Hyperspectral imaging
Nuance FX and EX imaging systems

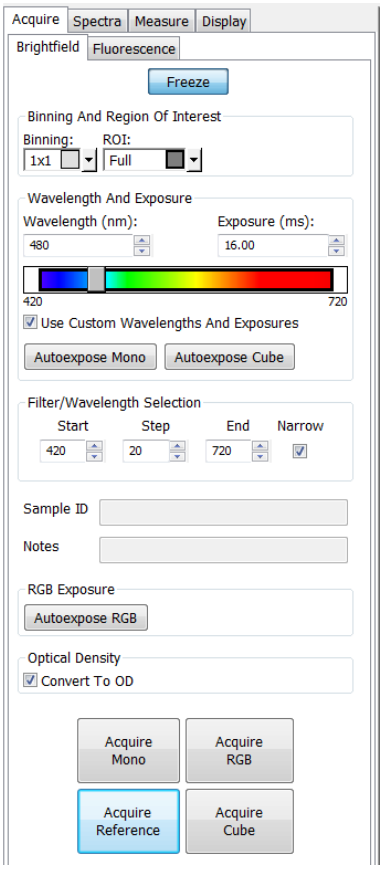
Introduction

The Nuance Multispectral Imaging System is an advanced imaging platform that facilitates the separation and analysis of overlapping signals in biological tissues. It is designed for use in complex imaging tasks where multiple fluorophores or chromogens are present within a sample. This system is particularly useful in fields like histology, pathology, immunohistochemistry, and molecular biology, where the accurate quantification of multiple markers is critical for understanding cellular processes.

In biological research, the study of complex systems often requires the simultaneous detection of several molecular markers, each associated with different biological targets. Traditional imaging systems often struggle with cross-talk between these signals, especially when markers are co-localized. This cross-talk can significantly reduce image clarity, leading to inaccurate quantification and misinterpretation of results.

The Nuance Multispectral Imaging System addresses these challenges through multispectral imaging and spectral unmixing technologies. By capturing images across a range of wavelengths, the system is able to separate signals based on their unique spectral profiles, even when these signals are overlapping spatially. This results in highly accurate visualization and quantification of each individual marker.

Acquiring Brightfield



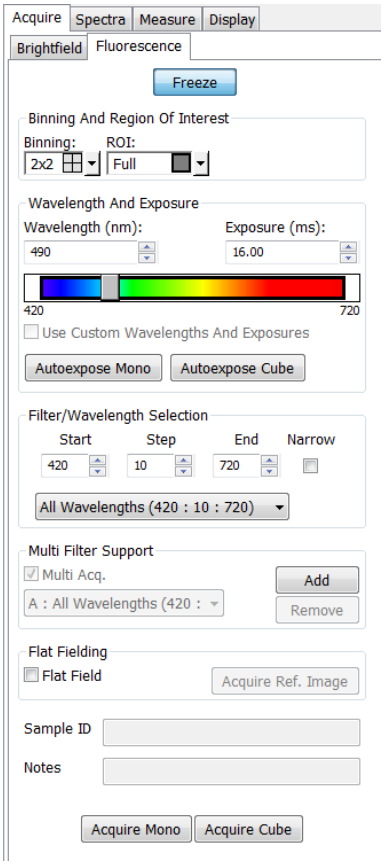
1. Freeze / live: Ensure light is diverted to camera and click “live” to display image on screen.
2. Acquire Reference: If converting to OD, move slide to empty field (Empty field should be free of tissue, cells or debris) and acquire reference frame.
3. Autoexpose and Acquire:
   a) Monochrome acquisition
      - Select wavelength of interest
      - Autoexpose Mono
      - Acquire Mono
   b) RGB (Red-Green-Blue) acquisition
      - Autoexpose RGB
      - Acquire RGB
   c) Image cube acquisition
      - Select wavelength range
      - Autoexpose Cube
      - Acquire Cube
4. Binning: Combines multiple pixels into a single pixel (reduces final resolution but increases acquisition speed).
5. Region of Interest: Select all or part of the field to acquire.
6. Sample ID / Notes: Include additional sample notes (acquisition date and parameters are automatically recorded).

Spectral Libraries

In order to ensure accurate unmixing of component signals from image cubes it is necessary to generate pure spectral libraries from single stained samples. Bring one unstained slide (for fluorescence only). For

BF and fluorescence also bring one single-stained slide for each stain or fluorophore used on experimental samples. When acquiring image cubes for each of these, use the same filter and wavelength selections as will be used for the experimental samples.

Acquire Fluorescence



1. Freeze / live: Ensure light is diverted to camera and click “live” to display image on screen.
2. Acquire Reference: To correct for uneven field illumination, move slide to area of uniform illumination and acquire reference frame. Flat Fielding makes it possible for the Nuance software to create better, more evenly bright mono images and image cubes in fluorescence. If you acquire fluorescence images or cubes without Flat Fielding, you may notice that the outer edges of acquired images are slightly darker than the rest of the image. Before acquiring a mono image or spectral cube, acquire a reference - or background - image. This requires a fluorescence sample slide that is evenly bright across the field of view. Once you have taken a reference image, it will be saved with the current Nuance protocol, if you save the protocol.
3. Autoexpose and Acquire:
    a) Monochrome acquisition
        - Select wavelength of interest
        - Autoexpose Mono
        - Acquire Mono
    b) Image cube acquisition
        - Select wavelength range
        - Add multiple filters (optional)
        - Autoexpose Cube
        - Acquire Cube
4. Binning: Combines multiple pixels into a single pixel (reduces final resolution but increases acquisition speed).
5. Region of Interest: Select all or part of the field to acquire.
6. Sample ID / Notes: Include additional sample notes (acquisition date and parameters are automatically recorded).

Manual Compute Spectra

Identifying Mixed Spectra

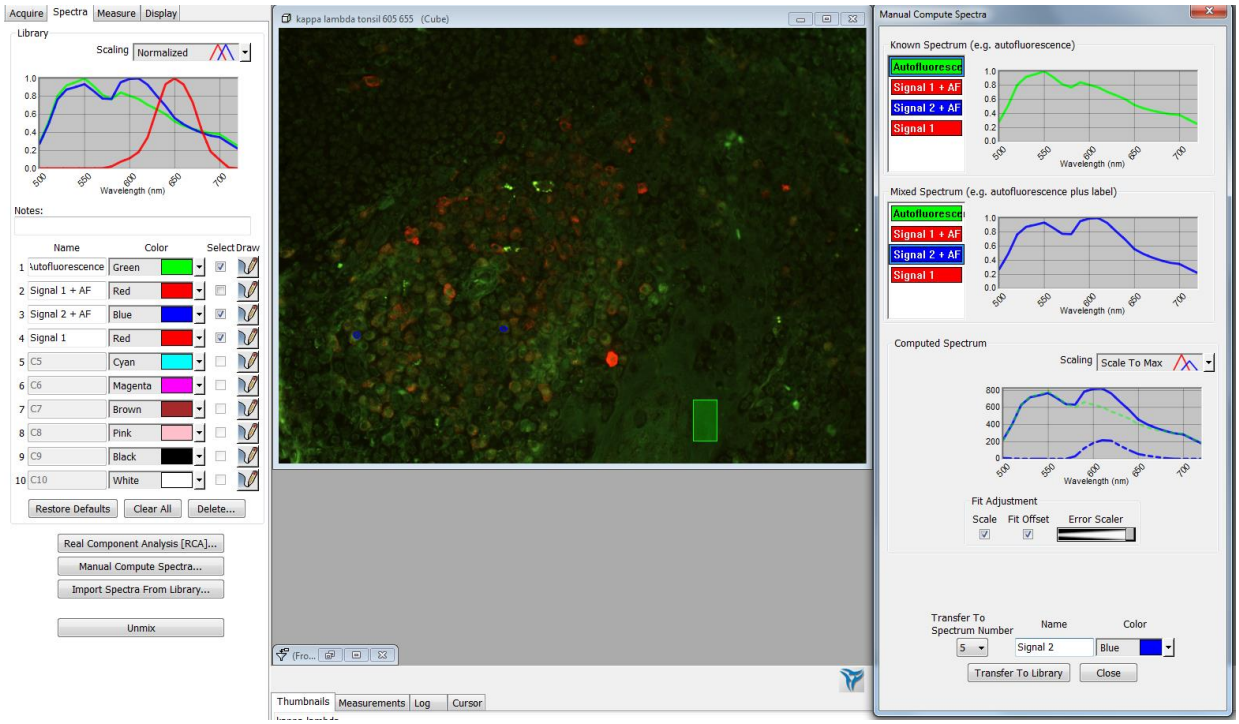
1. Click Draw then select a region on the image corresponding to the desired signal.
2. Name the selected spectrum (e.g. autofluorescence, DAPI, etc.)

Notes: Repeat steps 1-2 to identify each component signal, selecting a new number, color and label each time. Ideally each mixed spectrum added to the spectral library from single stained samples (autofluorescent signal would come from unstained sample).

Generating Pure Spectral Library

3. Click Manual compute spectra
    a) Select known spectrum Known signal is the autofluorescence generated in steps 1 and 2 from an unstained sample or background region and will be subtracted from the mixed signal.
    b) Select Mixed spectrum: Mixed spectra added to the spectral library during steps 1 and 2 using single stained samples or by carefully selecting regions positive for only one of the fluorescent stains.
    c) Computed Spectrum
    i. Error scaling: set graph as “scale to max” and adjust scale to line up mixed and known spectral curves in regions outside signal area.
    ii. Use “fit offset” to enhance weak signals
    d) Transfer to library, repeat until all signals have been separated.

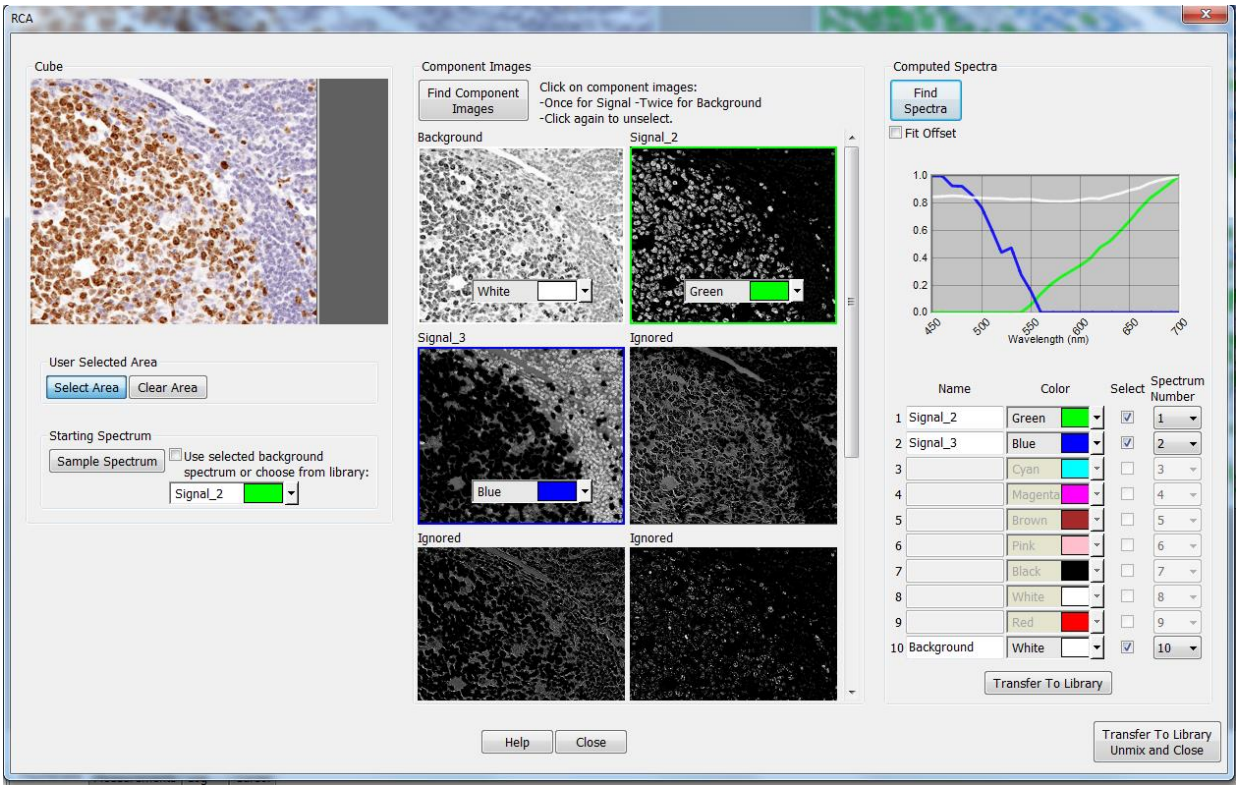
4. Select signals and click Unmix: Once spectral library is generated all image cubes can be unmixed.



Real Component Analysis

After acquisition click Spectra tab and then click Real Component Analysis (RCA) to begin

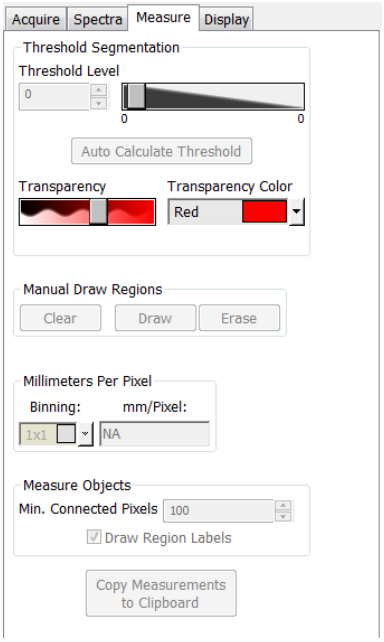
1. Starting Spectrum (Optional): If background signal has already been added to library (manually or by importing from spectral library), select this signal as starting spectrum.



2. Click Find component images: After clicking, find images below with clear signal (white indicates bright signal), then assign label (1 click labels as “Signal_#”, 2 clicks labels as “Background”, 3 clicks resets to “ignored”)

3. Click Find spectra: Click “Fit offset” for weak signals
4. Signal Labels:
    a. Select each unique signal (check box)
    b. Give each a name (e.g. autofluorescence, DAPI, etc.)
    c. Change the color if necessary (this will change how the spectral graphs look but will not change the image appearance)
    d. Choose the spectrum number to which you would like to transfer each spectrum.
5. Click Transfer to library, Unmix and Close: This will transfer selected spectra to the indicated spectrum number in your active library (overwriting any spectra currently occupying these slots).

Threshold Segmentation Analysis



1. Threshold level: adjust the slider to set the minimum signal intensity for pixels to be classified as positive.
2. Minimum connected pixels: sets smallest size of positive region of interest (ROI).
3. Manual segmentation: Can draw/copy/move/000e ROIs.
4. Threshold mask: set the color and transparency of the mask used to indicated positive ROIs.
5. Set Scale: Indicated mm/pixels if known (use fiduciary to determine if unknown).

For each ROI the following are reported:

- Avg. signal or Avg. signal/sec
- Total signal or Total signal/sec
- Max signal
- Area (pixels and mm^2)
- Major/minor axis
- (x, y) coordinates of ROI center

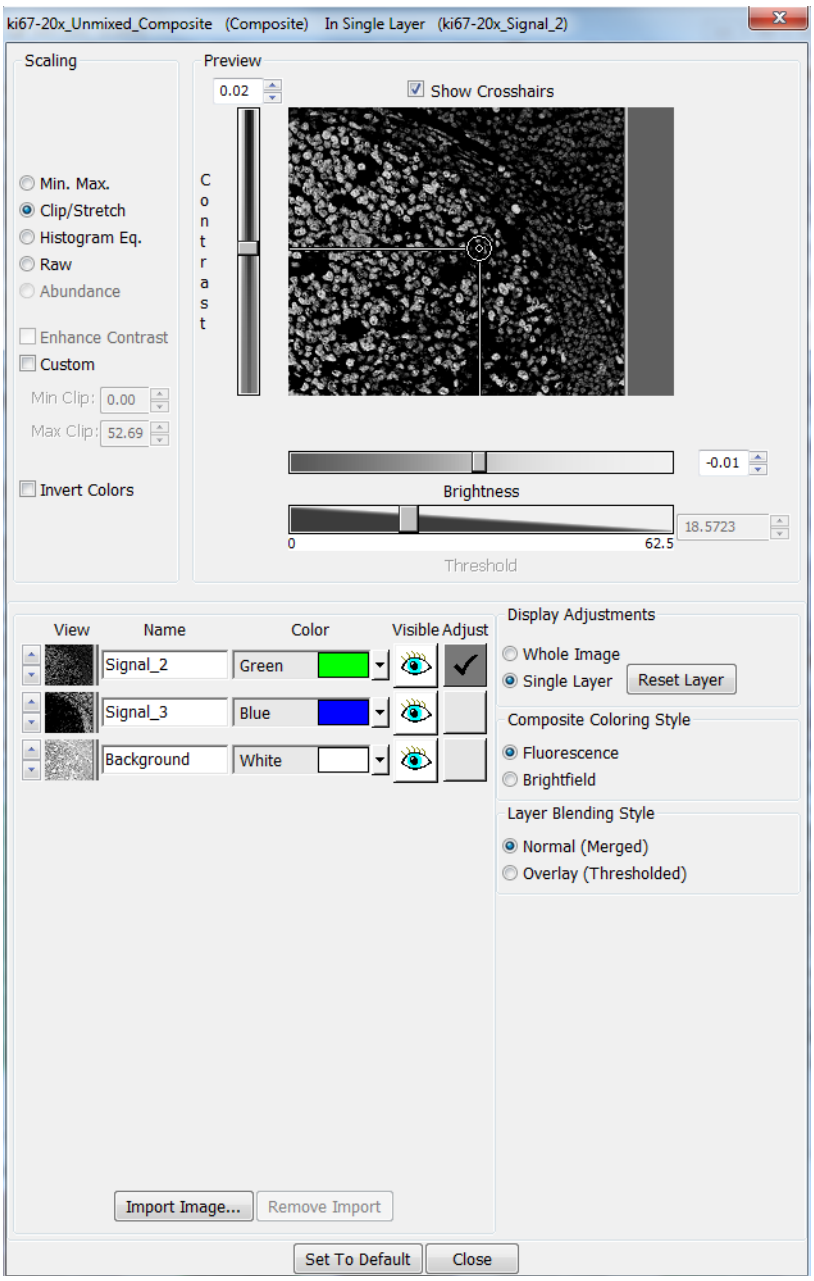
Data can be copied/pasted/exported to:

- Microsoft Excel
- GraphPad Prism
- Other spreadsheet software

Display Control Utility

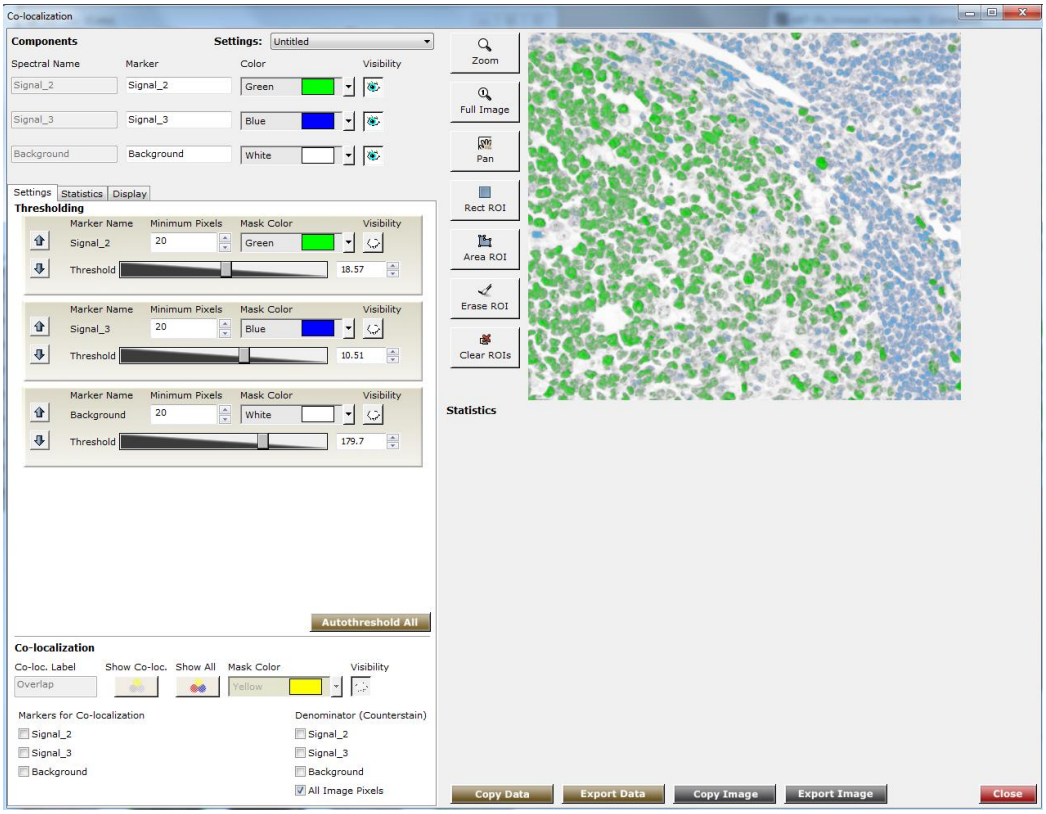
1. Select the correct Composite Coloring Style.
2. Display adjustments:
    a. Select whether to adjust the whole image or single layer.
    b. For single layer adjustments check the layer to be adjusted. You can also select which layers are visible here.
3. Scaling:
    a. Min-Max (minimum pixel intensity - 0; maximum pixel intensity - 255)
    b. Clip stretch (0.01% lowest pixel intensity - 0; 0.01% highest pixel intensity - 255)
    c. Custom clip stretch (can change % of lowest/highest intensity pixels stretched to 0/255)
    d. Abundance scales all pixels to maximum in composite image (preserves relative abundance)
4. Brightness/contrast tool: Drag crosshairs to simultaneously adjust brightness and contrast. Or adjust sliders separately.

For bright field ensure that background color is set to white. By default, layer blending is set to *Merged*, in which component images are mixed based on intensity. To prioritize certain signals, switch to *Overlay* mode. Here component threshold masks are layered on top of each other, and you can choose to highlight a select signal by placing it on top.



Colocalization Analysis

1. Settings: If you have saved previous colocalization setting these can be loaded here. File must contain same # of spectra as the unmixed composite image to be analyzed.
2. ROI selection: Use the ROI draw tools to highlight part of the unmixed composite image for colocalization analysis. By default, the entire image is selected.
3. Thresholding: For each component signal set the minimum signal intensity (Threshold), set the minimum object size (Minimum Pixels), and set the Mask color and Visibility (optional).
4. Colocalization:
    a. Identify colocalization marker(s).
    b. Set denominator/ROI (i.e. full image or region of image).
5. Statistics: Reports % colocalization, pixel counts, and component stats in given ROI.
    a. Export data to Microsoft Excel/GraphPad Prism/Other spreadsheet software.
6. Save Settings for future use.


Co-localization
Components
Settings:
Untitled
Spectral Name
Marker
Color
Visibility
Signal_2
Signal_2
Green
Signal_3
Signal_3
Blue
Background
Background
White
Settings
Statistics
Display
Thresholding
Marker Name
Minimum Pixels
Mask Color
Visibility
Signal_2
20
Green
Threshold
18.57
Signal_3
20
Blue
10.51
Background
20
White
179.7
Autothreshold All
Co-localization
Co-loc. Label
Show Co-loc.
Show All
Mask Color
Visibility
Overlap
Yellow
Markers for Co-localization
Signal_2
Signal_3
Background
Denominator (Counterstain)
Signal_2
Signal_3
Background
All Image Pixels
Zoom
Full Image
Pan
Rect ROI
Area ROI
Erase ROI
Clear ROIs
Statistics
Copy Data
Export Data
Copy Image
Export Image
Close