Opal Polaris 780 is a two-step process that is necessary to complete after all other Opal fluorophores have been used, as this is an antibody-based staining step, and heating of the slide cannot be performed after it has been applied to the marker of interest (Step 13).

| | | |
|---|---|---|
| **Step 1** | Slide Preparation | |
| **Step 2** | Epitope Retrieval | |
| **Step 3** | Blocking | ***Repeat steps 3-7 for each primary antibody and corresponding Opal fluorophore, with the exception of Opal 780 (see steps 8-13).*** |
| **Step 4** | Primary Antibody Incubation | |
| **Step 5** | Introduction of Opal Polymer HRP | |
| **Step 6** | Signal Ampliftcation | |
| **Step 7** | Antibody Stripping | |
| **Step 8** | Blocking | |
| **Step 9** | Primary Antibody Incubation | |
| **Step 10** | Introduction of Opal Polymer HRP | |
| **Step 11** | Introduction of Opal TSA-DIG | |
| **Step 12** | Antibody Stripping | |
| **Step 13** | Opal Polaris 780 Signal Generation | |
| **Step 14** | DAPI Counterstain and Mount | |

**FIGURE 3.** Opal 7-Color and Opal 9-Color Workflow Schematic.





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