Spectral Unmixing: an *In Vivo* Case Study

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Introduction

Spectral unmixing has emerged as a pivotal technique in preclinical optical imaging, enabling the separation of overlapping spectral signals from multiple fluorophores or chromophores, improving quantification of molecular probes and reducing tissue autofluorescence interference in applications like tumor detection and physiological monitoring. This technique is particularly vital in modalities such as fluorescence imaging, where spectral overlaps from biological tissues complicates signal isolation. This white paper presents the process of spectral unmixing for an in vivo fluorescence imaging (FLI) case study, using a MILabs OI system and Imalytics Preclinical Software. For a detailed description of general spectral unmixing process and suggestions on how to setup a study, please refer to the MILabs technical note "**Spectral Unmixing with MILabs OI system**".

Data acquisition setup

The data used in this document to showcase the spectral unmixing process were kindly provided by the Small Animal Imaging Facility at the University of Alabama at Birmingham, supported in part by the Preclinical Imaging Shared Facility grant P30CA013148 and instrumentation grant S10 OD030465-01.

The goal of this case imaging session was to successfully perform spectral unmixing on several tumor-bearing mice with two fluorophores injected (AngioSense680 and IRDye800), taking into account autofluorescence.

5 mice were imaged together in a 5-mouse bed using the MILabs Optical Module on the MILabs Docking Station; the imaging and post-processing steps are the same for acquisitions carried out on the Main System. Each mouse had a tumor in the right mammary fat pad, and the mice were injected and placed as described in Table 1. No negative control mouse, without fluorophore injections, was included in this imaging set.

For a reliable spectral unmixing process, it is important to choose wisely the filters to be used during acquisition and use appropriate excitation/emission filter sets that cover the emission spectra of both fluorophores present plus the autofluorescence spectrum. For this reason,

Name	Position in the bed	Received probes
M1	Far left	AngioSense680 alone (*used as positive control*)
M2	Mid-left	Antibody-IRDye800 alone (*used as positive control*)
M3	Center	combination AngioSense680 + antibody-IRDye800
M4	Mid-right	combination AngioSense680 + antibody-IRDye800
M5	Far right	combination AngioSense680 + antibody-IRDye800

Table 1. Fluorescence settings in the acquisition interface. Filter selection is indicated in red.

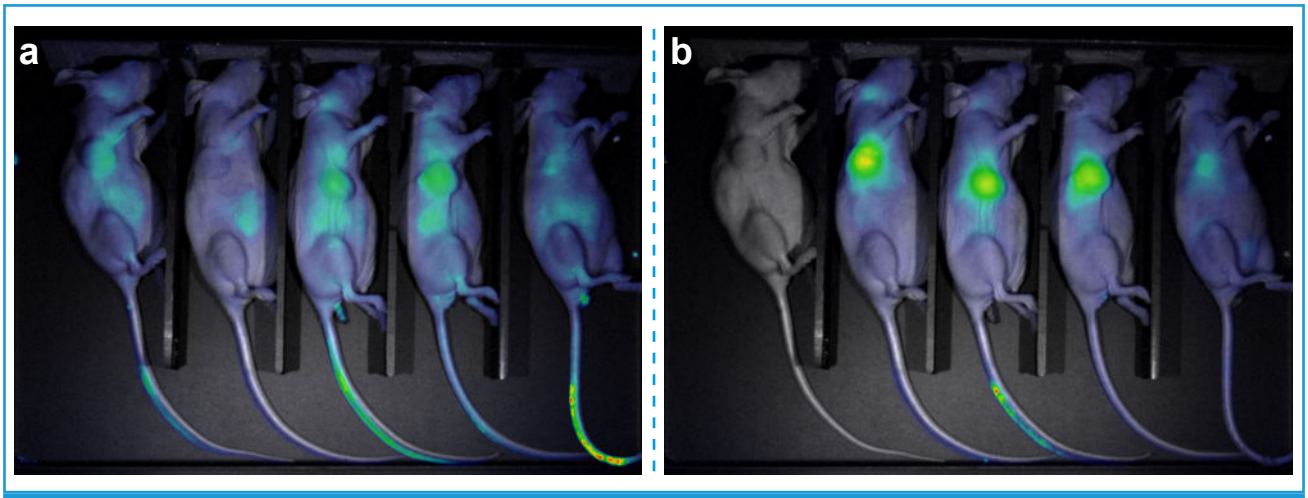

Figure 1. Acquired overlayed FLI images using (a) channel 1 (625/661), and (b) channel 2 (769/832).

images were acquired in the following channels (excitation/emission bandpass filters used):

- 625-15/661-20 (best fitting AngioSense680) (Fig. 1a)
- 769-41/832-37 (best fitting IRDye800) (Fig. 1b)
- 625-15/832-37
- 640-14/775-46

The two numbers representing each filter are the central wavelength and the bandwidth in nanometers.

Please be aware that, even if in this case they were not present, it is always strongly recommended to include *negative controls* (e.g. animals without any fluorescent probes) to allow quantification and compensation for autofluorescence and other tissue intrinsic signals, improving the reliability and interpretability of unmixed results. Without strong negative controls, unmixing algorithms risk over-attributing autofluorescence or nonspecific signals to the target probe, potentially inflating apparent biodistribution or contrast, negatively affecting the spectral unmixing process.

Spectral unmixing process

The entire spectral unmixing process for acquired multi-channel images was carried out using Imalytics Preclinical software. This functionality can be found in the software menu under the tab "*Unmixing*" (for version 3.0.X) or "*Modules*\ *Unmixing*" (for versions 3.1.X). Two Imalytics tutorials ("Spectral unmixing" and "Spectral unmixing MILabs") are available for consultation within the software [1-2].

As first step, AngioSense680, IRDye800 and animal autofluorescence spectra were extracted. To do this, we started by opening the photographic reference "Photo.tiff" as Underlay, followed by the FLI images of all the channels mentioned in the previous paragraph as Overlay (four channels). Then different regions, containing clear positive optical signal for each fluorophore (and autofluorescence), were selected (Fig. 2).

In this example, having 2 fluorophores co-present and considering autofluorescence, the steps needed to correctly set up the spectra extraction are the following:

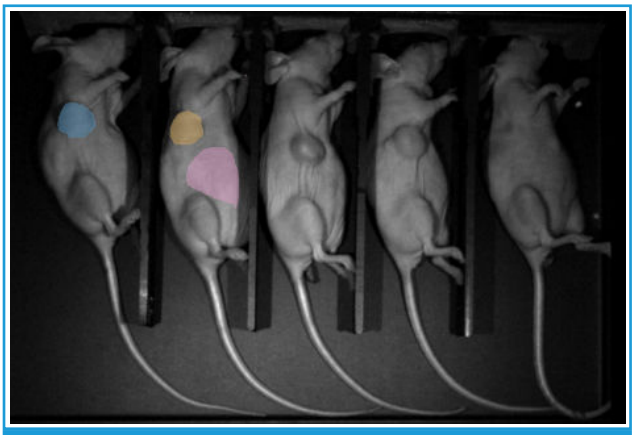

Figure 2. Class segmentation for spectra extraction: blue for AngioSense680, orange for IRDye800 and pink for autofluorescence).

- create one class for AngioSense680 and select a ROI on the first animal (injected only with that fluorophore) in an area with a positive signal (e.g. tumor)
- similarly, create another class for IRDye800 and select a ROI on the second animal in an area with positive signal (e.g. tumor)
- create another class and select respective ROI to extract spectra for animal auto-fluorescence
- the Overlay layer with the four channels and the class segmentation can be both saved (*File* > *Overlay* > *Save overlay as* , and *File* > *Segmentation* > *Save segmentation*…)

After the ROI selection, the spectra was extracted by simply going to the "*Unmixing*" menu tab and selecting "*Save class spectra*" (Fig. 3). Then the mean intensity of each class in each channel was automatically computed; the result was saved as a *.spectra* file.

Once completed the spectra extraction, to perform the spectral unmixing, we navigated again to *Unmixing* > *Spectral unmixing* (Fig. 3) and selected the file with the extracted spectra previously saved.

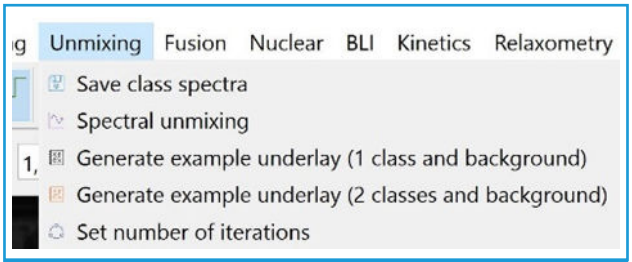

Figure 3. Imalytics unmixing menu.

Results and quantification analysis

The unmixed results obtained for the first two channels, the single fluorophores, are shown in Figure 4. These unmixed images can be visualized individually to assess the distribution of each fluorophore and can be overlaid to provide insight into their spatial colocalization and potential interaction.

ROIs were drawn on each mouse to include the tumor area for analysis. Signal total intensity values in each ROI were calculated via the software menu "*Statistics* > *Class statistics (underlay/overlay)*" (Fig. 5).

Results of the unmixed signals from the two fluorophores are reported for each animal in Figure 6.

References

[1] Imalytics White Paper Series: Spectral unmixing (multispectral images). 2025
[2] Imalytics White Paper Series: Spectral unmixing MILabs (multispectral images). 2025

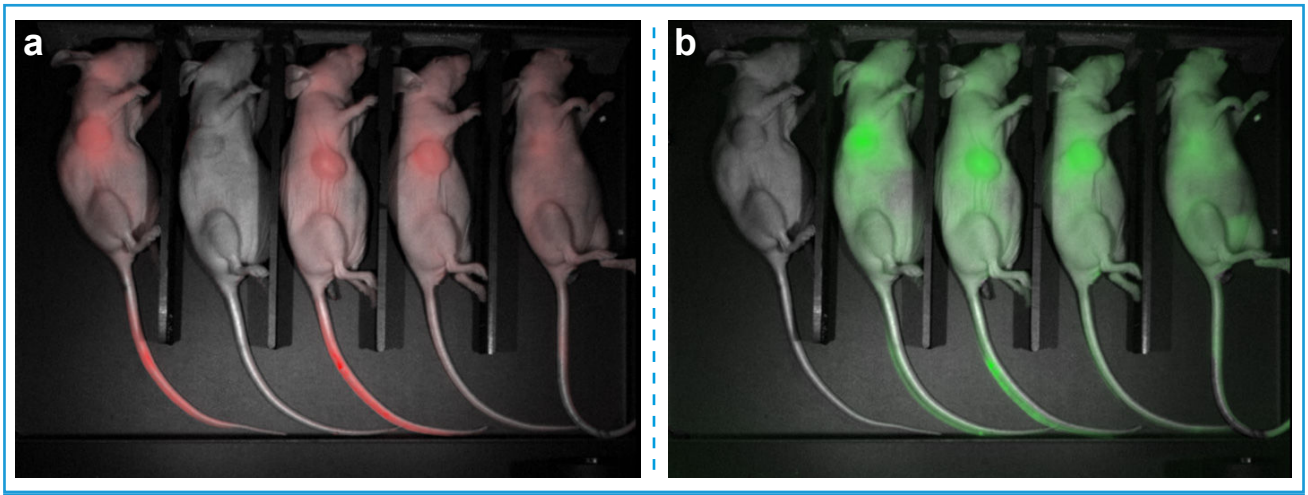

Figure 4. (a) Unmixed AngioSense680 channel, (b) unmixed IRDye800 channel.

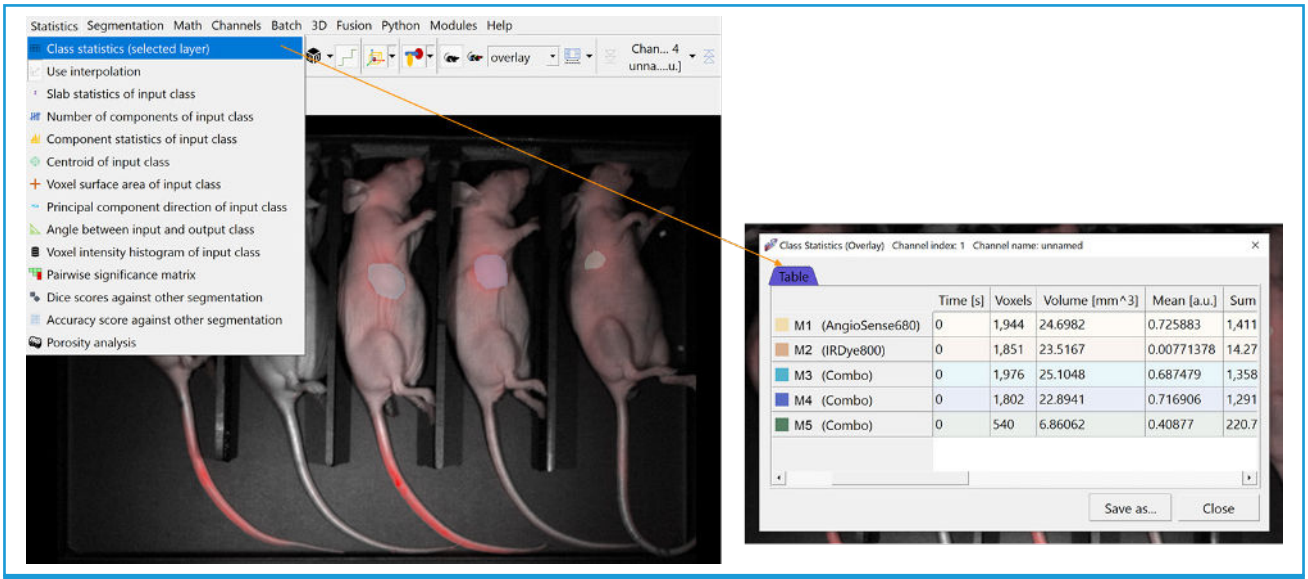

Figure 5. ROI quantification analysis.

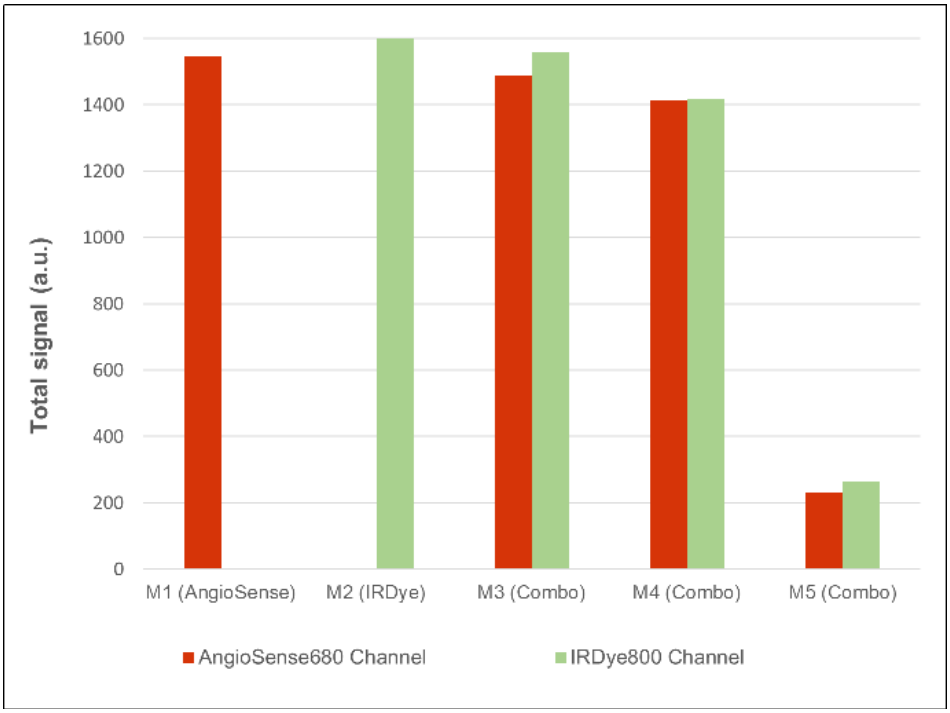

Figure 6. Total intensity results.