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The PhenoImager HT 2.0 System Streamlines Data Generation with On-instrument Spectral Unmixing

INTRODUCTION

WHY MULTISPECTRAL IMAGING (MSI)?

FORMALIN-FIXED PARAFFIN-EMBEDDED (FFPE) TISSUE POSES CHALLENGES FOR ACCURATE MULTIPLEXED IMMUNOFLUORESCENCE

Multiplexed Immunoflurescence (mIF) is a valuable tool for measuring the abundance and location of multiple colocalized analytes in complex anatomical samples. However, when working with formalin-fixed, paraffin-embedded (FFPE) samples, mIF faces certain challenges which can impact sensitivity and specificity. Tissue autofluorescence (AF) frequently hampers visualization of immunofluorescent markers in FFPE samples and decreases sensitivity by masking positive signals.[1] Additionally, crosstalk may occur due to overlapping fluorophore spectra, leading to false-positive signals bleeding through to other detection channels and decreasing specificity.[1] Addressing these challenges is crucial for obtaining accurate and reliable results when using mIF with FFPE samples for biomarker analysis and spatial localization within tissues.

The full spectrum of mIF imaging methods

Three main imaging methods (monochrome, RGB, multispectral; see Figure 1) are commonly utilized for mIF, and each is affected differently by the confounding factors of autofluorescence and signal crosstalk. Monochromatic imaging, which captures one color at a time, has significant limitations. It cannot measure multiple analytes simultaneously or differentiate signals from fluorophores with overlapping spectra. Particularly in FFPE tissues, monochromatic imaging struggles to accurately account for varying levels of autofluorescence, since autofluorescence is usually not uniform across tissue sections.[1]

Using a three-color camera employing red, green, and blue filters (RGB) enhances multiplex detection and provides more information compared to monochromatic imaging. However, most organic fluorophores have broad emission spectra and still exhibit considerable overlap, even with three-color RGB detection. Further, the problem of diverse autofluorescence signals across tissue sections remains a challenge for RGB imaging setups.[2]

In contrast, multispectral imaging (MSI) stands out for quantitative imaging in complex tissues with good spatial resolution (Figure 1). MSI captures fluorophore and autofluorescence emissions at multiple wavelengths

across the visible spectrum, resulting in a multispectral image.[1] By selecting optimal wavelength bands and employing spectral unmixing, MSI can accurately distinguish individual fluorophores from each other and from autofluorescence. Accurate spectral unmixing of a sample with complex mixtures of fluorophores relies on predetermining the spectral signatures (or spectral 'libraries') of fluorophores and autofluorescence obtained through computational modeling or empirical measurements using representative samples. This makes MSI a promising approach for overcoming challenges associated with autofluorescence and spectral overlap in mIF.
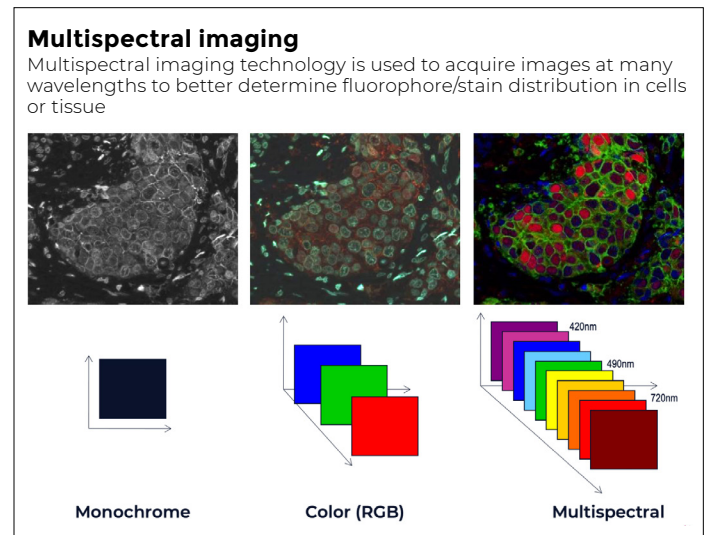
Multispectral imaging
Multispectral imaging technology is used to acquire images at many wavelengths to better determine fluorophore/stain distribution in cells or tissue

| Monochrome | Color (RGB) | Multispectral |

FIGURE 1. MSI provides increased signal-to-noise in quantitation compared to monochrome and RGB imaging methods. In both monochrome and RGB imaging, there is significant signal overlap between fluorophores and among fluorophores and autofluorescence background. MSI (right) significantly enhances the signal-to-noise ratio in quantitative imaging, effectively mitigating these challenges.

OPTIMIZED WHOLE-SLIDE OR FIELD-BASED MSI USING THE PHENOIMAGER HT SYSTEM

The PhenoImager HT imaging system has been designed for optimal performance with multispectral imaging (MSI). When performing multispectral whole slide scans, the system leverages Akoya's cutting-edge imaging workflow (formerly MOTiF) to acquire ultra-fast spectral measurements, a proprietary feature crucial for advanced analysis. This whole-slide imaging workflow incorporates eight meticulously designed paired excitation and emission bands, along with a six-channel illuminator to generate optimal spectral information for tissue autofluorescence and a subset of dyes, including Akoya's Opal™ fluorophores. These components collectively ensure the capture of exceptional spectral information, eliminate selection biases, and make re-analysis easy and reproducible because representative fields of view do not need to be pre-selected prior to imaging.

Moreover, the PhenoImager HT system excels in capturing emission spectra of MSI fields with an impressive range of over 40 wavelength bands. This capability is made possible by integrating a liquid crystal tunable filter into the imaging path, allowing for a comprehensive mapping of the complete fluorophore response, encompassing both shape and amplitude. This unique attribute empowers the utilization of a wide array of fluorophores or chromogens, offering flexibility without the necessity for meticulous filter selection.

SPECTRAL UNMIXING IS KEY FOR ACCURATE QUANTITATION OF FLUOROPHORE SIGNALS

Upon obtaining imagery through either whole-slide or field-based multispectral imaging (MSI), the contributions of individual fluorescence spectra from each fluorophore are separated using spectral analysis algorithms in the unmixing process. The end result is a series of images, each depicting the abundance of a specific fluorophore within the overall composite image.

The significance of MSI and the subsequent unmixing process is particularly evident when attempting to quantify these distinct signals. Traditional quantification methods, especially conventional fluorescent detection without unmixing, grapple with considerable challenges. In such systems, the signal from each filter band is typically assigned to one component without accounting for potential spectral overlap or varying autofluorescence across the field of view. This conventional approach can indeed quantify a limited number (1-4) of spectrally distinct fluorophores with moderate accuracy. However, when dealing with scenarios where fluorophore spectra overlap or autofluorescence varies across the sample, these conventional systems fall short in providing accurate and reliable quantification. The power of MSI and its advanced analytical algorithms lies in surmounting these hurdles, unlocking a new level of precision and fidelity in fluorescence signal quantification.

Spectral unmixing on the PhenoImager HT system boosts signal clarity

The PhenoImager HT system's proprietary MSI and spectral unmixing technology precisely characterizes the spectral signature of each fluorophore. This precision paves the way for exceptionally accurate spectral unmixing and quantitation while effectively eliminating autofluorescence (Figure 2).

The cornerstone of reliable unmixing lies in the PhenoImager HT system's unmixing library. This repository contains precise representations of each fluorophore's emission spectrum, and the autofluorescence spectrum derived from an unstained sample. Leveraging patented methods for spectral unmixing, the system clearly separates fluorophore signals, enabling users to assign the real contribution of each fluorophore to each pixel in an image with increased signal-to-noise ratios (Figure 3).
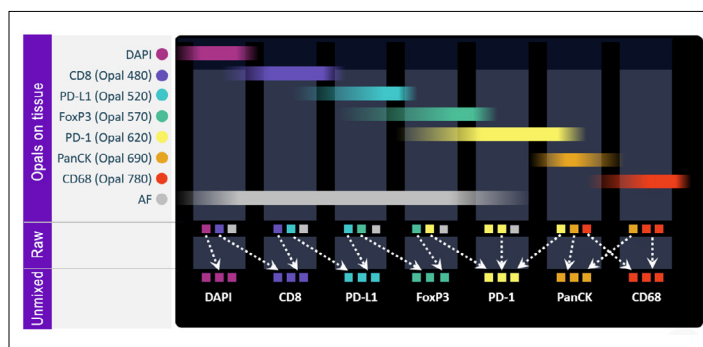

FIGURE 2. How spectral unmixing works on the PhenoImager HT system. Example shows a 7-color, 6-plex immunofluorescence panel. Six markers are detected with 6 different Opal dyes, plus DAPI as a nuclear counterstain, each emitting fluorescence over a range of wavelengths shown (top). These ranges overlap, and, while conventional MSI systems might capture all 7 signals, they would not be able to accurately quantify these signals, due to spectral overlap/bleed-through and inability to accurately subtract autofluorescence signal. The "raw" panel (middle) shows 3 boxes for each emissions channel. These are emissions that are likely to overlap. The arrows represent the process of spectral unmixing facilitated by proprietary software (bottom) whereby the emissions are reassigned to the correct channel.
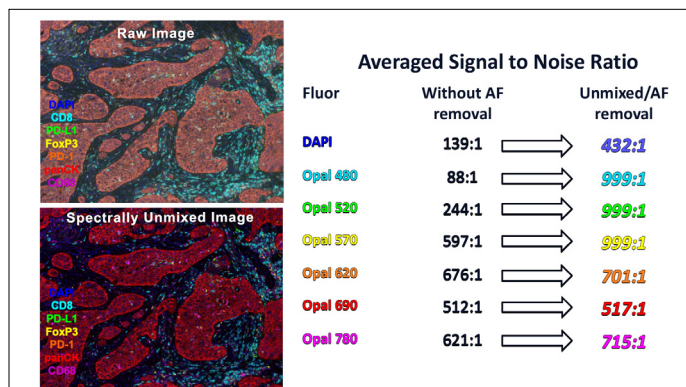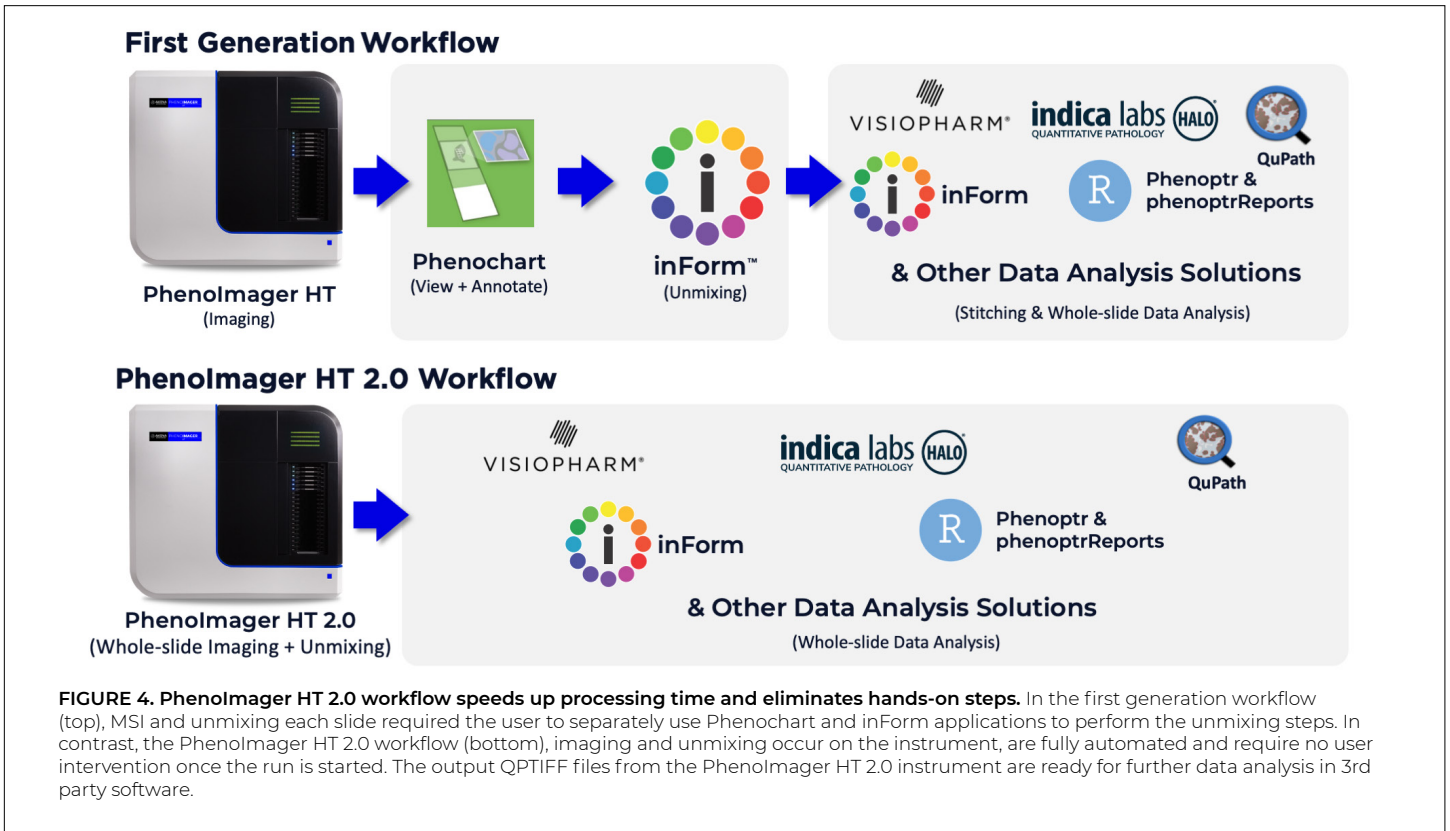

Averaged Signal to Noise Ratio		
Fluor	**Without AF removal**	**Unmixed/AF removal**
DAPI	139:1	432:1
Opal 480	88:1	999:1
Opal 520	244:1	999:1
Opal 570	597:1	999:1
Opal 620	676:1	701:1
Opal 690	512:1	517:1
Opal 780	621:1	715:1

FIGURE 3. Unmixing improves signal-to-noise ratios for fluorophore signal quantitation. A seven-color image was acquired on the PhenoImager HT system using a Lung Cancer 6-plex panel. Compared to the raw image, the spectrally unmixed image shows higher signal-to-noise ratios across a range of wavelengths, enabling accurate quantitation (AF - autofluorescence).

FIGURE 4. PhenoImager HT 2.0 workflow speeds up processing time and eliminates hands-on steps. In the first generation workflow (top), MSI and unmixing each slide required the user to separately use Phenochart and inForm applications to perform the unmixing steps. In contrast, the PhenoImager HT 2.0 workflow (bottom), imaging and unmixing occur on the instrument, are fully automated and require no user intervention once the run is started. The output QPTIFF files from the PhenoImager HT 2.0 instrument are ready for further data analysis in 3rd party software.

Streamlining spectral unmixing with PhenoImager HT 2.0 integration

In earlier workflows for whole slide imaging and spectral unmixing, users initially obtained raw scans using the PhenoImager HT instrument. Subsequently, these raw images were annotated to indicate regions of interest (ROI) for spectral unmixing, which was then performed by inForm™ software. The resulting individual unmixed ROI tiles could be analyzed individually or re-stitched for additional whole slide analysis. This process of creating a seamlessly stitched, unmixed whole-slide image for spatial analysis required users to manually perform several steps, resulting in significant processing time (Figure 4, top).

With the PhenoImager HT 2.0 instrument software upgrade, this entire workflow has been automated on the PhenoImager HT instrument (Figure 4, bottom). The PhenoImager HT 2.0 software offers users the flexibility to load an unmixing library, enabling automated spectral unmixing immediately after each scan. This functionality operates concurrently with the acquisition of subsequent scans, seamlessly integrating the parallel processing of scanning and spectral unmixing. This integration adds only an additional 8 minutes, equivalent to the time required to unmix the last acquired scan, to most standard scanning runs (20X, 15mm x 15mm).

ENABLING AUTOMATED UNMIXING WITH THE PHENOIMAGER HT 2.0 WORKFLOW

This new automated unmixing workflow includes loading an unmixing library into the PhenoImager HT 2.0 software. An unmixing library is a collection of library spectra for

each fluorescent component present in the sample (typically, Opal dyes and tissue autofluorescence). This unmixing library is created in inForm software utilizing well-established spectral extraction methodologies carried over from the first-generation workflow.

The initial step of enabling the automated spectral unmixing workflow with HT 2.0 involves the user selecting between 'synthetic' spectra for the fluorescent dyes (which are modeled theoretically) or spectra that were measured from their own samples and instrument using inForm's patented spectral extraction. Algorithm (see Figure 5).[3] Opting for measured spectra enhances unmixing accuracy.
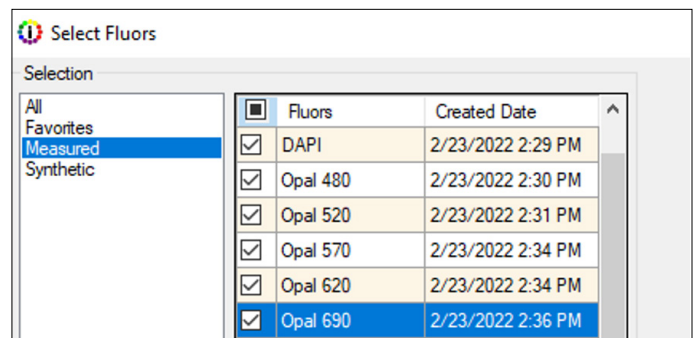

FIGURE 5. Selection of synthetic or measured spectra.

Next, the user proceeds to load a representative scan of an unstained slide, to be used to extract an autofluorescence library spectrum using the AF tool in inForm (see Figure 6). The autofluorescence slide scan should have the same imaging bands as the scans to be acquired in unmixed format, and the tissue type should be representative of the experimental samples.

Following the successful extraction of a reference autofluorescence spectrum from the loaded scan, the user exports the collection of spectra as an unmixing library file to be loaded in the PhenoImager HT 2.0 software (Figure 7).
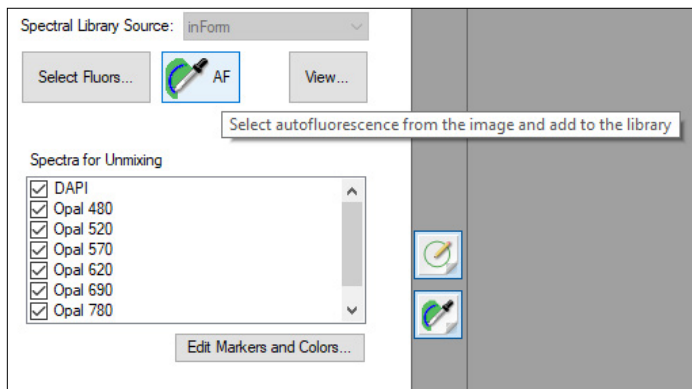

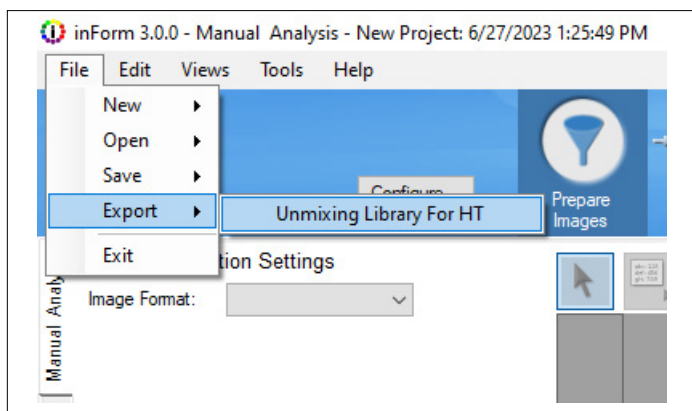**FIGURE 6. Extracting autofluorescence library spectrum**.

FIGURE 7. Exporting the unmixing library using the inForm software. The user chooses the menu item "Export" and selects "Unmixing Library For HT" to save an unmixing library based on the currently selected spectra and marker names.

Once the unmixing library has been saved, it can be selected during the scan protocol setup process on the PhenoImager HT 2.0 system user interface, so that the system can perform the on-instrument unmixing steps in parallel with imaging (Figure 8). Marker names can be edited in this step, so that the same unmixing library can be used for different MSI experiments.

PHENOIMAGER HT 2.0 SYSTEM DELIVERS ADDITIONAL IMPROVEMENTS TO SENSITIVITY, DATA QUALITY, AND FLEXIBILITY

Improved data reproducibility and accuracy through enhanced focus algorithm: The system now applies a larger Z- search range toward the interior of the slide to accommodate variability in mounting media thickness. Furthermore, the improved algorithm also reduces the impact of failed focus points near the edge of tissues and improves error handling.

Advances in Sample Detection: By minimizing background effects in the fluorescence overview scan, the system now features more sensitive tissue detection, including improved detection of multiple tissue pieces that may vary in brightness.

Flexibility with three data format options: During initial scan protocol setup on the PhenoImager HT 2.0 system, users can now choose one of three image format options, two of which are new additions. All three formats are compatible with Phenochart 2.0, inForm 3.0, as well as select, widely used third-party software such as QuPath, Visiopharm and Indica Labs HALO. The PhenoImager HT 2.0 user guide contains documentation on these formats so that individual users can also develop their own analysis routines.
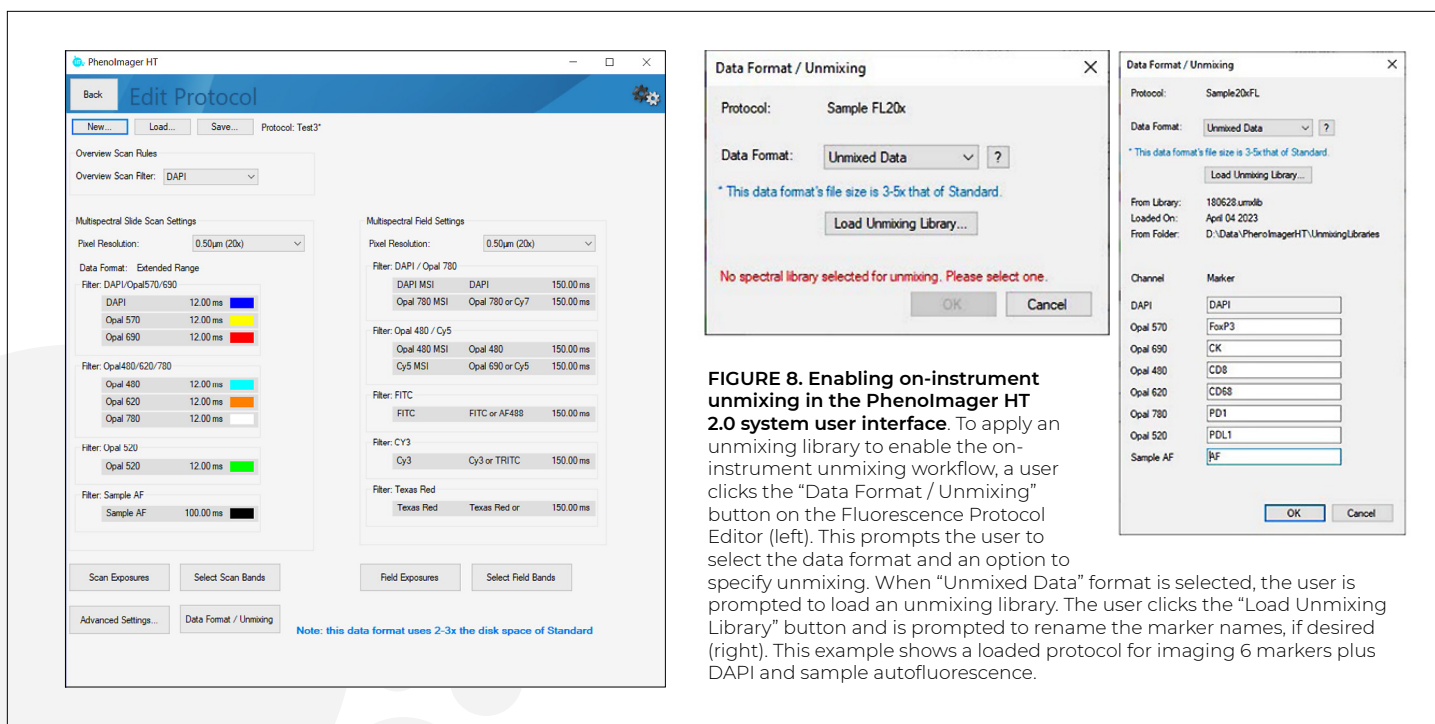

FIGURE 8. Enabling on-instrument unmixing in the PhenoImager HT 2.0 system user interface. To apply an unmixing library to enable the on-instrument unmixing workflow, a user clicks the "Data Format / Unmixing" button on the Fluorescence Protocol Editor (left). This prompts the user to select the data format and an option to specify unmixing. When "Unmixed Data" format is selected, the user is prompted to load an unmixing library. The user clicks the "Load Unmixing Library" button and is prompted to rename the marker names, if desired (right). This example shows a loaded protocol for imaging 6 markers plus DAPI and sample autofluorescence.

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CUSTOMER SPOTLIGHT

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PhenoImager HT for high-throughput studies
Our team has actively supported nearly 60 different clinical trials, spanning 19 different cancers, over the past 18 months. The Akoya Biosciences PhenoImager HT instrument works extremely well not only for high-throughput analysis, but also bespoke analysis, offering insights specific to the clinical questions posed by a trial. Using this instrument has enabled us to quickly get to the bottom of what the clinical trial is trying to achieve, while helping eliminate concerns on whether the technology used suits a particular sample.

Impact of PhenoImager HT 2.0
Along with high throughput multispectral imaging, this latest iteration (HT2.0) can perform real-time spectral unmixing during data capture from the patient sample. This presents a significant advantage as large datasets – comprising high-resolution images of up to hundreds of gigabytes in size – can be quickly saved, transferred, and analysed. As a result, our lab's analysis time has been cut by half, without compromising on accuracy, ensuring that the research output remains of a high standard. Overall, this next generation of Akoya's technology has been crucial in expediting our analysis process.

The three data formats are as follows – note that the two new formats generate file sizes that are larger than the standard 8-bit QPTIFF file:

- **Standard 8-bit QPTIFF:** 8-bit; can be unmixed separately in the inForm application. This is the same format as in the original PhenoImager HT workflow.

- **NEW DEFAULT FORMAT--Extended Range QPTIFF:** 12-bit data in a 16-bit QPTIFF format. This new option yields a file format that can be unmixed in inForm software. This format has a higher dynamic range and is intended to provide ~3-fold protection against saturation

for unexpectedly bright samples (Figure 9). This feature significantly improves the initial fluorescent scan's success rate, saving users time by eliminating the need for rescans due to saturation.

- **NEW--Unmixed QPTIFF:** This format results from the application of a user-selected unmixing library to unmix an Extended Range QPTIFF. Users can choose the unmixing library before scanning, allowing for automatic unmixing immediately after scanning. Additionally, the system preserves a backup copy of the Extended Range QPTIFF, ensuring accessibility to raw data if needed.
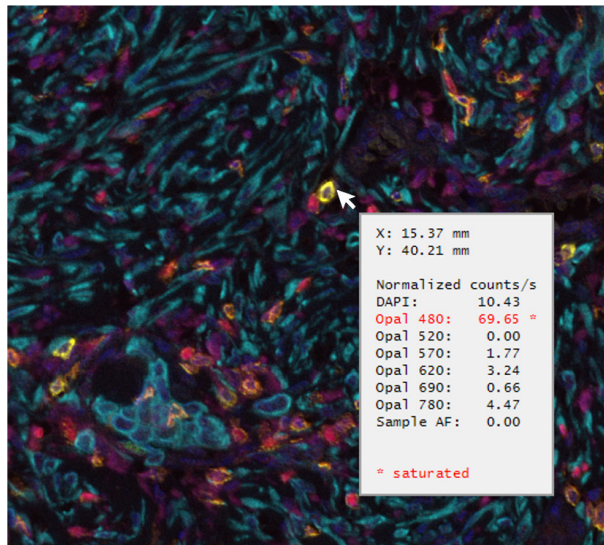
Standard 8-bit scan
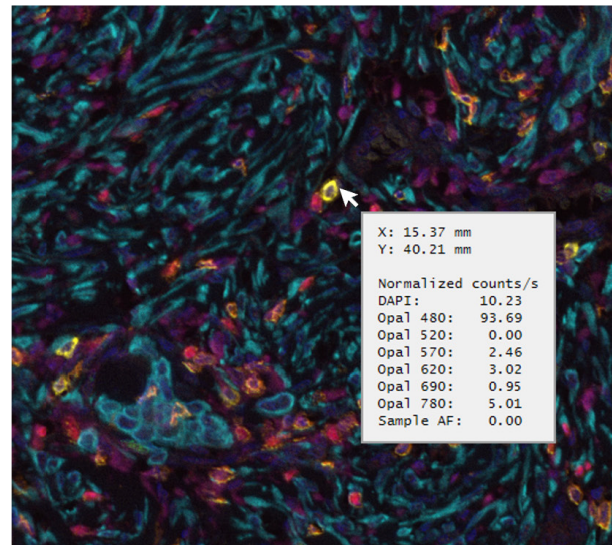
Extended Range 16-bit scan

FIGURE 9. Extended range data format provides higher dynamic range to reduce the likelihood of saturation. The 8-bit scan (left) shows a saturated spot with Opal 480 (indicated by cursor arrow). In contrast, there is no saturation at the same spot in the corresponding extended range 16-bit scan (right).

Comparative Validation of the PhenoImager HT 2.0 Workflow

To validate the performance of the faster, automated PhenoImager HT 2.0 workflow, we conducted an evaluation using replicate slides generated from serial sections of the same sample. This comparative analysis involved the new streamlined PhenoImager HT 2.0 workflow and the first generation PhenoImager HT workflow, allowing for a comprehensive assessment of performance. In this study, two replicate slides each from serial sections of an ovarian tumor microarray were stained with a 6-plex panel with markers for CD8 (Opal 480), PD-L1 (Opal 520), FoxP3 (Opal 570), PD-1 (Opal 620), PanCK (Opal 690), and CD68 (Opal 780). Subsequently, both sets of slides underwent

scanning and unmixing utilizing the respective workflows. The resulting data was analyzed using inForm software to compute critical parameters such as tumor area (refer to Figure 10a and 10b) and cell density per marker (as illustrated in Figure 10c and 10d) per core.
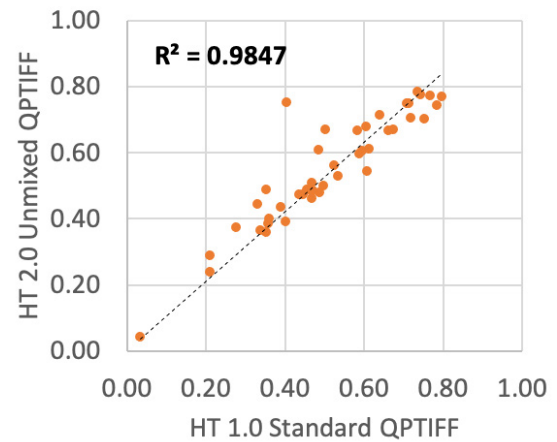
In each comparative analysis, the results per core exhibited high correlation. This validation underscores the accuracy and reliability of the PhenoImager HT 2.0 workflow, reaffirming its potential as an advanced and efficient analytical tool for accurate spatial phenotyping and spatial signature development.
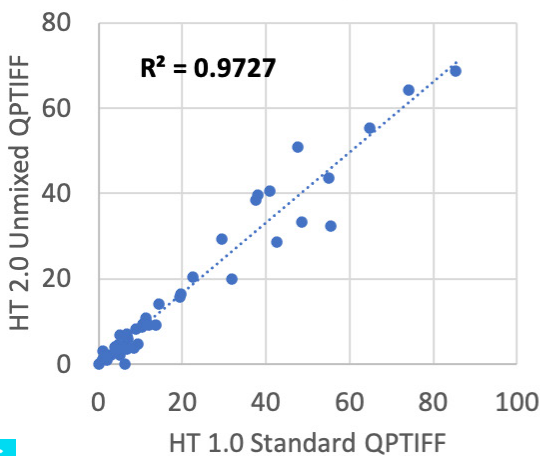
APPLICATION (VALIDATION DATASET)
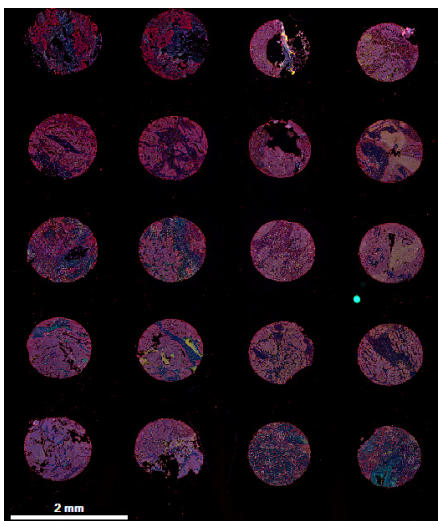
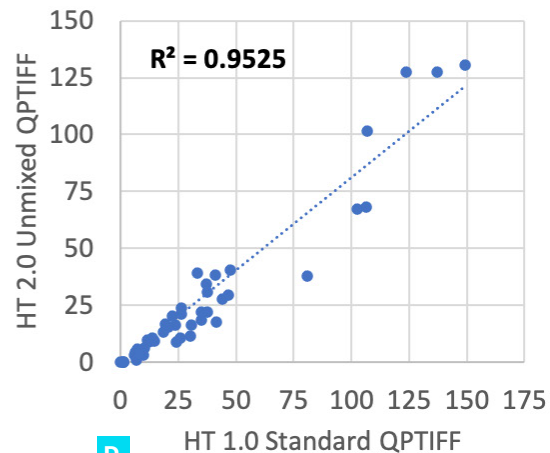**FIGURE 10. Calculated tumor area (b) and cell density per marker (c, d) results were highly correlated between slides scanned and unmixed using PhenoImager HT 2.0 vs first generation workflows.** For the PhenoImager HT first generation workflow, the standard (8-bit) QPTIFF image file was acquired with Vectra Polaris 1.0.13 software and unmixed and analyzed with inForm 2.6.0. For the PhenoImager HT 2.0 workflow, the unmixed QPTIFF file was generated by PhenoImager HT 2.0 software. The PhenoImager HT 2.0 condition was analyzed with inForm 3.0. Data included two serial section scans. Spatial phenotyping using inForm software was performed using separately trained algorithms using the same standard operating procedure.

Conclusion

The PhenoImager HT 2.0 system represents a highly scalable cutting edge workflow, delivering up to 5-fold in time savings and increased accuracy through automation and imaging improvements. This upgrade adds even more value to the PhenoImager HT system, the first spatial biology platform to meet reproducibility requirements for clinical applications.[4] With the introduction of the PhenoImager HT 2.0 system upgrade, this mIF workflow gains a substantial enhancement, empowering its capability to effectively support multisite trials and streamline clinical research laboratory processes by making the development of spatial signatures faster and easier than ever.

References

1. Mansfield JR. Multispectral imaging: a review of its technical aspects and applications in anatomic pathology. Vet Pathol. 2014 Jan;51(1):185-210.

2. Stack EC, Wang C, Roman KA, Hoyt CC. Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. Methods. 2014 Nov;70(1):46-58.

3. Covered by US Patents 7,555,155; 7,953,264; 8,280,140; 8,639,043; 10,126,242; 11,307,142 and patents pending.

4. Taube JM, Roman K, Engle EL, Wang C, Ballesteros-Merino C, Jensen SM, McGuire J, Jiang M, Coltharp C, Remeniuk B, Wistuba I, Locke D, Parra ER, Fox BA, Rimm DL, Hoyt C. Multi-institutional TSA-amplified Multiplexed Immunofluorescence Reproducibility Evaluation (MITRE) Study. J Immunother Cancer. 2021 Jul;9(7):e002197.