Certainty in Imaging: Isolate Autofluorescence to See What You Have Been Missing

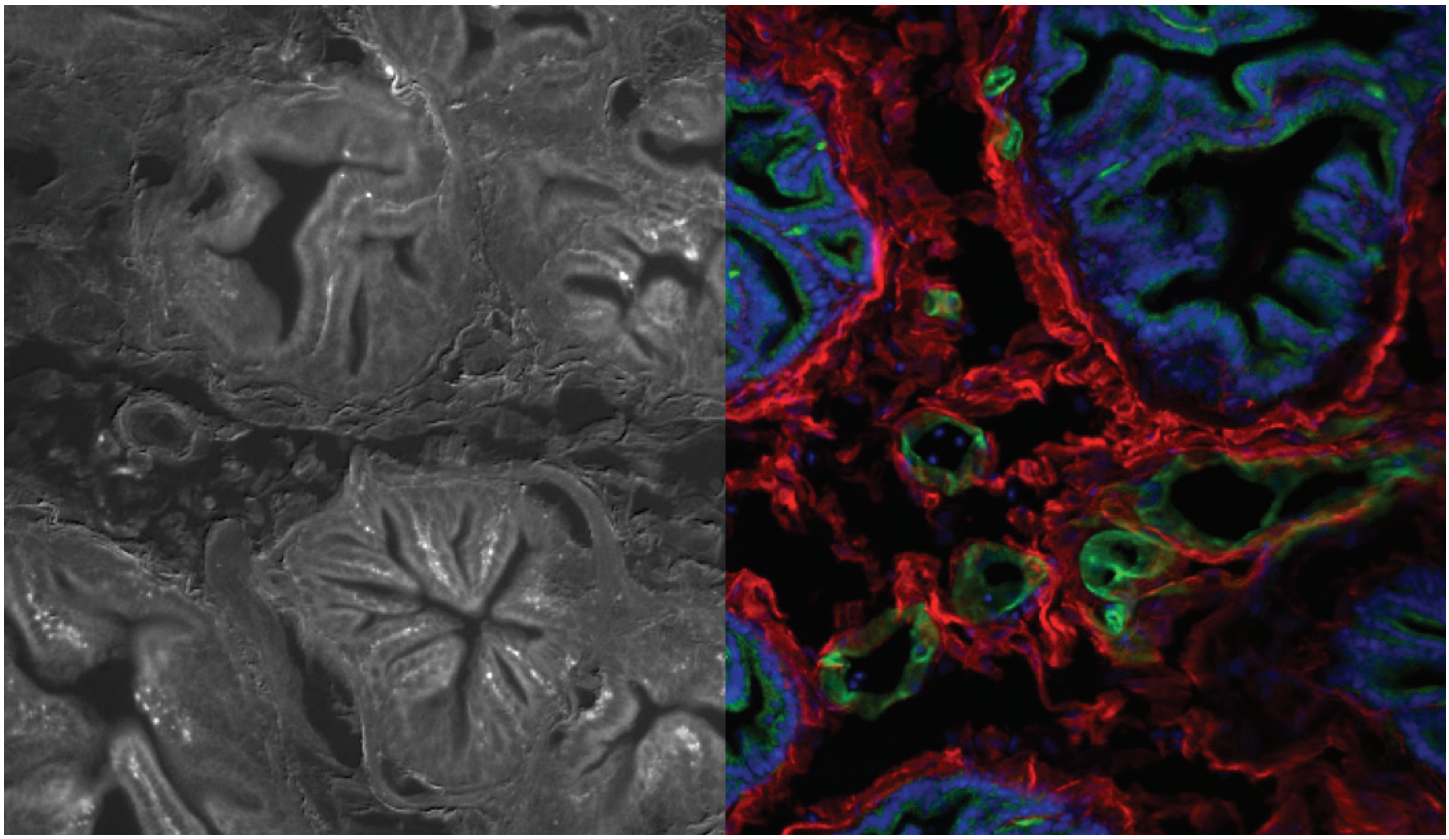

FIGURE 1. Prostate tissue stained with two markers plus DAPI, the left side showing autofluorescent signal that was isolated & removed during unmixing. CD31-AF488 was used for visualizing blood endothelial cells and gp38-AF568 for visualizing fibroblasts. Lymphatic endothelial cells stain positive for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

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 has successfully overcome this pervasive problem using multispectral imaging & spectral unmixing, the ability both isolate autofluorescence to a discreet channel & also to isolate each of the biomarkers of interest regardless of their intensity or any spectral overlap.

Learn more about the confounding effects of autofluorescence and how Akoya has mitigated this issue to unlock the full impact of spatial biology.

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, we acquire images using the Vectra Polaris® MOTiF™ workflow (although you could use the multispectrally equipped Mantra™ manual microscope as well; Fig. 3). Unmixing and analysis of images were done with inForm® v2.4.8 followed by phenoptrReports for spatial analysis.

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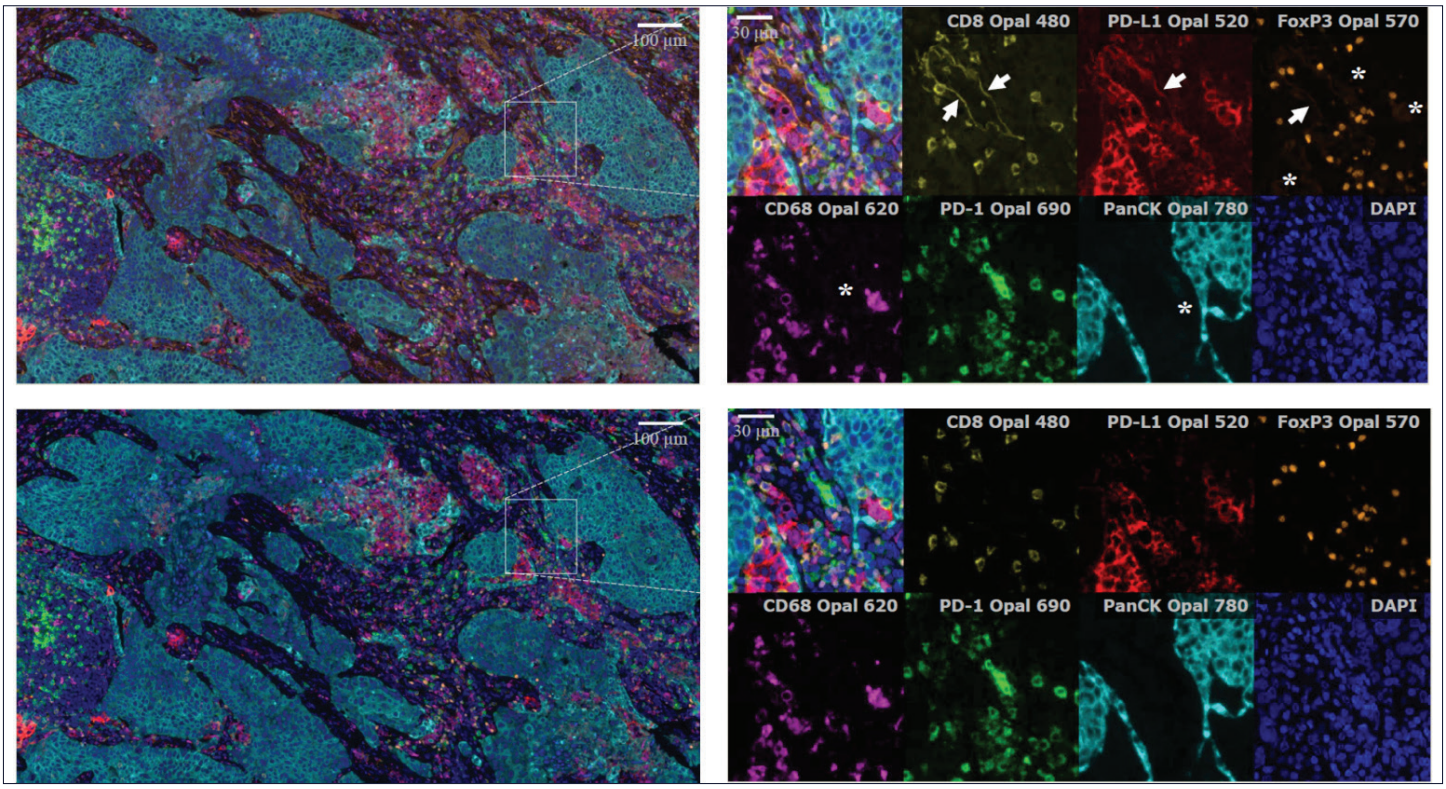


FIGURE 2. Conventional narrowband scan acquired with bandpass filters (top) vs. unmixed multispectral imaging (bottom) using Opal™ fluorophores. Arrows indicate autofluorescence contamination; asterisks indicate crosstalk from a spectrally adjacent band..

How Multiplex 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 mIF (mIF is short for "multiplex immunofluorescence" and commonly used in publications to describe this IHC process).

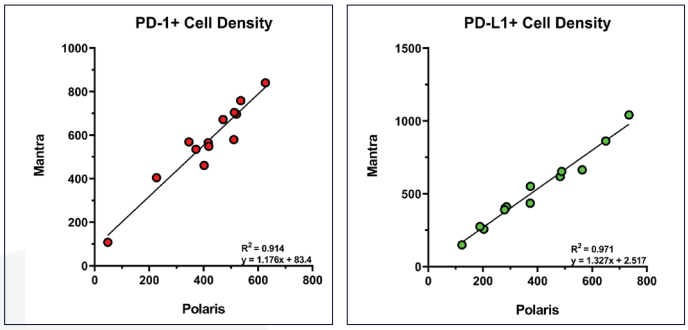


FIGURE 3. Either tool works: Graphs represent a subset of a concordance analysis featuring PD1 & PDL1 cell densities on both the Mantra™ and Vectra® Polaris™ imaging systems.

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 its important to use project specific samples to build your spectral library.

Once we have a representative autofluorescence (commonly abbreviated as 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 all together.

Why Other 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, our 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 (Fig. 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), our technique achieves robust spectral unmixing, which 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

We quantitatively analyzed various human FFPE tissues (Lung, Skin, Brain and Prostate) stained with Opal and non-Opal fluorophores. Images show representative regions of interest (ROIs), before and after spectral unmixing (Fig. 4), while the table (Fig. 5) 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 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.

SIGNAL VS. NOISE RATIO	
Channel	**SNR Fold Increase**
DAPI	3.1x
CD8 with Opal 480	11.4x
PD-L1 with Opal 520	4.1x
FoxP3 with Opal 570	1.7x
PD-1 with Opal 620	1.04x
panCK with Opal 690	1.01x
CD68 with Opal 780	1.15x

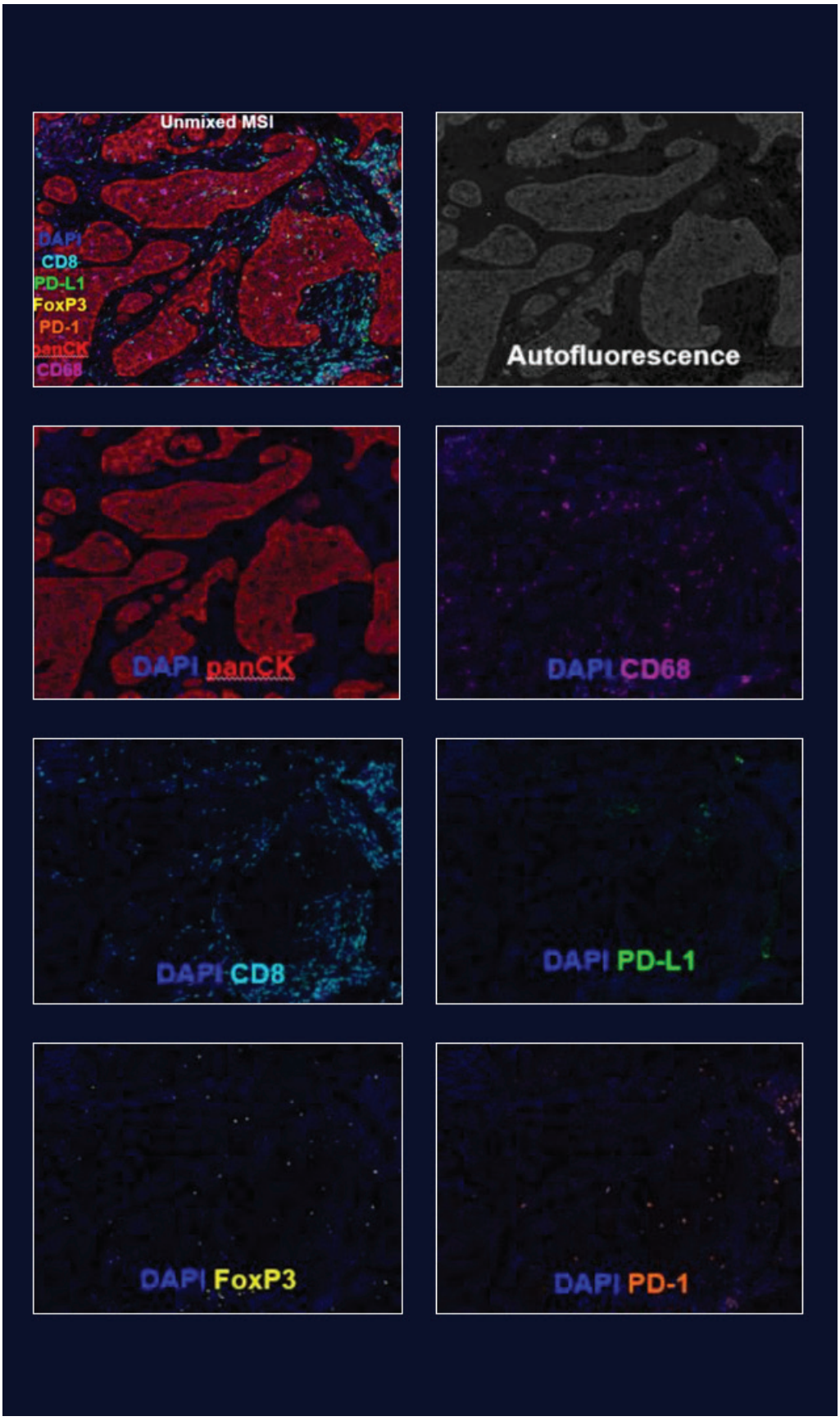


FIGURE 4. Autofluorescence in fluorescent imaging decreases signal to noise ratio, generating uncertainty in data analysis regardless of the number of biomarkers. **FIGURE 5.** Significant fold improvement measured in signal to noise ratio from this lung tissue sample as a result of spectral unmixing.

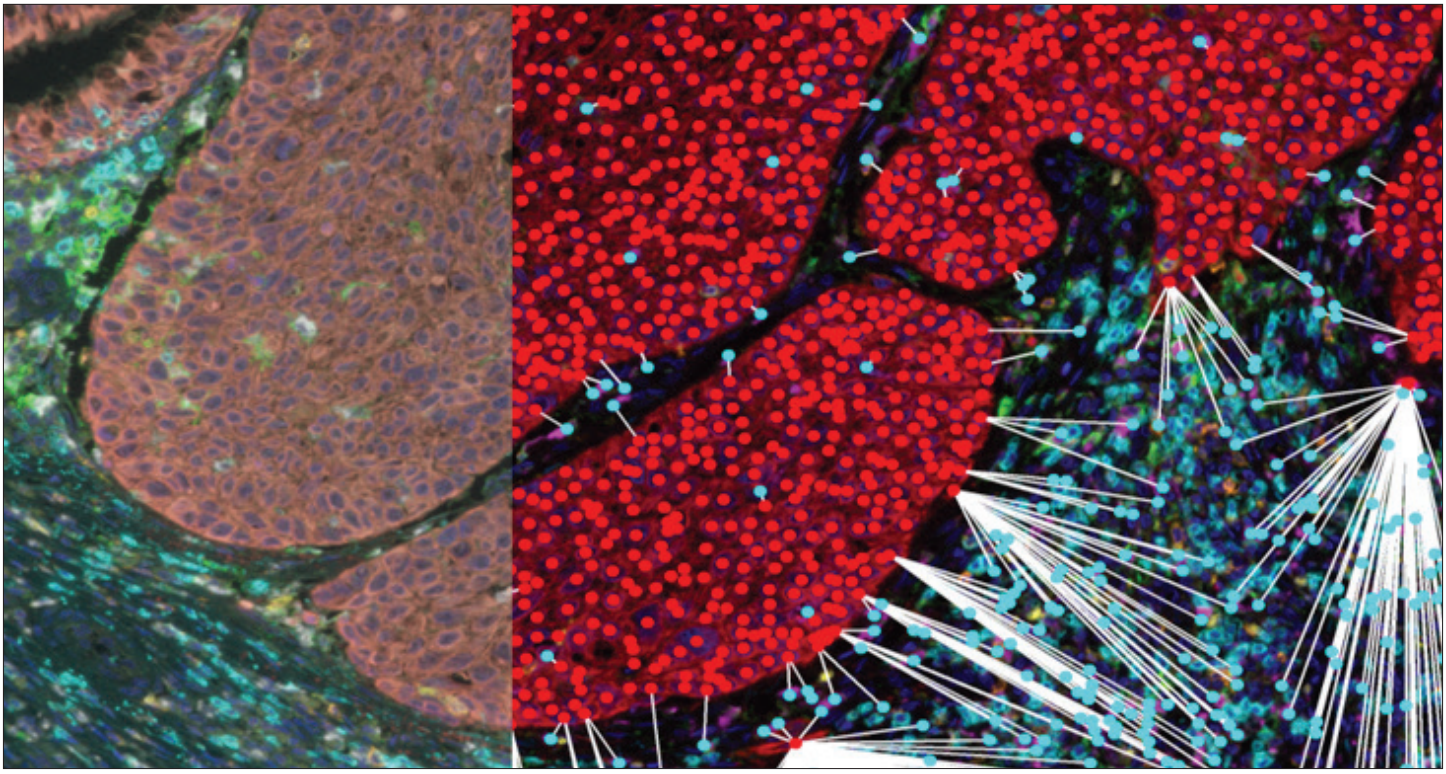
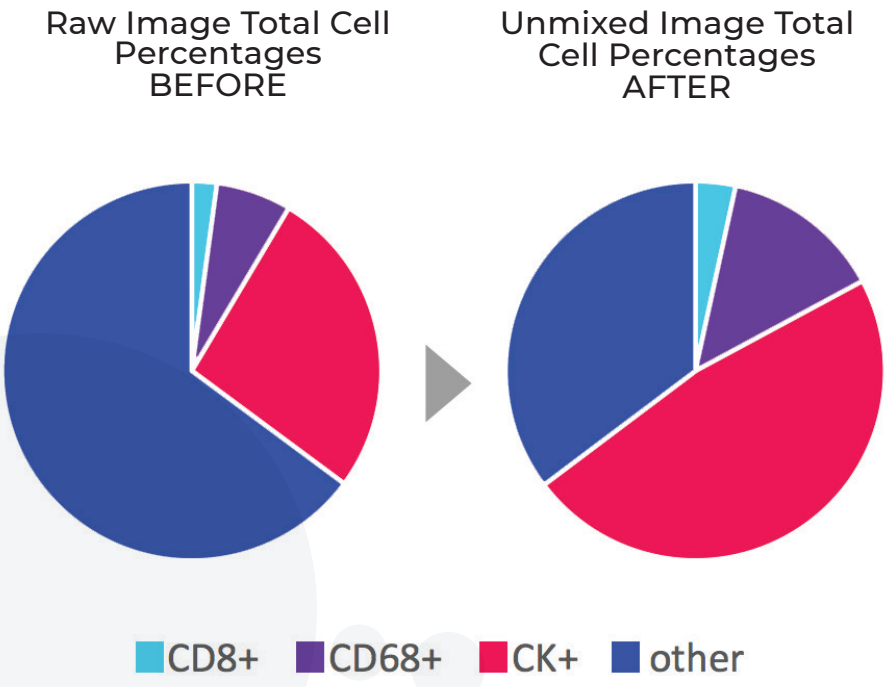


FIGURE 6. Multispectral imaging is the second of three steps in the Phenoptics process, preceded by Opal reagent staining and followed by whole-slide image analysis. On the left side, all raw signal is mixed together. On the right, umixed imagery has been segmented & phenotyped using inForm machine learning then spatially analyzed to reveal the communication networks that exist between cellular neighborhoods.

Errors in Phenotyping Mixed Images

Unmixing signal from AF prior to automated phenotyping increases accuracy of your results. In this example, we analyzed a lung cancer sample before and after unmixing and AF isolation.



It Used To Be Rocket Science

The year was 1999 and we were on a mission to Mars. A winding road had led us to the aerospace community after developing a multispectral imaging system. As we prepped our device to sustain a rocket launch, we were now in a position to image and spectrally unmix Mars' mysterious landscape and fields of stardust in order to discover and explore whole new worlds where enhanced signal-to-noise ratios and data precision were essential. As our R&D continued the scientific community began to recognize the value of this technology in a multitude of applications; from agriculture and topography to chemistry and biology. Whether exploring planetary neighbors or cellular neighborhoods, multispectral imaging turns out to be essential if you want to get in the business of measuring light precisely and let that precision drive discovery.

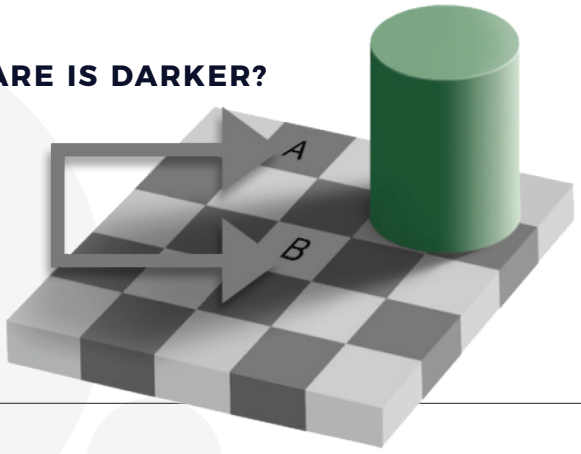
FIGURE 7. The problem. Arrows in the raw lung cancer image point to AF that is being incorrectly identified as CD8. When we unmix AF into its own channel (shown in the unmixed image as gray), we can tell the difference.

I CAN'T BELIEVE MY EYES!

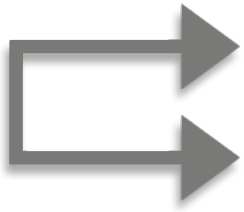
Humans are not good at visually measuring wavelengths/intensity of light but rather the brain infers this information to help in decision making. In this way, we cannot trust our eyes to see reality, but instead we generate a story about reality based on our perception of the world. What we perceive 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's one reason why it's important to employ quantitative methods when interpreting imaging data.

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**.



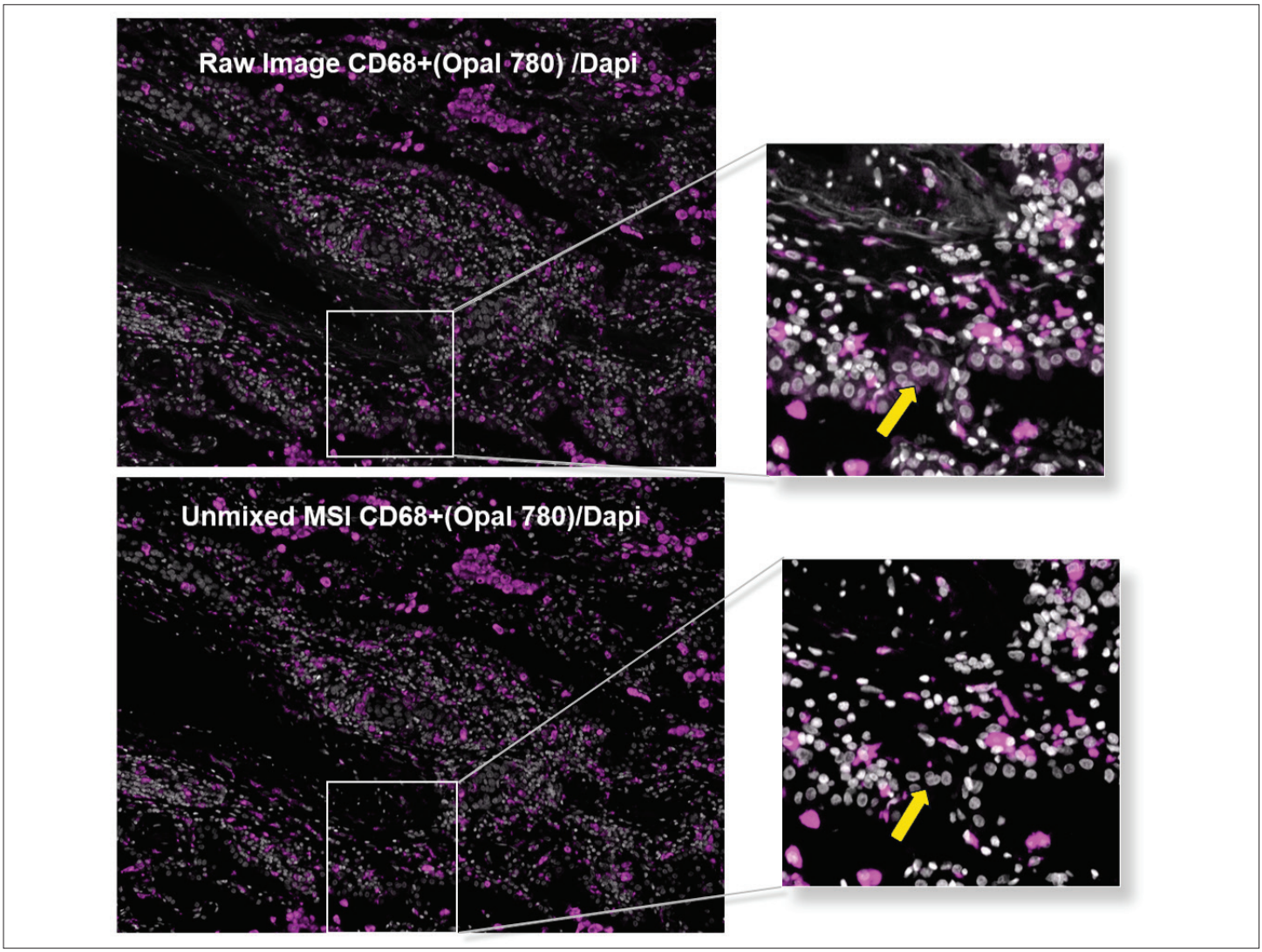


FIGURE 8. Looking in the far red channel at CD68 we find that AF has obscured cells from view, the raw image misses nearly half the true macrophages.

We found that raw imagery of cellular phenotypes can be difficult to accurately quantify, even when you use fluors that are spectrally far apart (Fig. 9). In our example, CD8+ proved difficult to find in the cyan channel (Opal 480) due to AF contamination compared to unmixed phenotyping results (Fig. 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 (Fig. 8).

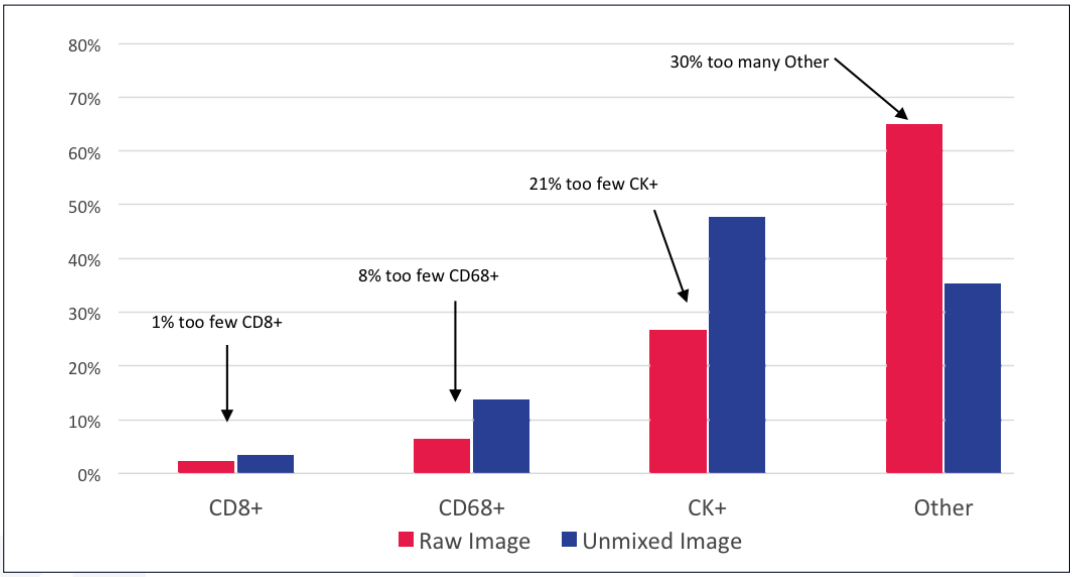


FIGURE 9. Results of unmixing experiment

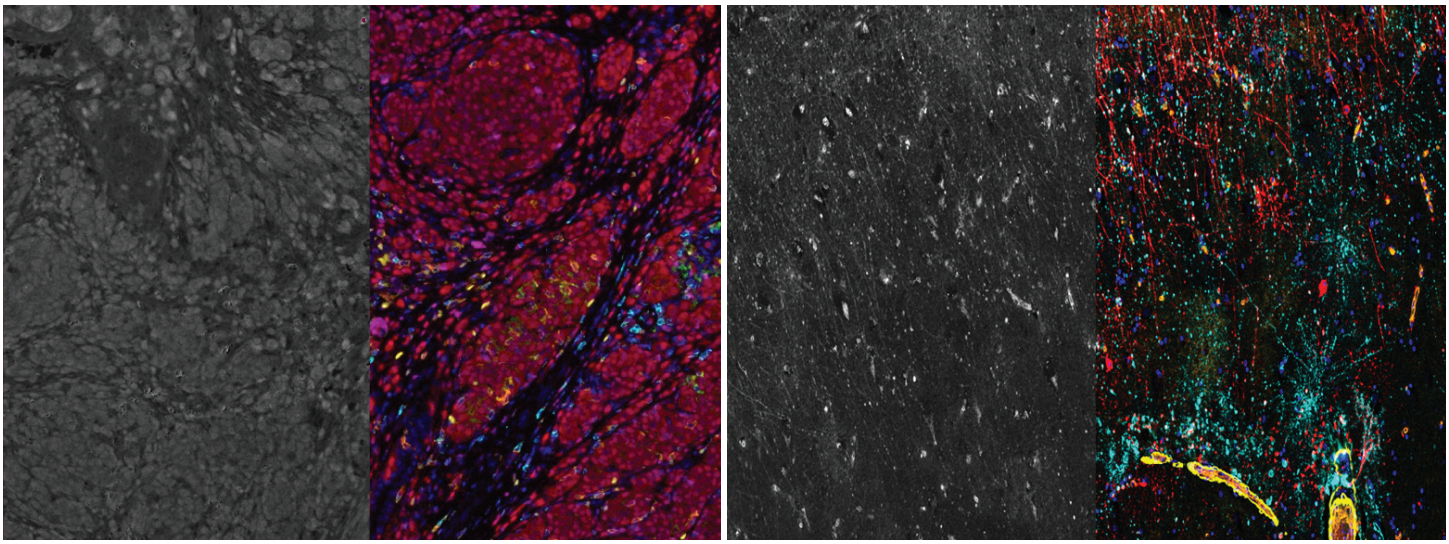


FIGURE 10. Right half of image is unmixed, left half is autofluorecent signal that was removed during unmixing; Melanoma (Opal™ Polaris 7 color Plug-n-Play Melanoma Kit) and **FIGURE 11.** Brain tissue (Opal™ Detection, 6 colors).

The closer we look, the more complicated the biology is that we see. For predictive accuracy, we want to be quantitative in our calculations of spatial interaction. This informs us of the communication networks existing between cellular neighborhoods. The impact of raw image error (Fig. 9) is compounded by analysis of complex and rare cell phenotypes within tissue samples, especially those containing high AF signals (Fig. 12).

Impact Of AF on Different Tissues Shown in Signal to Noise Fold-Change Resulting from Unmixing

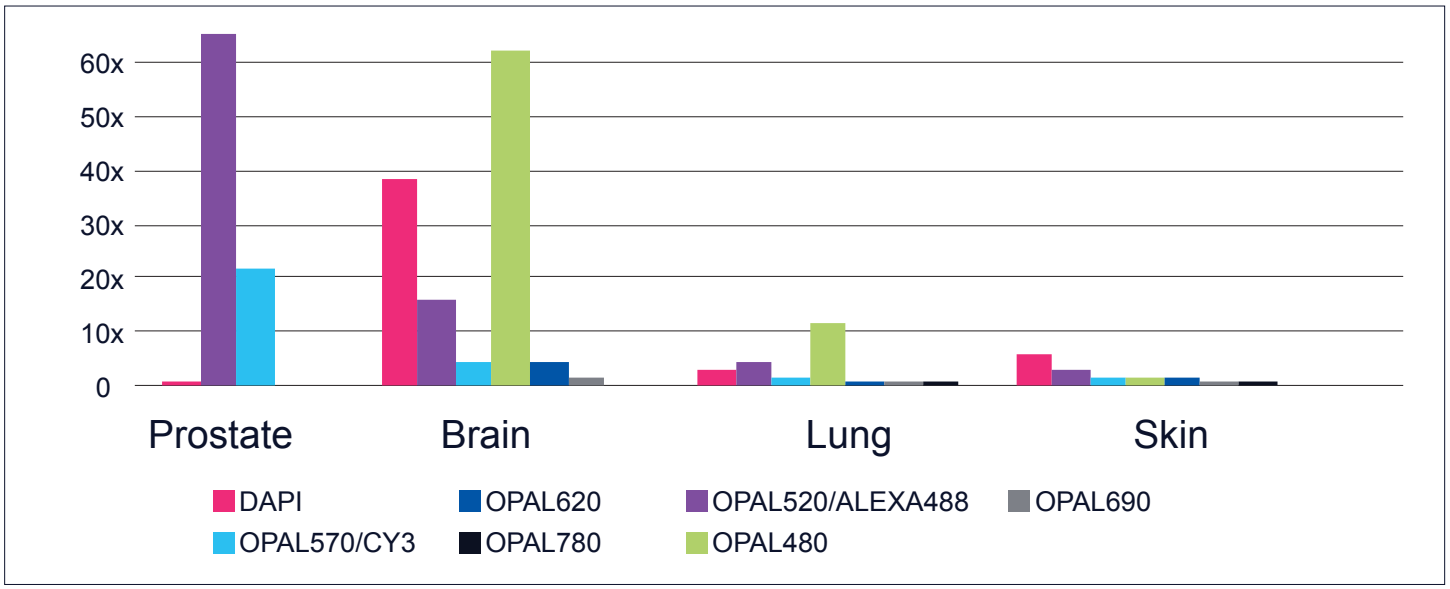


FIGURE 12. All tissue has AF, some more than others.

Limitations of traditional immunofluorescence	Advantages of multispectral unmixing
· Limited to 3-4 markers	· Go beyond 4 markers
· Autofluorescence background	· Removal of background autofluorescence
· Spectral bleed through	· Remove spectral bleed through
· Signal-to-noise ratio low	· Signal-to-noise ratio high
· Crosstalk with antibody species	· No Ab species cross-reactivity with Opal
· Low confidence in quantitative analysis of data	· Reliable and quantitative analysis of data

CONCLUSION

The goal of multispectral imaging and unmixing is not only to being able to assess multiple biomarkers, but also to be able to generate quantitative information—information in which the user can have the utmost confidence.

Even in applications that require the labelling of a more limited number of biomarkers where fluorophores are separated and spectral overlap is less of a concern (Fig. 1: Prostate cancer, where only two markers and counterstain were used), the removal of background autofluorescence is a tremendous advantage.

The problem is not just in highly autofluorescent tissue and in tissue with weakly expressing biomarkers, across the board multispectral unmixing has proven vital for data accuracy and is unlocking the promise of spatial biology.

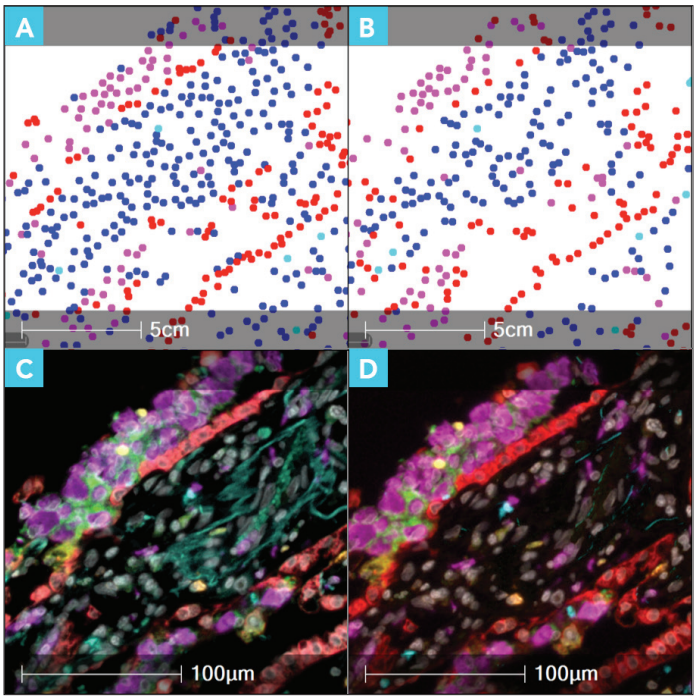


FIGURE 13. Raw image data on the left, unmixed on the right. Multispectral unmixing allows for autofluorescence and background removal and quantitative pathology.

IMAGERY COURTESY OF:

- **PROSTATE (FIG. 1)**—Tabea Sturmheit, Ph.D. Lymphocyte Activation Unit, San Raffaele Scientific Institute, Milan
- **BRAIN (FIG. 11)**—B.R. Huber MD, Ph.D. Dept of Neurology, Boston University; CTE Center, Boston University School of Medicine; VA Boston Healthcare System
- **LUNG (FIG. 4) AND SKIN (FIG. 10)** —Peter Miller, Vice President Research & Development, Akoya Biosciences, USA

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