

Opal Multiplex IHC Assay Development Guide and Image Acquisition Information

Phenoptics Research Solutions

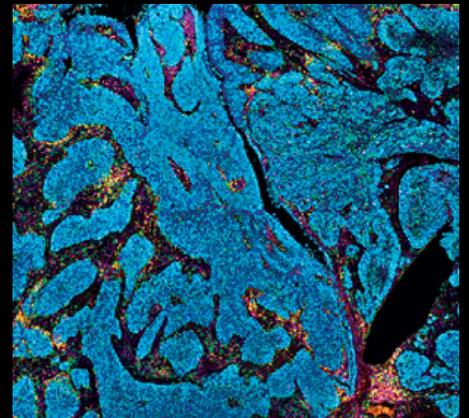
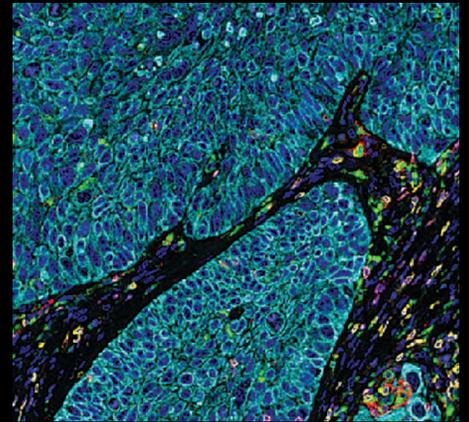
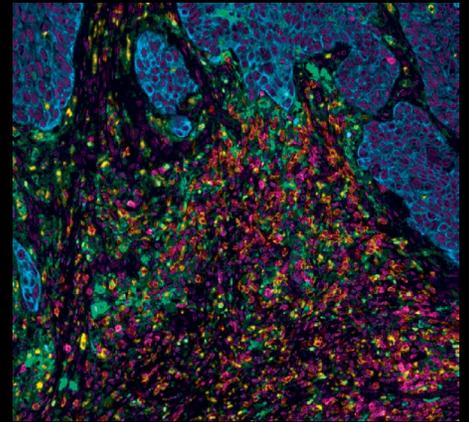
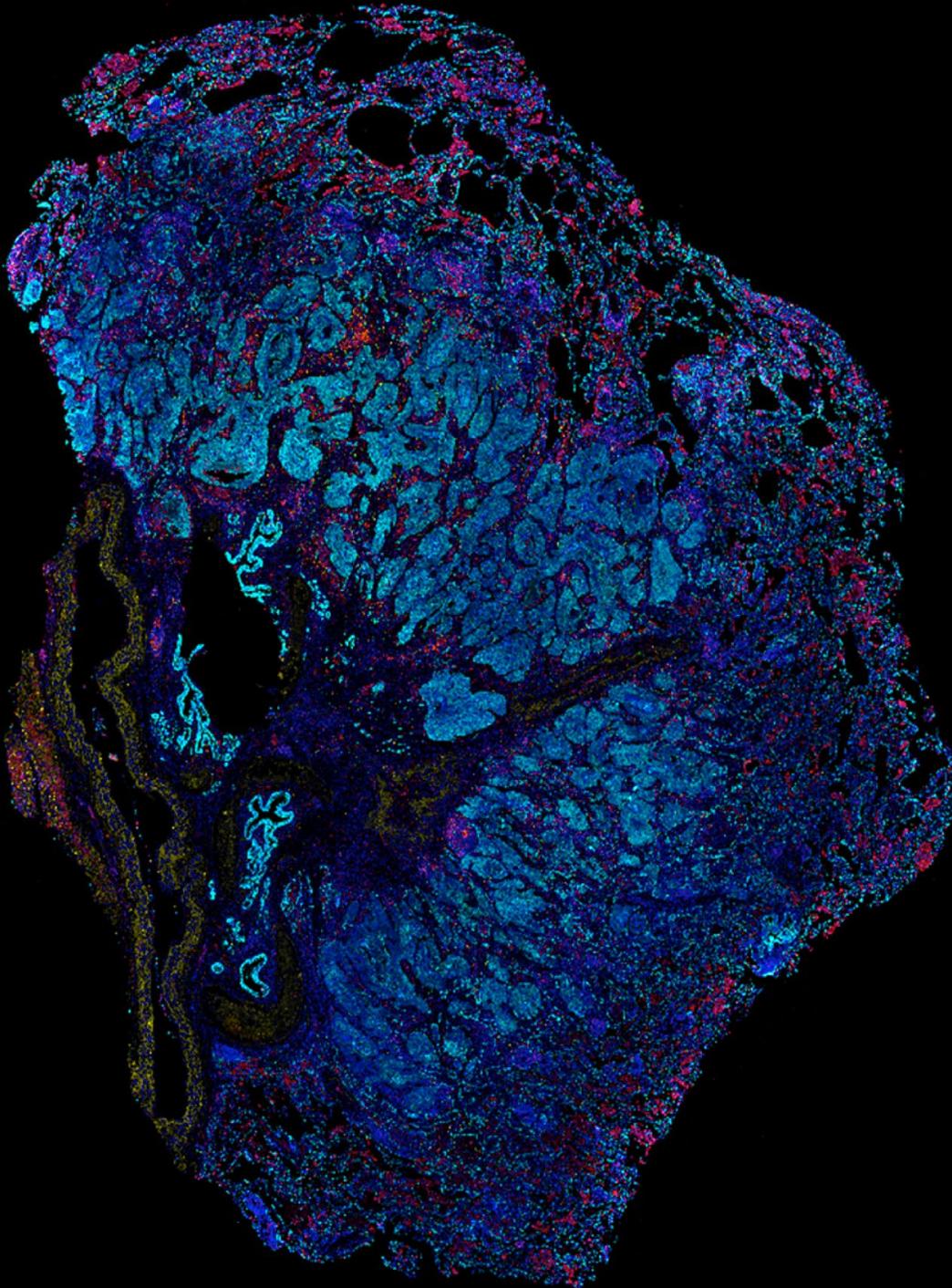


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■ Preface

Welcome to the world of Phenoptics and Opal reagents!

This guide will act as your reference for all things pertaining to our Opal product line, including assay development and optimization using both manual and automated techniques, product notes for each imaging system, an image analysis guide, and frequently asked questions/troubleshooting. The goal of this document is to act as a starting point for new Opal users to help guide them in their set-up of their first panel, and to assist veteran Opal users with integration of the new Opal Polaris 480 and 780 fluorophores.

We are continually updating our Opal staining methodology, as well as adding exciting new reagent platforms to help improve the Phenoptics workflow while simultaneously extending its performance capabilities. As such, please be advised that this is a living document that will be continually updated with new insights and information. We recommend that you regularly check our website for updates.

We strongly advise all research laboratories who are integrating Opal into their multiplex IHC workflow to consider utilizing the information provided in this guide to become confident in Opal immunohistochemistry assay development, fluorescence imaging using the Vectra or Mantra platforms, and image analysis using inForm, Visiopharm, or HALO.

As always, if you have any issues or concerns during the Opal optimization process, please do not hesitate to reach out to us at support@akoyabio.com so we may assist you.

Happy staining!

Phenoptics 2.0 – An Overview

What is Phenoptics? Akoya's Phenoptics workflow enables imaging and analysis of up to eight immunofluorescent markers plus DAPI within intact tissue sections. Quantitative analysis of multiple cellular phenotypes and cell-to-cell interactions can be observed within their native spatial constructs, a feat that can be extremely difficult to observe by other imaging modalities. The complete Phenoptics workflow incorporates three aspects: Opal reagents, image acquisition hardware, and tissue analysis software (Figure 1).

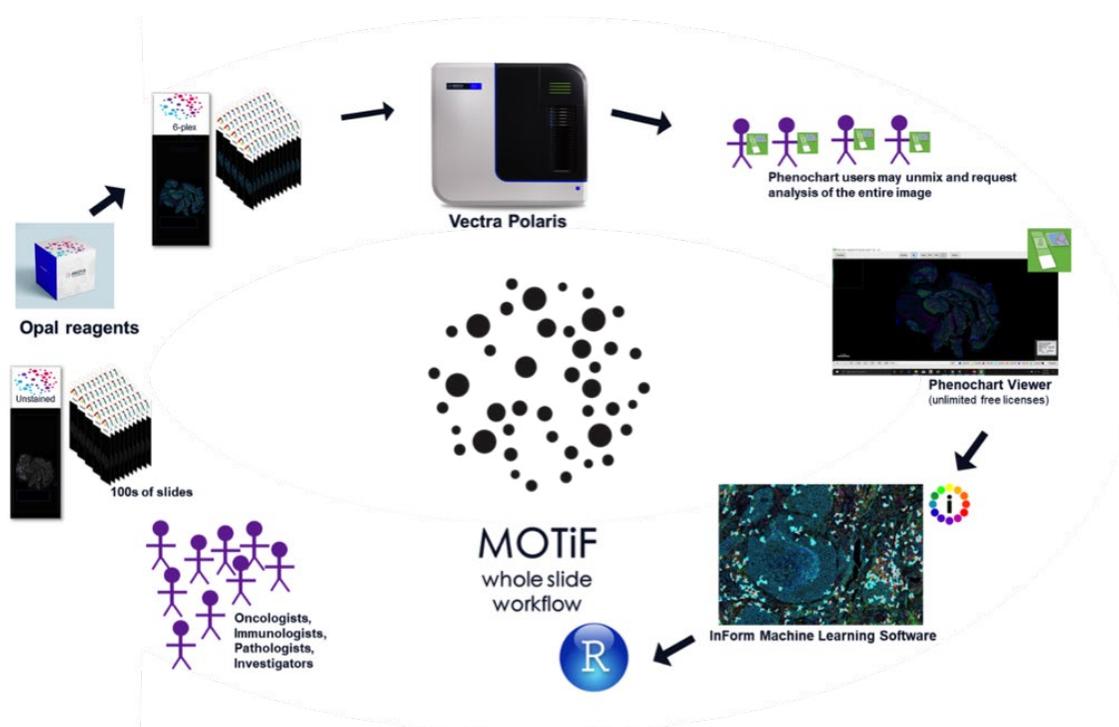


FIGURE 1. MOTiF Whole Slide Workflow. Our MOTiF™ technology generates unmixed whole slide scans of up to 7-colors in less than 10 minutes. Images captured using our MOTiF technology allow for files to be annotated in Phenochart and directly opened in inForm for quantitative analysis.

Opal Assay Kits and Reagents

Opal allows for the use of any standard unlabeled primary antibody, including multiple antibodies raised in the same species. The Opal polymer HRP is applied after, using Tyramide Signal Amplification (TSA) to amplify IHC detection by covalently depositing fluorescent molecules in close vicinity to that targeted antigen. After labeling is complete, antibodies are removed in a manner that does not disrupt the Opal fluorescence signal, allowing for the next target to be detected without antibody

cross reactivity. Opal enables development of multiplexed assays with balanced, quantitative signal for both rare and abundant targets of interest.

Opal assay kits are available for purchase with up to seven colors, including DAPI counterstain. The Opal staining protocol is similar to standard IHC that uses diaminobenzidine (DAB); primary antibodies are detected with an HRP-conjugated secondary antibody and a detection substrate. As such, many of the reagents and materials used in this process will be familiar to current IHC users.

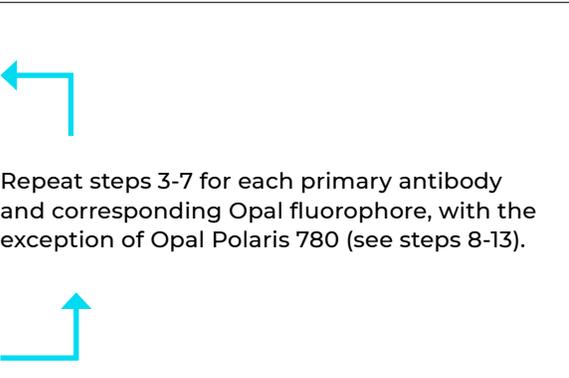
STEP 1	Slide Preparation		
STEP 2	Epitope Retrieval		
STEP 3	Blocking	 <p>Repeat steps 3-7 for each primary antibody and corresponding Opal fluorophore, with the exception of Opal Polaris 780 (see steps 8-13).</p>	
STEP 4	Primary Antibody Incubation		
STEP 5	Introduction of Opal Polymer HRP		
STEP 6	Signal Amplification		
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 2. Iterative staining flowchart for Opal Multiplex IHC Assays. Opal Multiplex assays are based on traditional IHC staining workflows.

Opal Fluorophore Kits

Fluorophore	Opal Multicolor IHC Kits			Excitation (nm)	Emission (nm)	Cap Color
	4-color	7-color	Polaris 7-color			
Spectral DAPI	✓	✓	✓	368	461	Blue Dropper
Opal Polaris 480			✓	450	500	Purple
Opal 520	✓	✓	✓	494	525	Green
Opal 540		✓		523	536	Yellow
Opal 570	✓	✓	✓	550	570	Red
Opal 620		✓	✓	588	616	Brown
Opal 650		✓		627	650	Orange
Opal 690	✓	✓	✓	676	694	Clear
Opal Polaris 780			✓	750	770	Orange

Of note, Opal reagents come as a lyophilized powder that must be reconstituted to a stock solution using 75uL of DMSO, provided in the kit (Opal 780 is reconstituted in 300uL diH₂O). Additional Opal reagent packs are available to increase your panel capacity for detection of nine colors.

Opal IHC Detection Kits	Part #
Opal Polaris 7-Color Manual IHC Kit	NEL861001KT
Opal Polaris 7-Color Automation IHC Kit	NEL871001KT
Opal 7-Color Manual IHC Kit	NEL811001KT
Opal 7-Color Automation IHC Kit	NEL821001KT
Opal 4-Color Manual IHC Kit	NEL810001KT
Opal 4-Color Automation IHC Kit	NEL820001KT
Opal 4-Color Anti-Rabbit Manual IHC Kit	NEL840001KT
Opal 4-Color Anti-Rabbit Automation IHC Kit	NEL830001KT

NOTE: Each IHC Detection Kit includes the following: Opal Fluorophores, Spectral DAPI, Opal Polymer and 1xPlus (Automation) Amplification Diluent HRP.

Opal IHC Panel Kits	Part #
Opal 7 Immunology Discovery Kit (CD4, CD8, CD68 + 3 open channels)	OP7DS2001KT
Opal 7 Tumor Infiltrating Lymphocyte Kit (CD4, CD8, CD20, FoxP3, CD45RO, panCK)	OP7TL3001KT
Opal 7 Solid Tumor Immunology Kit	OP7TL4001KT
Opal 4-Color Lymphocyte Kit (CD4, CD8, CD20)	OP4LY1001KT
MOTiF™ PD-1/PD-L1 Panel: Auto Lung Cancer Kit	OP-000001
MOTiF™ PD-1/PD-L1 Panel: Auto Melanoma Kit	OP-000003

NOTE: Each Opal IHC Panel Kits include the following: listed primary antibodies, Opal Polymer HRP, Antibody Diluent / blocking solution, Opal Fluorophores and their amplification diluent, Spectral DAPI, and the necessary antigen retrieval / stripping buffer.

Individual Reagent Packs

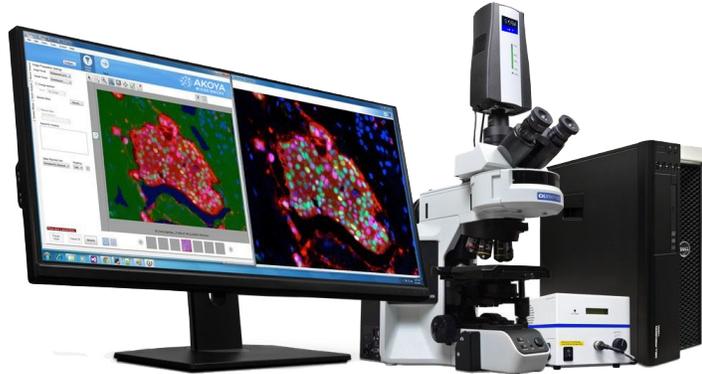
Opal Fluorophore Reagent Packs	Part #
Opal Polaris 480	FP1500001KT
Opal 520	FP1487001KT
Opal 540	FP1494001KT
Opal 570	FP1488001KT
Opal 620	FP1495001KT
Opal 650	FP1496001KT
Opal 690	FP1497001KT
Opal Polaris 780	FP1501001KT

Image Acquisition Platforms

Multispectral imaging with spectral unmixing eliminates fluorophore crosstalk and increases signal-to-noise ratio by capturing each fluorescent spectral signal while isolating the tissue autofluorescence to allow for true signal quantification. The Mantra® 2, Vectra® 3, and Vectra® Polaris quantitative pathology imaging systems from Akoya Biosciences deliver high quality data where morphological context is preserved down to the sub-cellular level.

To see Akoya's suggested reading materials regarding our imaging systems, please go to our website; under our Phenoptics Image Resources you will easily be able to access product notes written about each imaging system.

More detailed information can be found in the Mantra 2, Vectra 3 and Vectra Polaris Product notes located here: [Mantra-Vectra-Instruments](#).



Mantra 2

The Mantra quantitative pathology workstation has full multispectral capabilities for fluorescence and brightfield samples and is set up for 'point-and-shoot' workflows where slides are handled manually with a standard manual microscope stage. Mantra can also image up to 9 colors. The microscope is otherwise entirely automated for objective changes, filter changes, and image acquisition. The Mantra workstation is ideal for labs getting started with multispectral imaging, supporting workflows where individual 10x, 20x, or 40x fields will suffice, and for assay development, allowing higher throughput systems such as Vectra Polaris to be freed up. It incorporates multispectral imaging technology, image acquisition and analysis with the inForm software, and can be used with a variety of stains including Akoya's Opal reagent kits.



Vectra 3

The Vectra 3 provides multispectral and automated imaging of up to 6 slides to detect and measure weakly expressed and overlapping biomarkers within a familiar digital pathology workflow. Tissue sections or TMAs can be labeled with up to seven immunofluorescent (IF) or immunohistochemical (IHC) stains such as Opal™, or with conventional stains such as H&E and trichrome. The Vectra 3 can also be used with the Phenochart™ whole slide contextual viewer with annotation capability where pathologists and technicians can navigate around slides and identify areas of interest for high-resolution multispectral acquisition.

Vectra Polaris

The Vectra Polaris is a state-of-the-art multispectral imaging system that enables the detection and downstream quantification of multiple overlapping biomarkers as the signals are unmixed from one another and from autofluorescence. Novel optics provide best-in-class walkaway automation, requiring less touch-time to batch process large numbers of slides. With a capacity of 80 slides and dynamic loading, this system incorporates high speed digital whole slide scanning in a platform capable of 10-40x magnification in brightfield or fluorescence to allow users to visualize, analyze, quantify, and phenotype immune cells in situ with enhanced security and reliability.



Inside the instrument, Akoya's renowned liquid crystal tunable filter (LCTF) technology generates unmixed, annotated regions-of-interest (ROI) of up to 9-colors for deeper interrogation of biology, and our MOTiF™ technology generates unmixed whole slide scans of up to 7-colors in less than 10 minutes. With our simplified workflow, once hand-selected ROI are chosen, there is no need to return to the slide for multispectral image acquisition. This shortens image acquisition and analysis of large contiguous areas and provides a permanent record of the spectrally unmixed whole slide for future analyses as understanding of underlying biology evolves.

Software Analysis Programs

Our Phenoptics workflow offers a complete range of software programs to aid in image analysis. With these programs, quantitative assessment of your slides has never been easier. Use trainable machine learning algorithms to phenotype cells, recognize morphologic regions of the tissue (e.g., tumor, dermis, stroma, and inflammation), provide cell counts and densities within each region, and determine per-cell and per-subcellular compartment intensity values. Data may be further analyzed using phenoptr and phenotrReports to generate many types of phenotype-based reports, and to map and visualize the spatial relationships between selected cellular phenotypes.

Phenochart

The Phenochart whole slide contextual viewer enables viewing and annotation of Brightfield and fluorescent digital slides scanned on the Vectra 3 and Vectra Polaris Automated Quantitative Pathology Imaging systems. With the new live unmixing preview, built-in synthetic spectra are used to unmix the acquired images without having to open the images in the inForm image analysis software, allowing for more rapid determination of tissue and staining quality. Images captured using our MOTiF technology allow for files to be annotated in Phenochart and directly opened in inForm for quantitative analysis.



inForm Tissue Finder

inForm Tissue Finder contains a powerful unmixing engine that enables the separation and measurement of weak and spectrally overlapping markers in multiplex assays. Automated, trainable algorithms permit detection and segmentation of specific tissues and identification of multiple phenotypes within a tissue sample. inForm is required to spectrally unmix all the fluorophores and autofluorescence on any image acquired on a Vectra or Mantra system; users then have the option to export unmixed TIFF images for analysis using third-party software like ImageJ, QuPath, HALO, and VIS.

Definitions

Term	Definition
Multiplex Slide	Study samples and positive/ negative control tissue that are labeled with all markers of interest plus DAPI nuclear stain. Can observe immune and tumor cell interactions, as well as assess any interference or crosstalk.
Monoplex Slide	Control tissue (either tonsil or representative study samples) are labeled with only one marker in the panel and counterstained with DAPI. These are necessary for assessment of staining performance and comparison to established standards. <ul style="list-style-type: none"> · Monoplex slides should be developed in such a way that the appropriate number of microwave treatments (MWT) is applied for each target. (e.g., your monoplex slide for your third biomarker in sequence should experience antigen retrieval, two MWT before the addition of your antibody, secondary, and Opal, and four MWT after staining.)
Library Slides	Spectral library slides each stained with a single fluorophore and made from reliable positive control tissue (i.e. tonsil) or study tissue type if possible. <ul style="list-style-type: none"> · These will be necessary for creating an accurate unmixing library for analysis of monoplex and multiplex slides. A set of library slides consists of: <p>SINGLE STAINED CONTROL SLIDES:</p> <ul style="list-style-type: none"> · One slide stained for each Opal fluorophore without DAPI. We recommend to use an antibody marking an abundant epitope for each Opal fluorophore (e.g., CD20) · One slide only with DAPI and no Opal fluorophore. <p>AUTOFLUORESCENCE CONTROL SLIDE:</p> <ul style="list-style-type: none"> · One completely unstained representative study sample for collection of an autofluorescence spectrum. The unstained slides should be processed in the same way as the other slides, omitting both the Opal fluorophore and DAPI.
Panel	Primary antibodies and their associated Opal pairings run in a designated staining order on FFPE tissue to produce a multiplex image.
MWT	Microwave treatment (other HIER alternatives could be used if carefully validated)
DAPI	4',6-diamidino-2-phenylindole. A blue-fluorescent nuclear stain.
DAB	Diaminobenzidine. A chromogen that is used in IHC to detect and saturate the marker of interest.
AR	Antigen Retrieval buffer. Comes in a pH of 6 or 9 to aid in exposing epitopes of interest.
DMSO	Dimethyl Sulfoxide.
RT	Room temperature.
MSI	Multispectral Imaging
MOTiF	Rapid whole slide MSI technology exclusive to the Vectra Polaris.

Quick Start Guide

Multiplex Assay Panel Table

Use the table provided below for to keep track of your assay panel development.

Project Name: _____

Date: _____ Tissue(s): _____

Researcher: _____

Order	Antibody	Supplier	Clone/ Lot	Catalog #	Dilution Factor	Opal Pairing	AR
1							
2							
3							
4							
5							
6							
7							
8							

Notes: _____

Opal Assay Development Overview

Opal Multiplex Staining High-Level Overview Schematic

The entire Opal assay development and optimization procedure is summarized as below:

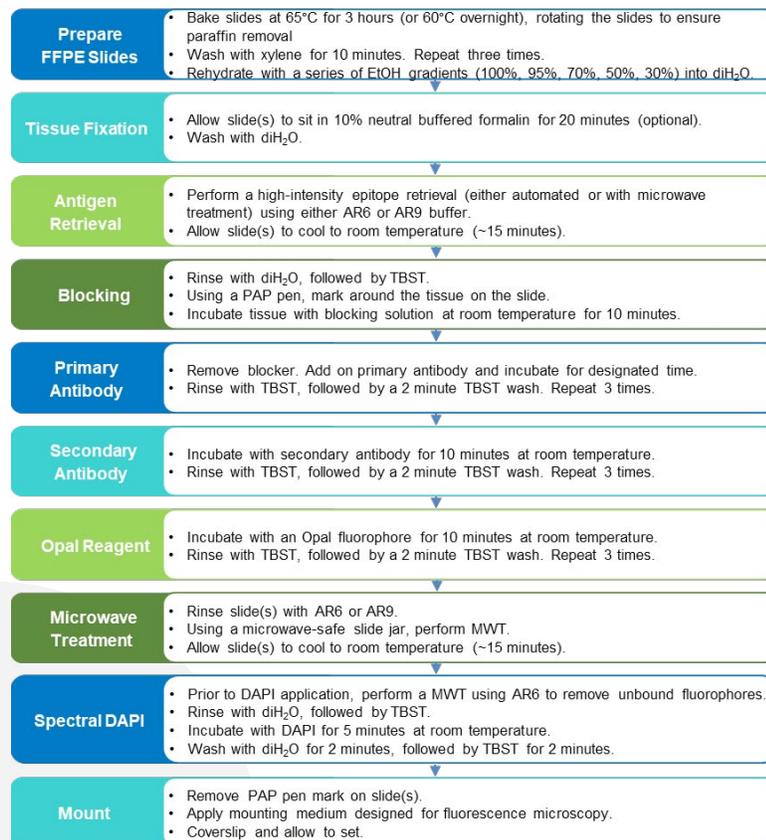
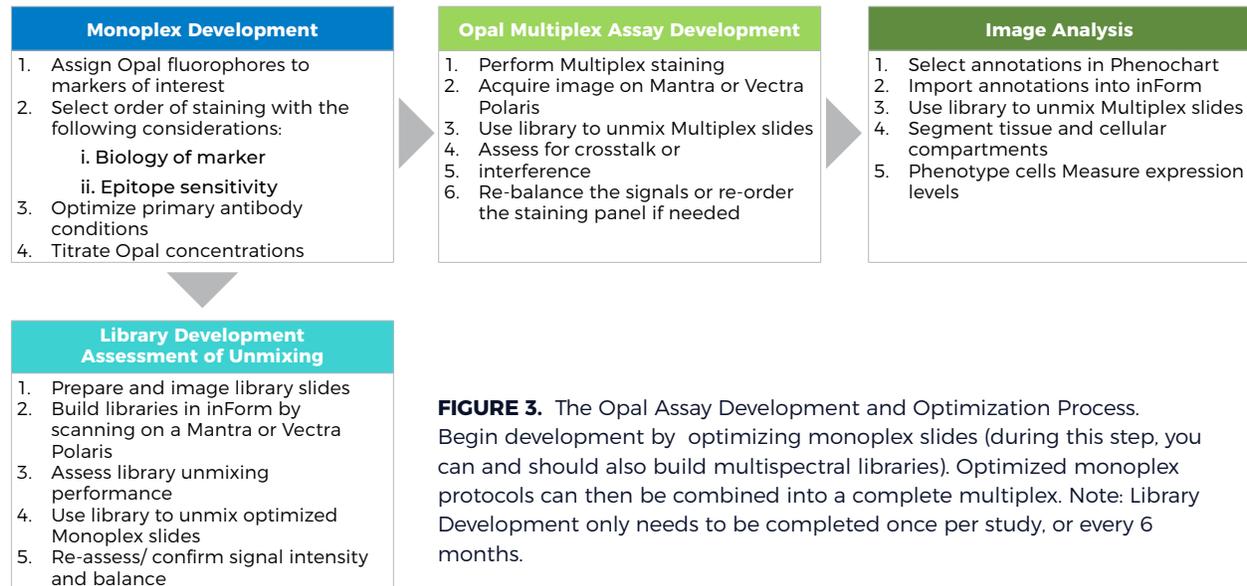


FIGURE 4. Detailed staining flowchart for manual Opal Multiplex assays. This process is independent of development and makes up the step by step instructions for performing the staining.

Required Materials and Reagents

The materials and reagents listed in the table below are required to perform Opal staining.

Materials and Reagent Purchased from Akoya:	Additional Required Materials and Reagents:
Opal Fluorophores	Isotype Control Antibodies
Antigen Retrieval (AR 6 and/or AR9)	Primary Antibodies*
Antibody Diluent/ Block	Positively charged slides
Secondary Antibody HRP Conjugate	Glass coverslips, #1.5
Spectral DAPI	Products for deparaffinization
Opal Polymer	Xylene
1x Plus (Automation) Amplification Diluent HRP	EtOH (100%, 95%, 70%)
	Slide incubation/ humidity tray
	Hellendahl staining jars◊◊
	Microwave oven with carousel (Rated 1000-1200 watts) or other preferred method
	Library slides
	Mounting Medium (recommended ProLong™ Diamond from Thermo Scientific)
	Hydrophobic barrier pen (PAP pen)
	Ultrapure, peroxidase-free water (recommended autoclaved MilliQ)
	TRIS-buffered saline with Tween® 20 (TBST)
	10% Neutral Buffered Formalin

*Any species, unlabeled and validated for use with IHC.

◊Critical for MWT because they hold enough AR buffer to ensure that slides do not dry out. Opal Slide Processing Jars from Akoya are non-breakable and hold up to 14 slides.

◊◊Necessary for manual staining.

■ Panel Design Considerations

Heat Induced Epitope Retrieval (HIER) is used to:

- Quench endogenous peroxidase activity
- Antigen retrieval
- Remove bound antibodies from a target

Stripping and HIER Options:

- Laboratory-grade microwaves that include temperature probes: 20 mins @ 98°C (time and temperature TBD by user => Check the stripping control section) (e.g., EZ Retriever from Biogenex and Biowave from Pelco)
- Conventional household microwaves with Inverter technology: Warm at max power until it boils (~1 min) and put it at 20% or so in order to heat more gently over 15 mins
- Waterbath (98°C - 20 mins minimum)
- Steamer (10-20 mins in watersteam so ~100°C)
- Pressure cookers - Biocare or household (Time TBD by the user)
- Any HIER platform you currently use for IHC retrieval . E.g: the PT Module from DAKO

NB: Please read the Stripping control section to learn how to validate your stripping method of choice

If and only if, you use different primary antibody species AND secondary only reactive to the corresponding species to reveal: You can reveal one primary Ab at a time with specific secondary HRP & quench HRP residues between each secondary Ab addition by using Hydrogen peroxide (3% - increase as needed/ tissue tolerated)

PLEASE NOTE: *This only quenches HRP and cannot be used for stripping primary antibodies. Using that method different primary antibodies species will be required to play on secondary ab cross-reactivity*

Drop-out controls: Antibody stripping is not always 100% efficient. It is important to empirically determine if previously bound antibodies are removed/denatured appropriately, as to ensure signal is not crosstalk. **Please refer to the stripping control section above.**

You will need to run 5 control slides, with the following parameters:

- **Slide 1:** First complete sequence, denature. Second sequence, without primary antibody (but with secondary and detection.)
- **Slide 2:** Second complete sequence, denature. Third sequence, without primary antibody (but with secondary and detection.)
- **Slide 3:** Third complete sequence, denature. Fourth sequence, without primary antibody (but with secondary and detection.)
- **Slide 4:** Fourth complete sequence, denature. Fifth sequence, without primary antibody (but with secondary and detection.)
- **Slide 5:** Fifth complete sequence, denature. Sixth sequence, without primary antibody (but with secondary and detection.)

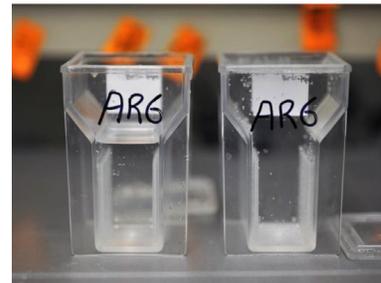
If any signal is present in the channel without a primary antibody, denaturing time or temperature needs to be increased. We recommend trying both 97 C and 100 C for denaturing

Microwave Setting Recommendations

Prior to developing your first panel, it is recommended to determine your specific microwave heating parameters, as cycles differ between brands of microwaves. Most conventional home microwaves work well for microwave treatment (MWT) in the Opal staining process. Users should validate microwave performance independently as outlined below prior to any Monoplex slide development (for first panel only to establish microwave parameters).

Microwave Treatment Protocol

1. Place slide vertically into an Opal Slide Processing Jar. Fill the jar completely with your select Antigen Retrieval buffer.
2. Place jar in microwave and bring sample to a boil. Determine the exact conditions needed to microwave your sample at a constant boil for 20 minutes.



- a. First determine the time it takes your microwave to reach 100°C (boiling point) at 100% power by calculating using a temperature probe and blank slides.
- b. Next, microwave the slides at 20% power for 15-minutes. After 15 minutes,

check the buffer level. If it is below the jar neck and tissue is exposed, either reduce power or reduce time until buffer does not go below the neck.

Monoplex Development Considerations

Monoplex slides should be developed with the appropriate number of MWTs to assess the robustness of your antigen, antigen retrieval, and the intensity of the Opal fluorophore.

- **Note:** ALWAYS keep microwave clean and clear of debris.
- **Note:** NEVER leave the microwave unattended when in operation.
- **Note:** OPAL POLARIS 780 SHOULD ALWAYS BE PLACED LAST IN THE STAINING ORDER SEQUENCE, as it should not be exposed to MWT.

Library Slide Development

The goal of this step is to create a set of Library slides, each stained with only one fluorophore (Opal or DAPI). Also included should be one slide with no fluorophores to represent autofluorescence. These slides will be used to create a spectral unmixing library.

To create library slides, we recommend using reliable positive control tissue (i.e. tonsil) or study tissue type if possible. Using CD20 on tonsil since CD20 is ubiquitous, uniform, and highly expressing. Emission spectra collected from these slides are less subject to issues related to sparse and/or low signal levels. A significant benefit of this approach is that library slides can be reused for several months when stored in optimal conditions (dark at 4°C) and support standardization across studies, a goal for translational research.

If using CD20 or any abundant marker, it's very important to keep the signal in the Opal recommended range within the same range as your multiplex signal intensities.

Another approach would be to use the monoplexes created for your panel development (without DAPI counterstain) if the marker is expressed enough, i.e. at least 40 cells in the field of view used for spectrum extraction.

MOTiF and MSI note: MSI does require good spectral libraries (sometimes in the same tissue type) and this is easy to build when working on your monoplex slide development by omitting DAPI addition. For MOTiF, there is a fantastic synthetic library.

Manual Opal Staining

Monoplex Assay Development

The first step in the development of a Multiplex panel is defining the proper staining parameters for each of the individual antibodies and Opal pairings, known as Monoplex slides. Of the four stages in the Opal process (Monoplex, Library, Multiplex, Analysis), this can be the most technically challenging. When users have successfully completed their first panel development, other panels are easier to optimize.

Panel Design

Pairing Opal Fluorophores to Primary Antibodies

Opal fluorophores need to be paired to each marker. To help in your selection process, we suggest taking into account the following:

1. Co-expression: If you are interested in investigating co-expression of markers on a specific cell type, then select fluorophores that are not spectrally adjacent.
2. Rare vs. Abundant markers: Low expressers should be assigned to brighter fluorophores, while more abundant markers should be allotted to dimmer fluorophores (see table below).

Opal Brightness Rankings		
Fluorophore	Mantra and Vectra Polaris	Vectra 3
Opal Polaris 480	Highest	N/A
Opal 520	Highest	Highest
Opal 540	Medium	Medium
Opal 570	Medium	Medium
Opal 620	Medium	Medium
Opal 650	Highest	Highest
Opal 690	Low	Low
Opal Polaris 780	Lowest	N/A

FIGURE 5. Opal Brightness Rankings

Staining Order

Choose a staining sequence based on the previous factors discussed, including for each marker the amount of antigen retrieval required, its biology, and the abundance of it. Once selected, prepare Monoplex protocols that include the appropriate number of heat treatments (HIER) before and after Opal detection, to mimic tissue exposure that will occur in the multiplex protocol. We have found including these HIER steps during this Monoplex optimization step greatly decreases the amount of rebalancing and adjustments needed for the multiplex protocol.

1. Antigen retrieval: The order of immunostaining can change signal intensity. Some epitopes become more exposed after successive rounds of HIER and result in greater signal when detected later in a multiplex protocol (depends on clone used more than marker). And reversely, Some rare epitopes may be degraded by multiple exposures to heat and should be placed early in the staining order.
 2. Biology: Some Opal fluorophore signal intensity can also be affected by HIER (i.e., attenuation of Opal 520 and Opal 570). Designing your monoplex slides with the correct number of HIER can help determine any corrections needed on Opal concentration and assay order prior to running the multiplex.
 3. Co-localization: If possible, arrange the order so sequential antibodies do not colocalize in the same cellular compartments within the same cells. This can help when issues arise, including incomplete stripping.
- As previously mentioned, Opal Polaris 780 should be allocated for the last position in the staining order sequence and as it is the dimmer dye, it should be paired with the most abundant marker.

Optimizing Your Monoplex Slide

Determining Primary Antibody Dilution Factors

Once a panel design has been chosen, the next step is the Monoplex optimization process. Similar to traditional IHC, this process includes determining the optimal antigen retrieval methods, primary antibody dilution factors, and incubation times. Opal reagent concentrations can be titrated to change brightness and signal intensity. We recommend experimenting with antigen retrieval methods to determine which yields the highest staining intensity, using serial sections to compare.

Use the following methods below to help determine optimal primary antibody concentrations.

1. Vendor-specified Primary Antibody Dilutions (Akoya Recommended)

- Create a set of 6 slides for each antibody.
- Label three 1.5mL tubes from the stock primary antibody.
 - Dilute your Ab stock according to the ab vendor or as defined through previous IHC validation.
 - Create a 1:2 and a 1:4 dilution from the vendor-recommended concentration or optimal DAB concentration
- Boil three slides with AR6 and the other three with AR9 for 20 min.
 - Allow to cool to RT (~15min)
- Following AR, perform a blocking step and place primary antibody on the tissue sections and allow to incubate.

	IHC defined	1:2 DF	1:4 DF
AR6	Slide 1	Slide 3	Slide 5
AR9	Slide 2	Slide 4	Slide 6

FIGURE 6.

- Wash and apply the HRP Polymer followed by the Opal reagent of your choice at a concentration of 1:100,
- Image the tissue sections and assess each set of parameters for the appropriate staining pattern. If imaging using MOTiF, images can be viewed in Phenochart using the live unmixing preview.

If there is no significant difference in staining between the different parameters, we recommend proceeding with a 1:2 dilution factor from IHC optimal concentration for primary antibodies and an AR6 for antigen retrieval.

2. Use conventional IHC and visual assessment:

For each antibody, optimize and stain positive control slides, as you normally would in the course of your IHC protocol development, seeking the appropriate staining pattern, which typically means selecting staining parameters that lead to specific, intense, captures the full dynamic range of expression with continuous levels of intensity, shows

complete membrane staining for surface markers, and has low background. Then, stain serial sections using primary antibody concentrations determined or conventional IHC and with the Opal fluor assigned above during panel design, using Opal dilution of 1:100 if staining manually and 1:150 if staining automatically (except for Opal Polaris 780, which is diluted 1:25 in antibody diluent/block).

3. Opal Dilutions for Optimal Signal Intensity

It is recommended that the range for all Opal fluorophores be between 10-30, with the exception of Opal Polaris 780 where the range can be between 1-10, as much dimmer than the other dyes.

Aside of this signal intensity recommendation, we advise a signal-to-background ratio greater than 10, which should be easily achievable with Opal amplification.

For additional information and even more rigorous assessment, please use the following study: (<https://www.jove.com/t/58390/automated-multiplex-immunofluorescence-panel-for-immuno-oncology>) Surace, M., DaCosta, K., Huntley, A., Zhao, W., Bagnall, C., Brown, C., Wang, C., Roman, K., Cann, J., Lewis, A., Steele, K., Rebelatto, M., Parra, E. R., Hoyt, C. C., Rodriguez-Canales, J. Automated Multiplex Immunofluorescence Panel for Immuno-oncology Studies on Formalin-fixed Carcinoma Tissue Specimens. *J. Vis. Exp.* (143), e58390, doi:10.3791/58390 (2019).

If using spectrally close dyes in a 8-plex panel, most signal intensities should stay below 20 as much as biologically possible (especially for OPAL 540 and OPAL 650 dyes). You can adjust signal level by diluting further the OPAL dyes, e.g. 1:200 instead of 1:100. (or concentrate if too low count).

Here we describe how to adjust fluorescence intensity levels.

1. Have an autofluorescence slide created with project specific tissue type.
 - a. Slide is created in the same manner as monoplex slides, however, does not receive an Opal dye or DAPI.
2. Create a library in inForm.
3. Create a new inForm project.
 - a. Add in the individual optimized monoplex slides and autofluorescence slide.
4. Select the dropper tool to acquire the autofluorescence spectrum (see Fig.7).
 - a. Use the autofluorescence slide and select (select by drawing a line over brightest region) the brightest regions (often RBCs) (NB: stay on tissue only).
5. Load in your library Spectra for Unmixing.
 - a. Click on Select Fluors...
 - b. Choose from your own library or from the label 'Synthetic.'
 - c. Once loaded, select 'Edit Markers and Colors...' and label the paired fluorophores with the markers of interest.
6. Once finished, select "Prepare All" to spectrally unmix the Monoplex images.
7. For each of the Monoplex images use the inForm information cursor to observe the signal intensity of the respective fluorophores on each slide.
 - a. View the brightest and weakest signals on positive cells on the slide to see if they are within range (Fig. 9).
8. Once the range has been determined, adjust the Opal dilution as necessary to increase or decrease fluorescence intensity signals to get within the Target Brightness range.
 - a. Signal intensity is directly proportional to Opal concentration.
 - i. Example: If intensity is 50 normalized counts, change the Opal dilution from 1:100 to 1:200 to lower normalized counts.
 - ii. We recommend trying three dilutions: 1:150, 1:200, and 1:250 to get a sense of signal intensity changes. If necessary, more dilutions can be used.

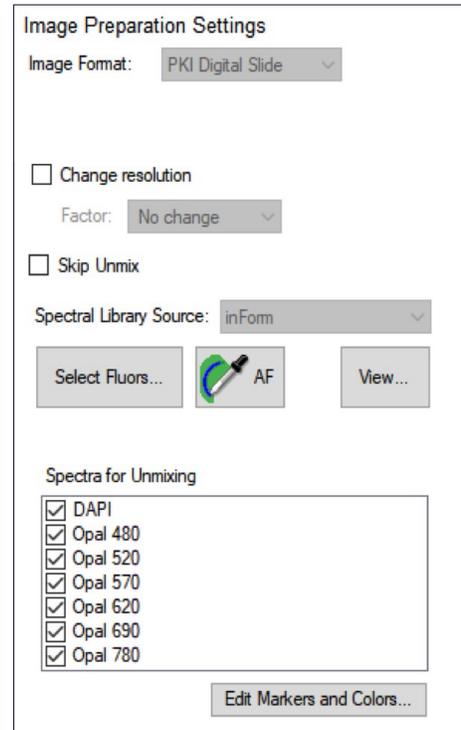


FIGURE 7.

- iii. Opal detection can be diluted substantially (anywhere from 1:100 to 1:2000).
9. If signals are still weak at an Opal concentration of 1:100, try the following:
- Use Opal at 1:50, however, we recommend against using dilutions <1:50 as this can lead to antigen sheltering or unmixing issue.
 - Opal 690 is a relatively weak fluorophore, so it is often used at 1:50.
 - Opal TSA DIG at 1:100, Opal Polaris 780 in antibody diluent at 1:25
 - Additional AR sessions.

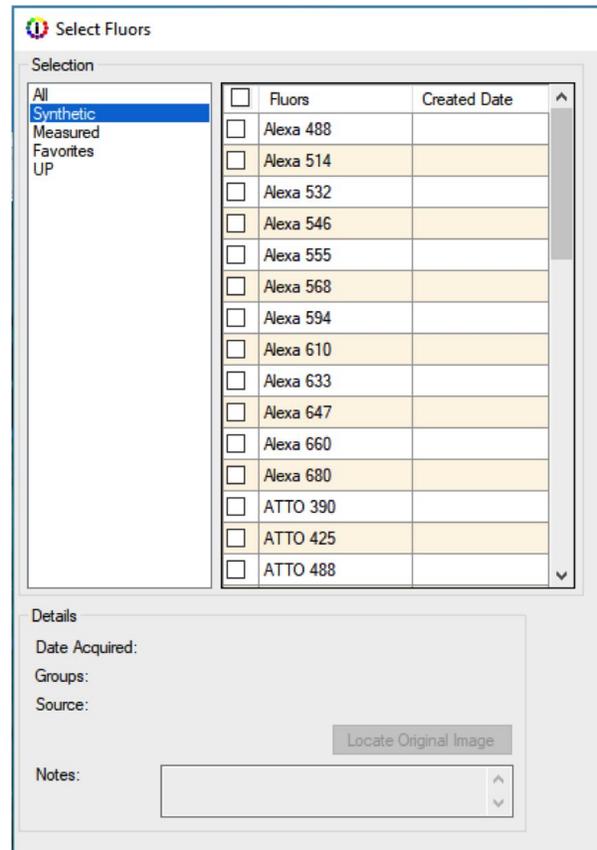


FIGURE 8.

- Some tissue has heavy mucin interference, including colon and lung tissue. Certain antigens benefit from being placed later in the multiplex panel sequence.
- Use more aggressive antigen retrieval methods.
 - E.g. certain PD-L1 clones do better under these conditions and benefit from being placed later in the multiplex panel.
 - We also observed that several AR6 equal a harsher AR9 pre-treatment. Hence those antibodies requiring AR9 prior to staining are often working well when placed at the end of the sequence
- Longer primary antibody incubation times.
 - This can include extending the time from 20 to 40 min, or incubating overnight at 4°C.

Note: This may increase non-specific signal.
- Use a different primary antibody clone.
- Double check to ensure your positive control tissue is positive for the marker of interest.

- i. In some extenuating circumstances, positive control tissues may not express your marker of interest. Double check your antibody binding with cell pellets or other additional means.
10. Once your signal is within the correct Target Brightness range, assess signal-to-background ratio to confirm it is 10:1 or higher.
- a. To determine signal-to-background, measure the signal levels in tissue areas where there appears to be non-specific signal. If background is too high (>10%), try to correct using these steps:
 - i. Add an additional blocking step.
 - 1. Use 3% hydrogen peroxide to quench bound HRP or different serum/ protein blocking buffer.
 - ii. Use alternate clones or reduce primary antibody concentration.

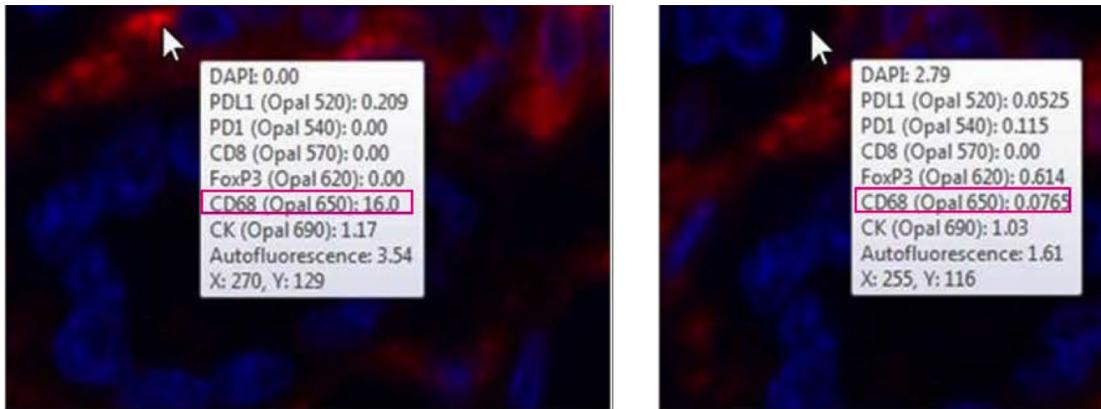


FIGURE 9.

Factors that affect Opal counts recommendations		Can address by adjusting [Opal TSA]	Can address by adjusting [Primary Ab]
Reasons to want higher counts	Crosstalk from neighbor channel is high ('balancing')	✓	✓
	Crosstalk from AF is high	✓	✓
	Used for an expression marker – want larger dynamic range	✓	sometimes
Reasons to want lower counts	Crosstalk from this channel to neighbor channel is high ('balancing')	✓	✓
	Spectral shifts observed at high intensity	✓	✓
	Insufficient stripping	x	✓
	Interference/blocking of subsequent colocalized marker staining	sometimes*	sometimes*

FIGURE 10. Considerations for Opal intensity counts while balancing signals during optimization. Higher intensity counts (30-50) can be beneficial in channels such as Opal Polaris 480 when working with highly autofluorescent tissue. Lower counts (1-10) can be beneficial when working with abundant antibodies with high affinity to their epitopes.

Quantitative Assessment

Following Monoplex optimization, the protocols will be implemented onto representative study samples. This step is undertaken because expression levels may be different from positive control tissue (if you didn't use representative study tissue). If signals are lower on study samples compared to positive control tissues, we recommend changing the Opal dilution to increase signal. If that is not enough, consider switching AR methods or increasing the primary antibody concentration. Under rare circumstances, re-optimization may need to be done. Once the Monoplexes have been successfully validated on study samples, you can combine everything into a full multiplex run.

Opal Multiplex IHC Development

Once your Monoplex slides have been optimized, it is time to transition them to into a Multiplex panel. The Multiplex workflow is a combination of all the Monoplex protocols, keeping a single DAPI application until the end.

Recommendations and Tips for Manual Multiplex Panel Development

- 6- or 7-color assays may require leaving slides overnight while in process. Here are three options for the overnight step, in order of preference:
 - Post-microwave cooling in AR buffer at room temperature.(N.B. Close lid with parafilm to prevent evaporation
 - Overnight primary antibody incubation at 4 °C, when verified in monoplex assay development.
 - Storing in blocking solution at 4 °C.
- It is rare that the order of AR6 and AR9 together in an Opal multiplex IHC assay impacts the outcome.
- Using the Multiplex Assay Design Table (at end of this document) to mark the completion of each step significantly helps to reduce errors and redundancy in your staining workflow.
- During Assay Development, it can be helpful to review your staining under a microscope after each Opal fluorophore application.
 - The best time to do this is immediately after MWT for antibody removal, using a coverslip mounted with water. If there is a problem with the staining, it may possible to repeat at this point. At later stages, it will become more difficult to evaluate staining without spectral unmixing, because more than one fluorophore may be visible in a given epi-illumination cube.

If you find signals much lower in study samples, we suggest changing Opal dilution to restore signals. If this is not sufficient, you may want to increase primary concentration. In rare cases, the positive controls are so different from the actual study samples, requiring you to revisit the first step.

Automated Opal Staining

The basic premise of the Opal Multiplex assay does not change when performing it on an automated platform. It is still necessary to perform all of the appropriate assay development steps, including antibody optimization (running DAB single stains for antibody titration and antigen retrieval buffer parameters, antibody-Opal fluorophore pairings, running monoplexes with the correct number of antibody stripping steps incorporated into your protocol, etc.)

The guidance above for assay development is applicable for all platforms. The largest difference between running the Opal Multiplex assay manually and via an automated platform is the method used to strip previously bound antibodies from the tissue. Therefore, there is an additional set of controls we suggest you run when optimizing Opal Multiplex on an automated platform. Please refer to the stripping validation recommendations above.

In term of Opal dilution we advise :

- 1:150 starting dye dilution if using Leica BOND RX autostainer
- 1:50 if using the Roche Discovery Ultra platform

NOTE: *If you intend to utilize your automated platform as your primary method of staining, it is preferable to accomplish all of your optimization activities automated. Optimized manual assays are not “plug-and-play” into automated protocols. Manual and automated concentrations for both primary antibodies and Opal fluorophores can be different.*

Create 6-plex based on above empirical findings minding antibody application order (as determined by running Monoplexes with the correct number of antibody stripping steps written in), antibody concentration, Opal-antibody pairing, Opal reagent concentration, and denaturing parameters for each antibody in the panel.

■ Opal Image Acquisition

Please follow the directions provided in your respective instrument User Manual. For any questions regarding Image Acquisition, please reach out to your regional TAS or to Akoya's Support team at support@akoyabio.com.

Tips for Image Acquisition

Exposure Times

- When setting exposure times for your project, use slides that contain expression of as many markers as possible. Be sure to review a few slides to ensure times are accurate.
 - For each marker/ filter, move around the slide and autofocus in each new area. If an area of red appears (signal is saturated), click the 'Autoexpose' button to adjust the exposure for the filter.
 - For MOTiF images, it is important that your signal intensities are not saturated.
 - o Repeat for all filters. When complete, these times will be used for all slides in that project.

Tissue Imaging

- Certain PAP pens exhibit intrinsic fluorescence that can interfere with whole slide scanning. Best practice is to take care in removing PAP pen prior to coverslipping. Circling should ONLY be done with blue (not black) sharpie and only on coverslip side of slide

Image Analysis Overview

The following walk-through will provide you with a brief introduction to Phenochart and inForm Tissue Finder to help support your assay and project development. There are many ways in which these software platforms can be utilized. For the purposes of this Assay Development Guide, we will only be highlighting Akoya recommended settings. More information on each of the respective platforms, including in-depth User Manuals, can be found at www.akoyabio.com/phenopticsm/software.

Phenochart Workflow

Our Phenochart slide viewer software is optimized to work with the Vectra 3 and Vectra Polaris, including our MOTiF workflow and .qptiff files. You can use this tool to assist in viewing and navigating whole slide scans, select areas for further review, annotate regions for analysis, or select stamps for MSI.

Here we provide an overview of the features available for your workflow. More detailed information can be found in the Phenochart User Manual located at www.akoyabiosciences.com under Phenoptics → Software → Phenochart.

Viewing Acquired Images

When you first open Phenochart, you must Login. Click the Login button on the upper right-hand corner. Type in a username and press OK. Next, select Load Slide in the upper left-hand corner.

A Load Slide pop-up will appear. Go to the location your slides are located and select the slide you wish to view. When selected, it will be highlighted in blue. If you need to change browser locations, go to the Gear Icon (red box) in the right-hand corner and click on it. Go to “Change Browser Location” to change the file folder. When you are ready, double click on the blue highlighted slide to view it.

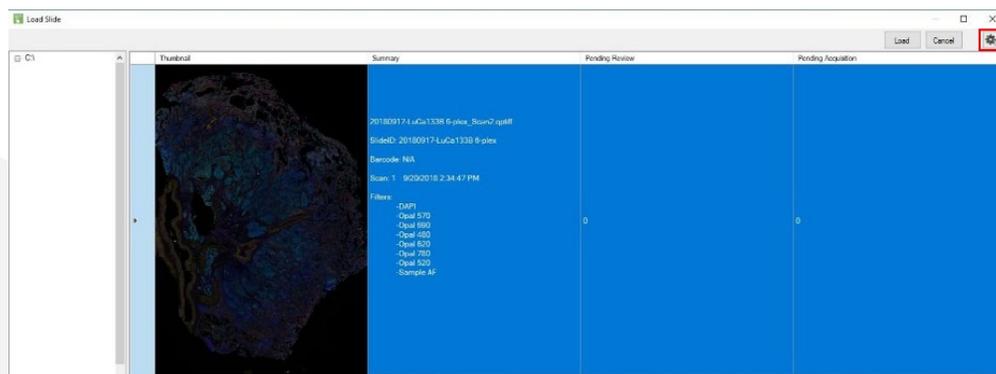


FIGURE 11. Clicking Load Slide will allow you to select a slide to view and choose regions to annotate. Selecting the Gear icon (red box) will allow you to change folder location, customize information columns, and reset the columns to default.

If you would like to view different columns of information, under the gear icon select Set Column Visibility. This will allow you to customize what information is viewable when you are looking to Load Slides. The Reset Display button will convert the columns back to their default size and display.

Live Unmixing Preview

Once the image has been selected, if the image was acquired via Multispectral Slide Scan on a Vectra Polaris, you can use the Unmixing button to preview a live unmixed image of your slide using synthetic Opal spectra, including autofluorescence isolation. This is only a preview, however, and images must still be fully unmixed in inForm.

To Unmix in Phenochart, select the Unmixing button and change the view editor from None (no unmixing) to Opal + AF (unmixing components and autofluorescence isolation).

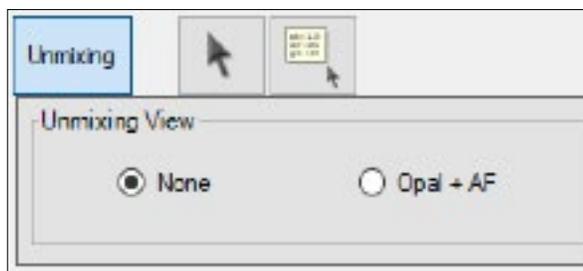


FIGURE 12. Selecting Unmixing will allow you to toggle between your raw image (None) and a live unmixed preview, including autofluorescence isolation (Opal + AF).

Brightfield and Fluorescence Rendering

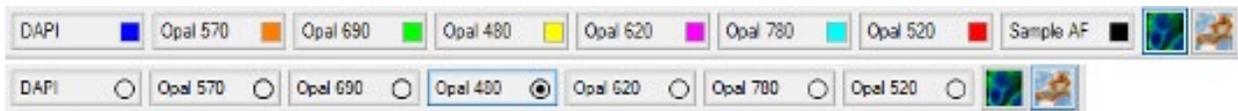


FIGURE 13. Top image is all the components associated with the fluorescence rendering of the image. Bottom image is the view observed with Brightfield rendering.

When Opal + AF is selected, two lower buttons in the right-hand corner will appear, allowing you to toggle between Fluorescence rendering, where components can be turned on and off individually, and Brightfield rendering (new to Phenochart version 1.0.11), which only shows one component at a time with simulated DAB and Hematoxylin counterstain. Brightfield colors can be changed by going to the Gear Icon and selecting Change Brightfield Colors. The Brightfield view may be a useful resource for pathologists to use when selecting ROI for analysis.

Reviewing Fluorophore Intensity for Opal Optimization

A new addition in Phenochart version 1.0.11 is the ability to visualize normalized fluorophore intensity counts. This can help expedite your Opal Optimization of your Monoplex and Multiplex panels, as is commonly done using inForm (link to section). After Unmixing your image components, select the Intensity Cursor to visualize coordinate information and intensity values from where your mouse hovers on. The normalized counts factor in exposure times. This is the same normalization that would be performed as default by inForm. If red text displays in the intensity cursor text box, this means that the selected pixel is saturated in the indicated channel(s).

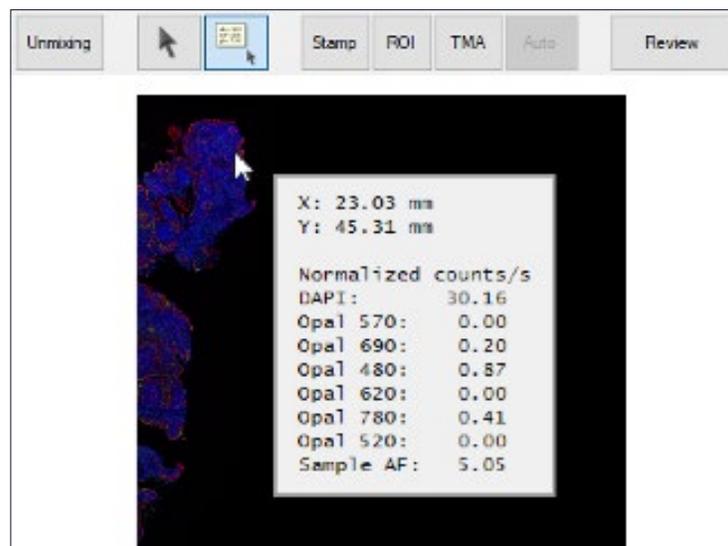


FIGURE 14. Select the Intensity Cursor and hover over any region on the slide to view normalized counts on unmixed imagery. This can help to determine if there is potential crosstalk, spectral bleed-through, or if signals are too weak or bright.

Selecting Annotated Regions for inForm Projects

An annotation is a region selected for further MSI acquisition or for analysis in inForm. There are several options to select from, including Stamp, ROI, TMA, and Auto. We will review Stamps, as this is the most often used selection. For more information on the other forms of annotations, please view the Phenochart User Manual resource.

Stamps

There are five different kinds of stamps:

1. Choose **Acquisition** to take multispectral fields of the stamp area the next time this slide is on the Vectra™.
2. Choose **Review** to create stamps that must be reviewed before they can

be acquired. Vectra will only acquire multispectral fields of accepted stamp annotations.

3. Choose **Push to inForm** to launch the selected stamp area into inForm™. This option will only allow stamps at the scan's native resolution, and is only available for slides scanned with Vectra 3, and when inForm 2.2 or higher is installed on your machine.
4. Choose **inForm Projects** to select stamp areas for analysis in inForm. Load the .qptiff into inForm 2.4.3 or later to analyze these fields. These stamps can only be made at native resolution, and only on slides scanned with Vectra Polaris.
5. Choose **inForm Batch** to select stamp areas for batch analysis in inForm. Batch process the .qptiff in inForm 2.4.3 or later to analyze these fields.

Option 4 and 5 are the most commonly used for .qptiff files acquired on the Vectra Polaris. When you select inForm Projects, you can choose the field size. We recommend 1x1, with at least five fields selected. The selected regions will automatically be saved and appear in inForm when loaded. Repeat this for all slides as needed. inForm Batch is used for batch analysis and the annotations can overlap those selected for inForm Projects.

inForm Workflow

Our inForm Tissue Finder (version 2.4.6 and greater) is an adaptable software program that can be used to unmix, segment, and phenotype your acquired multispectral images. Here we take you through the inForm workflow using Akoya's recommended configuration.

Starting A New Project

Once inForm is initiated, the first screen to appear will indicate in the upper left-hand corner title bar that it is a New Project. Everything else will be grayed out. To begin, Configure your analysis workflow by selecting the Configure button (Fig. 15).

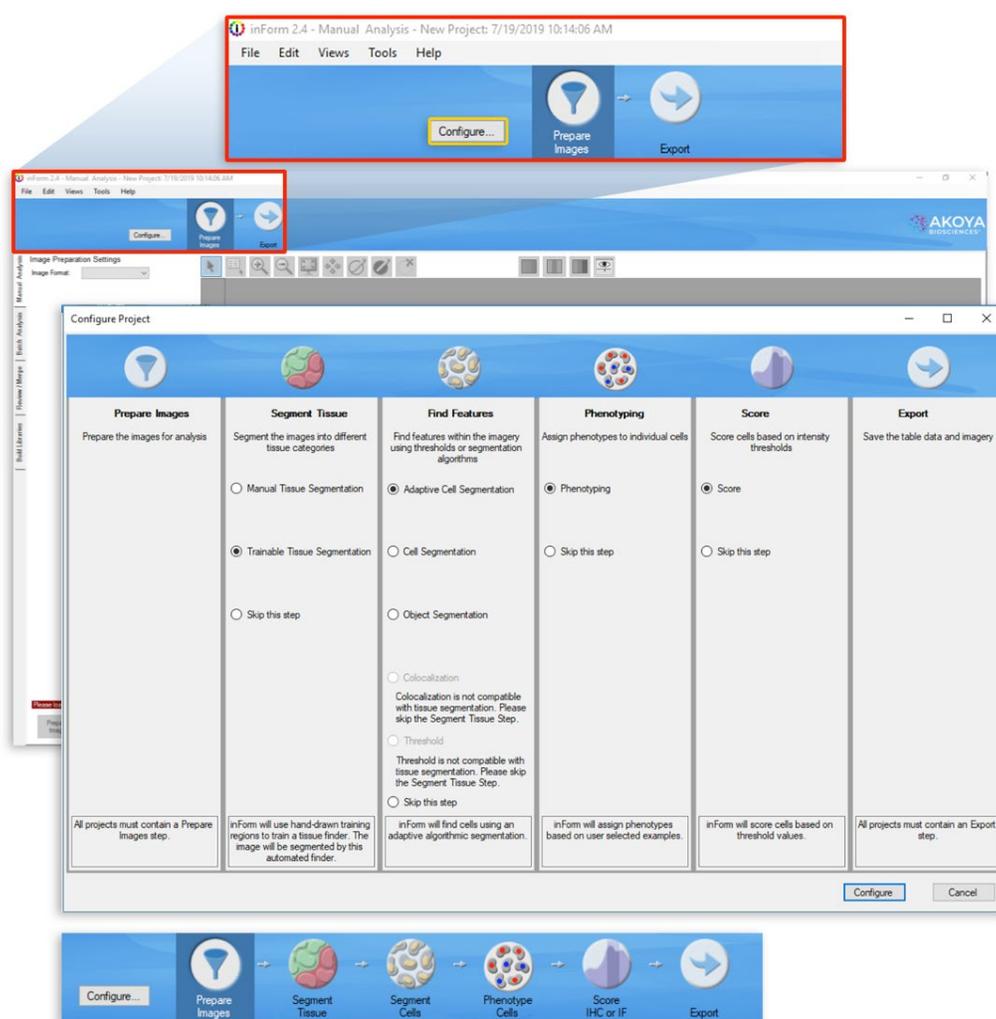


FIGURE 15. (A) Select Configure (yellow) to create your project workflow. A pop-up option will allow you to select which steps you would like to include. Akoya's recommended workflow is selected in the above figure. (B) When your steps have been chosen, hit Configure. Your project workflow steps will now be shown in the top blue bar.

When configuring your workflow, Akoya recommends the following for each step:

1. Prepare Images
2. Segment Tissue: Trainable Tissue Segmentation
3. Find Features: Adaptive Cell Segmentation
4. Phenotyping
5. Score
6. Export

Each part of this workflow is adaptable to your research needs, and steps can be skipped if they are not necessary to your data analysis. All steps can be skipped except for Prepare Images and Export, which unmixes your raw .im3 and .qptiff files, which are then exported as composites and/or component TIFFs that can be used for subsequent analysis on third party software.

When done selecting your workflow, click Configure. Your selections will now be visible in the blue header bar. A dark blue box will color around the step that you are currently on in the workflow.

Prepare Images

To bring your acquired images in for analysis, go to File → Open → Image. Find the file with your acquired scans and load either Multispectral .im3 files or PKI Digital Slide .qptiffs into the project. For PKI Digital Slides, be sure to have selected inForm Project annotated regions of interest in Phenochart before proceeding. Be sure to also load in your Autofluorescence slide as well. The Image Format and Sample Format will be automatically selected based on the image type loaded into the project.

Unmixing of Fluorophores and Autofluorescence

When your images are uploaded into the project, it is recommended to save your project before moving onward. Next, you will hit the Select Fluors button to choose which library you would like to use for unmixing. Synthetic spectra and AF are automatically selected for qptiff images or you can select your previously measured library. When you select each fluor, its spectrum will appear to the right of the fluorophore selection panel. When all fluors have been selected, click OK.

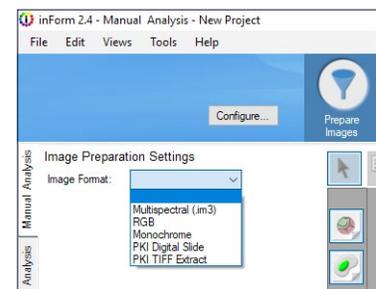
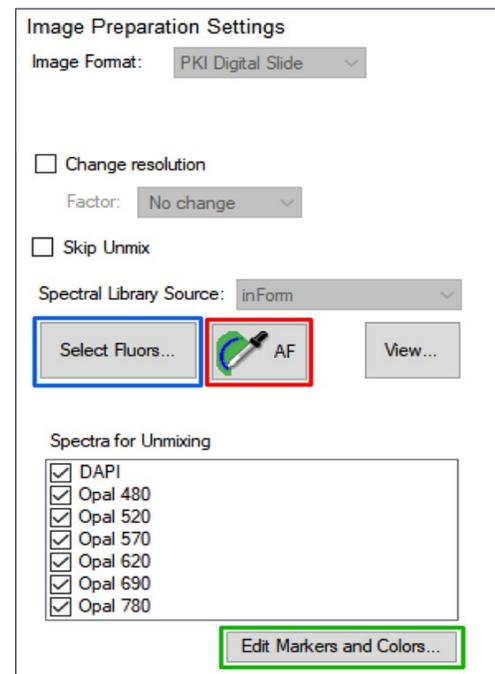


FIGURE 16. Select the Image Format to choose which files you will be analyzing.

Next, to isolate the Autofluorescence spectrum, select the AF eye dropper icon. Once selected, take the dropper and select a representative tissue region on the autofluorescence image, making sure to avoid any pixels not containing tissue. When done, either select Prepare Image or Prepare All located in the lower left-hand corner. If you still observe Autofluorescence in your image, be sure to go back and re-select a new area on the AF slide. Of note, be sure to go to the View Editor on the top of the icon bar and select the Eye Icon (far right of all icons). Go down to the Autofluorescence color and change it from Dark Slate Grey to Black. You will need to do this every time you change the AF region selected.



You can also Edit Markers and Colors for your fluorophores by selecting the button under Spectra for Unmixing. Once all images have been prepared, you can move on to the Segment Tissue button by selecting the button at the top workflow schematic, or by hitting the Advance button at the lower left-hand side of inForm.

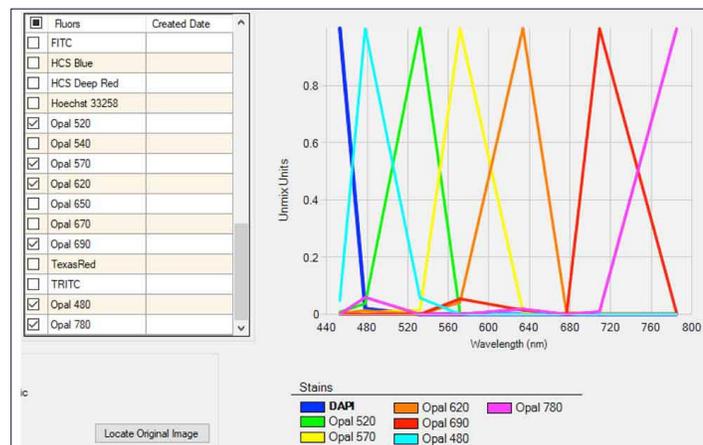


FIGURE 17. (A) Select Configure (yellow) to create your project workflow. A pop-up option will allow you to select which steps you would like to include. Akoya's recommended workflow is selected in the above figure. (B) When your steps have been chosen, hit Configure. Your project workflow steps will now be shown in the top blue bar.

Tissue Segmentation

Under Segment Tissue, and in particular, the Trainable Tissue Segmentation module, the program will utilize a user-trainable algorithm for tissue segmentation based on morphology as well as specified markers. In addition to following select steps, we also provide some suggestions for optimal tissue segmentation.

First, you will need to define a minimum of two tissue categories. Select New, and a Category box and color will populate in the Tissue Category Box. You can define this as any label (some suggestions are: Tumor, Stroma, All Tissue, Necrosis, Fat,

Other, and Background). Use the minimum number of tissue categories needed to accomplish your scientific objective. You can put multiple tissue architectures into one 'other' category (e.g., stroma, necrosis, fat, etc.) that in a particular case you might not be interested in.

When your tissue categories are defined, you will need to add training regions in order to train your tissue segmenter to recognize your tissue regions of interest. To do this, you will need to draw on the tissue example areas associated with the respective categories. The Draw button will be selected to show which tissue category you are training for. Draw training regions in each of the respective categories. Your tissue training regions do not need to cover the entire tissue section, only capture enough for the algorithm to detect the pattern. It is often helpful to have a few training regions that go right up to and follow the edge of tissue categories. Training regions do not need to be in contact with one another. When finished drawing at least one trainable section for each tissue category, you will move on to Components for Training, Pattern Scale, and Segmentation Options.

Use only those 'Components for Training' that are informative in detecting tissue categories of interest. For example, DAPI, autofluorescence, and cytokeratin (or other tumor marker) should be used for differentiating certain tumors from stroma. The segmentation accuracy of your tissue will be affected by the selected Pattern Scale, and a higher accuracy does not necessarily mean better results. For this option, you can select from Small to Maximum. Typically for most tumors, Akoya recommends Large, but you can increase or decrease this depending on the scale of your tissue regions and how accurate you want your tissue boundaries to be.

Segmentation Resolution does not affect the training (i.e., you can change it without needing to re-train, however, you will need to re-segment. Changing the resolution removes any phenotyping training, so be confident in your segmenting before moving forward). The finer the resolution, the less pixelated the segmentation borders will be. Akoya recommends keeping the resolution to Medium during training for speed purposes, then changing to Fine after you are finished training the tissue segmenter. There is a trade-off between pattern scale, segmentation accuracy, and resolution of segmentation. Evaluate changes to these parameters to find optimal segmentation results.

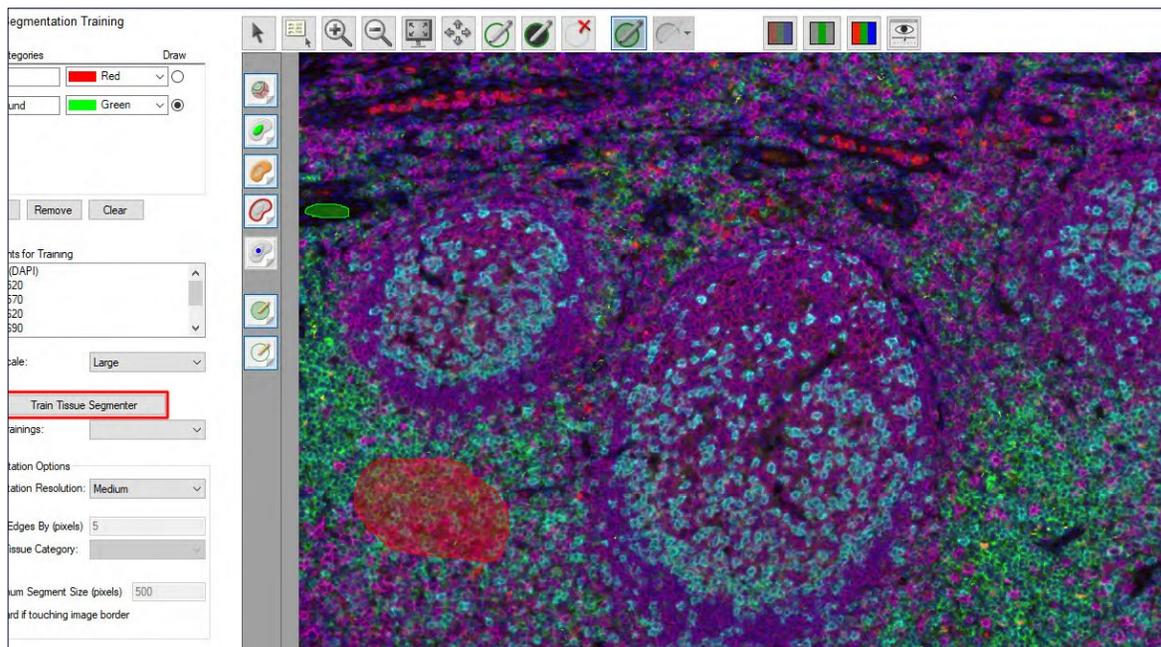


FIGURE 18. For Tissue Segmentation, add in your Tissue Categories of interest (blue box) and draw training regions for each of them on an image in your project. Choose your Pattern Scale sizing (medium is recommended when starting), then include additional Segmentation Options to help further optimize your tissue segmentation. When finished, click on Train Tissue Segmenter (red box).

When you have your training regions and parameters set, select Train Tissue Segmenter. When the algorithm training accuracy stabilizes or reaches 100%, click done and then select Segment Image or Segment All to view the results. You may need to re-train the classifier, create more training regions, and/or change your Segmentation Options and Pattern Scale to achieve optimal results. Of note, Segmentation accuracy (Training %) may never reach 100%. Click done when there is no longer progress being made. Visual inspection of multiple images is the best option of assessing segmentation accuracy.

When satisfied with your tissue segmentation, move on to Cell Segmentation.

Additional recommendations for defining your tissue segmentation training algorithm

- Training is usually performed most efficiently using quick iterations and making adjustments until optimal results are obtained. Make sure to draw your training regions carefully so you don't overlap categories (i.e., including some stromal tissue in a tumor training region). Draw several training areas for each category and evaluate segmentation result. Refine by adding new training areas to address any miscategorized tissue.

- It may be worth noting that sometimes retraining on the same regions can improve segmentation results (even if the accuracy value doesn't change). However, as soon as you change any settings above the Recent Trainings box, previous segmentations are cleared.
- Segmentation options can be adjusted after correct segmentation to refine borders and resolution.
- Minimum Segment Size (pixels) and Trim Edges functions can be quite helpful for filtering out small segmentation errors and fine-tuning segmentation to the edge of tumor areas or small areas of background.
- Try to capture full range of tissue morphology in training regions, for each category, including areas with weak and strong expression of tissue markers.
- If samples are very diverse, it may be necessary to create different segmentation algorithms for different samples.
 - For example, if the algorithm is getting most things correct, but the user finds that additional training on a problematic sample results in the segmenter getting stuck at a low level of accuracy.

Cell Segmentation

The Segment Cells section allows users to segment the different cellular compartments, including nuclei, cytoplasm, and membrane using individual components. There are multiple options for cell segmentation, including the classic inForm Cell Segmentation, as well as the new Adaptive Cell Segmentation, which uses algorithms to account for variations in staining and background levels within and across images to identify cellular compartments from multiple image planes. These features provide improved segmentation performance when images contain mixtures of cell types with differing nuclear morphologies. Segmentation of all cellular compartments (i.e. nuclei, cytoplasm, membrane) also supports better phenotyping.

For this overview, we will focus on Adaptive Cell Segmentation.

Nuclear Segmentation

The first cell compartment you will want to segment is the cell nuclei. Under Components, select Add... From the dropdown menu, select DAPI then click on the ellipsis button to configure the component. The Typical Intensity slider can be used to adjust the threshold used to detect nuclear pixels.

- A real-time preview window will appear when using the Typical Intensity configuration. The goal is to find the appropriate level that detects all the nuclear signal while excluding non-specific background.
 - To help with this, turn off the tissue segmentation overlay and all channels except for the ones that you are using to help in segmentation.
 - The preview window can be moved around the image for further assessment.
 - This threshold is adaptive and measured relative to nearby background. Increase this value if too much background is being detected as nuclear. Lower this value if faint nuclei are being missed. At this point, do not focus on whether nuclei are correctly split from each other (splitting will be addressed in subsequent steps). Only focus on whether the correct pixels are being detected as nuclear.

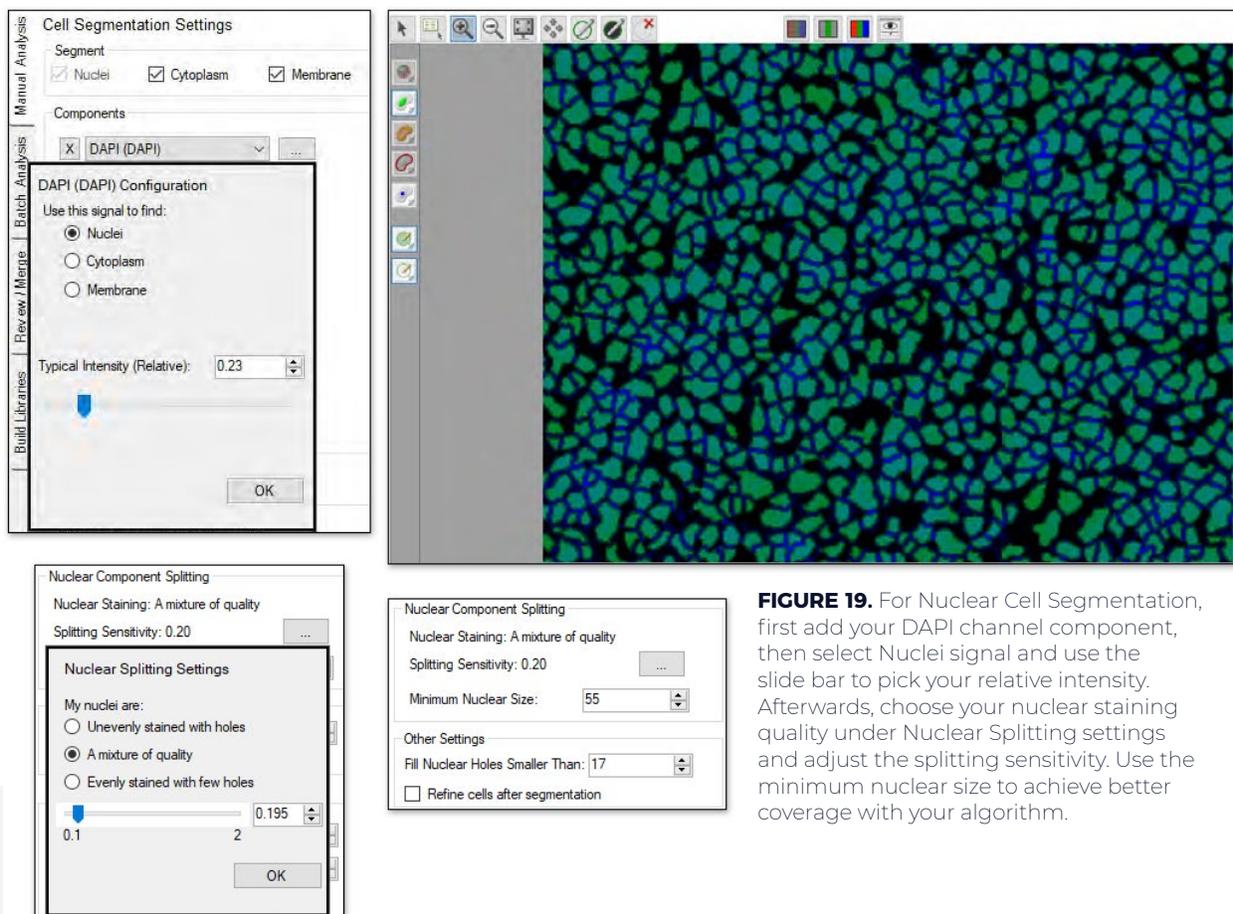


FIGURE 19. For Nuclear Cell Segmentation, first add your DAPI channel component, then select Nuclei signal and use the slide bar to pick your relative intensity. Afterwards, choose your nuclear staining quality under Nuclear Splitting settings and adjust the splitting sensitivity. Use the minimum nuclear size to achieve better coverage with your algorithm.

When satisfied with the signal coverage, click OK. You can toggle back and forth between the nuclear segmentation mask using the green “Show/Hide the Nuclear

Segmentation Map” button from the map icon bar to the left of the image. Next, use the Nuclear Component Splitting settings to assist further with segmentation. Select the ellipsis button to help configure splitting sensitivity. Under Nuclear Splitting Settings, choose the appropriate nuclei staining quality of your image: unevenly stained with holes, a mixture of quality, or evenly stained with few holes. When not sure, select a mixture of quality. Use the slider bar to adjust the splitting threshold: the lower the value, the more splitting will occur. Toggle back and forth with the nuclear segmentation mask overlay to ensure you are getting accurate splitting. Select OK, then set your Minimum Nuclear Size (in pixels). This setting will ignore splitting lines that generate nuclei smaller than this size as well as removing individual cells that fall below this limit.

TIP: Changing image to hematoxylin pathology view makes evaluation of cell segmentation accuracy much easier

Afterwards, use the Other Settings option to help further refine your nuclear segmentation.

- If you find that some nuclei contain empty ‘holes,’ increase the Fill Holes Smaller Than value until those holes are filled.
- If you find that the detected cells are more jagged or angular than desired, check the Refine cells after segmentation box to compact the cells into rounder shapes.

Once finished, you can then go back to Components and add additional components to aid with nuclear cell segmentation. For each, select which compartment the signal should be used to find and choose if you want to use the signal to assist in nuclear splitting.

- It can be helpful to use markers with well-defined membrane staining to aid in nuclear splitting for improved phenotyping results.
- You may also want to include additional nuclear markers, such as FoxP3, if the staining is blocking the DAPI signal resulting in missed nuclear segmentations.

Membrane/ Cytoplasm Segmentation

Following nuclear segmentation, go to the Cell Segmentation Settings box and select Cytoplasm and Membrane. Under Components, select Add..., then select a component. When adding in a component, Akoya recommends first selecting an abundant membrane marker. Afterwards, click on the ellipsis button and choose either the membrane or cytoplasm designation. You do not have to have a component selected for cytoplasm in order to segment it.

Under the Membrane and Cytoplasm Settings box, set the desired cytoplasm thickness and membrane search distance, as well as the membrane settings.

- Adjust the Cytoplasm Thickness to define the distance (in pixels) from the boundary of each cell's nucleus to the outer boundary of its cytoplasm. For each cell, all pixels between the nuclear boundary and the cytoplasm outer boundary will be defined as cytoplasm. This distance should be adjusted based on visual inspection of the results, so that cytoplasm segmentation approximately matches the average size of cells.
- Adjust the Membrane Search Distance to define the maximum distance (in pixels) from the boundary of each cell's nucleus to search for membrane signals. If positive signals are detected between the nucleus boundary and the Membrane Search Distance, the membrane will be drawn where the positive signal is detected. If no positive signal is found between the nucleus boundary and the Membrane Search Distance, the membrane will be drawn at the nearest of three stop points: membrane search distance, a neighboring cell membrane, or cytoplasm thickness.

Akoya recommends that your Membrane Search Distance should be at least double your Cytoplasm Thickness (ex. Membrane Search Distance =

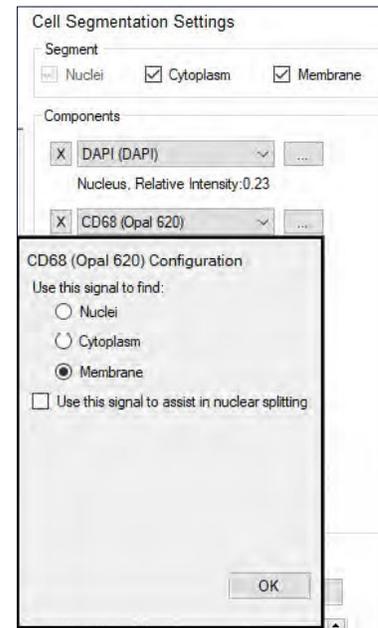


FIGURE 20. Select the component you want to use for membrane and/or cytoplasm segmentation, then select the appropriate designation.

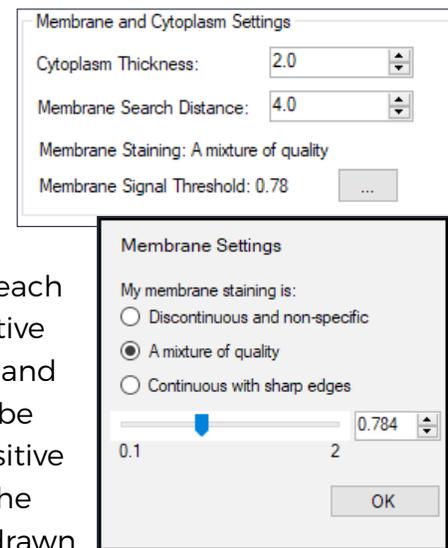


FIGURE 21. Using visual assessment, adjust the Membrane and Cytoplasm Settings so the algorithm detects them appropriately.

4; Cytoplasm Thickness = 2).

- By setting the Membrane Search Distance to be larger than Cytoplasm Thickness, you allow the algorithm to search a large distance for true positive membrane signals, but to collapse back to a smaller cell size if no positive signals are found. For this added flexibility, you may want to include Cytoplasm segmentation even if you are not directly interested in the Cytoplasm compartment.
- Afterwards, click the ellipsis button to configure the sensitivity of membrane signal detection. Select the best description of the staining quality of the configured membrane components, similar to the Nuclear Staining quality. Then use the slider bar to adjust the membrane detection threshold. Lower values will result in detecting weaker signals.
- Hit Segment All to segment all the images in your project, then hit Advance or select Phenotype Cells to move forward to the next step.

You will want to optimize these parameters as much as you can before moving on to phenotype training. Changing any of the tissue or cell segmentation parameters erases phenotype training cell selections, so it is highly recommended to review the tissue and cell segmentation algorithms for all of the images to be analyzed before proceeding. This can be done within an inForm project for a small number of images but may require batching for a large number of images. If problematic images are discovered, they can either be discarded or pulled into the inForm project for further training.

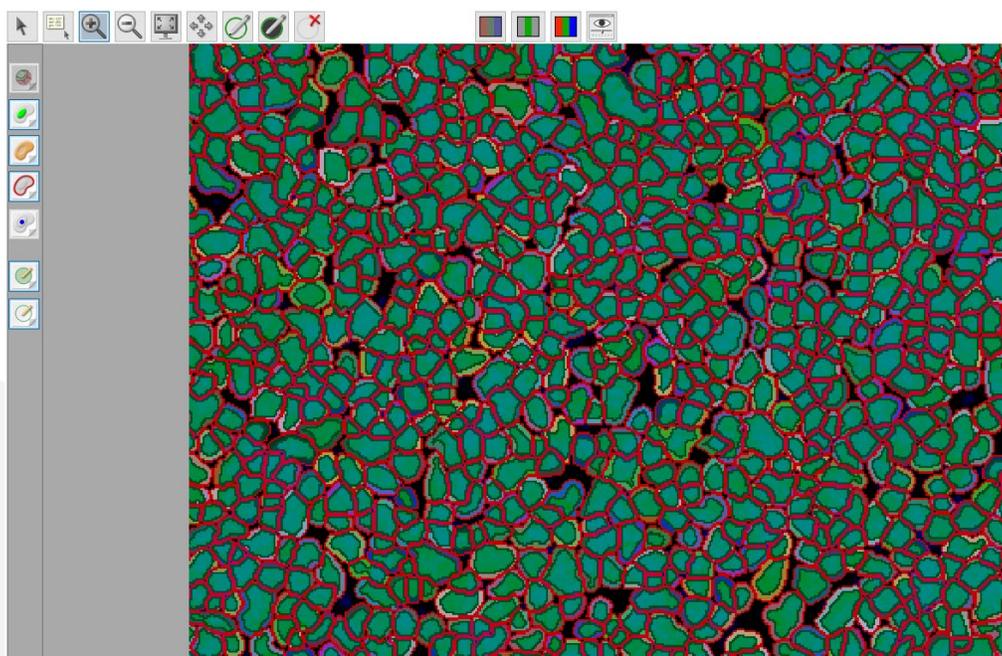


FIGURE 22. Image of successful cell segmentation of all components: nuclear, cytoplasm, and membrane.

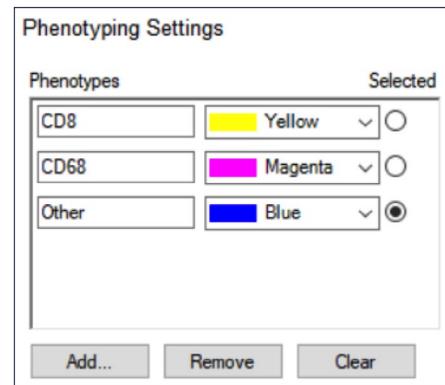
Phenotyping

inForm's phenotyping is a user-trainable feature, similar to Adaptive Cell Segmentation, that uses learning algorithms to identify and classify user-identified cell phenotypes. Prior to beginning your phenotyping, Akoya recommends that you verify that your tissue and cell segmentation parameters have been adequately optimized. This is important because in order to achieve accurate phenotyping, proper cell segmentation is necessary.

Add Phenotypes for Training

Under Phenotyping Settings, select Add... A Create New Phenotype box will appear.

Type in the name of the phenotype of interest (making sure to include either "+" or "-" at the end) and designate a respective color. Akoya recommends using a color similar to the fluorophore color designations selected when preparing images. Be sure to include only phenotypes that are necessary to achieve your measurement goal.



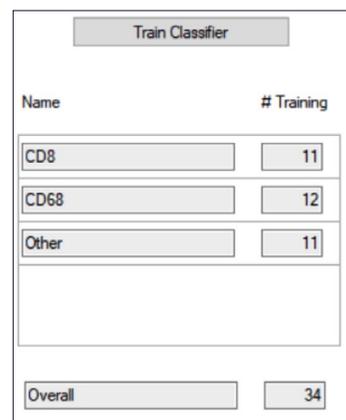
Phenotypes	Selected
CD8	<input type="radio"/>
CD68	<input type="radio"/>
Other	<input checked="" type="radio"/>

FIGURE 23. Phenotypes added in to the project for training. To ensure accurate training, include an 'Others' category to encompass cells not being phenotyped in the project.

Multiple Phenotyping Projects

If you have a specific project that has several co-expressing phenotypes, it may be prudent to sub-divide the inForm project to avoid the complexity in training and the need to cover all marker combinations. To do this, save the project and rename it after completing both the Tissue and Cell Segmentation (it is critical that the tissue and cell segmentations are the same across projects). This will act as your primary project file from which you can create multiple smaller projects that encompass different phenotyping classifiers. Save these projects under different names and batch all images through each one. Of note, it is important to include an 'Other' cell category for all remaining cell types within each of the sub-projects.

All data can be consolidated using phenoptrReports to reveal cell counts and co-expressors.



Name	# Training
CD8	11
CD68	12
Other	11
Overall	34

FIGURE 24. The number of cells identified for training can be observed in the training box. A minimum of five cells per phenotype is required for algorithm training. When finished, click Train Classifier.

Training Classifiers

Using the View Editor, turn off all channels except for those being used in your training set to better identify the markers of interest. Select the Edit Phenotypes button (icon of a cell with a blue dot and arrow), and on your image, left click on a cell and choose a respective phenotype designation. If nothing appears, you may not be within a cell boundary. To help with identifying cell boundaries, view the cell segmentation overlay on your image.

- Make sure to check the cell segmentation of cells included in the phenotype training set to avoid training on poorly segmented cells (i.e. under-split cells where the nucleus crosses two different phenotypes), which can skew the results.
- Make sure to train on negative cells that neighbor positive cells since the shared membrane can result in false positives.
- Any pre-existing phenotype training will be lost if tissue or cell segmentation parameters are changed.

For cancer immunology studies across multiple samples, we have found that identifying roughly 5 to 30 examples of each cellular phenotype to start are necessary for reliable classifier training. This may involve an initial training with 5 to 10 for each phenotype, followed by 3 to 5 iterations to assess results. Training may be done on several images within the project to achieve the best training algorithm. New cells added during each iteration improve phenotyping. With a well stained, abundant sample, Akoya training sets range between 50 to 150 examples for each marker, with the most training cells in the “other” category.

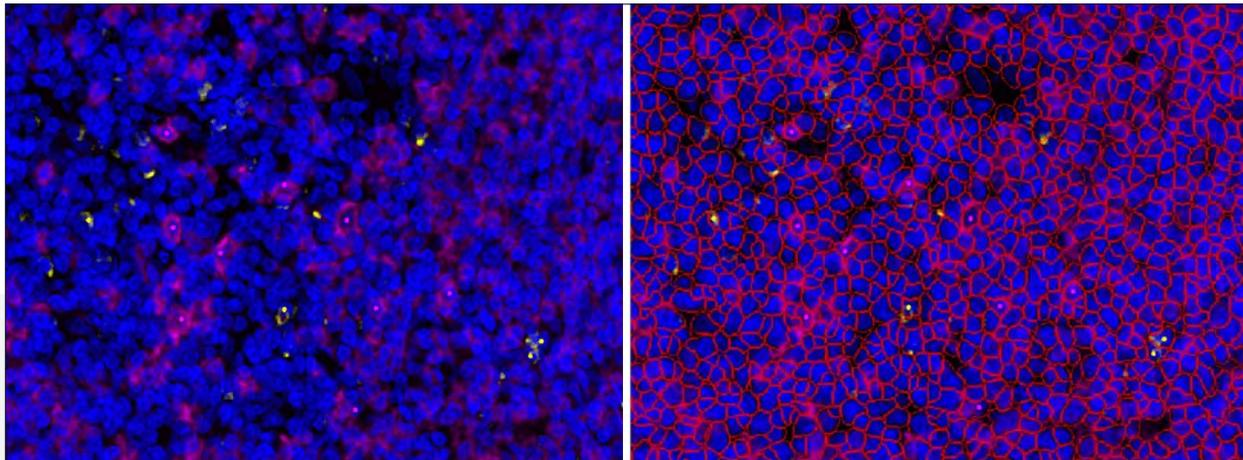


FIGURE 25. When looking for cells to phenotype, it is recommended to turn off non-relevant channels in the view editor (left). To further assist with this process, it may be helpful to view the cell segmentation overlay (right). Retraining a Classifier

Following the initial training, inForm will assign all segmented cells with a phenotype. When your cursor hovers over the trained cell, a phenotype designation will appear along with a percent confidence. If cells appear to be trained incorrectly, left-click on the cell to change its classification. Cells that were used in the training algorithm display a confidence of 100%.

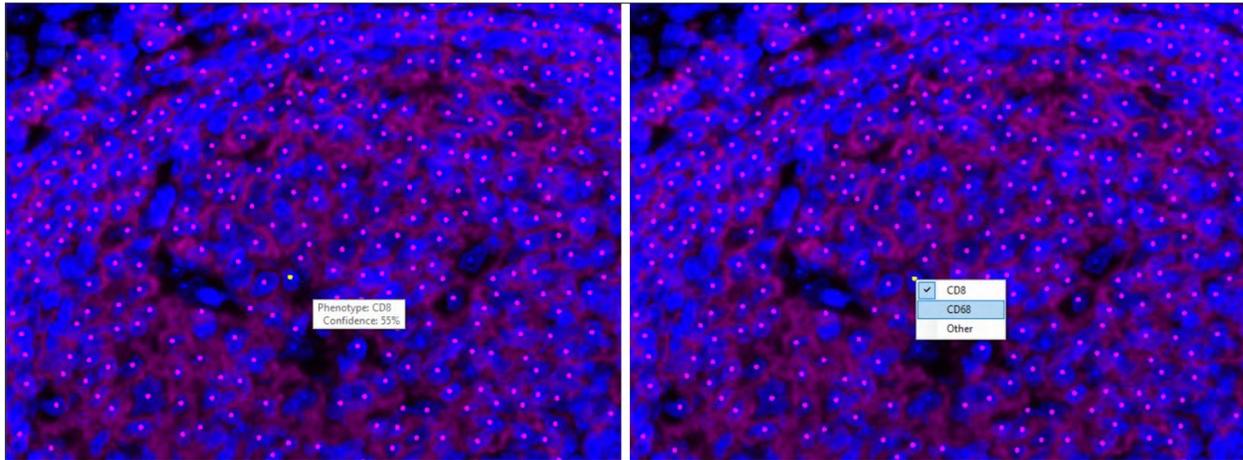


FIGURE 26. Following classifier training, be sure to review your images. If a cell has been incorrectly phenotyped (left), hover over it and left click to change its designation (right). The more cells that are user-identified, the more accurate the algorithm becomes.

- Be sure to review cellular areas that have significant overlap or variable staining over multiple images (select Phenotype All to view phenotyping across all images in the project). The more cells correctly identified, the more accurate the training algorithm.

Scoring IHC or IF

This inForm module is used to assess staining intensity at varying levels, similar to visual inspection of chromogenic staining. For this section, it is important to have properly segmented the cell nuclei, cytoplasm, and membrane in your images.

Tissue Category and Scoring Type

To start, the tissue category and scoring type will need to be selected.

- Tissue Category: Selection is based off of tissue segmentation chosen in an earlier module. Only tissue categories that have cells segmented within them will be available for selection.

- Scoring: There are five histogram score types that can be selected depending upon your analysis needs. Akoya recommends using the 0-3+ (4-bin) option for scoring, particularly with clinical samples and distinct markers of interest, such as PD-L1.

Marker Settings

To analyze an expression marker, first select which compartment the marker is located (nuclei, cytoplasm, or membrane). Next, designate the component you are going to be scoring. This is where it is helpful to have your fluorophores labeled with the marker of interest.

To identify your Threshold Max, click on the Auto button. This will specify the maximum value for each of the threshold bins (sliders) so be sure to set it on your brightest image. If the 4-bin option is selected, you will set three thresholds to receive values for the 4 bins:

0/1+ threshold: All objects below this threshold will be placed in the zero bin.

1+/2+ threshold: All objects below this threshold but above the 0/1+ threshold will be placed into the 1+ bin.

2+/3+ threshold: All objects below this threshold but above the 1+/2+ threshold will be placed in the 2+ bin. Anything above this threshold is included in the 3+ bin.

To obtain accurate scoring, the user should adjust the Threshold (slider bar) for each bin based upon visual assessment of the images. It is recommended to space the thresholds for

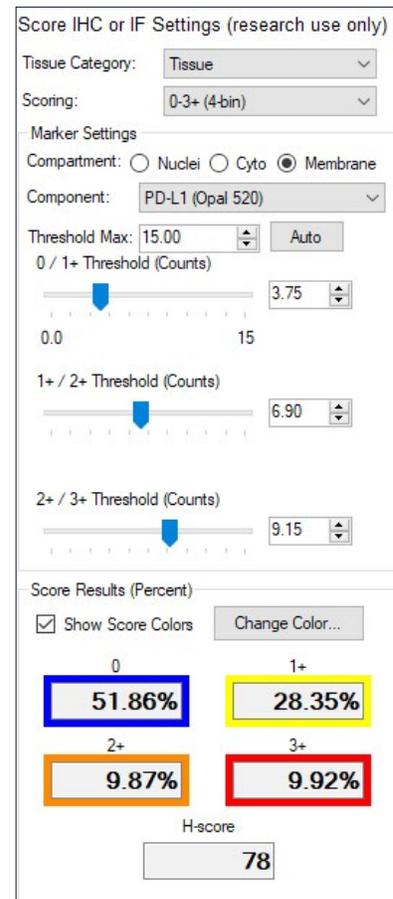


FIGURE 27. Example of settings used to characterize PD-L1 expression in tonsil tissue.

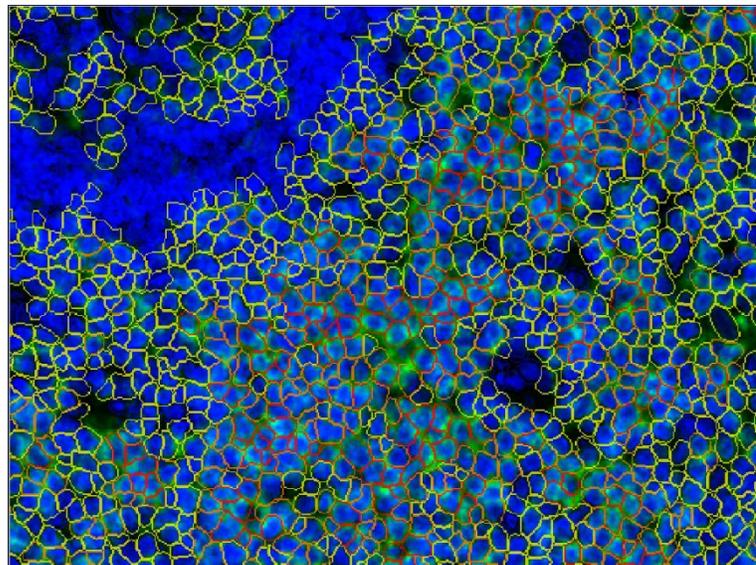


FIGURE 28. Visual inspection of the thresholds for each bin is key to ensure accurate scoring. In this example, PD-L1 (green) is a strongly prevalent marker in tonsil, and much of the staining falls into the 1+ bin.

each bin equidistant from each other. Score Results reports the percent positivity of the cellular compartments within each bin and an H-Score (calculated from the percentages in each bin, ranging from 0-300).

To visualize the results on the image, select Show Score Colors. Colors can be changed to provide better visualization on the imagery by selecting Change Color... The option to return the colors back to their original scheme can be accomplished by selecting Reset in the Change Color box.

An important note: if you have multiple projects as a result of Phenotyping, only one module for Scoring IHC or IF needs to be completed in one of the projects. All data will be aggregated in phenoptrReports.

inForm Project Data Export

When all of the modules in the respective inForm project have been completed, it is time to export the data. There are several options that can be chosen for export. Here, we will cover the selections Akoya recommends that will work with phenoptrReports.

Image Export Options

Before exporting the data can occur, an Export Directory must be selected. Export Directory must be an empty folder.

Under Image Export Format, you can choose between TIFF or JPEG.

Next, select the Images to Export. Akoya recommends RGB with Tissue Segmentation Map, RGB with Phenotype Map, RGB with Scoring Map, and Composite Image, however, the user may select as many as they would like. If you will be further analyzing your images with the phenoptrReports spatial map viewer, you will also need to export the Component Images (multi-image TIFF).

Using the View Editor, go to Data Displayed and drop down to Cell Segmentation Data. This data is important for analysis in phenoptrReports. The following figure (Fig. 29) displays the recommended components that should be selected at a minimum. If your sample is a TMA, be sure to also include Show Core ID.

Tables to Export

Select the necessary data tables. Typically, the Cell Segmentation Data and Score Data tables are selected.

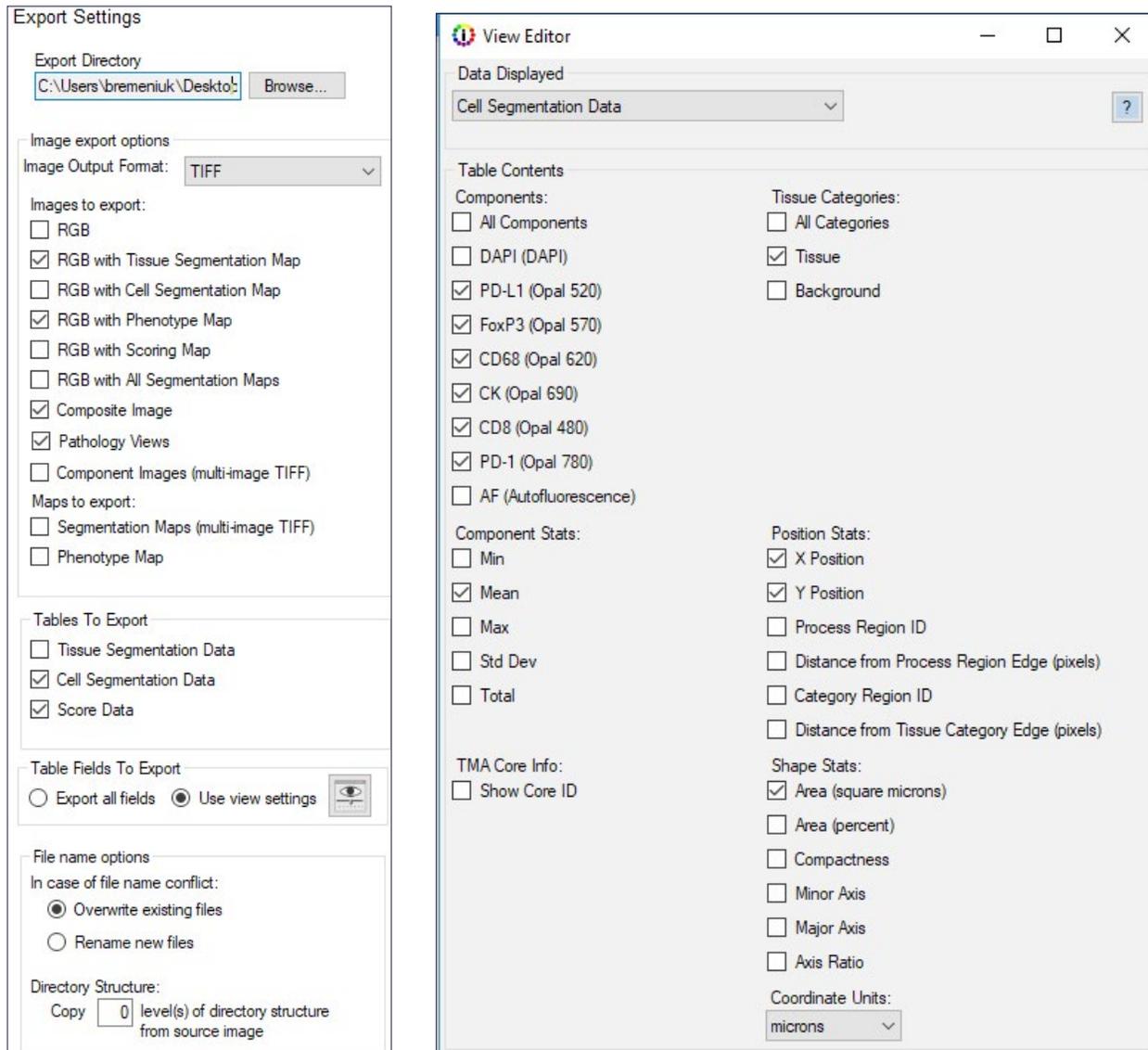


FIGURE 29. Recommended Export Settings (left) and Cell Segmentation Data components (right) to be used in phenoptrReports.

Table Fields to Export

Akoya recommends choosing the 'Use View Settings' option, which is based on the View Editor settings that were used in the project. Be sure to select all fields you think you may need to avoid the need to re-batch.

File Name Options

Lastly, select whether the project should Overwrite existing file data if a duplicate is found, or if the program should Rename all new files added. For data integrity purposes, Akoya recommends Overwriting existing data.

■ Staining Performance Review and Evaluation

Signal Balance Assessment

When transitioning a multiplex panel, signals can alter from what was previously observed on monoplexes, resulting in an unbalanced signal. Ideally, signal levels should be within a factor 3 of each other, particularly for spectrally adjacent fluorophores. Large disparities (>5-fold differences) in signals for spectrally adjacent fluorophores can lead to problems with interference and cause unmixing artifacts. To check for signal balance, unmix the images in inForm, or use the latest iteration of Phenochart, and hover the info cursor over bright areas corresponding to each fluorophore. Compare the values of all listed fluorophores to observe the expression range for the marker in question and assess the value against a bright spot. If signal levels of any fluorophores have increased or decreased to be more than 3-fold different than an adjacent fluorophore, the signal will need to be re-balanced, usually by adjusting the signal of the outlier fluorophore.

- Note: Outliers in signal can occur for a variety of reasons, such as tissue folds or fixation artifacts. Not all anomalies are caused by unmixing issues.
- Reasons we have seen unexpected changes in signal during the creation of the multiplex protocol after a robust monoplex development include unforeseen physiology (i.e., two markers co-localizing unexpectedly) and high variability in study samples. Staining sequence should be re-assessed.

Interference Assessment

Overstaining with an Opal fluorophore can interfere with later applications of Opal fluorophores in the multiplex panel. For that reason, signal levels should usually remain below 30 for multiplex controls. There are two ways to check for interference.

1. Visual Confirmation: Turn the respective channels on and off in inForm (or Phenochart) that are associated with the fluorophores in question. Signal loss will appear in one channel due to the presence of signal in another, resulting in a “hole” observed in the staining pattern that is filled by the interfering signal. Confirm the staining patterns of the markers of interest to ensure accuracy of diagnosis.

- a. Quantitative Assessment: Stain serial sections of a TMA with monoplex and multiplex protocols, and using inForm, measure signal levels within each TMA core and in appropriate cells and cellular compartments. Plot average monoplex against multiplex signals for the same cores. The fit of a straight line through the scatter plot should yield an R-squared value of at least 0.8.

Crosstalk Interference

Crosstalk can be the result of inadequate stripping of antibodies during MWT or HIER. This problem, while rare, may be related to unusually high affinity primary antibodies. To assess for crosstalk in your staining:

1. Visual Confirmation: Turn the respective channels on and off in inForm (or Phenochart) that are demonstrating overlap. Images should appear to have an additive effect when channels are overlaid on one another. Confirm the staining patterns of the markers of interest to ensure accuracy of diagnosis.
 - a. Quantitative Assessment: Stain serial sections of a TMA with monoplex and multiplex protocols, and using inForm, measure signal levels within each TMA core and in appropriate cells and cellular compartments. Plot average monoplex against multiplex signals for the same cores. The fit of a straight line through the scatter plot should yield an R-squared value of at least 0.8.
2. Spectral Crosstalk: Ensure your staining order separates spectrally adjacent dyes in both sequence and expression (i.e., we would not recommend staining CD8 with Opal 540 followed in sequence immediately by CD3 with Opal 570 as this would lead to a high probability of crosstalk). To assess for spectral crosstalk:
 - a. Hover over your multiplex image and your appropriate library image. Confirm the spectra for the fluorophores in question are the same.
 - b. Compare your most recent library with the library slides shipped with the Vectra Polaris. The spectra should be close. If not, this can indicate a poor spectral extraction.

Validation

Human visual inspection remains the gold standard for confirming stain localization and specificity, tissue segmentation, cellular compartments, and cellular phenotypes. Pathologist should be consulted to confirm staining and tissue morphologies, particularly in regard to clinical samples. Reference images can be acquired from peer-reviewed publications as well as online resources like the Human Protein Atlas 3 and GeneCards 4.

Opal Assay Quick Start Guide

Preliminary Steps

1. Bake slides for at least 1 hour or overnight at 65 °C
2. Wash slides in xylene for 10 minutes, 3 times
3. Wash slides in 100% Ethanol for 10 minutes
4. Wash slides in 95% Ethanol for 10 minutes
5. Rinse slides in 70% Ethanol
6. Rinse slides in distilled Water
7. Incubate slides in NBF for 20 minutes (some tissues may need longer times)
8. Rinse slides in distilled Water
9. Rinse slides in AR 6 or 9 (use the AR solution that will be used for the first antibody)
10. Repeat step 9 Opal Detection

	ANTIBODY 1	ANTIBODY 2	ANTIBODY 3	ANTIBODY 4	ANTIBODY 5	ANTIBODY 6
Antigen						
Species						
Dilution						
Inc. Time						
Inc. Temp.						
Opal fluor						
AR6 / AR9	Fill jar AR 6 or 9					
	AR temperature h	Micro 45" high				
	HIER time:	Micro 15' 20%				
	Cool at least 15'					
	Rinse water					
	Rinse TBST					
	PAP pen encircle					
	Block 10'					
	Antibody	Antibody	Antibody	Antibody	Antibody	Antibody
	Rinse TBST					
	TBST 2'					
	TBST 2'					
	TBST 2'					
	HRP 10'					
	Rinse TBST					
	TBST 2'					
	TBST 2'					
	Opal 10'					
	Rinse TBST					
	TBST 2'					
	TBST 2'					
	TBST 2'					
Use AR6 or AR9 as specified in next col	Rinse AR 6 or 9					

■ Final Steps

- Fill jar with AR solution pH 6
- Heat slides in microwave for 45 seconds at high power, solution should boil
- Heat slides in microwave for 15 minutes at 20% power
- Allow slides to cool on bench for at least 15 minutes
- Rinse slides in distilled Water
- Rinse slides in TBST
- Incubate slides with DAPI solution for one minute
- Wash slides in TBST for two minutes
- Wash slides in distilled Water for two minutes
- Allow slides to dry
- Apply fluorescent mounting medium to the slides
- Apply coverslip to the slides

References

1. Toth, Zsuzsanna E., and Eva Mezey. "Simultaneous visualization of multiple antigens with tyramide signal amplification using antibodies from the same species." *Journal of Histochemistry & Cytochemistry* 55.6 (2007): 545-554. (<http://jhc.sagepub.com/content/55/6/545.short>)
2. Stack, Edward C., Chichung Wang, Kristin A. Roman, and Clifford C. Hoyt. "Multiplexed immunohistochemistry, imaging, and quantitation: A review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis." *Methods* 70, no. 1 (2014): 46-58. (<http://www.sciencedirect.com/science/article/pii/S1046202314002837>)
3. Uhlén, Mathias, et al. "Tissue-based map of the human proteome." *Science* 347.6220 (2015): 1260419. (<http://www.proteinatlas.org/>)
4. GeneCards®: The Human Gene Database (<http://www.genecards.org>)

Additional Resources

Please visit www.akoyabio.com/support for additional resources, including FAQs and publications.

■ Notice

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FOR MORE INFORMATION CONTACT:

Akoya Biosciences, Inc.

100 Campus Drive, 6th Floor

Marlborough, MA 01752, USA

Phone: +1 855-896-8401

Fax: +1 855-404-0061

Email: support@akoyabio.com

Web site: <https://www.akoyabio.com/company/contact-us>



AKOYA
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Akoya Biosciences, Inc.

100 Campus Drive, 6th Floor

Marlborough, MA 01752, USA

www.akoyabio.com