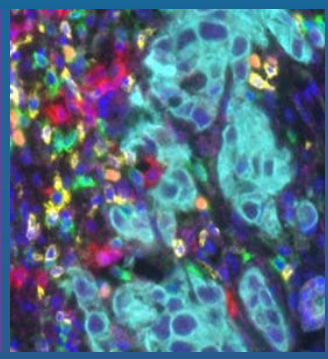
HUMAN HEALTH

ENVIRONMENTAL HEALTH

PHENOPTICS RESEARCH SOLUTIONS



Assay Development Guide

PerkinElmer®
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PHENOPTICS MULTIPLEX IHC DETECTION, IMAGING AND ANALYSIS

What is Phenoptics?

PerkinElmer's Phenoptics workflow enables imaging and analysis of up to six immunofluorescence markers within intact tissue sections (Figure 1). Quantitative assessment of cellular phenotype and activity becomes possible in a way that is similar to flow cytometry, while simultaneously providing tissue context and information about cell-to-cell interactions that is difficult or impossible to obtain by other methods. The Phenoptics workflow incorporates three essential elements.

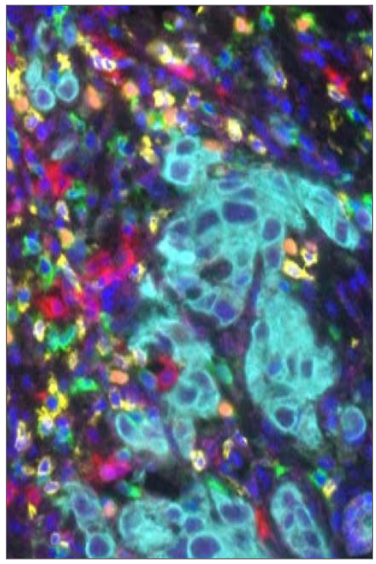

Figure 1. Breast cancer tissue section, Opal 7-color IHC. Red: CD20. Yellow: CD8. Green: CD4. Magenta: PDL1. Cyan: cytokeratin. Orange: FoxP3. Blue: nuclei.

- **Opal™** is a method for multiplex fluorescent immunohistochemistry in formalin-fixed, paraffin-embedded (FFPE) tissue. It allows use of standard unlabeled primary antibodies, including multiple antibodies raised in the same species. The basic approach was inspired by a protocol published by Zsuzsanna Tóth and Éva Mezey[1]. The current method involves detection with Opal reactive fluorophores that covalently label the epitope. After labeling is complete, antibodies are removed in a manner that does not disrupt the Opal fluorescence signal. This allows the next target to be detected without fear of antibody cross reactivity. Opal enables development of multiplexed assays with balanced, quantitative signal for rare and abundant targets.
- **Multispectral imaging** eliminates fluorophore crosstalk and interference from tissue autofluorescence, allowing precise measurement of each fluorescence signal within a tissue sample. **Vectra®** and **Mantra™** multiplexed biomarker imaging systems from PerkinElmer are recommended for unmixing of spectrally overlapping fluorophores as well as tissue autofluorescence. Separation of all of the fluorescence signals within the sample is essential for quantitative measurement.
- **inForm™ Tissue Finder™** image analysis software determines per-cell and per-subcellular compartment intensity values. This information is used in combination with user-trainable machine learning algorithms to phenotype cells, recognize morphologic regions of the tissue (e.g., tumor, dermis, stroma, inflammation), and provide cell counts and densities within each region. Data may be exported from inForm for additional analysis such as distance mapping between selected cellular phenotypes. In addition to fluorescent composite images, inForm provides simulated brightfield monoplex IHC from the same data for improved visual interpretation.

It is important to confirm that each step in the Phenoptics workflow meets criteria for success because information from earlier steps will affect execution and optimization of later steps. Research laboratories performing this method should be proficient with conventional immunohistochemistry assay development, fluorescence imaging (preferably on the Vectra or Mantra platforms), and image analysis programs. Advanced measurements like spatial relationship calculations require familiarity with R, TIBCO Spotfire®, or similar data analysis programs.

Signal intensity (as measured on the Vectra or Mantra systems) for all Opal fluorophores will be proportional to expression if assay development and exposure time guidelines are followed [2].

For evaluation of more than six IHC targets in one sample, two panels may be developed that include one or more common landmark markers such as a tumor marker. The landmarks may be used to help with alignment of images across serial sections.

This document offers advice and best practices for implementing the entire Phenoptics workflow, from Opal assay development through imaging and analysis. It is intended to be a starting point. We encourage anyone who is implementing the method to build on this and welcome any comment.

Slide Definitions

Successful optimization of a multiplex assay requires three different types of slides.

- **Monoplex.** Control tissue slides are labeled for one marker with a single Opal fluorophore and counterstained with DAPI. They are necessary for assessment of staining results and comparison to established standards.
- **Library.** Spectral library slides stained with a single fluorophore and made from relevant control tissue will be necessary for accurate unmixing and analysis of your monoplex and multiplex slides. The following slides will be required.
  - One control tissue slide stained for each antibody-Opal fluorophore combination under optimized conditions, without DAPI.
  - One control tissue slide stained with DAPI alone.
  - One unstained control tissue slide, for assessment of autofluorescence. The unstained slides should be processed in the same way as the other slides, omitting both the Opal fluorophore and DAPI.
- **Multiplex.** Control tissue slides are labeled for all of the markers in the multiplex panel and counterstained with DAPI. These slides allow assessment of the multiplex assay for interference and crosstalk.

Phenoptics Assay Development Flow Chart

1. Primary Antibody Titration

- Select antibodies
- Pair antibodies with Opal fluorophores
- Evaluate staining pattern
- Optimize antibody concentration for appropriate autoexposure times with Opal detection
- Optimized single stain slides may be used as library slides

2. Library Development and Assessment of Opal Monoplex Results

- Create DAPI and unstained library slides
- Build and evaluate library using optimized single stain Opal, DAPI, and unstained slides
- Create monoplex slides
- Image monoplex slides, unmix, and develop simulated IHC view Evaluate intensity and specificity for each monoplex assay

3. Opal Multiplex Assay Development

- Select order of detection
- Perform multiplex stain (with DAPI)
- Confirm non-interference and crosstalk in multiplex images
- Rebalance or re-order if needed

4. Image Analysis

- Tissue segmentation
- Cell segmentation
- Phenotyping cells and measuring expression levels
- Validation

Opal Reagents and Materials

Opal kits are available with up to seven colors, including DAPI counterstain.

Fluorophore	Opal Multicolor IHC Kits				Excitation Max	Emission Max
	4-color	5-color	6-color	7-color		
Spectral DAPI	✓	✓	✓	✓	358 nm	461 nm
Opal 520	✓	✓	✓	✓	494 nm	525 nm
Opal 540		✓	✓	✓	523 nm	536 nm
Opal 570	✓		✓	✓	550 nm	570 nm
Opal 620		✓	✓	✓	588 nm	616 nm
Opal 650				✓	627 nm	650 nm
Opal 670	✓				650 nm	670 nm
Opal 690		✓	✓	✓	676 nm	694 nm

The Opal staining protocol is similar in many respects to standard immunohistochemistry (IHC) that uses diaminobenzidine (DAB). Tissue is incubated with unlabeled primary antibodies. These are detected with HRP conjugated secondary antibody and a detection substrate. For this reason, many of the reagents and materials used in this process will be familiar to anyone who performs IHC currently.

Catalog numbers for Opal kits and some ancillary reagents may be found in the Supplementary Information at the end of this document or at www.perkinelmer.com/opal.

Required Reagents

- Opal Multicolor IHC Kit. Kits include DAPI and AR6 (antigen retrieval, pH 6) buffer that is optimized for the protocol.
- Primary antibodies for targets of interest.
  - Select antibodies for specificity to the target and other performance considerations. Unlike other multiplexed approaches, there is no need to limit selection based on species or to source labeled primary antibodies.
  - Select antibodies previously validated for IHC when available. Usually, they will work as well or better with Opal.
  - In some cases, antibodies that do not work for standard IHC will work with Opal.
- AR9 (antigen retrieval, pH 6) may work better for some targets, particularly markers that are expressed within the nucleus.
- Isotype control antibodies for confirmation of assay specificity.
- Antibody diluent/block.
- Secondary antibody HRP conjugate.
  - Opal Polymer HRP Ms + Rb is recommended for work with human tissue and primary antibodies from mouse or rabbit.
  - Please contact us for options with non-human tissue and antibodies from other species.
- 10% neutral buffered formalin (NBF).
- TRIS-buffered saline with Tween®20 (TBST) wash solution (0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween®20).
- Ultrapure, peroxidase-free water. Autoclaved Milli-Q® water works for this purpose. Other options should be validated.
- Mounting medium for fluorescence—without DAPI or other counterstain. Users should validate independently. Here are some options that have worked in our laboratories.
  - VECTASHIELD® HardSet Antifade Mounting Medium from Vector Labs.
  - ProLong® Diamond from Thermo Scientific.

Required Materials

- Microwave oven with a carousel and 10 power settings, rated at 1000-1200 watts. Most conventional home microwaves work well for MWT in the Opal process. Panasonic® products with Inverter® technology have more precise power control and are recommended if purchasing a new microwave oven. Users should validate microwave performance independently.
- Hellendahl type staining jars are critical for MWT because they hold enough AR buffer to ensure that slides do not dry out. Opal Slide Processing Jars from PerkinElmer are non-breakable and hold up to 14 slides.
- Baths and solvents for deparaffinization and rehydration of FFPE tissue. Xylene is recommended for deparaffinization. Histological grade ethanol is required for rehydration.
- Slide incubation/humidity tray for incubation steps.
- Hydrophobic barrier pen.
- Glass coverslips.
- Control tissues.
- Charged slides.

Primary Antibody Titration

Once primary antibodies have been sourced for the targets of interest, single analyte Opal assays must be developed for each antibody in appropriate control tissue. Optimized single-fluorophore Opal slides may be used as library slides.

Opal Fluorophore-antibody Combinations

Select an Opal fluorophore for detection of each target. Here are some considerations for antibody-fluorophore pairing.

- Most antibodies can be optimized to work with any Opal fluorophore.
- More abundant targets should be detected with the far red Opal dyes: Opal 650, Opal 670, or Opal 690.
- For highly autofluorescent tissue like spleen or brain, Opal 520 may work better for detection of abundant proteins.
- When possible, avoid locating spectrally adjacent fluorophores within the same subcellular compartment of the same cells. For example, certain T cells can coexpress CD3 and CD8 within the membranous compartment. Avoid using Opal 520 and Opal 540 for these two markers. CD3 and CD8 may be detected together with Opal 520 and Opal 570 instead.

Microwave Optimization

Microwave treatment (MWT) is used in the Opal method to quench endogenous peroxidase activity, for antigen retrieval, and to remove antibodies after a target has been detected. Timing for each step in the procedure may have to be modified for the microwave oven that you are using. Slides are placed vertically in an Opal Slide Processing Jar, which is then filled to the top (~140 mL) with AR6 or AR9 buffer and covered loosely. One jar is placed in the microwave at a time, near the edge of the carousel to ensure even distribution of energy (Figure 2). The microwave procedure consists of two steps.

1. 100% power until the boiling point is reached. The time for this step may have to be increased or decreased depending upon the performance of the microwave in your lab This will usually take 45-90 seconds.
2. 20% power for 15 minutes.

Do not operate the microwave oven unattended and keep the oven chamber clean and clear of debris.

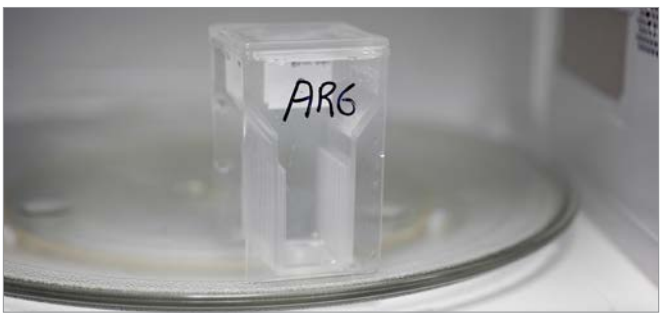

Figure 2. Opal slide processing jar placed near the edge of the carousel in a microwave oven.

Standard Opal Monoplex Protocol Overview

1. **Deparaffinization.** Heat slides in a dry oven at 55-60 °C at least one hour, positioned to allow drainage of melting paraffin. Wash slides with xylene for 10 minutes, three times. Hydrate through an ethanol gradient ending with a distilled water wash (Figure 3).

Figure 3. Deparaffinization process.

2. **Slide Fixation.** Fix tissue in NBF for 20 minutes followed by a distilled water wash. Some tissues, such as skin, will benefit from longer fixation to assure adhesion to the slide and control for under-fixation. Rinse in distilled water.
3. **Antigen Retrieval.** Rinse slides with AR6 or AR9 solution as appropriate for the target being detected. Place slides in an Opal slide processing jar and fill with AR6 or AR9 solution. Perform MWT and allow slides to cool to room temperature on the bench for at least 15 minutes (Figure 4).
4. **Blocking.** Rinse slides with water and then with TBST. Encircle the area of tissue to be stained with a hydrophobic barrier pen. Incubate the tissue with antibody diluent/blocking solution for 10 minutes at room temperature.

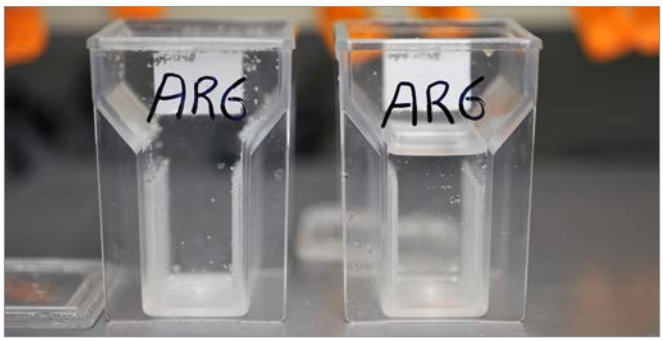

Figure 4. Slide processing jars before (left) and after (right) MWT. Although some evaporation has occurred, the tissue is still covered with AR solution.

5. **Primary antibody incubation.** Remove the blocking solution and then apply the primary antibody solution to the tissue (Figure 5). Antibody concentration and incubation time must be optimized for Opal detection. See Optimization of Primary Antibody Concentration for Opal Detection for more detail on this.

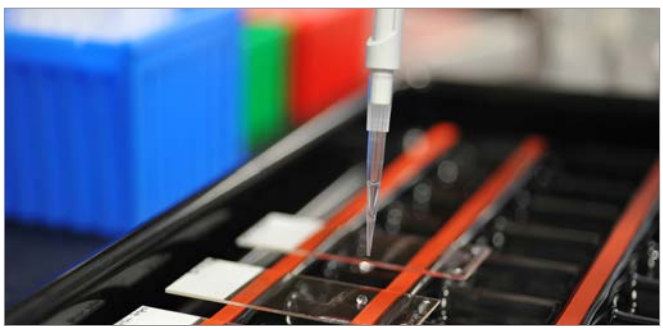

Figure 5. Application of primary antibody.

6. **Secondary Antibody Incubation.** Apply the secondary antibody solution to the tissue. Opal Polymer HRP should be incubated for 10 minutes at room temperature. Most other HRP labeled secondary antibodies are incubated for 60 minutes at room temperature. Rinse slides with TBST and then wash with TBST two times, three minutes each.
7. **Opal Fluorophore Incubation.** Apply Opal fluorophore solution to the tissue and incubate for 10 minutes at room temperature. Wash slides with TBST three times, two minutes each.
8. **Antibody Removal.** Rinse slides twice with AR6 solution. Place slides in an Opal Slide Processing Jar and fill with AR6 solution. Perform MWT and allow slides to cool on the bench for at least 15 minutes (Figure 4).
9. **Spectral DAPI.** Rinse slides in distilled water and then in TBST. Incubate slides in DAPI solution for five minutes at room temperature. Wash slides with TBST for two minutes, and then with distilled water for two minutes. (Skip this step where appropriate for single stain library slides.)
10. **Mount.** Apply mounting medium for fluorescence microscopy and coverslip.

Optimization of Primary Antibody Conditions for Monoplex Opal Detection

The goal of optimization is to generate balanced signal for each marker in the panel. Vectra or Mantra auto exposure times may be used as a guideline and should generally fall between 50 and 200 milliseconds for optimized assays.

- Initially, most antibodies are evaluated at 0.4, 0.2 and 0.1 µg/mL. (It may be necessary to contact the vendor for initial concentration of the antibody.)
- If there is an established IHC/DAB method, try a 1:5 dilution from that concentration. Further dilution may be necessary.
- If antibody concentration is not available and there is no established IHC method, try three or more 3-fold serial dilutions from an initial dilution.
- If the exposure time is under 50 milliseconds, dilute the primary antibody further.
- If the signal intensity is too low or the exposure time is too long consider these options.
  - Switch to HRP polymer reagent (i.e. Opal Polymer HRP) if not currently using.
  - Consider alternate antigen retrieval strategies.
  - Try additional rounds of MWT – some antigens become more exposed under these conditions. This is often observed in tissues with mucin like lung and colon.
  - Try longer incubation times for the primary antibody, even overnight at 4 °C. Note that this may increase non-specific signal.
  - Switch to a different antibody clone. Consult the literature for recommendations.
  - Increase the primary antibody concentration. Note that this may also increase non-specific signal.

Library Development and Assessment of Opal Monoplex Slides

In addition to the optimized Opal single-stain slides, DAPI-only and unstained slides will be required for library development. These slides should otherwise be processed in the same way as the Opal slides.

The instructions below apply to the Vectra and Mantra Systems. Users with other fluorescence microscopy systems employing the Opal 4-color kit should refer to the documentation for those systems with respect to filters and settings for imaging DAPI, fluorescein, Cy3 and Cy5.

General Recommendations for Imaging Opal Slides with Mantra or Vectra Imaging Systems

- Always image using the standard spectral bands for all epi-fluorescence filters available: DAPI, FITC, CY3, Texas Red, and CY5. This applies for all slides including the monoplex spectral library slides and the unstained autofluorescence sample. Acquiring spectra across the full wavelength range is essential for building spectral libraries and correctly unmixing signals from Opal slides.
- Make new spectral library and autofluorescence slides for each study. The spectra from each stain can be stored in the 'Stain Store' in the inForm program
- Always enable saturation protection by checking the Exposure Correction box (Figure 6). This protects against unexpectedly bright signals by reducing exposure when necessary. InForm adjusts for differences in exposure times at each wavelength in unmixing, ensuring that results may be compared between samples.
- Use the lamp at 10% power for imaging Opal slides.

Figure 6. The Exposure Correction box in Vectra enables saturation protection and should always be checked for acquisition of Opal images.

Signal bands for each Opal fluorophore are shown in Figure 7 below. Use the autoexpose function (Figure 8) when the fluorophores present on the slide emit within the current signal band. Set the exposure time to 150 milliseconds when no fluorophores on the slide emit within the current signal band.

Filter / Signal Band	DAPI	FITC	Cy3	Texas Red	Cy5
Fluorophore					
Spectral DAPI					
Opal 520					
Opal 540					
Opal 570					
Opal 620					
Opal 650					
Opal 670					
Opal 690					

Figure 7. Signal bands for DAPI and each Opal fluorophore are indicated by the colored boxes. Use the autoexpose function when fluorophores are present that emit within the current signal band.

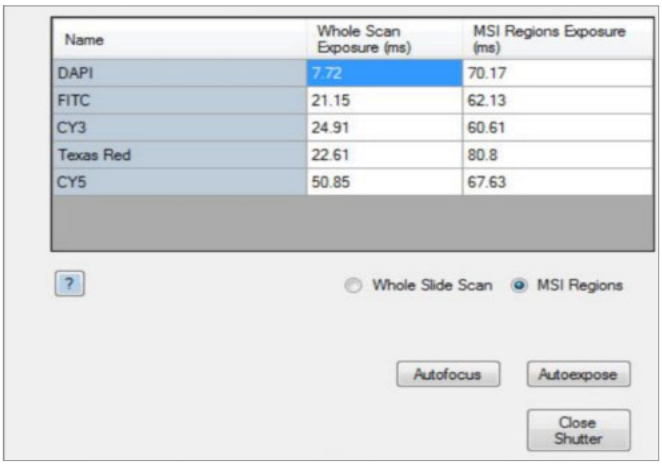

Figure 8. The Vectra 3 Fluorescence Set Exposure control panel. Generally, autoexposure times for optimized Opal monoplex assays range from 50 to 200 milliseconds for the MSI Regions.

Library Development

1. Acquire images from the single fluorophore and unstained autofluorescence spectral library slides prepared earlier using exposure times set in previous step.
2. Extract each Opal fluorophore spectrum using inForm 2.1 (or later) automated tools in Build Libraries tab (Figure 9).

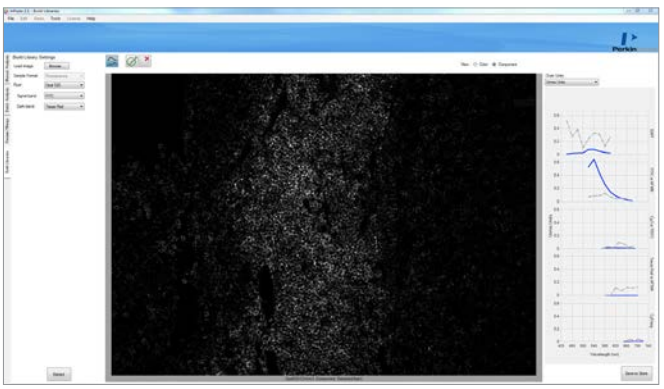

Figure 9. Spectral library slide image for a single flurophone. Spectrum is extracted into the stain store.

3. Save to 'Store' each fluorophore spectrum, preferably with a color that will make it easier to discern in composite view and with a meaningful group tag (Figure 10).

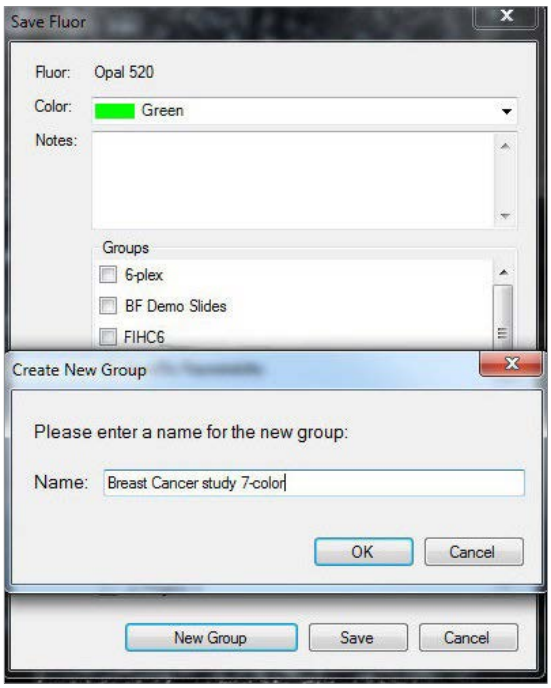

Figure 10. Saving Opal fluorophore spectrum to the library, with a meaningful group tag.

Library Assessment

Test the library by unmixing the spectral library slides, looking for crosstalk.

- In Manual Analysis, load images of the spectral library slides including the autofluorescence (AF) unstained sample.
- Select the library spectra created earlier.
- Collect the autofluorescence signature from the AF unstained sample with the AF sampling tool. Capture auto-fluorescence only from areas with tissue, avoiding areas without tissue. (Figure 11) Include red blood cells in your autofluorescence samples if present. In some cases, we find that capturing a pure red blood cell fluorescence spectrum leads to more effective unmixing.
- Unmix and inspect the composite images for crosstalk, in FL view with AF display unchecked.
  - For each monoplex sample in turn, disable that fluorophore's channel – it should go black.
  - The autofluorescence sample should be completely dark with AF channel off - toggle on/off to see localization of autofluorescence.
- Signals in other channels may be observed when the extracted spectra in inForm are inaccurate. Inaccurate spectra may be caused by non-specific staining or excess tissue autofluorescence.

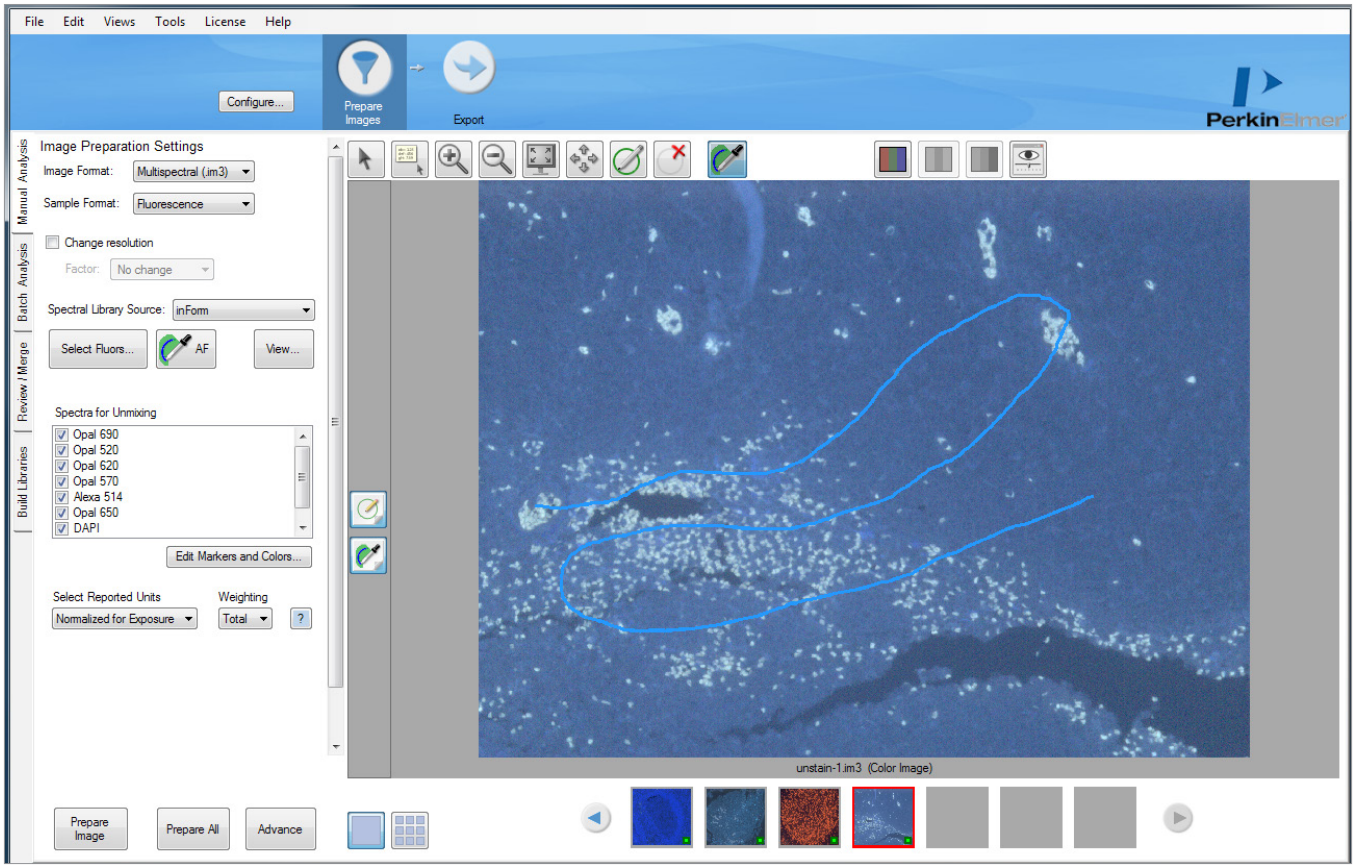

Figure 11. Collection of tissue autofluorescence signal.

Evaluate Intensity and Specificity of Opal Monoplex Slides

Develop a set of Opal monoplex slides using control tissue. These slides should each be stained for a single target using the associated Opal fluorophore under optimized conditions and counterstained with DAPI. These slides may be used for assessment of each monoplex assay and direct comparison to established standards in chromogenic IHC.

Acquire multispectral images of your optimized Opal monoplex slides that meet criteria for autoexposure. In inForm, spectrally unmix them using the library developed previously. Unmixed images may be used to evaluate intensity and specificity of signals and to compare distribution of stain to established standards.

- The information cursor in inForm is often a good way to assess signal intensity and specificity. Move the mouse cursor to an obviously positive cell and note the signal. In optimized assays, signal intensity on appropriate positive control tissue should be in the range of 5 to 20 for all targets (assuming that the lamp was set to 10% when the slide was imaged). Then, move the mouse cursor to an area of non-specific staining and note the signal. The signal-to-background ratio should be at least 10:1 (Figure 12).
- These images should look essentially the same as they would with conventional IHC, with specific and intense staining (Figure 13).

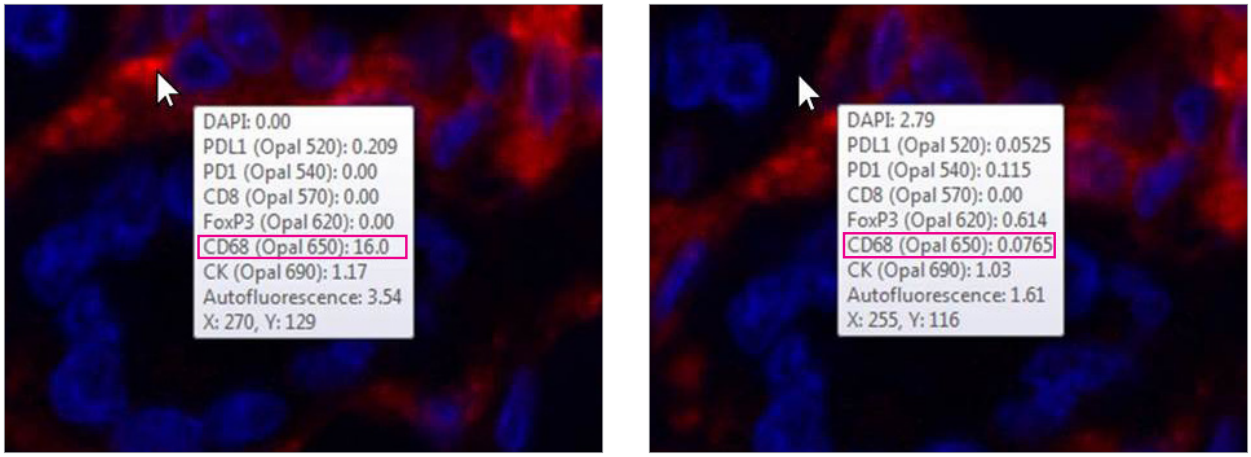

Figure 12. inForm information cursor showing signal and background intensity for Opal 650 monoplex stain. Opal 650 is visualized in red and DAPI in blue. Positive signal (shown on the left) should be at least 10 times higher than background (shown on the right). Signal intensity for all targets should usually be within 5 and 20 for relevant positive control tissue under optimized monoplex IHC conditions.

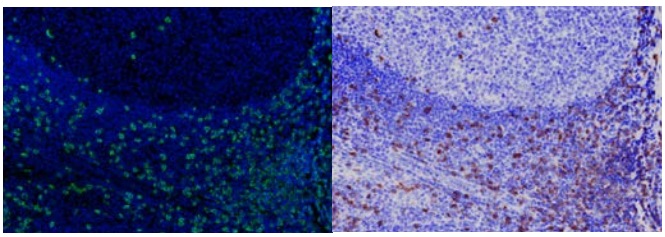

Figure 13. inForm views of monoplex CD8 in human tonsil labeled with Opal 520. The unmixed fluorescence composite view on the left visualizes CD8 in green and nuclei in blue. The simulated IHC view on the right allows for direct comparison of staining patterns with established standards.

Opal Multiplex Assay Development

The next step is to combine your verified monoplex Opal assays into a multiplex panel. The multiplex workflow is essentially the same as monoplex Opal detection. After MWT (Step 8 below), the slides may be incubated in blocking buffer again before introduction of another primary antibody (Figure 14).

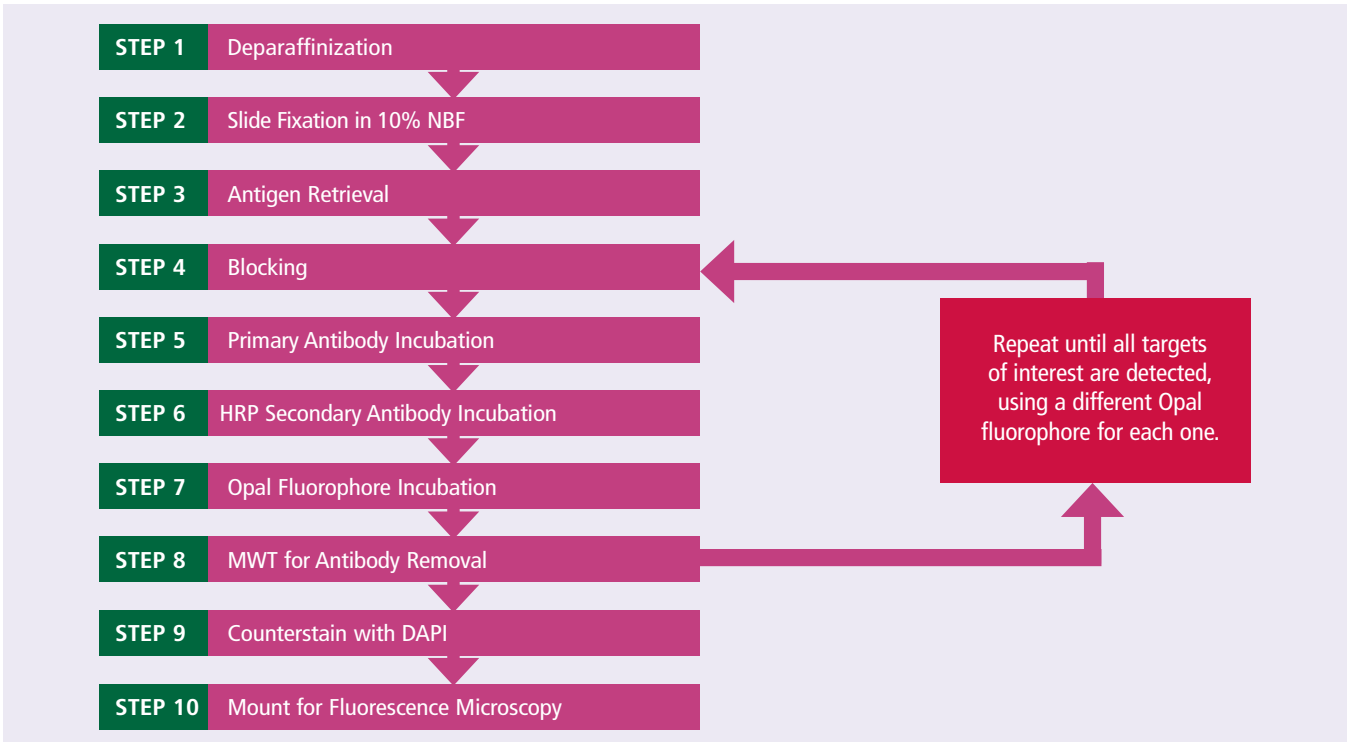

Figure 14. The Opal multiplex IHC workflow.

Here is some advice on multiplex method development.

- Generally, the order of marker application does not have a significant effect on outcome, but it can be helpful for optimization and debugging.
- If possible, arrange the order so successive antibodies do not colocalize in the same cellular compartments within the same cells. An example of this is CD3 and CD8 which can colocalize in the membranous compartment of some T cells. This can help debug issues such as incomplete stripping, contaminated antibody aliquots, etc.
- 6- or 7-color assays may require leaving slides overnight while in process. Here are three options, in order of preference:
  - Post-microwave cooling in AR buffer at room temperature.
  - Overnight primary antibody incubation at 4 °C.
  - Storing in blocking solution at 4 °C.
- If using both AR6 and AR9 together in an Opal multiplexed IHC method, the order of application does not seem to matter.
- It is very helpful to use a paper checklist and mark each step with a pen as it is completed (See Opal Detection Workflow Checklist in Supplemental Information).
- When developing an Opal assay panel, it can be helpful to review staining under the microscope after application of each Opal fluorophore. The best time to do this is immediately after MWT for antibody removal. You can check progress by viewing the sample under the fluorescence microscope, using a coverslip mounted with water. If there is a problem with the staining, it may possible to repeat at that 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 image cube.

Setting Acquisition Exposures for Imaging Opal Multiplex Samples

- Use multiplexed samples on Mantra or Vectra to determine exposure times. Select one or a few multiplexed study samples that contain positive expression for all markers.
- For each marker, move to an area that is positive; use the autoexpose button (Figure 8) to determine exposure for the epi-fluorescence filter corresponding to emission maximum.
- Repeat for all filters until all exposures are determined.
- These exposure times can now be used for acquiring images of multiplexed study samples.

Assess Signal Balance

When transitioning from monoplex to multiplex fluorescence, signals can increase or decrease individually, and can become unbalanced. Ideally, signal levels should be within a factor 3 of each other, particularly for spectrally adjacent fluorophores. Large disparities (>10-fold differences) in signals for spectrally adjacent fluorophores can lead to problems with interference and cause unmixing artifacts.

The best ways to check signal balance is to unmix images in inForm and use the 'information' cursor to hover over bright areas of each label and compare the values (Figure 15). These bright areas do not have to be in the same field. If signal levels of any fluorophores have increased or decreased to be more than 3 times larger or smaller than an adjacent fluorophore, you will want to do some rebalancing, usually by adjusting the signal of the outlier fluorophore.

In some cases, weak signal can be improved by moving a target to the end of the staining sequence. Some epitopes become more accessible with extra cycles of MWT/retrieval.

Other epitopes may be degraded by MWT. In those cases, signal may be enhanced by detection at the beginning of the staining sequence.

If a signal is too bright, it may be necessary to reduce primary antibody concentration to rebalance the multiplex assay.

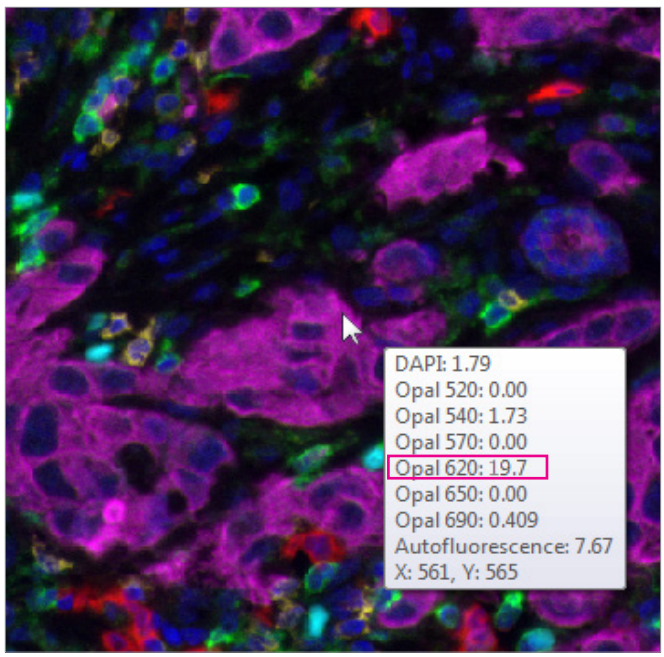

Figure 15. inForm information cursor showing signal intensity for Opal 620 in a multiplex tissue image.

Assess Interference in Opal Multiplex Slides

Overstaining with an Opal fluorophore can block later application of other Opal fluorophores, interfering with multiplexed results. For that reason, signal levels (Figure 15) should usually remain below 20 (when imaged with the lamp at 10%) for multiplex controls. There are two ways to check for interference.

- After spectral unmixing, visually confirm by turning layers off and on in inForm, using the view editor. This requires knowing what the staining patterns should look like, and looking for obvious loss of signal in one plane due to the presence of signal in another. This is often observed as a "hole" in the staining pattern that is filled by the interfering signal.

- **OPTIONAL:** For rigorous, quantitative assessment of interference, stain serial sections of a tissue microarray (TMA) with monoplex and multiplex protocols. Use inForm to measure signal levels within each core in appropriate cells and cell 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]

Assess Antibody Crosstalk

Crosstalk can come from inadequate stripping of antibodies during MWT. This problem is extremely rare and may be related to unusually high affinity primary antibodies. There are two ways to check for crosstalk.

- Visually confirm by turning layers off and on in inForm, using the view editor. This requires knowing what the staining patterns should look like, and looking for obvious addition of signal in one plane corresponding to localization in another plane.
- **OPTIONAL:** For rigorous, quantitative assessment of crosstalk, stain serial sections of a TMA with monoplex and multiplex protocols. Use inForm to measure signal levels within each core in appropriate cells and cell 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]

A potential remedy for antibody cross talk is to increase the length of time at 20% power for MWT (making sure that the slides do not dry out) or to repeat the MWT step.

Image Analysis

Tissue Segmentation

inForm Tissue Finder includes a user-trainable algorithm for tissue segmentation based on morphology as well as specified markers (Figure 16). Here are some suggestions for tissue segmentation.

- Training is usually performed most efficiently using quick iterations and making adjustments until optimal results are obtained. Draw a few training areas for each category and evaluate segmentation result. Refine by adding new training areas to address any miscategorized tissue.
- Use only those 'Components for Training' that are informative in detecting tissue categories of interest. For example, DAPI, auto-fluorescence and cytokeratin should be used for differentiating certain tumors from stroma.
- Use the minimum number of tissue categories needed to accomplish your scientific objective. You can put multiple tissue architectures into one 'other' category (stroma, necrosis, fat, etc.) that in a particular case you might not be interested in.
- Training regions don't need to cover the whole tissue area, only enough to capture 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 (Figure 17).
- There is a trade-off between pattern scale, segmentation accuracy, and resolution of segmentation. Evaluate changes to these parameters to find optimal segmentation result.
- Maximum Segment Size and Trim Edges functions can be quite helpful for filtering out small segmentation errors and fine tuning segmentation to the edge of tumor areas.
- Try to capture full range of tissue morphology in training regions, for each category, including areas with weak and strong expression of tissue markers.

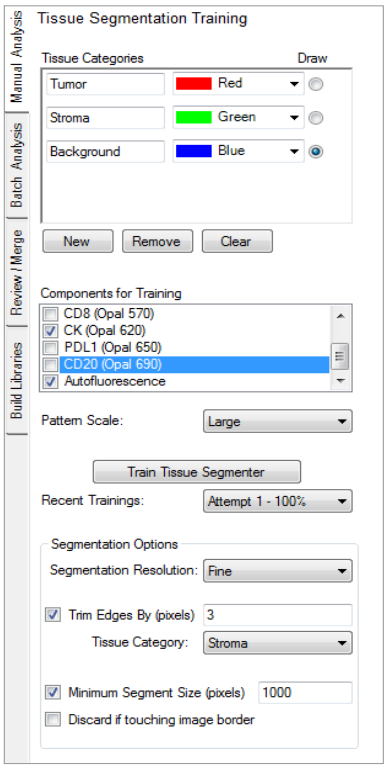

Figure 16. Tissue segmentation training controls.

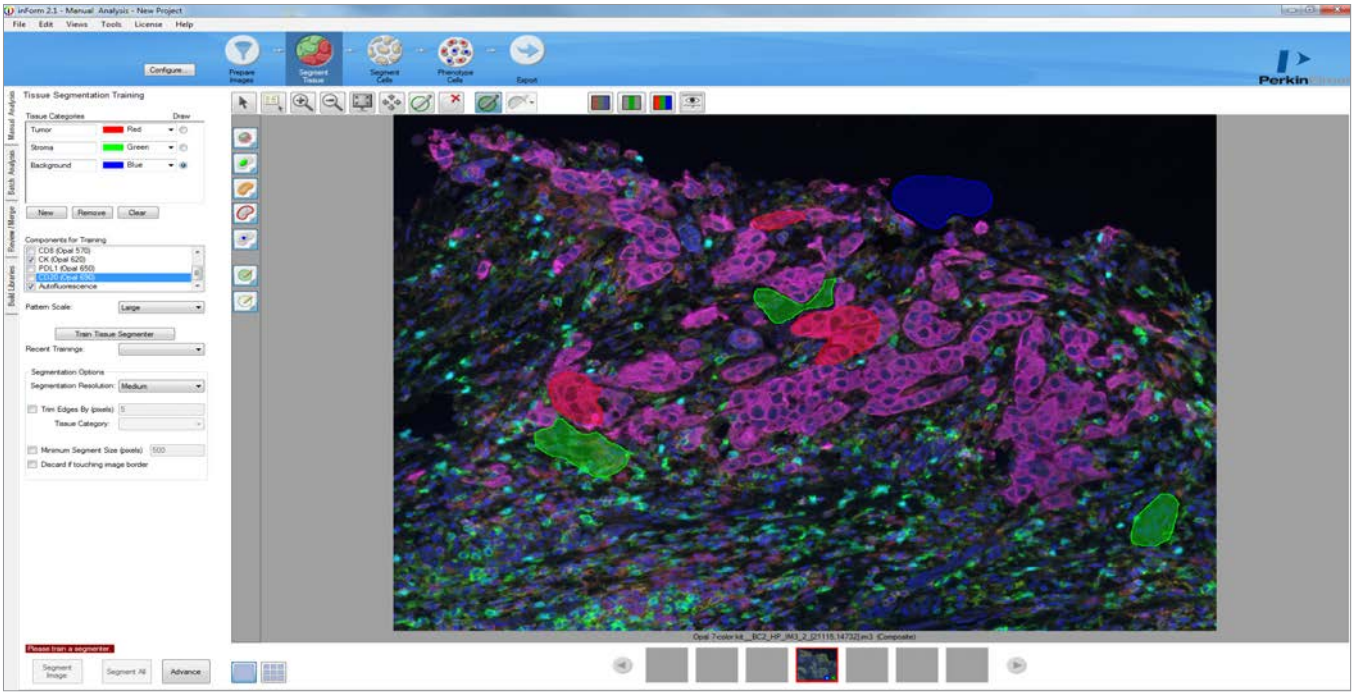

Figure 17. Tissue segmentation training example. Tumor training regions are shown in red, stroma in green, and background in blue.

Cell Segmentation

Here are recommendations for inForm cell segmentation analysis of Opal slide images.

- Use the Counterstain-Based approach.
- Segmentation of all cellular compartments (nuclei, cytoplasm, membrane) supports better phenotyping.
- Start by only selecting nuclei and optimize those parameters. This aids in visual assessment. Then move on to cytoplasm and membrane segmentation.

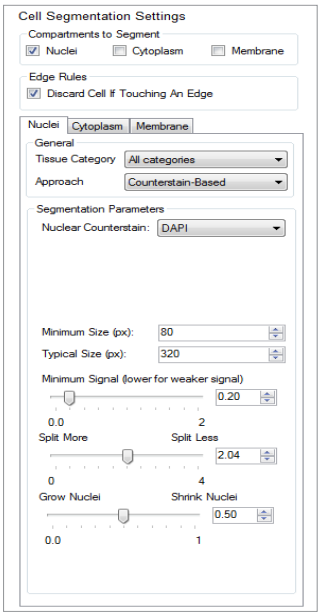

Figure 18. Cell segmentation training controls.

- Familiarize yourself with all parameters in the Counterstain-Based approach editor (Figure 18) by adjusting and observing the effect of each on segmentation (Figure 21).
- The default setting (for inForm 2.1 or later) are a good place to start, with the exception of the splitting slider which should be moved toward the middle.
- Optimize parameters as much as you can before starting phenotype training. Changing any of the tissue or cell segmentation parameters erases phenotype training cell selections.

Phenotyping

Here are recommendations for inForm cell phenotyping analysis of Opal slide images.

- Only phenotype what is needed to achieve your measurement goal – don't create unnecessary phenotypes. Include an 'other' phenotype category for all remaining cell types (Figures 19, 20).
- Make sure tissue and cell segmentation parameters have been adequately optimized before selecting phenotyping training cells. Any pre-existing phenotype training will be lost if tissue or cell segmentation parameters are changed.

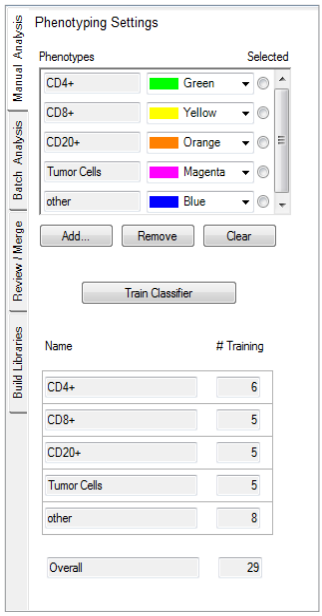

Figure 19. Phenotyping training controls.

- For cancer immunology studies across multiple samples, we have found that 5 – 30 examples of each cellular phenotype are necessary for reliable phenotyping. This may involve an initial training with 5-10 for each phenotype, followed by three to five iterations to assess results. New cells are added during each iteration to improve phenotyping.

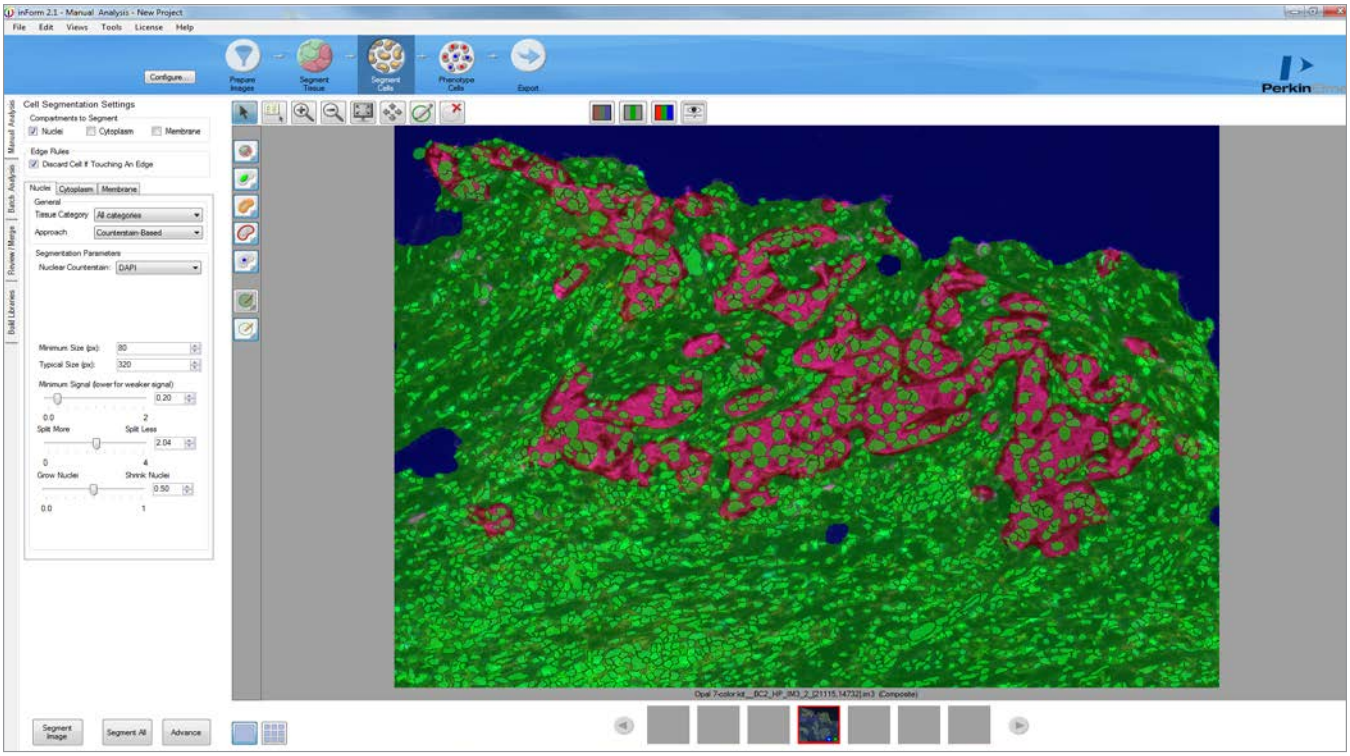

Figure 20. Cell segmentation training example. Tumor training regions are shown in red, stroma in green, and background in blue.

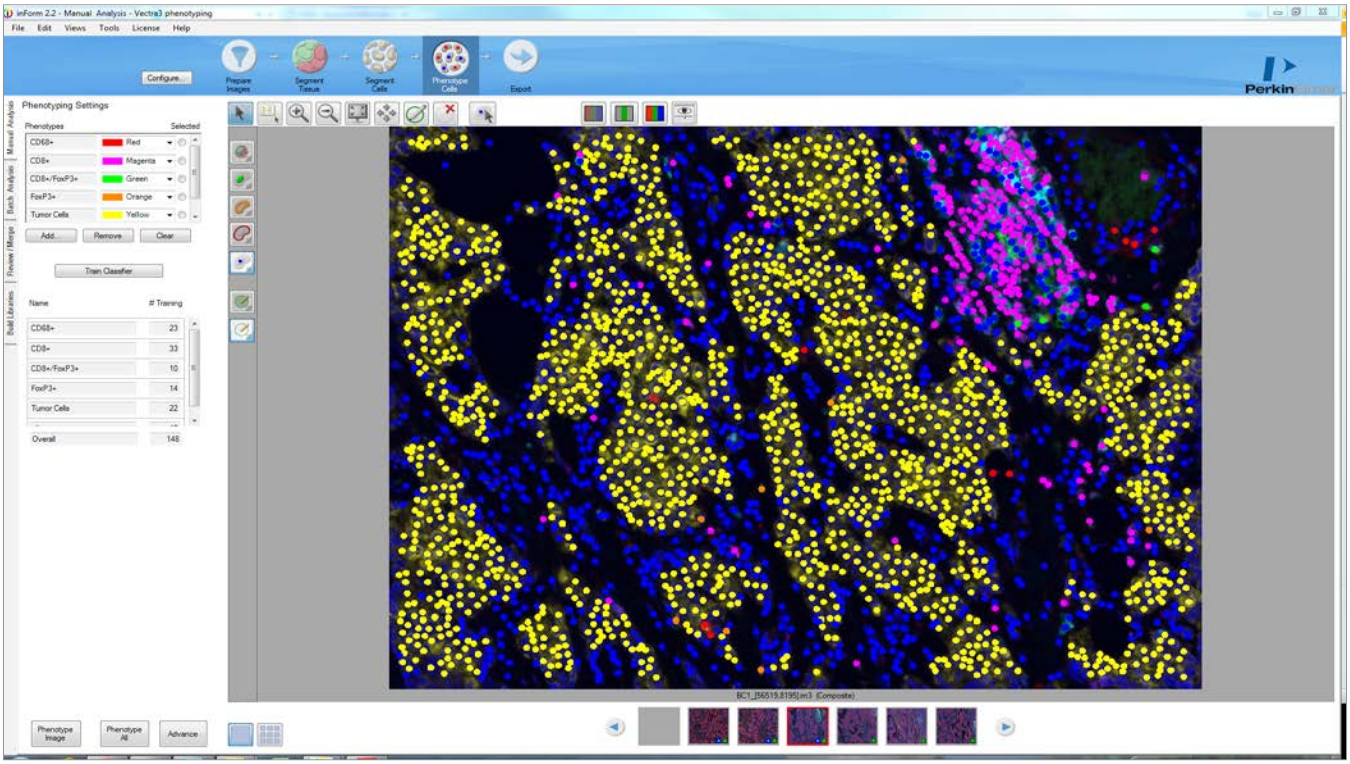

Figure 21. Phenotyping example. Cellular phenotypes are identified by colored dots.

Validation

Human interpretation of the imagery is still the gold standard for confirming stain localization and specificity, tissue segmentation, cellular phenotypes and cellular compartments. It is very important to have staining and segmentation results reviewed by someone like a pathologist who can recognize tissue architectures and morphologies. It is also very useful to reference against images from peer-reviewed publications as well as online resources like the Protein Atlas[3] and GeneCards[4].

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/)

Supplemental Information

Ordering Information

Opal Multiplex IHC Kits		
Opal 4-color IHC Detection Kit	50 slides	NEL794001KT
	250 slides	NEL794B001KT
Opal 5-color IHC Detection Kit*	50 slides	NEL795001KT
	250 slides	NEL795B001KT
Opal 6-color IHC Detection Kit*	50 slides	NEL796001KT
	250 slides	NEL796B001KT
Opal 7-color IHC Detection Kit*	50 slides	NEL797001KT
	250 slides	NEL797B001KT

Opal IHC Detection kits include Opal fluorophores, DAPI and AR6 buffer.
**Opal 5-, 6-, and 7-color kits require multispectral imaging. See options below.*

Opal Cancer Immunology IHC Panels		
Opal 7 Tumor Infiltrating Lymphocyte (TIL) Panel A Kit* (CD4, CD8, CD20, FOXP3, CD45RO, panCK)	50 slides	OP7TL1001KT
Opal 7 TIL Panel B Kit* (CD4, CD8, CD20, FOXP3, CD68, panCK)	50 slides	OP7TL2001KT
Opal 7 PD-1 Checkpoint Panel Kit* (PD-1, PD-L1, CD8, FOXP3, CD68, panCK)	50 slides	OP7CP1001KT
Opal 7 Immunology Discovery Panel Kit* (CD4, CD8, CD68, +3 open channels)	50 slides	OP7DS1001KT
Opal 4-color Lymphocyte Panel Kit (CD4, CD8, CD20)	50 slides	OP4LY1001KT

*Opal IHC Panel kits include primary antibodies, Opal Polymer HRP, Antibody Diluent, Opal fluorophores, DAPI, AR6 & AR9 buffer. *Opal 7-color kits require multispectral imaging. (Coming soon, please check www.perkinelmer.com/opal for the latest panels.)*

Figure 22. Opal 7-Color Immunohistochemistry (IHC) Kit.

Ancillary Reagents and Accessories		
AR6 Buffer, 10X	250 mL	AR600250ML
	4 x 250 mL	AR6001KT
AR9 Buffer, 10X	250 mL	AR900250ML
	4 x 250 mL	AR9001KT
Antibody Diluent, 1X	100 mL	ARD1001EA
Opal Polymer HRP Ms + Rb	50 mL	ARH1001EA
Opal Slide Processing Jars	4	STJAR4

Opal IHC Detection kits include Opal fluorophores, DAPI and AR6 buffer.
**Opal 5-, 6-, and 7-color kits require multispectral imaging.*

Opal Detection Workflow Checklist

- Check off every step once completed.
- If less than 6 antibodies are used, draw a line through the unused antibody columns on page 2 and write "N/A" over column to indicate steps were not performed.
- In the second row of the table, list the antigen for each antibody.
- In the third row of the table, list the species of the primary antibody.
- In the fourth row of the table, complete dilution factor for each antibody.
- In the fifth row of the table, complete the incubation time for each antibody.'
- In the sixth row of the table, complete the incubation temperature for each antibody.
- In the seventh row of the table, list the Opal fluorophore to be used for the antigen.
- In the eigth row of the table, circle AR6 or AR9 to indicate antigen retrieval conditions for each antibody.

Multiplex Assay Panel Name: _____

Antigen	Antibody Type	Clone #	Vendor	Catalogue #	Lot #

Preliminary Steps

1. Bake slides for at least 1 hour or overnight at 65 °C
2. Wash slides in xylene for 10 minutes
3. Repeat Step 2
4. Repeat Step 2
5. Wash slides in 100% Ethanol for 10 minutes
6. Wash slides in 95% Ethanol for 10 minutes
7. Rinse slides in 70% Ethanol
8. Rinse slides in distilled Water
9. Incubate slides in NBF for 20 minutes (some tissues may need longer times)
10. Rinse slides in distilled Water
11. Rinse slides in AR 6 or 9 (use the AR solution that will be used for the first antibody)
12. Repeat step 11

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	Fill jar AR 6 or 9	Fill jar AR 6 or 9	Fill jar AR 6 or 9	Fill jar AR 6 or 9	Fill jar AR 6 or 9
	Micro 45" high	Micro 45" high	Micro 45" high	Micro 45" high	Micro 45" high	Micro 45" high
	Micro 15' 20%	Micro 15' 20%	Micro 15' 20%	Micro 15' 20%	Micro 15' 20%	Micro 15' 20%
	Cool at least 15'	Cool at least 15'	Cool at least 15'	Cool at least 15'	Cool at least 15'	Cool at least 15'
	Rinse water	Rinse water	Rinse water	Rinse water	Rinse water	Rinse water
	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST
	PAP pen encircle	PAP pen encircle	PAP pen encircle	PAP pen encircle	PAP pen encircle	PAP pen encircle
	Block 10'	Block 10'	Block 10'	Block 10'	Block 10'	Block 10'
	Antibody	Antibody	Antibody	Antibody	Antibody	Antibody
	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST
	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'
	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'
	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'
	HRP 10'	HRP 10'	HRP 10'	HRP 10'	HRP 10'	HRP 10'
	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST
	TBST 3'	TBST 3'	TBST 3'	TBST 3'	TBST 3'	TBST 3'
	TBST 3'	TBST 3'	TBST 3'	TBST 3'	TBST 3'	TBST 3'
	Opal 10'	Opal 10'	Opal 10'	Opal 10'	Opal 10'	Opal 10'
	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST	Rinse TBST
	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'
	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'
	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'	TBST 2'
Use AR6 or AR9 as specified in next col.	Rinse AR 6 or 9	Rinse AR 6 or 9	Rinse AR 6 or 9	Rinse AR 6 or 9	Rinse AR 6 or 9	Rinse AR 6 or 9
	Rinse AR 6 or 9	Rinse AR 6 or 9	Rinse AR 6 or 9	Rinse AR 6 or 9	Rinse AR 6 or 9	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

RECORD OBSERVATIONS AND NEXT STEPS HERE

RECORD OBSERVATIONS AND NEXT STEPS HERE

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