AKOYA BIOSCIENCES®
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GUIDE TO

# Spatial Phenotyping in Clinical and Translational Research

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# Multiplex Imaging: Harnessing Spatial Biology to Bridge the IO Biomarker Gap

## Cancer immunotherapy has immense but still untapped potential

Immunotherapy using immune checkpoint inhibitors (ICI) has revolutionized the treatment landscape in oncology, but significant challenges remain. While ICIs have elicited durable responses for several intractable tumor types, response rates to ICI treatment have stalled at 20–30%. Without reliable tools with which to identify likely responders, patients who may have responded are not prescribed ICIs.

How can oncologists better stratify and select patients that may benefit from this mode of immunotherapy? The answer lies in choosing more predictive biomarkers of response.

In this eBook, we describe how protein spatial phenotyping with multiplex imaging enables efficient assessment of spatial phenotypic signatures, a class of immuno-oncology (IO) biomarkers with high predictive potential. We show that these biomarkers may be successfully deployed in clinical settings with standardized, reproducible workflows for multiplex imaging using multiplex immunofluorescence (mIF) technology.

### Spatial phenotypic signatures—Bridging the IO biomarker gap

Response to immunotherapy treatments is determined by activation of the host's immune system to fight tumor cells. Spatial architecture of the tumor microenvironment (TME) can strongly influence disease pathology, progression, and treatment response.

**Spatial phenotyping outperforms other biomarker tests in predicting immunotherapy response.**

A recent meta-analysis of more than 8000 samples, published in *JAMA Oncology*, showed that spatially resolved immunofluorescence/immunohistochemistry outperformed other biomarker testing approaches in predicting response to anti-PD-1/ PD-L1 treatments.[4]

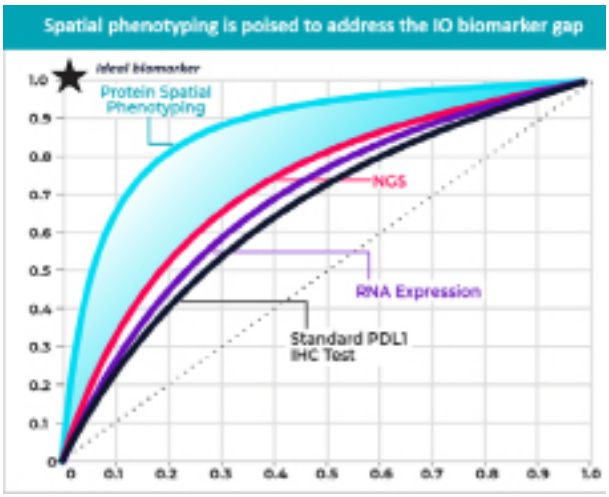


**FIGURE 1.** Relative accuracy of biomarker testing modalities in predicting response to anti-PD-1/ anti-PD-L1 treatments

Spatial phenotypic signatures represent a novel class of biomarkers that, by reporting spatial architecture, can reveal a tumor's biology, and thus how best to treat it. Spatial localization and quantification of tissue-specific proteins involved in immune activation enables identification of distinct cell types, their functional states, and how they are organized in space within the TME.

Mapping the organization and interactions of tumor and immune cells within the TME also provides the ability to better characterize intratumor heterogeneity, revealing "cellular neighborhoods." Cellular neighborhoods are localized regions within the TME where specific functions occur. Spatial phenotyping via quantitative imaging of these cellular neighborhoods across many tissues and cancer types generates spatial phenotypic signatures, which have been shown to be highly effective at predicting therapeutic outcomes.[2,3]

Hundreds of peer-reviewed publications have now validated spatial phenotyping assays using immunofluorescence-based multiplex imaging, generating methods that present many advantages over other biomarker testing modalities.[6,7]

## Limitations of conventional immunohistochemistry (IHC)

Conventional (brightfield) IHC is considered the gold standard for histological tissue antigen staining, but IHC assays for anti-PD-1/PD-L1 therapy exclude patients who may benefit from the use of these checkpoint inhibitors (false negatives) and include patients who do not respond (false positives). The failure of IHC in accurate prediction of IO response highlights IHC limitations:[1]

- Low plex capability: IHC is generally capable of labeling only a single or a few markers at a time. But robust phenotyping of the cells within the TME, and determining immune cell activation status, requires detecting multiple markers.
- Low reproducibility and subjective: IHC scoring can be heavily dependent on judgment calls of the operator.
- Low dynamic range: IHC often requires the saturation of stronger signals in order to detect weaker ones.

## Spatial phenotyping with multiplex imaging overcomes limitations

Multiplex imaging using immunofluorescence holds the promise of more quantitative, reproducible, precise, objective assays capable of delivering a wider dynamic range while simultaneously querying more markers for more powerful spatial phenotyping. Results are comparable with the results of monoplex IF and chromogenic IHC staining (Figure 2). A recent systematic review and meta-analysis comparing different biomarker modalities for predicting clinical response to anti-PD1/PD-L1 therapy demonstrated that protein spatial phenotyping with multiplex imaging had significantly higher diagnostic accuracy than other biomarkers including PD-L1 IHC (Figure 1).[4]

By utilizing multiple fluorophores with distinct emission spectra, multiplex imaging can simultaneously label a diverse set of antigens, giving it the power to characterize cell lineages with the same degree of accuracy as flow cytometry, while providing additional information such as spatial context. With conventional immunofluorescence-based multiplex imaging, as the number of biomarkers and their associated labels increases, so do the complexity and difficulty in separating biomarker presence and expression level. This is due to spectral overlap, where the emission spectrum of one fluorophore "bleeds" into

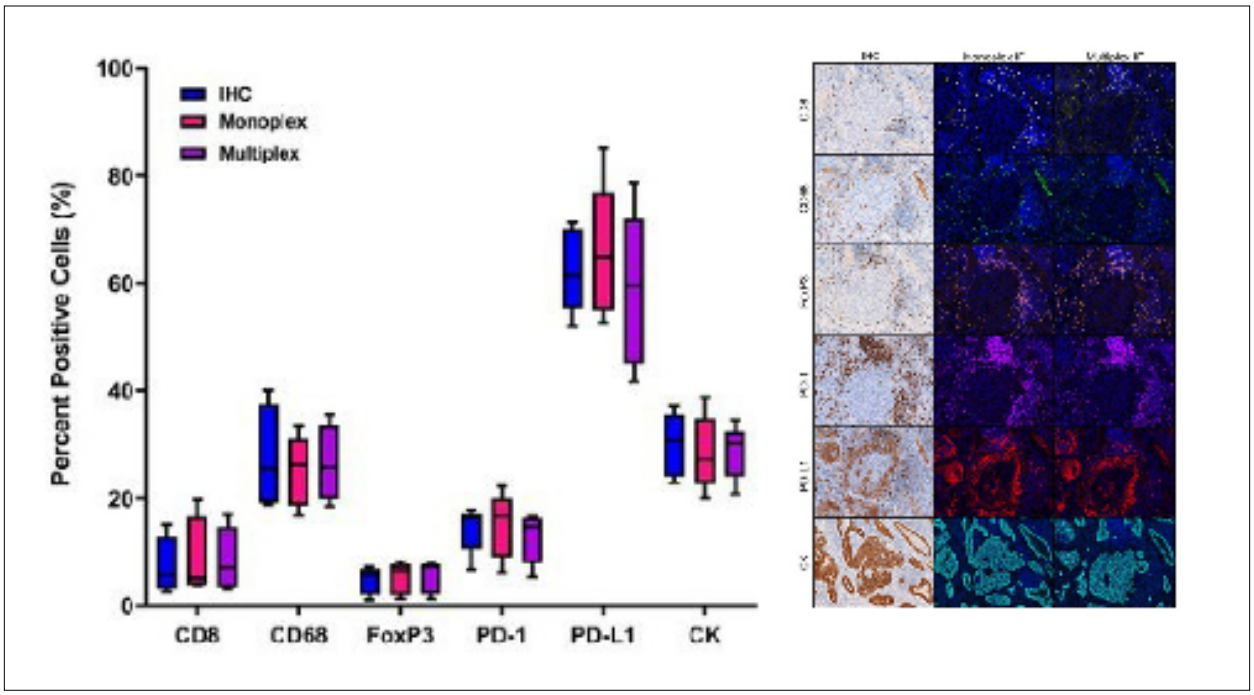


**FIGURE 2.** Multiplex imaging using immunofluorescence (IF) is comparable with monoplex IF and gold-standard chromogenic (DAB) IHC staining. Six markers were benchmarked across breast and non-small cell lung cancer (NSCLC) tissue samples (Image courtesy: Bethany Remeniuk, Akoya Biosciences. See Reference 5)

the detection channel of a neighboring fluorophore. This overlap contributes to a false positive signal.

To produce accurate imaging data using conventional immunofluorescence-based multiplex imaging, fluorophores must be chosen that are spectrally far apart from each other, thus setting the limits (i.e., biomarkers) and DAPI nuclear counterstaining. In addition, many biological samples are inherently fluorescent. This autofluorescence can also significantly affect the signal-to-noise ratio, further confounding reliable image analysis.

Next-generation multiplex imaging technologies, such as Akoya Biosciences' PhenoImager solution powered by patented multispectral imaging (MSI) technology, provide the analytical performance required to accurately quantitate up to nine colors in a single tissue sample (Figure 3). Employing advanced spectral unmixing algorithms to compensate for spectral bleed-through among channels and to isolate signal from background autofluorescence, this patented MSI technology allows for an increasing number of fluorophores to be imaged simultaneously while providing improved signal-to-noise ratio, more accurate phenotyping, and greater scoring accuracy **(see Chapter 4, "Certainty in Imaging")**.

## Spatial Phenotypic Signatures are novel biomarkers developed with Akoya Biosciences patented multispectral imaging (MSI) technology.

With a streamlined, automated, end-to-end workflow based on optimized Opal fluorescent dyes, rapid and accurate imaging systems (PhenoImager Fusion & PhenoImager HT ) and advanced analysis tools (inForm, phenoptr, and phenoptrReports), Akoya's

PhenoImager solution enables quick and accurate spatial phenotyping of tissues across the whole slide. This allows for the unbiased development of spatial phenotypic signatures that measure the interactions and cell densities of tumor and immune cells in the tumor microenvironment. Akoya's solution enables analysis of dozens of cell phenotypes and their spatial interactions from a single formalin-fixed, paraffin-embedded (FFPE) tissue section.

By validating and standardizing the multiplex imaging workflow, spatial phenotypic signatures can enter the clinic and make an impact on immunotherapy outcomes. The recent

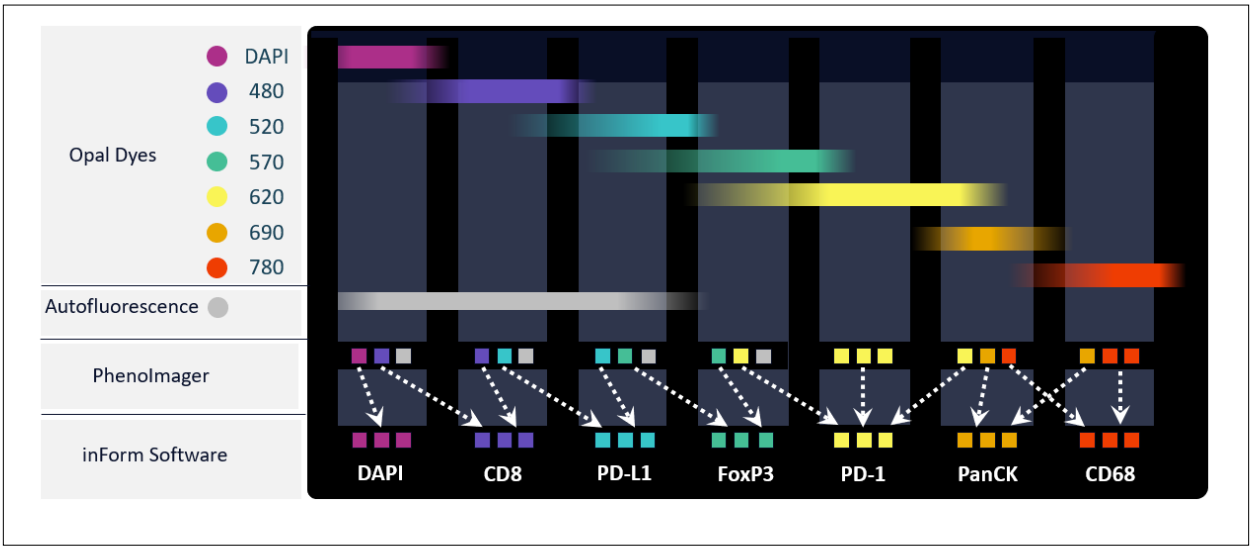


**FIGURE 3.** Akoya's patented multispectral imaging (MSI) technology with spectral unmixing enables more fluorophores to be imaged at once. A tissue is stained with Opal fluorophores and imaged using the PhenoImager slide scan protocol, using the inForm software, the exact spectral signature of each fluorophore is isolated to properly unmix each whole-slide composite image, as well as isolate and remove tissue autofluorescence (AF). Arrows represent the process of spectral unmixing (Source: Reference 10. Used under the terms of a CC BY 4.0 license )

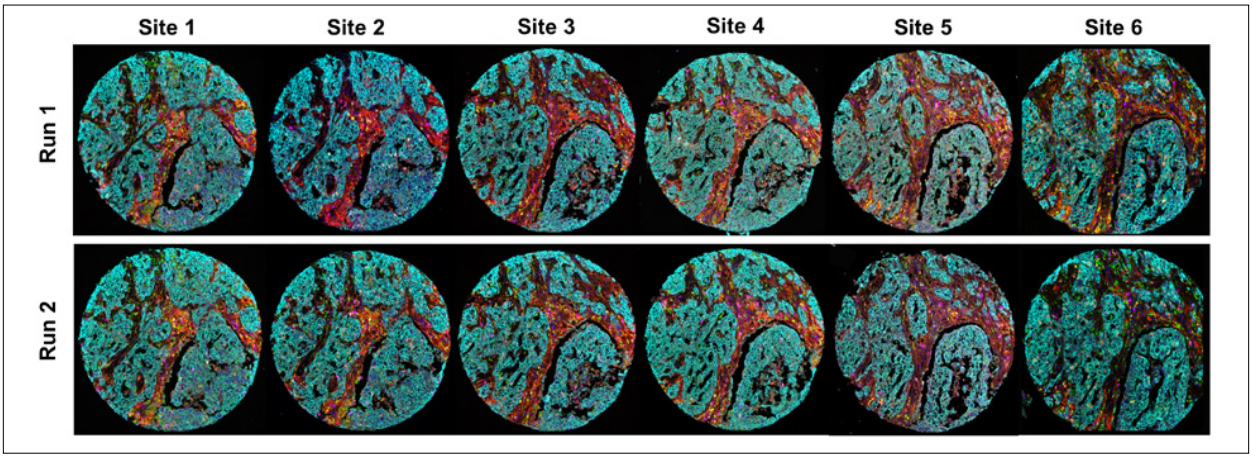


**FIGURE 4.** High reproducibility and robustness of mIF within and across assay sites. Intersite and intrasite concordance for automated multiplex imaging assay staining results of breast carcinoma TMA. The MITRE study established the high reproducibility and robustness of Akoya Biosciences' PhenoImager platform for spatial phenotyping in clinical and translational research (Image courtesy: Bethany Remeniuk, Akoya Biosciences. See Reference 5)

Multi-Institutional TSA-amplified Multiplexed Immunofluorescence Reproducibility Evaluation, or MITRE study, a multi-site collaboration that assessed the analytic performance of a multiplex immunofluorescence panel focused on the PD-1/PD-L1 axis, demonstrated its reproducibility and intra-laboratory and inter-laboratory concordance for multiple parameters (Figure 4).[5]

As more and more clinical laboratories apply this multiplex imaging-based spatial biology workflow to multiple cancer types, the PhenoImager platform moves toward being established as the standard method for generating robust and reproducible spatial phenotypic signatures, providing the level of accuracy and performance needed to support clinical trials and testing.[8,9]

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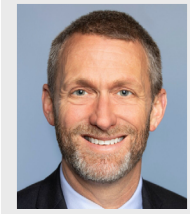
# Spatial Biomarkers and Multiplex Immunofluorescence in Immuno-Oncology

## Panel discussion with key clinical researchers

Recent developments in the study of spatial biomarkers continue to demonstrate the predictive power of this new class of biomarker assays. In a recent panel discussion, clinical researchers shared new strategies representing significant milestones that advance spatial biology into the realm of patient phenotyping and improve immunotherapy outcomes in the future.



### What is a spatial biomarker?

**Alexander "Sandy" Borowsky, M.D., Professor, Department of Pathology and Laboratory Medicine, UC Davis Health:**

Pathology is looking at the micro-anatomy of a histological section to make a diagnosis. There are currently two options for adding ancillary biomarker data on top of pathology data.

The first involves grinding tissue and measuring analytes. An instructive example is the biochemical assay for estrogen receptor assessment for breast cancer using tissue lysates. While the method is very quantitative, spatial context is lost, and distinguishing between tumor cells and normal cells

The other option is spatial phenotyping—looking at a biomarker directly on the slide, in the context of the structure of the histomorphology. For example, pathologists can see whether tumor cells or nearby cells are expressing the estrogen receptor and score appropriately. This is what we mean by spatial biomarkers—they must be measured directly on tissue section, in the context of the structure of the disease.

### Why is spatial phenotyping needed to predict therapeutic response of tumors?

**Kurt Schalper, M.D., Ph.D., Assistant Professor of Pathology & Director, Translational Immuno-oncology Laboratory, Yale Cancer Center:**

The tumor microenvironment (TME) is complex, highly organized, and varies across patients, and this is important when selecting patients for treatment.

Traditionally, immuno-oncologists have treated cancers with respect to their anatomical categorization—for example, breast cancer, lung cancer, or cervical cancer. In contrast to anatomical categorization, however, molecular subtyping has increased

This is where spatial phenotyping, the analysis of how cells organize and interact with each other, has the biggest value: It can pinpoint

the interactions within the TME where most anti-tumor immune responses occur. Employing spatial phenotyping methods in pharmacodynamic studies can also reveal the impact of a given treatment within the TME and the associated clinical response.

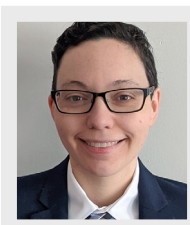
## Why measure protein expression versus other analytes for immuno-oncology research?

**Bernard A. Fox, Ph.D., Harder Family Chair for Cancer Research, Robert W. Franz Cancer Center; Earle A. Chiles Research Institute, Providence Cancer Institute:**

Evaluating proteins overcomes the uncertainty caused by different RNA transcripts being expressed differently under different conditions. The level of the protein expressed on the cell surface (for example, the intensity of PD-1 staining) can be the key determinant to gating which patients are more likely to have the best response. In studies of additional immune checkpoint interactions, it will be increasingly important to know what protein is expressed, the level of expression, and where the protein is expressed. So, it's very exciting to be able to assess these elements in patients using multiplex immunofluorescence (mIF) technology.

## What sort of spatial features are important in predicting clinical metrics?

**Liz Engle, M.S., Senior Laboratory Manager, John Hopkins Hospital, Maryland:**



As recently reported in *Science*, we used the AstroPath platform to analyze multispectral mIF imaging of the entire TME of melanoma patients—in-house samples as well as external site samples[1] We specifically examined spatial features like the co-expression of PD-1 on T cells and subtypes of T cells, and their relationship to PD-L1-expressing cells. We were able to identify FoxP3$^{+}$CD8$^{+}$PD-1$^{+}$ cells with just a six-marker panel. We examined the relationship of those cells to the tumor-stromal border, the relationship to different PD-L1 cells, and what these cells bound to—their nearest neighbors.

Our data show that the density of CD8$^{+}$FoxP3$^{+}$PD-1$^{low/mid}$ cells at the tumor-stromal border are a predictive marker of response to anti-PD-1 therapy, and CD163$^{+}$PD-L1$^{-}$ macrophages are associated with a lack of response. Combining those two cell phenotypes and a few other cell phenotypes have enabled us to completely stratify long-term response to anti-PD-1 therapy in melanoma patients.

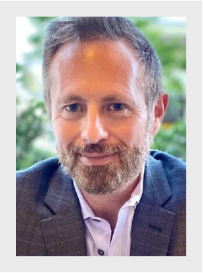
## What are some other indications for which a spatial biomarker approach using mIF has shown promise?

**SCHALPER:** Tertiary lymphoid structures and antigen presentation deficiency in tumor cells are two great examples of the power of spatial biomarkers.

Tertiary lymphoid structures (TLSs) are present in about one-third of cancers and are associated with better outcomes, both with immunotherapy and chemotherapy. TLS may provide independent, predictive information compared to biomarkers such as PD-L1 IHC or TMB.

Antigen presentation deficiency, on the other hand, is generally associated with lack of response and so provides an opportunity to integrate another marker that could potentially be non-redundant or non-overlapping with other signals.

Ultimately, using multiple spatial features may generate predictive models that are much better than either marker alone.



**Gavin Gordon, Vice President, Clinical Market Development, Akoya Biosciences:**
A recent *Nature Cancer* paper highlights the independent predictive capability of using mIF with Akoya's PhenoImager workflow as an independent predictor in several different data sets.[2]

## What is an example of an mIF biomarker strategy that could help advance how we think about biomarkers more generally?

**BOROWSKY:** Dynamics of on-therapy biopsy shows evidence of being a highly predictive biomarker of immuno-oncology protocol effectiveness. For example, monitoring regulatory T cell (Treg) activity, and Treg proximity to other T cells, can help guide therapeutic strategy. Suppressing Tregs can increase efficacy, after first confirming that Tregs are indeed the active component in suppressing the immune reaction against the tumor. It is critical to choose immunotherapy based on which of the patterns of immune evasion and/or immune suppression are active in an individual's tumor.

## What are the next steps toward analytical validation of an end-to-end mIF workflow?

**FOX:** The MITRE study was a very important step the ability to perform that testing.[3] Its follow-up study, called MITRE-2, has been designed to test all aspects of the technology independently of central review.

The initial MITRE study is likely to give more pathology groups the confidence that mIF is important for the future of oncology. mIF can help select for the patients that are most likely to respond. This may facilitate FDA approvals and reduce the costs associated with treating patients with inefficacious drugs.

## What are some of the challenges that face adoption of mIF workflows?

**GORDON:** Looking back at the history of current biomedical technologies can reveal a path by which mIF evolves from an emerging platform to widespread adoption. For example, early Sanger sequencing was insufficient for the challenges that, ultimately, next-generation sequencing (NGS) solved. Just like early translational studies on the sequencing of colorectal cancer and the National Cancer Institute's analytical validation studies of NGS, the AstroPath *Science* paper and the MITRE studies represent mIF's trajectory of going from technology platform to robust and clinic ready.

**FOX:** Using spatial biomarkers for clinical trial design may require additional biopsies to measure tumor environment changes with respect to treatment. Getting sufficient tissue for multiplex panels may be a challenge.

**ENGLE:** Another challenge may be standardizing mIF reagents in a way that is easily translatable to many labs.

**SCHALPER:** Widespread adoption of mIF will require three developments: First, the generation of standards must provide users the confidence that the assay is working. Second, a transition to the clinic must be supported by discrete panels with few markers that can be reliably stained, easily deployed, and clinically interpreted. Third, continued discovery of higher plex assays will help determine which markers should be incorporated into the next generation of clinical-grade panels.

**BOROWSKY:** Compared to chromogenic IHC, immunofluorescence offers a much bigger dynamic range and is more quantitative. But barriers include retraining pathologists to move to immunofluorescence, as well as simplifying data analysis and readout. These challenges are likely easily addressed.

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# Four Steps to Developing a Reproducible Multiplex Imaging Workflow

## Evidence is mounting for the robustness and predictive value of this technology

Spatial biomarker analysis utilizing multiplex imaging using immunofluorescence (mIF) is poised for wide adoption across discovery- and clinical-phase research. In particular, mIF using tyramide signal amplification with Opal dyes(mIF Opal-TSA) enables researchers to:

- Use the best primary antibodies regardless of species, without cross-reactivity
- Improve sensitivity, dynamic range, and resolution
- Achieve better correlation between protein expression and signal intensity
- Increase plexing for multiple biomarker detection

More and more laboratories are reporting optimized, reproducible workflows using mIF Opal- TSA, generating more evidence for the robustness and predictive value of this technology.

This chapter presents some of the collected learnings and best practices derived from the body of published protocols to date. Establishing reproducible workflows, encompassing antibody panel development, staining, image acquisition, and analysis, can save researchers time and ultimately make drug discovery and development more efficient..

### 1. Building the best antibody panel

Accurate and reproducible identification of distinct cell populations and their relationships within tissue using a mIF Opal-TSA workflow requires careful design, development, and optimization of the antibody panel for multiplex biomarker quantification.[1]

#### a) Start with pre-designed antibody panels

Choosing antibodies that will recognize desired targets with high sensitivity, specificity, reproducibility, and excellent performance in multiplex immunofluorescence assays, can seem daunting. Fortunately, pre-designed, optimized antibody panel kits, such as those from Akoya Biosciences, remove much of the guesswork for mIF assay development. These kits give new users an easy path to building an mIF panel by providing an optimized mix of primary antibodies recognizing key immune and/or tumor cell markers. Open channels give researchers the flexibility to add additional markers of their choice.

#### b) Select antibodies of choice

In collaboration with immunologists and disease specialists, identify the ideal set of

markers that can answer the biological questions at hand. What markers can unequivocally distinguish the target cell phenotype from surrounding tissues? Are there specific markers whose spatial distribution is important to understand for the study? Does your biological question involve colocalization of markers and/or interactions of complex cellular phenotypes?

If you have an antibody that works for traditional IHC, start with those. Otherwise, antibody search engines (e.g., CiteAb) are a good next step for discovering antibodies for desired markers. Search engines can help identify antibodies with the most complete validation data and links to citations backing performance. Consult with pathologists and assay specialists to gain further insight into how well-suited chosen antibodies are for mIF specifically. For mIF assays, monoclonal antibodies often provide lower background signal, higher specificity, and lower lot-to-lot variability than polyclonal antibodies.[2]

### c) Verify antibody panel selection with tissue controls

Choosing the right set of positive and negative control tissue samples is critical for developing an informative mIF assay. Again, collaborating with immunologists and disease specialists can help identify and source these tissues. Establish preliminary antibody specificity by testing individual antibodies on positive and negative cell lines, cell pellets, and tissue controls. Initial tests can be performed using higher-throughput immunoassays, such as Western blotting or immunohistochemistry (IHC). Further establish specificity by testing a range of both normal and diseased tissues, such as in defined tissue microarrays (TMAs).[2]

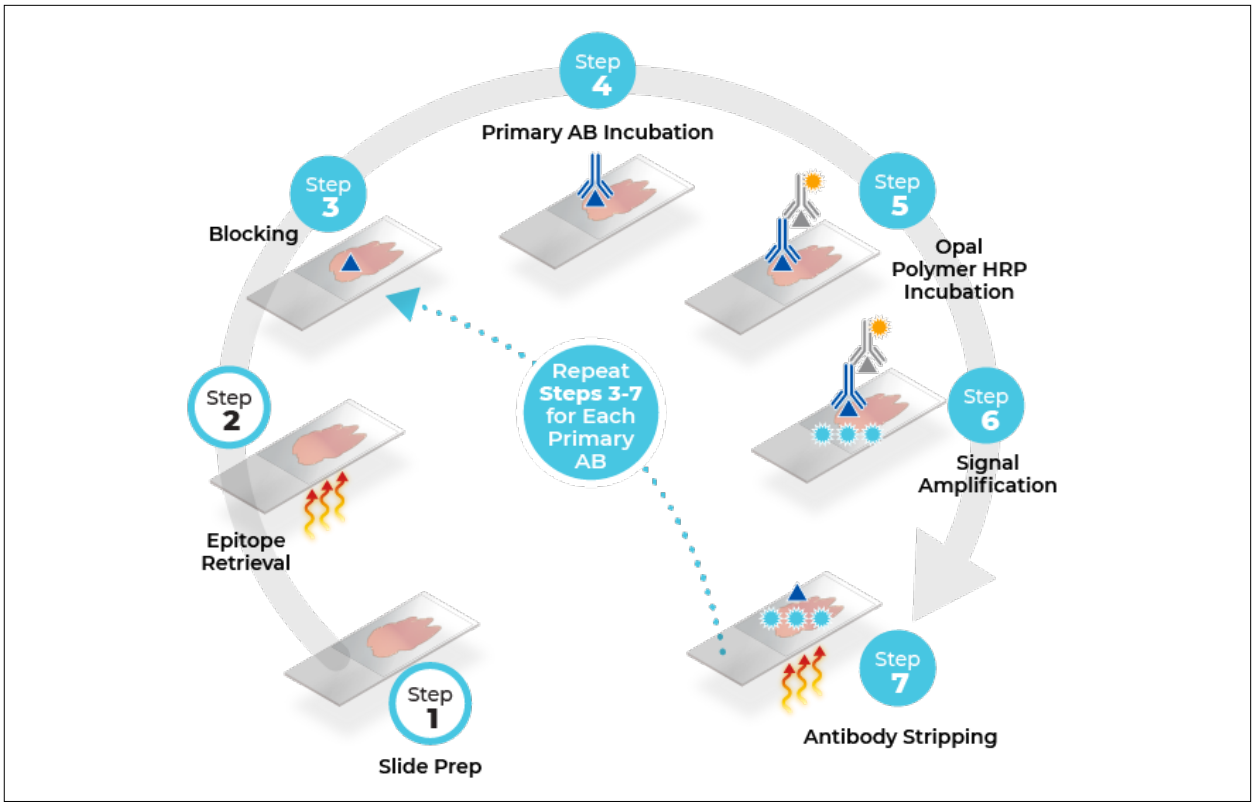


**FIGURE 1.** Iterative staining diagram for Opal Multiplex IHC Assays

Orthogonal verification and antibody cross-reactivity testing on other proteins within the same family as the target of interest can help identify any effects or artifacts directly related to the antibody. Establish antibody sensitivity by testing with cell lines that express the target of interest at different levels. On occasion, sensitivity may be improved by the pooling of antibody clones, usually with non-overlapping epitopes. Finally, test reproducibility by determining whether studies performed by multiple operators and/or using different antibody lots provide comparable results.[1]

## 2. mIF assay development and optimization

### a) Optimize first with mono-plex chromogenic IHC and IF

Develop single-plex assays first to gain an initial understanding of staining parameters, including tissue handling/fixation parameters, antigen retrieval conditions (pH and temperature), subcellular localization and staining patterns based on known positive controls, antibody and detection reagent titration, and other incubation and blocking conditions. Single-plex chromogenic IHC is the preferred starting method for this step unless the user has prior experience with IF.[2]

Once chromogenic IHC protocols have been established, perform single-plex antibody optimization using single-plex IF and the Opal-TSA detection workflow to verify that similar staining results from positive and negative tissues as was shown by chromogenic IHC. Start by using the same antibody dilutions for IF as for optimized IHC assays. However, the convention for IF is to pair the brightest Opal fluorophores with the weakest expressing biomarker and vice versa, so further antibody titration optimization may be required the desired tumor samples, human tonsil is commonly used as a tissue control for immune-oncology IF optimization to set the thresholds for the antibodies and disclose any discrepancies or background staining issues.[2]

### b) Multiplex IF optimization

First, determine the staining order for the mIF panel that generates similar staining patterns as those obtained with single-plex IF. Key factors to consider when determining the staining order include the abundance of the biomarker, antigen retrieval, and the ability of the epitopes to withstand heat-mediated stripping cycles (Figure 1). Staining order should minimize the umbrella effect, in which tyramide bound to a preceding marker sterically blocks recognition of an antigen in the same compartment. Starting the staining order with the least-concentrated antibody (most abundant antigen) and ending with the most-concentrated antibody may avoid the umbrella effect, although this is not universally true. Verify correct staining order by comparing results with IHC or single-plex IF. Umbrella effects can be reduced by increasing primary antibody concentrations, reducing fluorophore concentrations, or changing the staining order of antibodies.

Sometimes, mIF assay optimization reveals antibody cross-talk, when the signal for one antibody appears in the detection channel of another. In addition, many tissues are inherently fluorescent, and this autofluorescence can confound accurate detection of lower expressed biomarkers and may introduce false signal intensities. To reduce crosstalk and interference, change the staining order of the antibodies or increase the time of the antigen retrieval step corresponding to the antibody causing the interference.[3]

Multispectral imaging technologies that use spectral unmixing such as Akoya Biosciences **PhenoImager solution** can overcome these

issues by isolating autofluorescence into a discrete channel and isolating each biomarker of interest regardless of intensity or any spectral overlap.

First create a reference spectral unmixing library consisting of unstained and single-stained tissue sections. Determine the percentage of each intensity basis spectrum contributing to each pixel using the reference library. Extract the correct spectra excitation from each fluorophore and autofluorescence to verify the expected staining pattern. The ideal mIF assay yields images that balance signal intensity across all the markers in the panel, enabling reliable quantification.

## 3. Image acquisition and data analysis

Applying mIF to clinical applications requires optimizing the assay for high-throughput image acquisition, using the right imaging instrument and protocol.

### a) Select mIF imaging system

Whole-slide imaging is increasingly accepted as the most reliable imaging method.[6] As tissues exhibit significant cellular and spatial heterogeneity, especially for immune markers such as PD-L1 or CD8 infiltrates, it is essential to perform high-resolution multiplexed analysis across whole tumor sections instead of analyzing small regions of interest (ROI), as has traditionally been done. Analysis of small ROIs introduces significant variation, human bias, and errors in the assessment of disease-specific biomarkers.

Integrated mIF systems, such as the PhenoImager solution (Figure 2), enable multiplex-

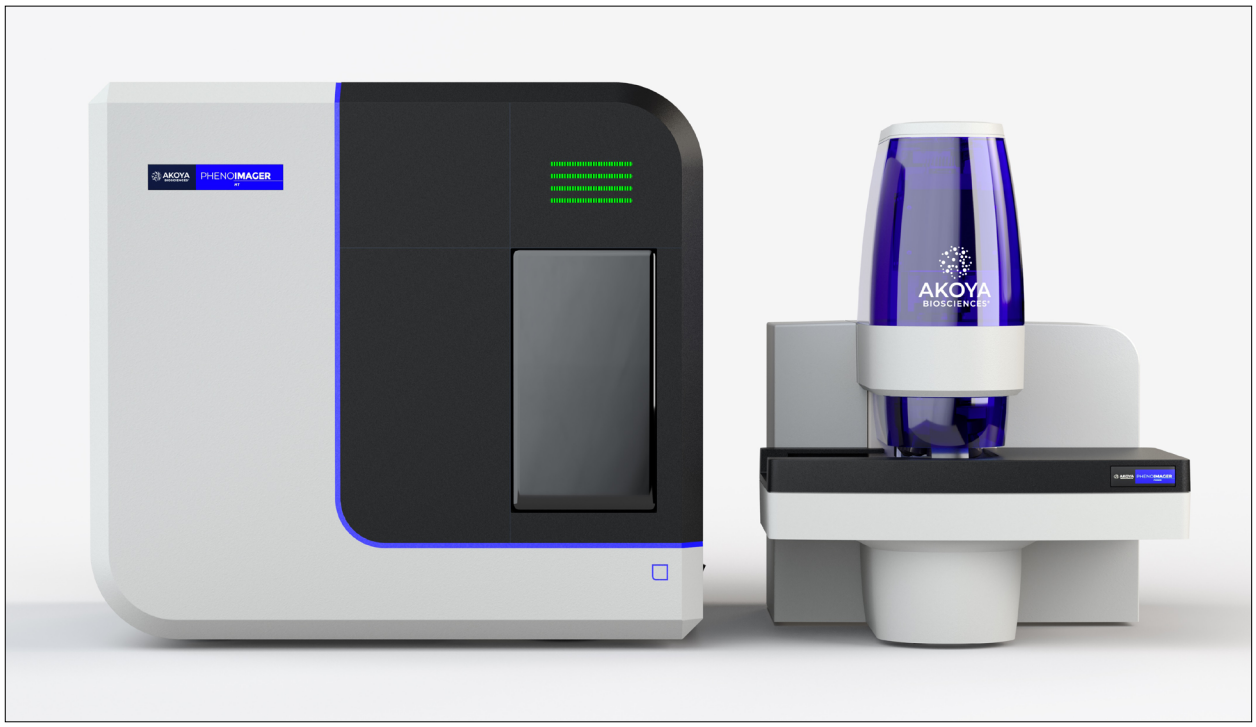


**FIGURE 2.** Multispectral imaging systems optimized for multiplex imaging include the PhenoImager HT (left) and PhenoImager Fusion (right)

ing coupled with digital analysis for high-resolution analyses on whole tissue slides.

Criteria for selecting an imaging system include:

- Spectral range
- Resolution
- Automation
- Whole slide imaging speed and throughput
- Multiplexing capability

**b) Develop image analysis method**

Following image acquisition, import the image files into a tissue analysis software such as **inForm** and spectrally unmix the image using the appropriate spectral library (Figure 3). The unmixed image files can then be further analyzed in inForm or a variety of commercial and open-source image analysis software packages. This software contains user-trainable protocols to automatically detect and perform tissue segmentation, nuclear/cell segmentation, and cell phenotyping using machine learning algorithms.

First, perform iterative testing on several images to optimize the classifiers, which are selected based on the type of segmentation or phenotyping needed. For example, cell segmentation can be classified using the DAPI counterstain that is used to identify the nuclei of every cell. Similarly, specific markers can be used to help to train the algorithm to identify the different cell phenotypes. Have the data reviewed by a pathologist familiar with the methodology to ensure accuracy, consistency and reproducibility across the samples. Cell segmentation and phenotyping data can then be used for more advanced analyses and visualizations, such as nearest neighbor analysis, cell densities, and heat maps. High dimensional data analyses, t-SNE, and UMAP plots, are also possible **(see Chapter 5, "Multiplex Imaging Analysis")**.

## 4. Testing the validated workflow: from staining to digital image

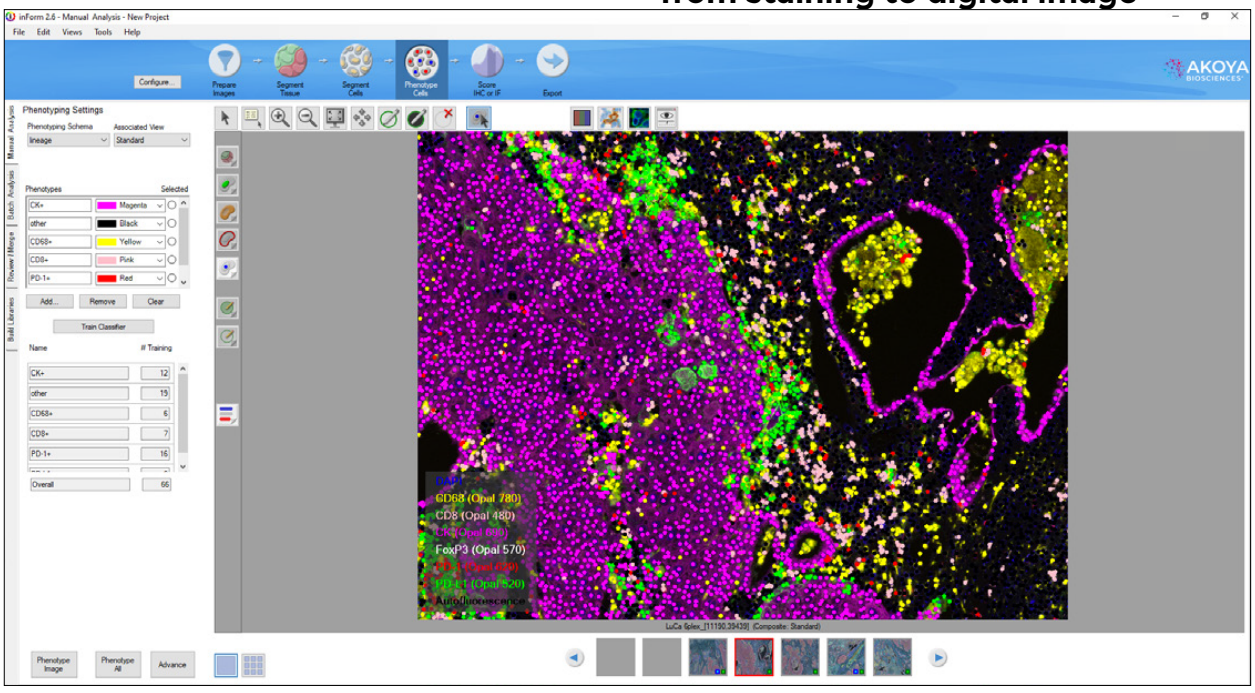


**FIGURE 3**. Akoya Bioscience's inForm software enables spectral unmixing, segmentation, and cell phenotyping

## analysis

Before mIF assays can be used to analyze experimental tissues beyond the control tissues, and ultimately translated to clinical applications, the end-to-end workflow must be standardized and validated. This validation entails both intrasite and intersite studies to demonstrate high concordance for staining intensity, cell density and expression assessments, and proximity measurements. Especially for use in clinical trials, validation requires mIF systems that can accommodate large numbers of samples, large areas of tissue, and reproducible image analysis.[5]

First, select the same TMA and control tissues that were used for antibody validation to perform staining workflow validation. Assess reproducibility of staining patterns for each marker across serial sections, taking into account that markers exhibiting highly heterogeneous expression levels may show section-to-section variation. Often, employing a third party to evaluate staining results generated by different groups may help guide efforts to establish reproducibility.[1]

Develop a standardized protocol for digital image analysis to minimize interobserver variability associated with subjective analysis. It is important to establish objective criteria and intensity thresholds for positive and negative signals, including examples of false positive and false negative signals. Detailed methodology for the computational image analysis algorithms used should be documented and used consistently across assays.

Finally, obtain consensus around statistical methods by which to compare assay performance with respect to the user, site, and experimental tissue type tested, as well as tissue collection and transport variables. mIF assays that pass the accepted criteria can be deemed sufficiently reproducible and robust for use in translational and clinical research.[5]

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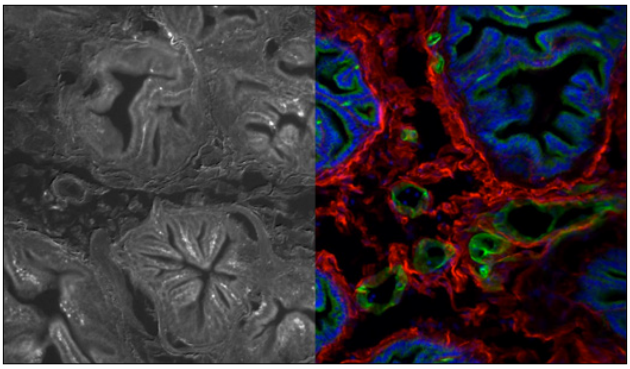


**FIGURE 1.** Prostate tissue stained with two markers plus DAPI, the left side showing autofluorescent signal that was isolated & removed during unmixing. CD31-AF488 was used for visualizing blood endothelial cells and gp38-AF568 for visualizing fibroblasts. Lymphatic endothelial cells stain positive for CD31 as well as gp38

Figure 1 image courtesy of Tabea Sturmheit, Ph.D. Lymphocyte Activation Unit, San Raffaele Scientific Institute, Milan

CHAPTER 4 *Certainty in Imaging*

# Certainty in Imaging

## Isolate autofluorescence to see what you have been missing

### Introduction

Autofluorescence, found abundantly in most formalin-fixed, paraffin-embedded (FFPE) tissue samples, has limited the full impact of image analysis by introducing false signal intensities, hiding lower expressed biomarkers and making tissue to tissue comparisons problematic.

Akoya Biosciences has successfully overcome this pervasive problem using multispectral imaging & spectral unmixing, the ability to both isolate autofluorescence to a discrete channel and also to isolate each of the biomarkers of interest regardless of their intensity or any spectral overlap (Figure 1).

### Autofluorescence robs signal

As a researcher, regardless of the tissue you work with, autofluorescence is hard to avoid. Some labs use chemical masking agents to try and hide autofluorescence. Others try to use complex algorithms to mitigate post-scan. Neither solution works very well.

To demonstrate an alternative, images were acquired using the PhenoImager HT™ (formerly Vectra® Polaris™) MOTiF™ workflow; the PhenoImager Fusion could be used, as well. Unmixing and analysis of images were done

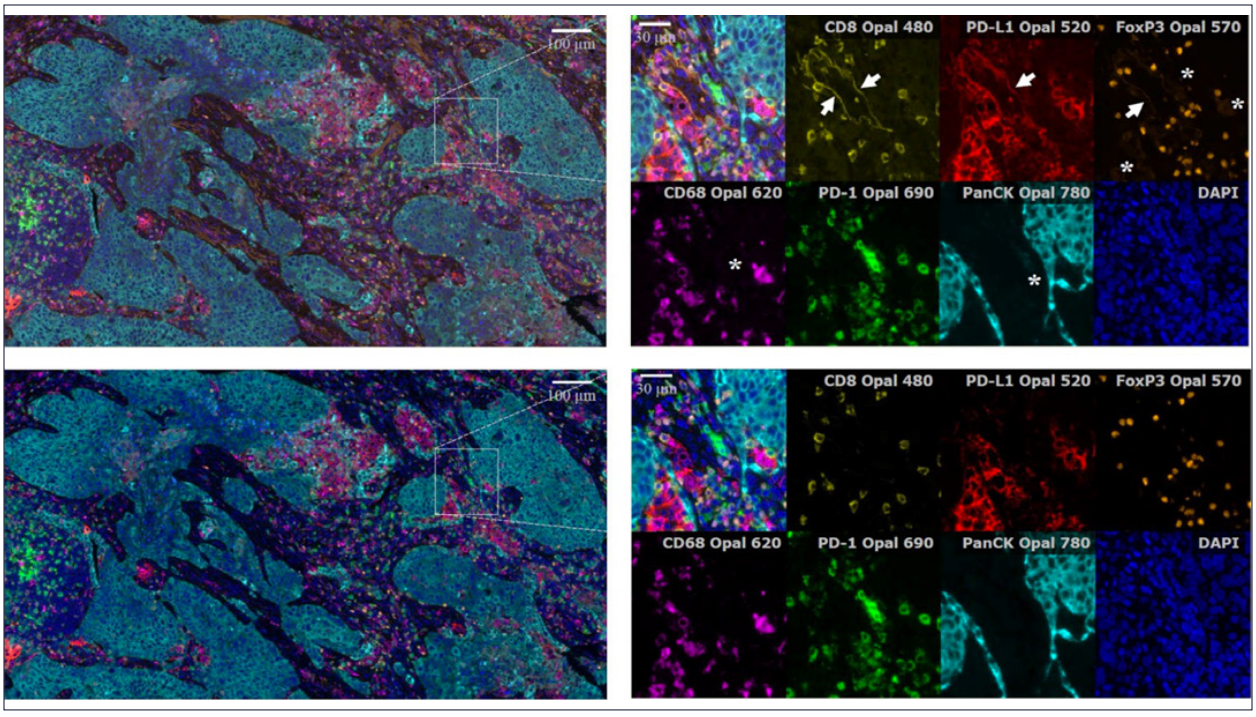



**FIGURE 2**. Conventional narrowband scan acquired with bandpass filters (top) vs. unmixed multispectral imaging (bottom) using Opal™ fluorophores. Arrows indicate autofluorescence contamination; asterisks indicate crosstalk from a spectrally adjacent band (Image courtesy: Bethany Remeniuk).

using inForm® v2.4.8 followed by phenoptr-Reports for spatial analysis.

## How multiplexing works

1. **Create a library.** To apply multispectral imaging to tissue, we start by setting up a spectral library for our experiment using the appropriate Opal™ MOTiF fluorophores. This is done by imaging examples of each tissue/ marker/fluor combination as single stains (without counterstain). This set of single stained images establishes a reference to unmix each subsequent color that will be imaged in multiplex immunofluorescence (mIF).

2. **Include an autofluorescence slide.** This library slide goes through all the mIF steps a regular multiplex sample would be exposed to, but does not get stained with any reagents. Each tissue type autofluoresces differently so it is important to use project specific samples to build your spectral library. Once we have a representative autofluorescence (AF) sample in our spectral library, it can be isolated from the rest of the spectra to dramatically increase signal over noise. This effect is akin to 'removing the haze' and can reveal important immune cells that conventional fluorescence imaging methods were not measuring accurately, or worse, missing altogether.

| Signal vs. Noise Ratio | | | |
|---|---|---|---|
| Channel | SNR Without AF removal | SNR Unmixed/ AF removal | SNR Fold increase |
| DAPI | 139.1 | 432:1 | 3.1X |
| CD8 (Opal480) | 88:1 | 999:1 | 11.4X |
| PD-L1 (Opal520) | 244:1 | 999:1 | 4.1X |
| Fox-P3(Opal570) | 597:1 | 999:1 | 1.7X |
| PD-1 (Opal620) | 676:1 | 701:1 | 1.04X |
| panCK(Opal690) | 512:1 | 517:1 | 1.01X |
| CD68 (Opal780) | 621:1 | 715:1 | 1.15X |

**FIGURE 4.** Significant fold improvement measured in signal to noise ratio from this lung tissue sample as a result of spectral unmixing.

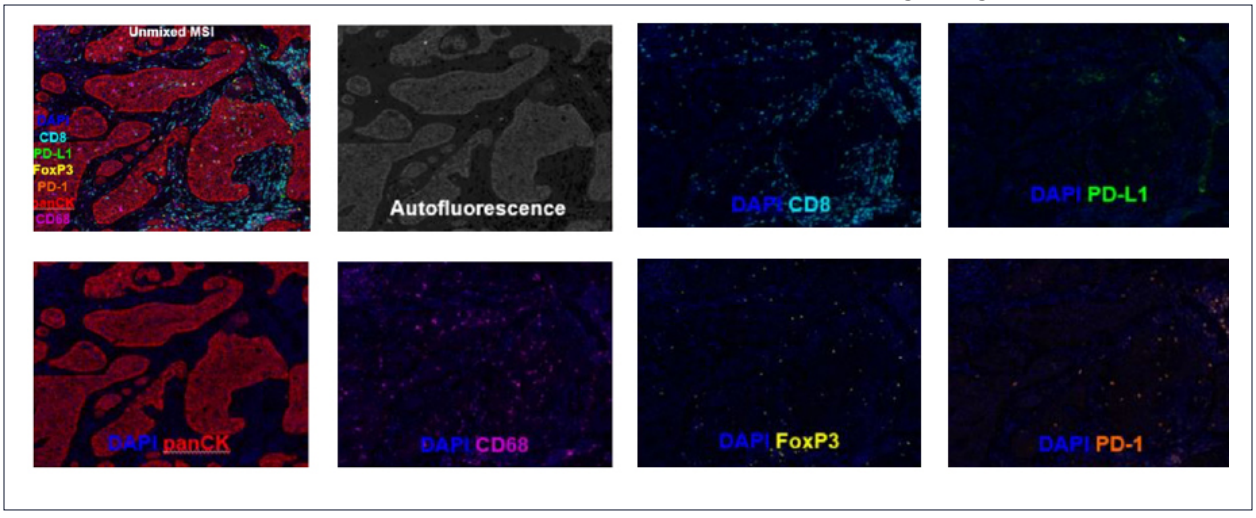



**FIGURE 3.** Autofluorescence in fluorescent imaging decreases signal-to-noise ratio, generating uncertainty in data analysis regardless of the number of biomarkers.

CHAPTER 4 *Certainty in Imaging*

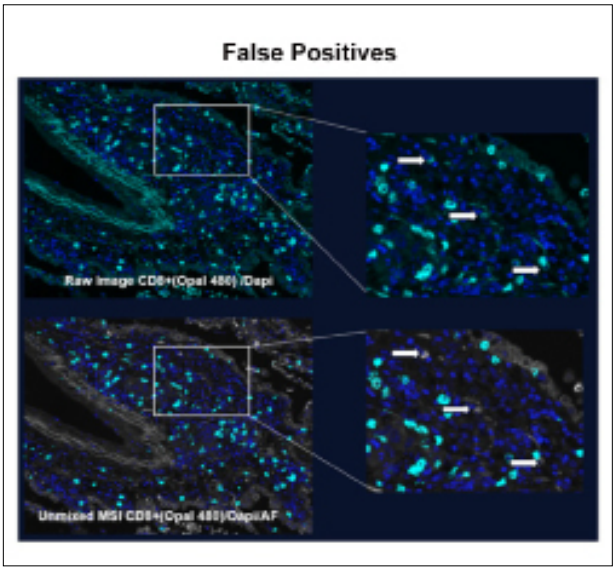



**FIGURE 5.** Arrows in the lung cancer raw image point to AF that is being incorrectly identified as CD8+. When AF is unmixed into its own channel (shown in the unmixed image as gray), we can visualize the difference.

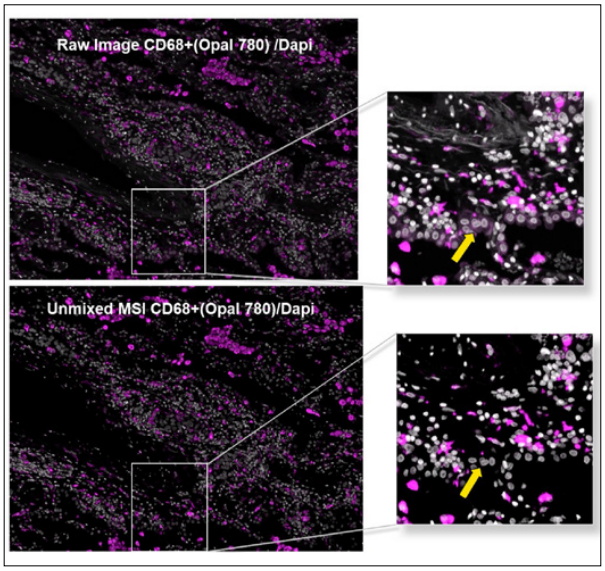
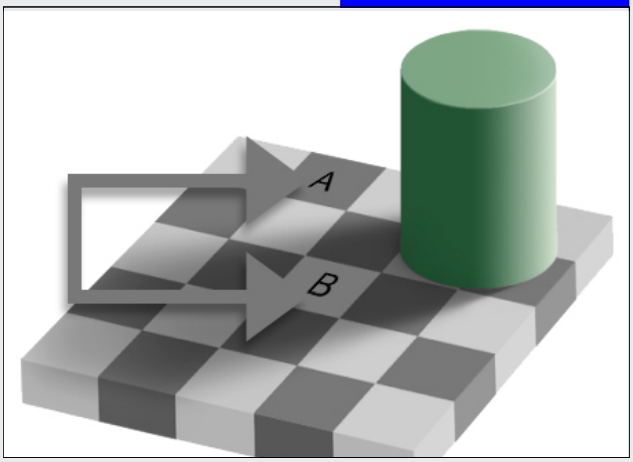



**FIGURE 6.** Looking in the far red channel at CD68, we find that AF has obscured cells from view, the raw image misses nearly half of the true macrophages.

## I Can't Believe My Eyes

Humans are not good at visually measuring wavelengths/intensity of light, but the brain infers this information to help in decision making. In this way, we cannot trust our eyes to see reality, but instead we generate a story about reality based on our perception of the world. What we perceive is a combination of visual inputs filtered through multiple areas of the brain and molded by past experience. In day-to-day life, seeing what we expect to see can increase efficiency, but in science, it can introduce error. That is one reason why it is immportant to employ quantitative methods when interpreting imaging data.

A or B–Rollover to See




### Which Square Is Darker: A or B?

In fact they are the same color, but the human eye takes context into consideration and incorrectly judges A as darker than B **(rollover the image above to see same color arrows with and without checker board background).**

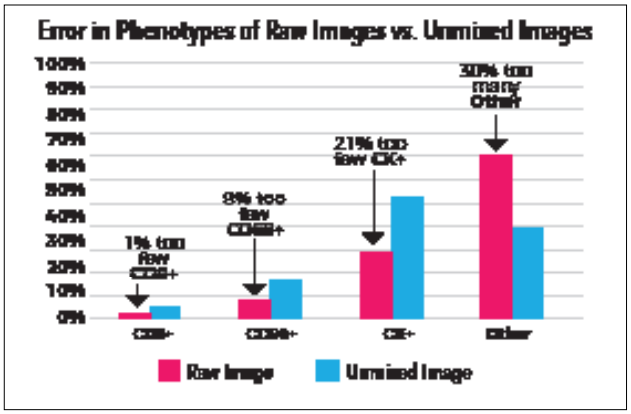



**FIGURE 7.** Results of unmixing experiment

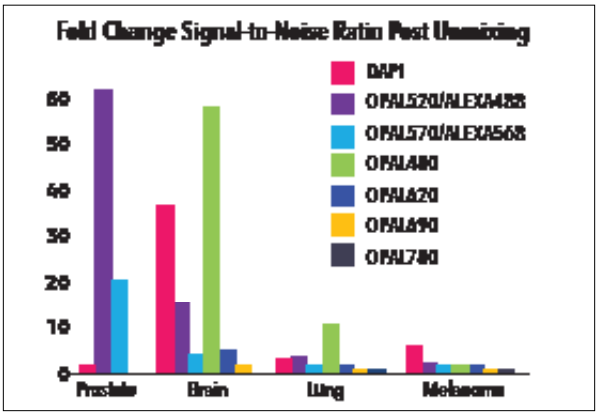



**FIGURE 8.** Impact of AF on different tissues. Signal-to-noise fold-changes resulting from unmixing.

## Why conventional systems struggle

To understand why it's sometimes hard to separate color from signal, let's look closer at current fluorescence imaging practices. Conventional systems utilize narrow bandpass filters that capture only a snapshot (typically the peak) of each fluorophore's emission spectrum. Multispectral imaging with spectral unmixing uses a different technique.

By utilizing a reference library of emission spectra for each fluorophore, Akoya Biosciences' approach allows users to gather each fluorophore's entire emission signature, not just a snapshot of the peak, enabling much more accurate unmixing and quantification, as well as the removal of autofluorescence (Figure 2).

Rather than using only a small portion of the fluorophore's emission spectrum, which is the technique employed by most traditional widefield fluorescence microscopes, Akoya Biosciences' technique achieves robust spectral unmixing. This makes each fluorophore's signal truly distinct from those of other fluorophores and enables the clear separation of signals, thus assigning the real contribution of each fluorophore to each pixel in the image.

## Unmixing experiment

Using Akoya Biosciences' technology various human FFPE tissues (lung, skin, brain and prostate) stained with Opal and non-Opal fluorophores were quantitatively analyzed. Images show representative regions of interest (ROIs) after spectral unmixing (Figure 3), while the table (Figure 4) illustrates the quantification of changes in the signal-to-noise ratio for each component. Signal-to noise ratios were calculated by measuring the pixel intensity of the top 99.9th percentile

| Limitations of traditional immunofluorescence | Advantages of multispectral unmixing |
|---|---|
| • Limited to 3-4 markers | • Multiplex greater than 4 markers |
| • Autofluorescence background | • Removal of background autofluorescence |
| • Spectral bleed through | • Removal of spectral bleed through |
| • Low signal-to-noise ratio | • High signal-to-noise ratio |
| • Low confidence in quantitative analysis of data | • Reliable and quantitative analysis of data |

pixels defined as 'Signal' and the bottom 10th percentile defined as 'Background' or 'Noise'. This was calculated for each ROI and averaged across the larger, multi-ROI sample for each tissue type.

We found that raw images sometimes overestimated a phenotype. In our example, almost half of all $CD8^+$ cells in our raw image were false positives from AF contamination in the cyan (Opal 480) channel compared to unmixed phenotyping results (Figure 5 and Figure 7). Alternatively, when we look at the $CD68^+$ cells in our far red (Opal 780) channel, we see that phenotyping calculations in our raw images greatly underestimates the total number of $CD68^+$ cells, missing almost half of our true positive macrophages (Figure 6 and Figure 7).

## Error in phenotypes of raw images vs. unmixed images

Both types of error could greatly impact any calculations of spatial interactions and the predictive accuracy of data. The impact of this error is compounded by analysis of complex and rare cell phenotypes within tissue samples, especially those containing high AF signals (Figure 8).

## Conclusion

Even for applications that require the labeling of a limited number of biomarkers, and in which fluorophores are well separated and spectral overlap is less of a concern, the removal of background autofluorescence provides a tremendous advantage (see Figure 1: Prostate cancer stained with 2 markers and DAPI counterstain).

The goal of multispectral imaging and unmixing beyond the assessment of multiple biomarkers, is to be able to generate quantitative information—information in which the user can have the utmost confidence.

The problem is not limited to tissues with high autofluorescence or tissues with weakly expressing biomarkers, across the board, Akoya Biosciences' patented multispectral imaging technology with spectral unmixing has proven vital for data accuracy and become the go-to-gold standard technique to achieve quantitative image analysis.

## Acknowledgement for their contribution and efforts

Agnes Haggerty, Grady Carlson, Carla Coltharp, Bethany Remeniuk, Roslyn Lloyd, Oliver Braubach, David Coulson, Ani Solanki, Andy Johnson, Victoria Duckworth, Lori Murray, Dan Walker - Akoya Biosciences.

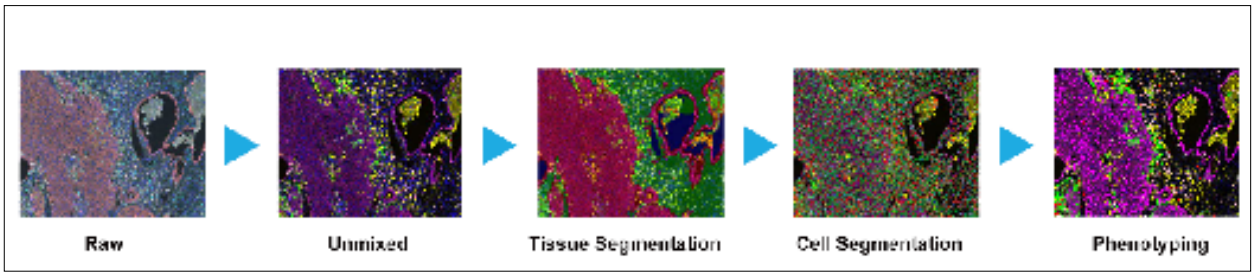



**FIGURE 1.** Workflow and general framework for multiplexed immunofluorescence image analysis using inForm software

CHAPTER 5 *Multiplex Imaging Analysis*

# Multiplex Imaging Analysis

## Obtaining objective and reproducible spatial signatures

The ultimate goal of multiplex imaging is to derive spatial phenotypic signatures, which define how cells organize and interact to influence disease outcomes. To achieve this goal at scale requires building an analysis workflow for multiplex imaging data that can be replicated within and across laboratories.

In this chapter, we outline a step-by-step path for obtaining informative spatial signatures starting from raw images generated by whole-slide multiplex imaging using immunofluorescence (mIF) combined with multispectral imaging technology.

### Pre-analytical quality control: Getting the most data out of images

Consulting a pathologist to confirm the quality of staining results is crucial for downstream analytical success.[1] Pathology quality assessment should answer the following questions:

- Does the pathology match the disease diagnosis, especially for rare tumors?
- Does the section contain enough diseased tissue/tumor tissue compared to non-tumor/ non-diseased tissue?
- What is the proportion of necrotic and noncellular material, and is it interfering with staining or interpretation?
- Are positive control markers showing expected staining intensity and localization?

### Choosing the right image analysis software and algorithm

Image analysis software improves the reproducibility of multiplex imaging because it enables automated analysis of characteristics that the human eye cannot always discern, such as cell shapes, dimensions of cell phenotype networks, weakly expressing and overlapping biomarkers within cells and cellular compartments, and vascular networks. However, because different image analysis systems and protocols can yield different results, it is important to harmonize and document these choices and algorithms when working across laboratories.

Consistent, objective analysis can be achieved by analyzing whole-slide images of whole tissue sections, using a standardized algorithm that treats events independently. Locking down a chosen algorithm does not always suit every tissue type or tumor type, so groups can employ a common model algorithm and apply small changes.

### Types of software

Image analysis software platforms can be classified into two groups, based on the level of user training required. Unsupervised software packages generally require minimal user training or computational skills but may offer less flexibility if higher order analysis for more complex studies is required. Supervised software packages allow users to perform more complex analyses but usually require both a higher level of training and user input.

Regardless of category, the chosen image analysis platform should feature:

- Ability to visualize whole-slide images billions of pixels in size
- High-throughput capabilities

- Ability to be evaluated and validated for regulatory data submissions

## Establishing the analysis protocol

One of the first post-acquisition decisions may simply be how many images to analyze for the training set used to generate an analysis algorithm. Possibly, the best way to capture comprehensive data on cell-cell interactions is to look at all (or most) of the images, but this may be a time-intensive process and in some cases, not necessary. Looking at randomized sub-samples might be a great way to measure, for example, the overall density of cell phenotypes. This needs to be done in a way to assure that images are truly randomly selected, avoiding the bias of choosing hotspots or other features.

Pathologists can help guide the selection, which is especially helpful for specific questions regarding certain tissue areas or features of interest.

## Tissue segmentation

After annotating raw, multispectral images and unmixing the images using the established spectral library (**see previous chapter**), the tissue must be segmented into regions of morphologically distinct architectures, such as tumor and stroma (Figure 1). Trainable pattern recognition makes this possible and often avoids prohibitively laborious manual identification of regions of interest.

Segmenting tissue identifies regions of tumor, non-tumor, and other (non-tissue areas). Tumor compartmentalization involves recognizing features based on the expression or absence of tumor-specific markers included in a panel, such as cytokeratin for a tumor/stroma segmentation.

Further segmentation can be achieved using other markers to define areas based on a feature of interest, such as B and T cell zones, tertiary lymphoid structures, ducts, islets, etc. While tissue segmentation is not a prerequisite for cell segmentation or cellular phenotyping, knowing the location and spatial distribution of different cell populations in a tumor has been demonstrated to predict response to treatment and further outcomes. For example, T cell populations within the tumor or the invasive margins, but not in the stroma, are associated with favorable prognoses in multiple cancer types.[3,4]

The markers used to identify compartments should also be flexible enough to train the image analysis software to easily identify areas to exclude (e.g., necrotic areas or areas without tissue) and can be iteratively refined by adding new training areas to address any misclassified regions. Once the tissue segmentation algorithm has been sufficiently trained, it can then be applied to a batch of similarly stained images.

Some image analysis software, such as **inForm Tissue Finder**, automates the detection and segmentation of specific tissue compartments through powerful patented pattern-recognition algorithms. Automation can improve the reproducibility of results and enables comparative studies of both multiple markers and multiple specimens.

## Cell segmentation

The next step, cell segmentation, identifies

single cells based on their nuclear stain, cytoplasmic, and membrane markers. Expressions of markers can then be read out on a per-cell and per-cell-compartment basis.

First, cell nuclei are identified using the intensity of nuclear DNA staining by 4',6-diamidino-2-phenylindole (DAPI). DAPI can be used alone or with other nuclear markers, for example, Ki67, a nuclear protein and a marker strongly associated with tumor cell proliferation and growth.

Nuclear staining can also be used in combination with cytoplasmic and membrane markers to better identify individual cells. Because every sample is different, adjusting for nuclear size and staining threshold parameters based on tumor type may be necessary when training the software to better identify and individualize cells. Using a universal membrane marker or a marker cocktail is recommended to optimize cell segmentation.[1]

## Cellular phenotyping

Initially, cell phenotypes are assigned using a single marker. Once cells are converted into addressable objects, users can perform more complex phenotyping, taking into account multiple marker positivity. Advanced machine learning approaches can automatically phenotype cells into user-defined categories.

The two categories of approaches used by most analysis software are machine learning and thresholding.

In machine learning, a classifier is an algorithm that categorizes data. For mIF image analysis using machine learning software such as inForm, a classifier must be trained to identify a phenotype of interest. Users select example cells to teach the classifier which are positive, and which are negative for a particular feature. Typically, users select 5–10 example cells, followed by iterative addition of more examples to refine the classifier for reliable cell phenotyping. Once training is complete, the algorithm can be applied to a batch of similarly stained images.

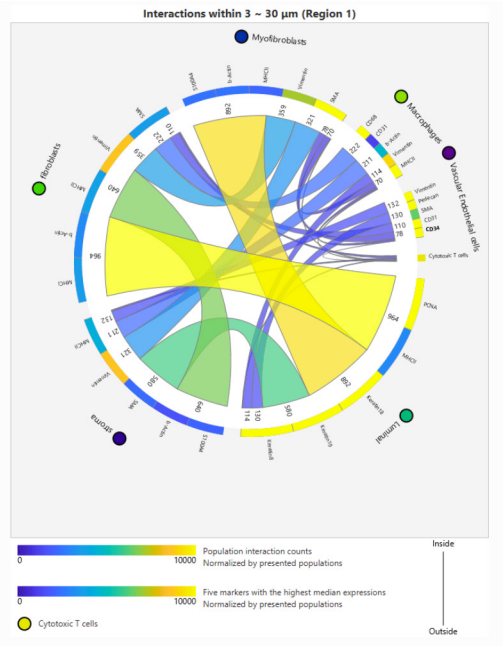



**FIGURE 2**. CIRCOS plots can be used to represent spatial interactions between populations

Machine learning is more robust and efficient than thresholding. Thresholding (or gating) involves choosing a fluorescence intensity above which a cell is defined as positive or negative. This approach is intuitive and may be useful for evaluating the expression of a limited set of markers, or with irregular staining patterns, or when classifying cells as low-, intermediateor high-expressors. Disadvantages of this method are that it is limited by segmentation noise and that the fluctuations in intensity may hinder batch analysis.

CHAPTER 5 *Multiplex Imaging Analysis*

## Spatial analysis

Putting cell phenotypes into their spatial context requires the measurement of a number of metrics that can ultimately yield spatial signatures of clinical outcomes. Below, we outline some of these metrics that can be analyzed using R and R studio-based statistical software packages, such as **phenoptrReports**.[6]

### Phenotypic matrices and proximity analysis

One way to visualize the spatial relationships between cell phenotypes in a sample is to create a phenotypic matrix, otherwise known as a distance matrix.[5] Spatial coordinates of two cell phenotypes are listed in a table, with one cell phenotype on the x-axis and the other on the y-axis. Each box of the table records the distance between the two phenotypes for every pair of cells. Extended to multiple markers and all the possible connections between

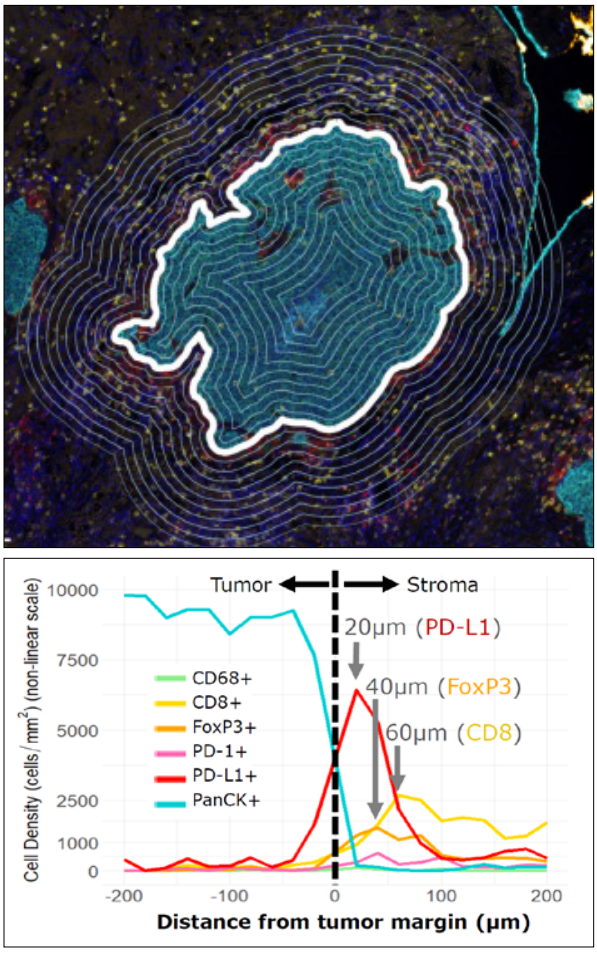



**FIGURE 3.** ( Top image) Cell density in bands (blue) from tumor margin (white). (Bottom image) Density of each marker within the bands shown. Peak densities for different markers are found at different distances outside the tumor margin (Image courtesy: Carla Coltharp, Akoya Biosciences)

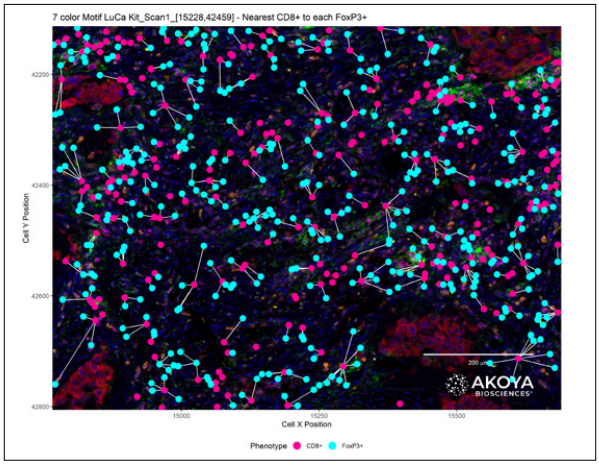



**FIGURE 4.** A nearest neighbor diagram shows cells in their spatial context. Pink dots represent CD8+ cytotoxic T-cells, and the teal dots, FoxP3+ regulatory T-cells. The lines connect each FoxP3+ cell with the nearest CD8+ cell (Image courtesy: Clemens Duerrschmid, Akoya Biosciences)

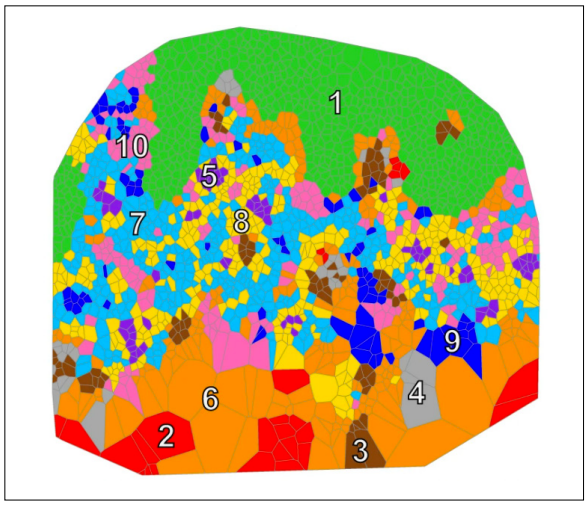



**FIGURE 5.** Voronoi diagram of 10 cellular neighborhoods identified in Cutaneous T cell lymphomas (CTCL) showing the spatial interactions between different cell types (Source: Reference 8. Used under the terms of a **CC BY 4.0** license)

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