# Spectral unmixing using Living Image software.

This guide will walk you through the steps of manually entering your sequences for the spectral unmixing procedure. The Living Image® (v4.3.1) software version includes an Autoexposure setting and an Imaging Wizard. For questions on how to use these two features please see the respective quick reference guide associated with these workflows. You can also find information pertaining to the use of these features in the Spectral Unmixing Wizard Setup reference guide. These features are designed to make setting up your sequences as easy as possible and we highly recommend that you take advantage of them when performing these steps.

1. If you are not using autoexposure, first determine the optimal imaging time for your subject using the specific excitation and emission filters for your particular fluorophore.

2. Adjust exposure time so as not to saturate – below 60,000 counts but to maintain more than 600 counts.

3. Click Sequence Setup and enter desired sequence in the Sequence Editor. It is recommended that the sequence should include filters which will excite the fluorophores specifically and then a background sequence.
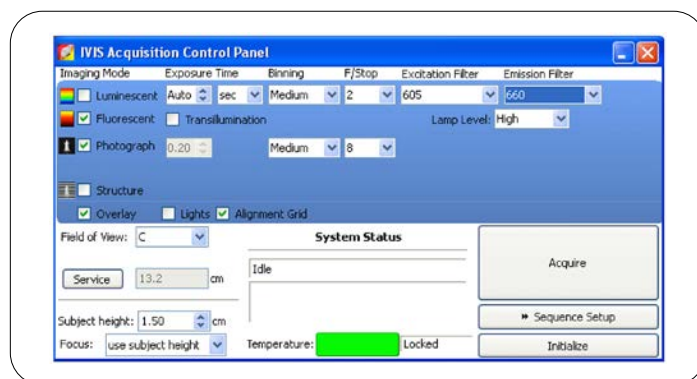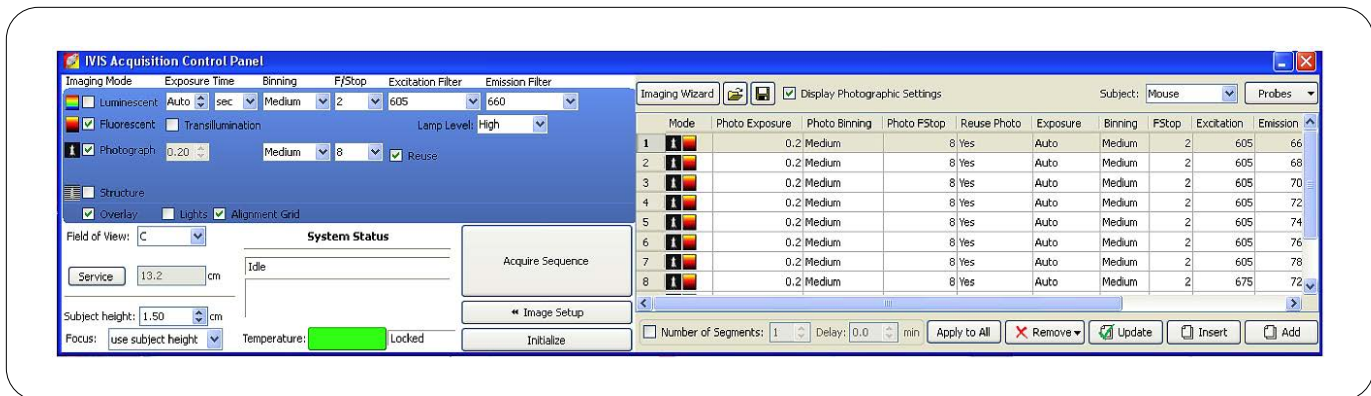
Let's take as an example, AlexoFluor 680 Peak
Ex. 679 nm/ Em. 702 nm

- Specific Sequence: Excitation 675 nm/
  Emission 720-780 nm (Remember we need 40-50 nm of
  separation between the excitation and first
  emission filter.)

- Background Sequence: Excitation 605 nm/
  Emission 660- 780 nm (We choose to excite at 605 nm in
  order to reveal the background autofluorescence before
  the AlexoFluor peaks, this allows the unmixing algorithms
  to more specifically recognize two separate sources,
  and unmixing is more efficient.)

Note: In the above example, we planned an emission scan
meaning that we are going to scan emission filters linearly
in order to discern the peak. However, you can also use
two emission filters coupled with multiple excitation filters
as well for an excitation scan.

Note: Make sure that the band gap between the excitation
and emission filters is sufficiently large so that the
excitation light does not leak through the emission filter
where it can be detected by the CCD. You should have
between 45-50 nm separating the two. This is the reason
why we did not include the pairing of Excitation 675 nm/
Emission 700 nm.

4. In the control panel, specify the settings for the
   fluorescence image (exposure time, binning, F/stop,
   excitation filter, emission filter).

Note: Autoexposure settings are available in LI4.3.1
and more information on this feature is available in the
Auto- Exposure Tech Note 2. It is recommended that you use
this feature when possible.

Note: Spectral unmixing can be performed with epi- or
transillumination modes of fluorescence. For spectral
unmixing in combination with transillumination, simply pick
your transillumination points and use with the desired

spectral unmixing sequence. See the Transillumination
Sequence Setup Quick Reference Guide for more information
on setting transillumination sequences.

5. Click Acquire Sequence.

Spectral unmixing:

1. Load the image sequence and switch units to radiant
   efficiency.

2. In the Analyze tab, select the images that you want to
   include in the analysis. Remember to check that each
   image remains within the 600-60,000 counts window. If
   there are saturated images or low intensity images,
   please deselect them and do not use them for analysis.

3. Select the methodology for Spectral Unmixing from the
   dropdown menu: Library, Guided, Automatic or Manual
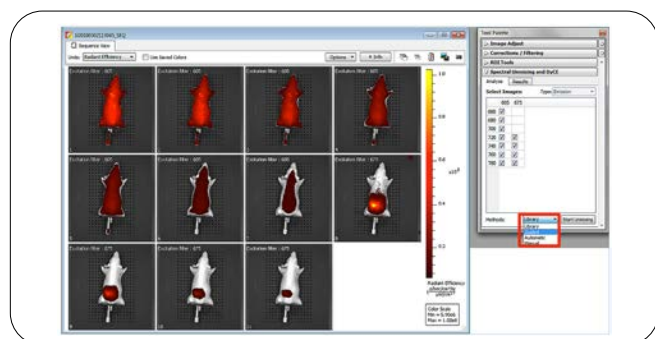
Special Note: A second subject with no fluorophore
present (negative control) is highly recommended to be
used as an autofluorescence only marker. Fluorescent
injectables will disseminate throughout the body and finding
an area with no fluorophore present will be impossible.
Also, autofluorescence varies slightly from one region of the
body to the next. Marking on a negative control animal in the
same anatomical position as the fluorophore positive animal
is a good idea.

Note: Filter selections MUST stay the same during longitudinal studies or any time two images will be compared. Using different filters during the spectral unmixing process will alter the data processed and will lead to a different result. Sequence acquisitions can be saved and reloaded through the Control Panel or Imaging Wizard, and it is recommended to use this feature when using the Spectral Unmixing process whenever possible to ensure experimental variation is kept to a minimum.

Proposed Usage: The new spectral unmixing modes of Manual and Guided give the user more control over what areas of the field of view will be used to define components of a mixed spectra. Automatic mode returns as a similar setup to the Living Image 4.2 Spectral Unmixing, where the software uses a pre-defined spectral profile for each component told that is in the acquisition.

Further, Manual and Guided can be used to create a Spectral Library file, a saved parameters file that can later be applied to spectral unmixing acquistions which have identical acquisition setups (longitudinal studies) using the Library mode. This eliminates any possible error when comparing unmixed images from different subjects while also allowing for more controlled unmixing parameters to be created. It is recommended that the user establish a Spectral Library using negative and positive control animals simulating the depth and location expected for their fluorophore.

Automatic: This mode compares intensity changes throughout the field of view that have resulted from the filter scan against the spectral profile of fluorophores available from the software's built-in spectral library. These fluorophores are chosen via a dropdown menu and if not present, the 'unknown' option can be chosen. Up to 4 component signatures may be selected. If the number of spectral components is unknown, the Principle Component Analysis (PCA) option can be activated where a statistical breakdown of the spectra will be displayed and a proposed number of components given.
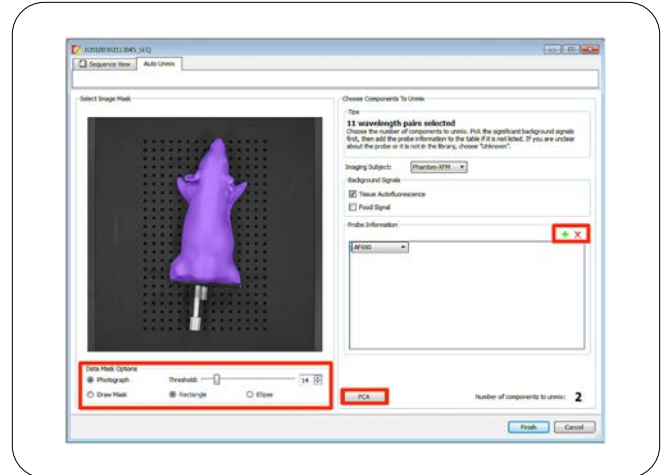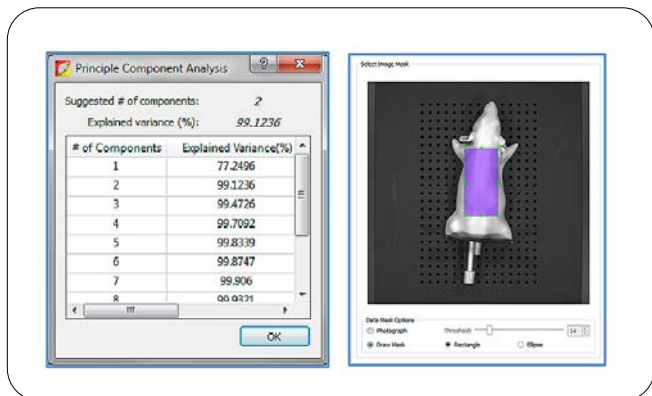

Once the correct number of fluorophores has been added to the Probe Information list via the green + button, threshold the subject from the stage by sliding the threshold bar. A pink color to be attributed to the subject, or you may use the Draw Mask option to draw a Rectangle or Ellipse around the area to be unmixed. Click Finish to proceed to the unmixed results.

Manual: The Manual spectral unmixing option gives the user full control to separate out every component in the acquisition through the use of spectral subtraction and negative controls. In this example, the red marked signal is actually AF680+autofluorescence while the green marked signal is autofluorescence only. Autofluorescence signal can be subtracted away from the AF680+autofluorescence component, leaving pure AF680 signal through a process called Compute Pure Spectrum. An overview of all sequence images, the ImageCube, will appear and gives a pseudocolor to all assumed separate signals in the field of view.
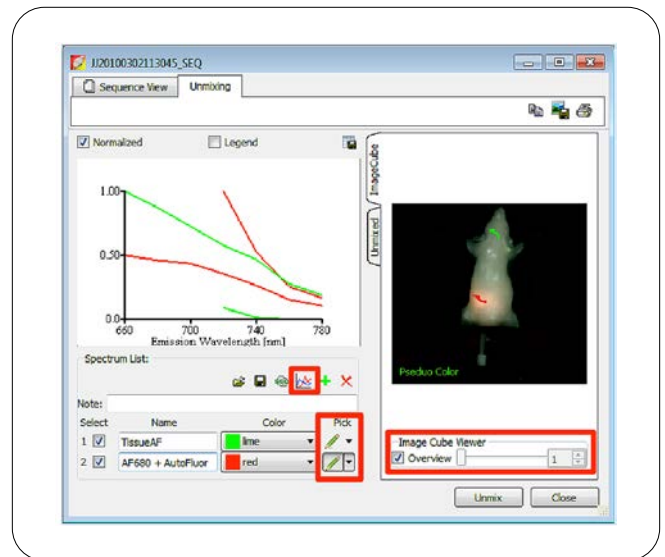
It may be helpful to uncheck the Overview button and scroll through the individual filter sets to find the location of the fluorophore signal. Use the Pen Tool to mark each component on the ImageCube. Once 2 known components have been marked (one will need to be background autofluorescence and is best to be from a negative control subject), launch the Compute Pure Spectrum interface by clicking the Graph icon. The Compute Pure Spectrum interface will allow you to define one marked spectra as a mixed spectrum and the second marked spectra as a known signature to be subtracted from the first.
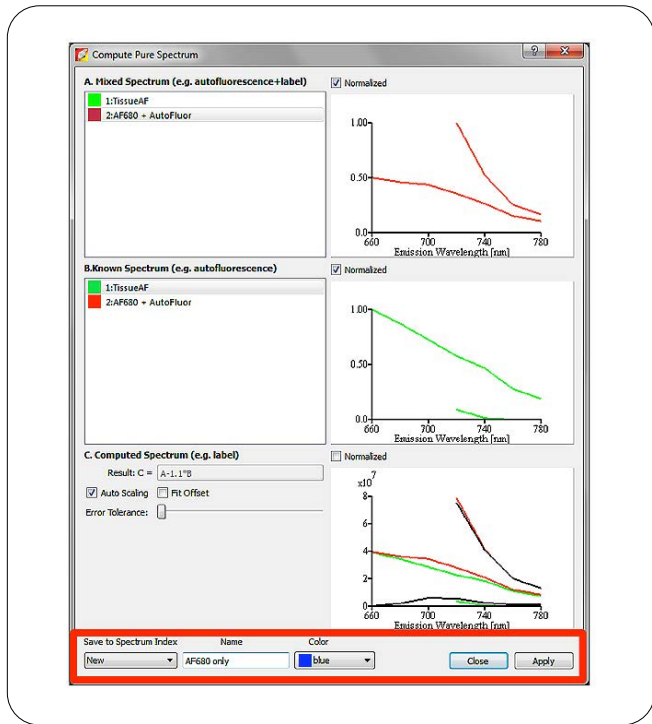

This will generate a 3rd component to the image, the AF680 only signal (colored here in blue). Additionally, previously saved Spectral Library components can be added in to the Unmixing Wizard by adding new component with the green + icon, choosing "Import" from the Pen Tool dropdown menu, and loading a Spectral Library file. This very powerful tool can be used to unmixed overlapping fluorophores with autofluorescence to yield very accurate unmixing results. See Library section for more information.
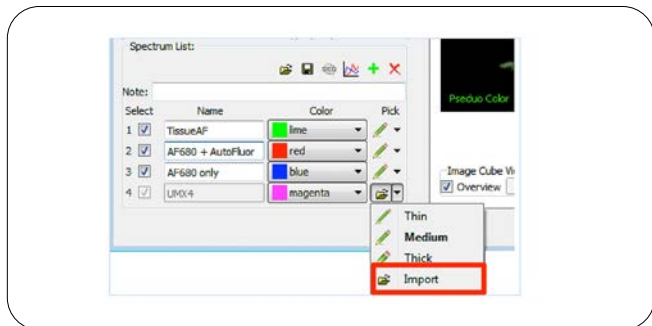

Once the AF680 only signal has been created through the Compute Pure Spectrum algorithm, it can be applied back to the original ImageCube to unmix AF680 as a completely separate component.

Make certain the correct components to be unmixed are marked with the check box, and click Unmix to retrieve the results. Once the unmixing has been verified, the components can then be saved as a Spectrum Library and applied to different imaging sessions which used an identical acquisition sequence.
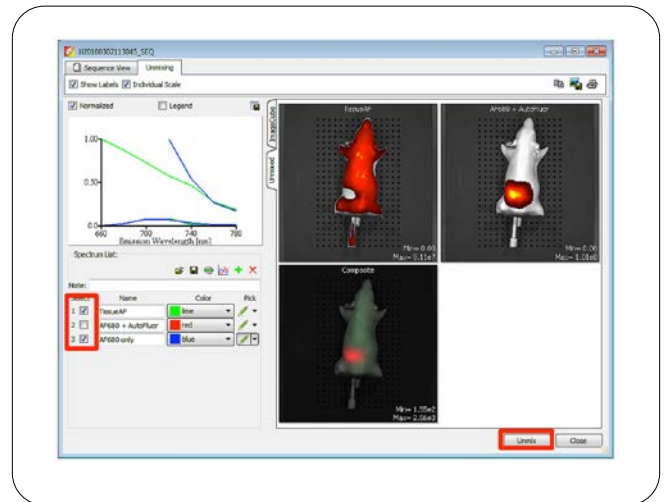
| Check box components to be unmixed
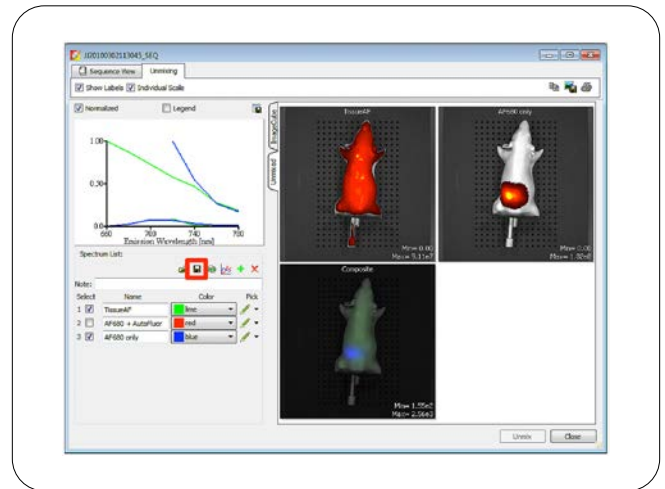
| Save unmixing parameters as a Spectral Library

Guided: Guided Spectral Unmixing assumes that you know where your fluorophore signals are originating from and that each fluorophore signal is mixed with only one background signal (e.g., fluorescent dye + autofluorescence). Guided option is primarily used for establishing Spectral Libraries with positive and negative control subjects, but can also be an unmixing tool for regionally-specific fluorescent signals (xenograft, orthotopic, etc).

To begin, select the Guided option from the dropdown menu and click Start Unmixing. Using the pen tool, mark the regions of interest for each component on the Spectrum List on the ImageCube, i.e., here we have drawn a red color over the AF680 + autofluorescence area and green color an area with only autofluorescence only background.

Once all fluorophores have been marked, click Next to view the unmixed results.
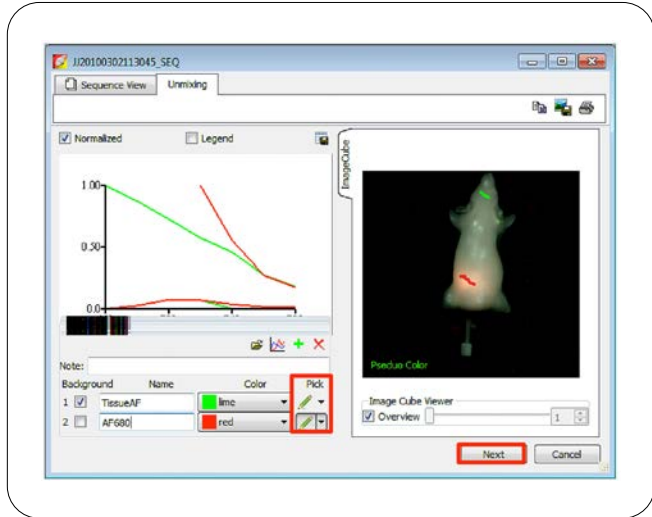
Library: The recommended protocol for Spectral Unmixing in longitudinal studies is to unmix fluorophores using a pre-established spectra profile, previously saved as a "Spectral Library" specific for one particular experiment (same filters, same subject orientation, etc). The Library spectral unmixing option will use a saved *.csv file to unmix the currently loaded image Sequence. If a Spectral Library does not already exist for your experiment, you can create one by using the Guided or Manual options with proper negative control and known fluorophore location in a positive control subject.

NOTE: Spectral Libraries can only be applied to sequences using the same filter combinations as were used during the creation of the Library.

If a Spectral Library does exist for the Sequence, begin unmixing by clicking Start Unmixing button. The Load Spectrum Library window will appear where your Spectral Library file can be applied to the Sequence. Check the "show qualified only" option to eliminate choices that do not pertain to the acquired sequence. Once the correct Spectral Library has been found, highlight the name in blue and click on "Apply". The sequence will automatically unmix using the methodology saved in the Spectrum Library file and results will be displayed.
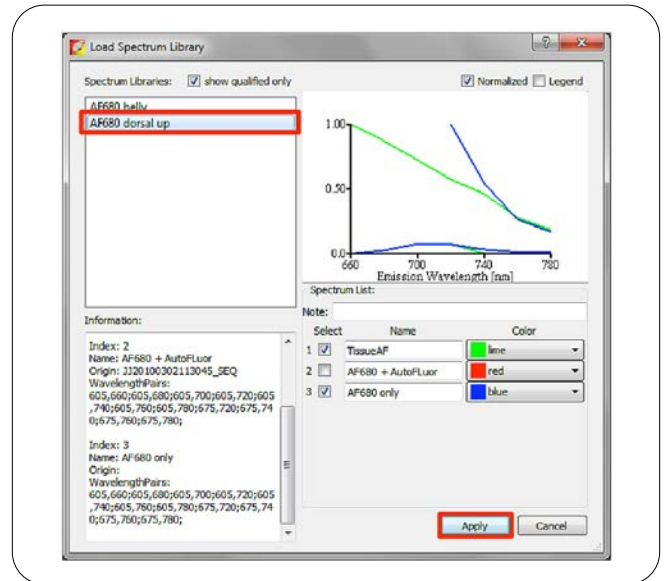
Results

The unmixed spectral images will appear as several Component images (one for each fluorophore unmixed) and a Composite image which will be color-coded for all fluorophores. Any image can be double-clicked to open in a separate window for analysis or image recording. Each image is auto scaled initially and each will have a label at the top center of the image. The Component images can be measured with Regions of Interest (ROIs) for quantification, while the Composite image will allow for alterations of the pseudocolor for a more appropriate presentation of the fluorophore source.

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