Imaging Facility
Biotechnology Resource Center
Cornell University
BRC_Imaging@cornell.edu

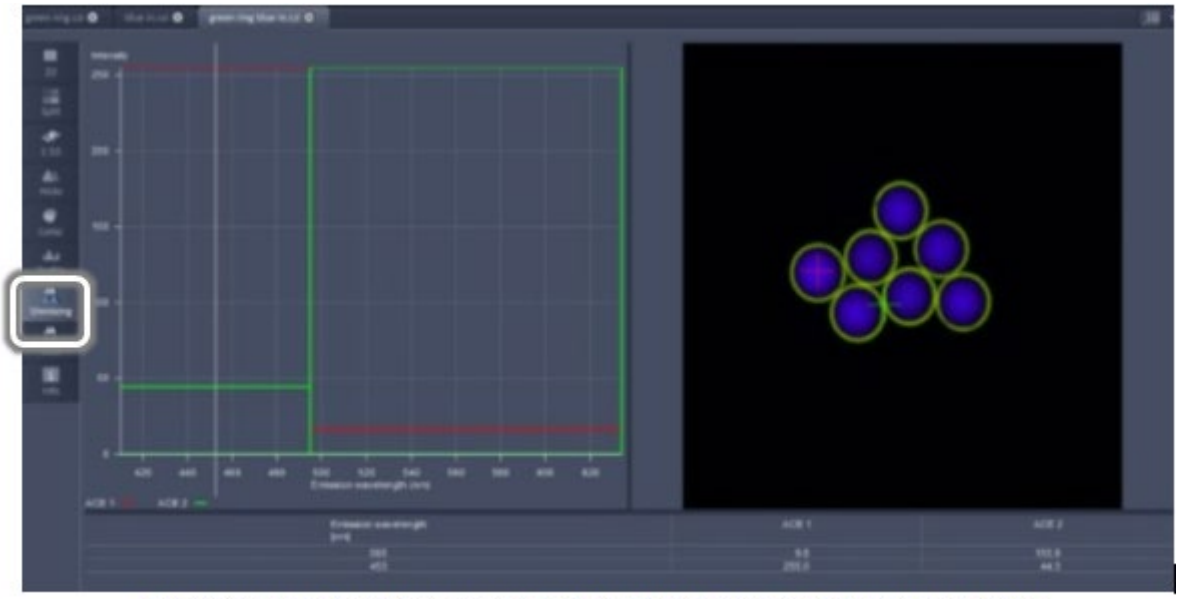
Linear Unmixing

Linear Unmixing analysis may be used to identify individual fluorophores within a complex mixture in a double-/triple-labeled specimen.

1. Under Light Path, click on the “Lambda Mode” button.
2. Select Spectral Collection range (emission wavelength).
3. Define spectral resolution. Use 9.7 nm or higher for simultaneous acquisition.
4. Select lasers with appropriate dichroics (MBS).
5. Do a Live Scan.
6. Use the *Gallery View* of the lambda image to balance detector gain, offset, scan speed, and laser power. Eliminate saturated and underexposed pixels in the image (use Range Indicator to view).
7. Acquire a reference spectrum (choose one method below):

   Automatic Component Extraction (ACE) - if your sample image has pixels that are not completely colocalized:

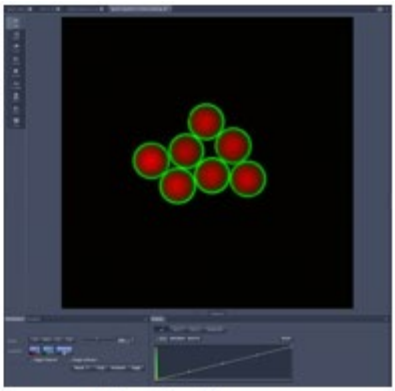
   a. After acquiring an image, go to the Unmixing tab on the left side of the image container.



Unmixing window shows spectral profile of dyes and 2-channel image

b. Select the Auto find/ACE button in the Unmixing window below the image; select number of components.
c. Identify background ROI using selection tools if needed.
d. Click *Linear Unmixing* button to unmix image.
e. Unmixed image is created in a new image tab.

Unmixing tab showing number of dyes to unmix using ACE


Unmixed image

Individual measurements with biological controls - for samples with a high degree of spectral emission/excitation overlap and colocalization (e.g. GFP and YFP)

a. Acquire an image for each of your control samples.
b. Click on Unmixing tab on the left side of the image container.
c. Select an ROI shape from the Unmixing control tab below image.
d. Click “Save to Spectra Database” to save reference spectra.
e. Repeat until all reference spectra have been collected.
f. Image your sample.
g. Load reference spectra.
h. Identify background ROI using selection tools if needed.
i. Unmix image.


Saving individual spectra from multi-channel image into Spectra Database