TECHNICAL NOTE

Brief overview guide on spectral unmixing.

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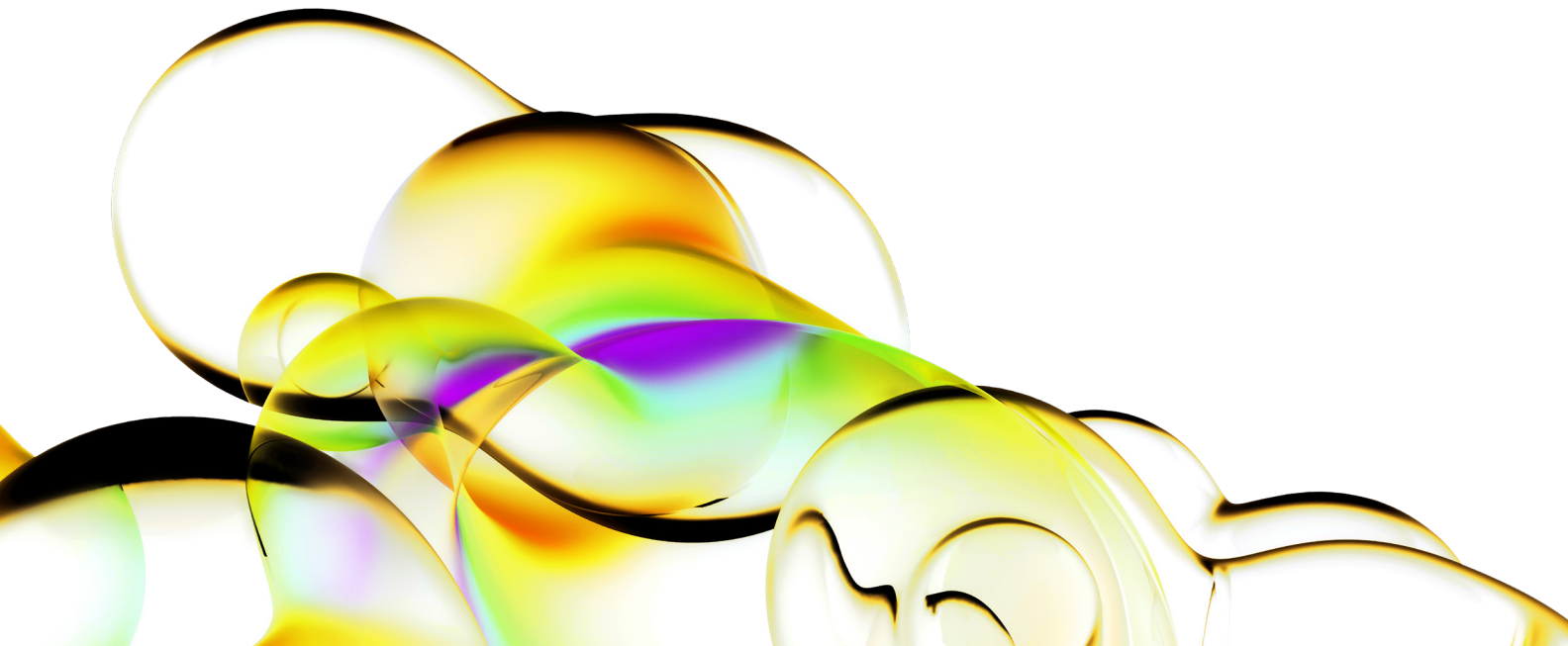
Spectral Unmixing (SPUM)

In fluorescence imaging, the presence of multiple spectrally-overlapping fluorophores in the subject can present challenges for interpreting imaging results. The Living Image® software SPUM tool is an effective way to isolate and quantify each individual fluorescent source for separate visualization and quantification. To perform SPUM, the user needs to have a proper study design to determine (A) a working acquisition sequence and to generate (B) a working spectral library for future SPUM analysis (Figure 1). The user can first take advantage of a small, but representative pilot study for this purpose and then apply the same acquisition sequence and library to the future, larger scale longitudinal studies. For acquisition sequence setup, the user can take advantage of the built-in Imaging Wizard which guides you through acquisition filter selection. For library generation, Living Image provides four methods for source isolation: Guided, Manual, Library and Automatic.

In this guide, we use the Guided method which is recommended for most cases.



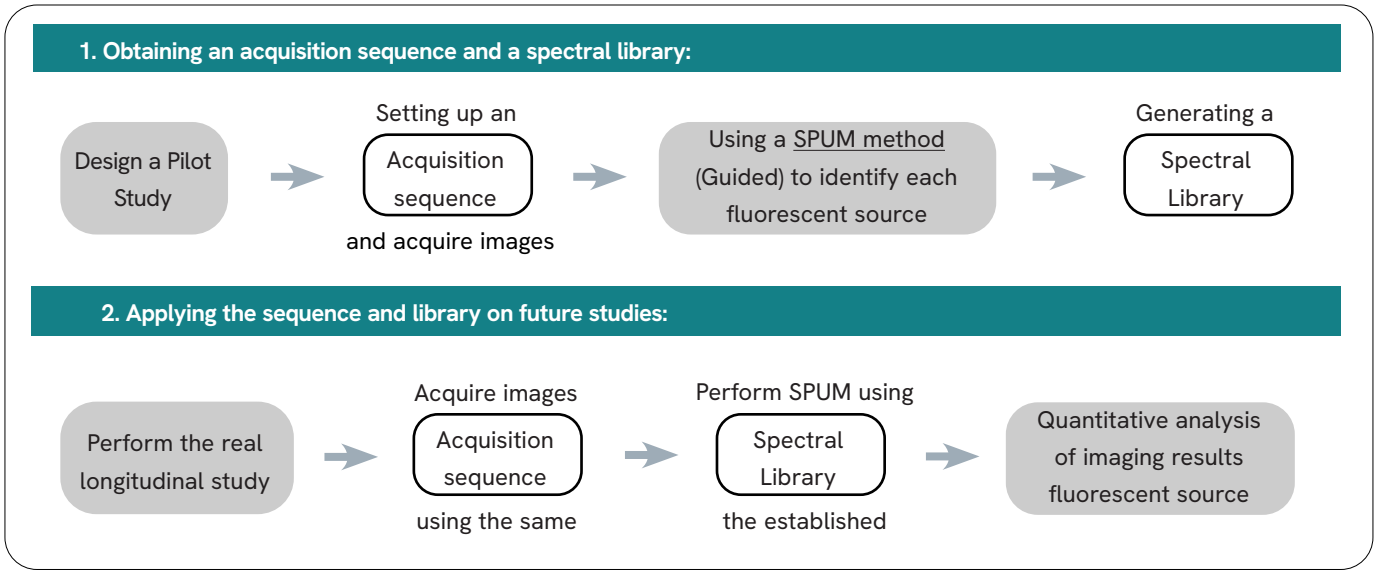


Figure 1. Analysis workflow for Guided SPUM.

Design a pilot study to generate a working SPUM library

In this example, the animals are maintained on alfalfa-free food which is highly recommended to prevent food-related autofluorescence. Figure 2 illustrates a typical pilot study design when two fluorescent agents IVISense™ MMP 645 (MMP645) and IVISense Bombesin Receptor 680 (BRS680) are systemically administered (i.e. iv injection) to target tumor. In particular, this case will need at the minimum 3 tumor-bearing mice (left three mice in Figure 2) to identify the following required regions for SPUM: a non-injected mouse to provide tissue autofluorescence (TAF), a second mouse injected with MMP645 to provide (MMP645 + TAF) and a third mouse injected with BRS680 to provide (BRS680 + TAF). As the IVIS® system has a 5-mouse capacity for planar imaging, it is recommended to include two additional mice that can be used for library validation. Both validation mice are injected with MMP645 + BRS680 (two purple mice in Figure 2). However, one is a tumor-bearing mouse for testing the targeting effectiveness of the agents. The other is a tumor-free mouse which evaluates whether the fluorescent agent has any native targets other than the intended tumor.

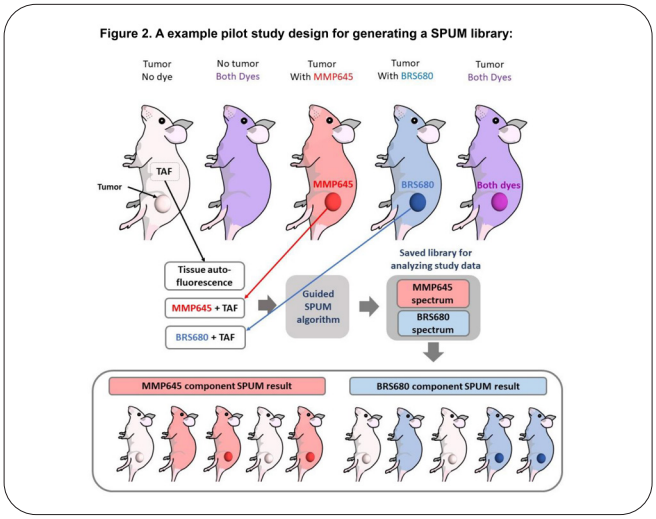


Figure 2. An example pilot study design for generating a SPUM library.

Using the imaging wizard to set up acquisition sequence

After establishing a representative pilot study, the first objective is to set up an acquisition sequence for generating a working spectral library. Acquisition sequence is a series of ex/em fluorescence imaging steps to cover all fluorescence sources present in the system. It is recommended to use the Imaging Wizard, which can guide you through the SPUM acquisition process (Figure 3). The wizard has a built-in library of fluorophores and imaging agents and can automatically configure corresponding ex/em filter sequence for you. For fluorophores not listed in the database, the user can manually input their excitation and emission peak wavelengths for sequence generation.

1. In the Imaging Wizard window, click on “Spectral Unmixing/ Filter Scan”, making sure “Epi-Illumination” is selected.
2. In the probes box, use the drop-down menu to select the fluorescent imaging agent and its corresponding ex/em peaks will show. This menu contains all Revvity imaging agents and other commonly used fluorophores. For fluorophores not listed, the user can select “Input Ex/Em” and manually input its peak wavelengths (nm).
3. Use the “Add” button for additional fluorophores. You can also click on the “Filter config” button to review and make change to filter selection, if needed.
4. When you finish, click “Next” and the Wizard will automatically generate a SPUM imaging sequence tailored for these set of fluorophores.

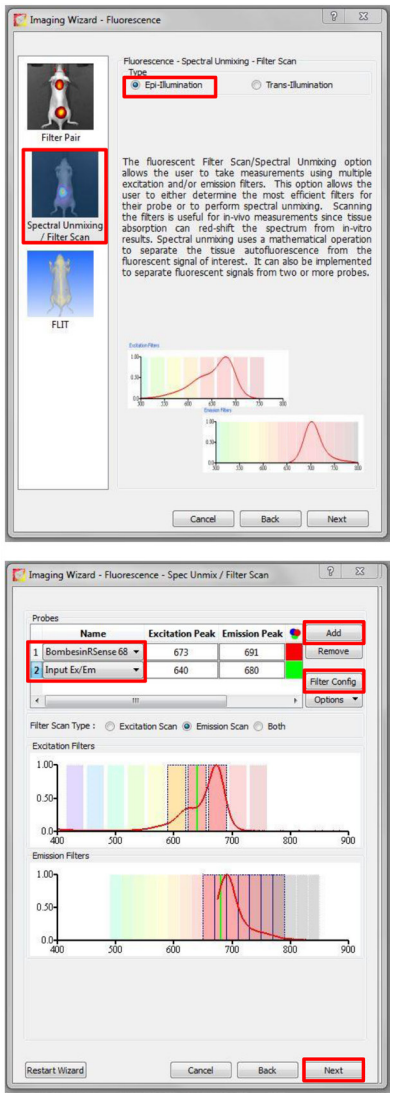


Figure 3. Automatic sequence setup using the Imaging Wizard.

Reviewing acquired images and SPUM method selection

After the image series is acquired, please click and expand the “Spectral Unmixing and DyCE” tab in the Tool Palette. You will see all the ex/em combinations present in the image series (Figure 4). On the “Units” drop-down menu, select “Radiant Efficiency” or “Counts” and review the data. Make sure there is no over exposure image in the series. There are four methods for SPUM: **Guided, Manual, Library and Automatic.** The Guided option is the recommended method and will be used in this guide. It is designed to separate each single fluorophore mixed only with the tissue auto-fluorescence (TAF) as is the case in most pilot studies. The Manual option gives users full control in situations that require resolving regions with more than two mixed components. Both the Guided and Manual methods can be used to create user-defined Spectral Library files, which can later be applied in the Library mode to analyze future datasets acquired using the same sequence. The Automatic option provides an unbiased way to quickly explore a SPUM dataset, especially when using fluorescence components whose spectra are included in the built-in library.

Figure 4. Loaded sequence dataset and SPUM analysis methods

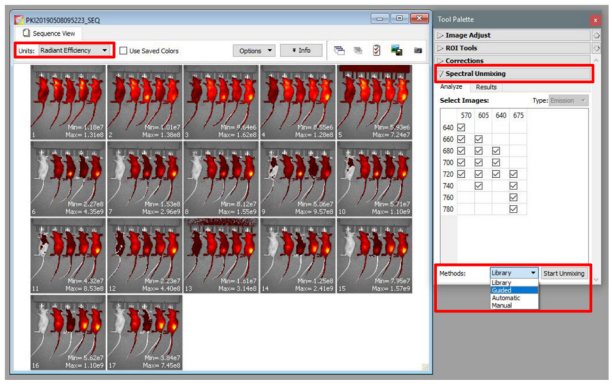


Figure 4. An example pilot study design for generating a SPUM library.

Guided SPUM library generation (recommended)

The Guided SPUM method assumes that you know where your fluorophore signals originate and that each fluorophore's signal is mixed only with one background source (e.g. TAF). The Guided option is primarily used for establishing a Spectral Library with positive and negative control subjects in a pilot study that is specifically designed according to this principle.

To begin:

1. To ensure accurate SPUM analysis, load the image sequence and check that each image remains within the 600-60,000 counts window. You can deselect saturated or low intensity images in the Analyze tab (Figure 4).

2. Switch display units to radiant efficiency. Select "Guided" from the Method dropdown menu and click "Start Unmixing" (Figure 4). An overview of all sequence images, the ImageCube, will appear and give a pseudocolor to all assumed separate signals in the field of view (Figure 5).

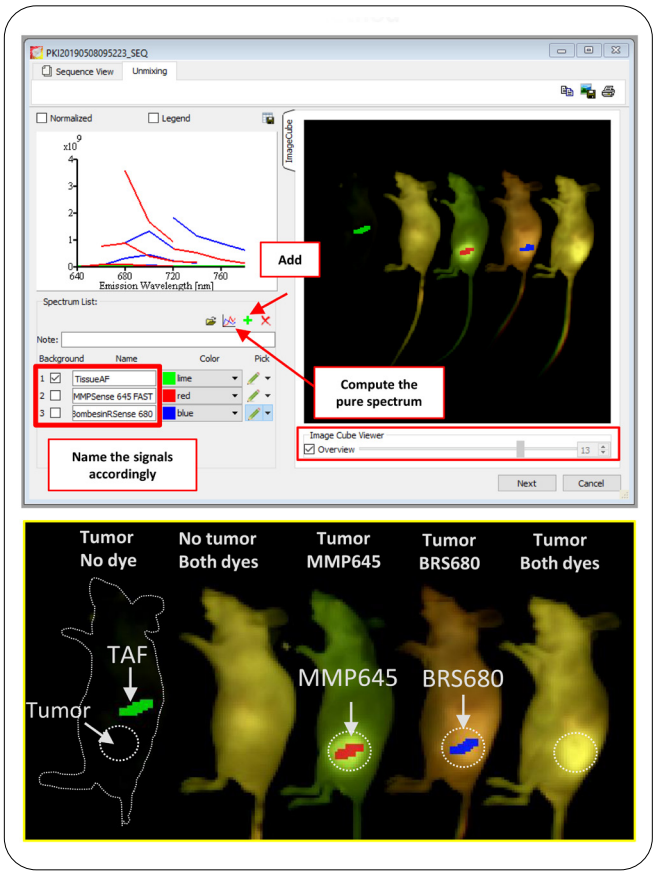


Figure 5. Guided SPUM method.

3. Using the pen tool, first mark the region of TissueAF (green) on ImageCube. Use the "Add" button repeatedly to add more components on the ImageCube. Name each component accordingly. Of note, since the Guided mode will automatically subtract TissueAF spectrum, the two components were named "IVISense MMP 645" and "IVISense Bombesin Receptor 680" in this example (Figure 5).

4. Once all fluorophores have been identified, click Next to view the unmixed results (Figure 6).

5. The calculated SPUM spectra can be saved in a Spectral Library and applied to different imaging sessions which used the identical acquisition sequence.

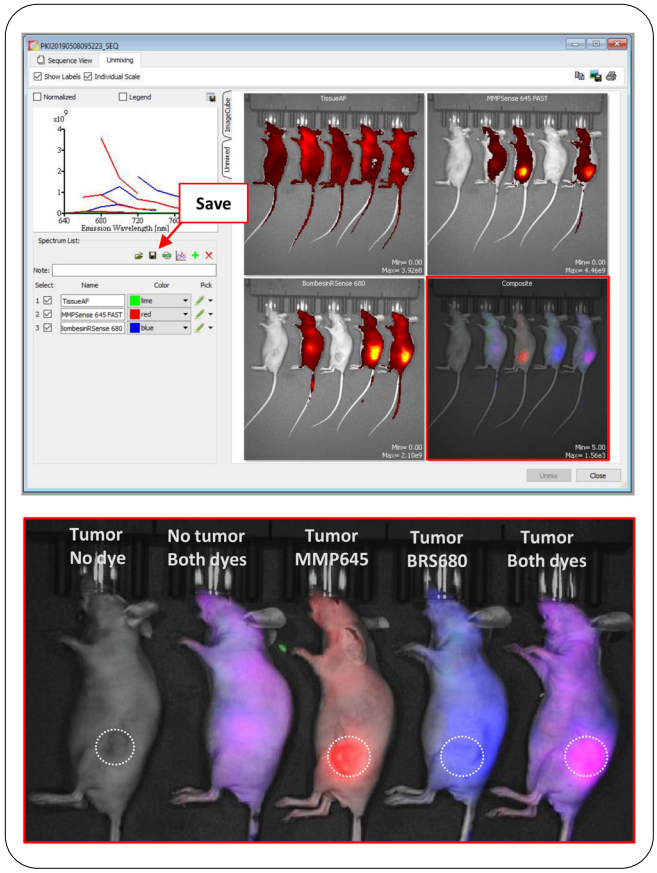


Figure 6. SPUM results using the guided mode.

SPUM vs conventional fluorescence imaging of two systemic probes

Figure 7 demonstrates the effectiveness of SPUM when imaging two systemically administrated, tumor-targeting fluorescent agents: IVISense MMP 645 (MMP645) and IVISense Bombesin Receptor 680 (BRS680) (Figure 7A). In this example, the mouse #1, #3, and #4 are required for library generation and the #2 and #5 mice provide validation. It is not surprising that, as both agents have closely related fluorescence profiles, the conventional single ex/em filter pair imaging fails to distinguish them (Figure 7B, No SPUM), and we saw considerable cross-talk signal in mouse #3 and #4. After applying SPUM, both fluorescent signals were separated, and more importantly each fluorescent component was precisely quantified (Figure 7C, %C: percentage of control). SPUM effectively eliminated all cross-interference (essentially zeroing the irrelevant probe for each channel), making the quantitative assessment more accurate and providing probe background levels for each channel.

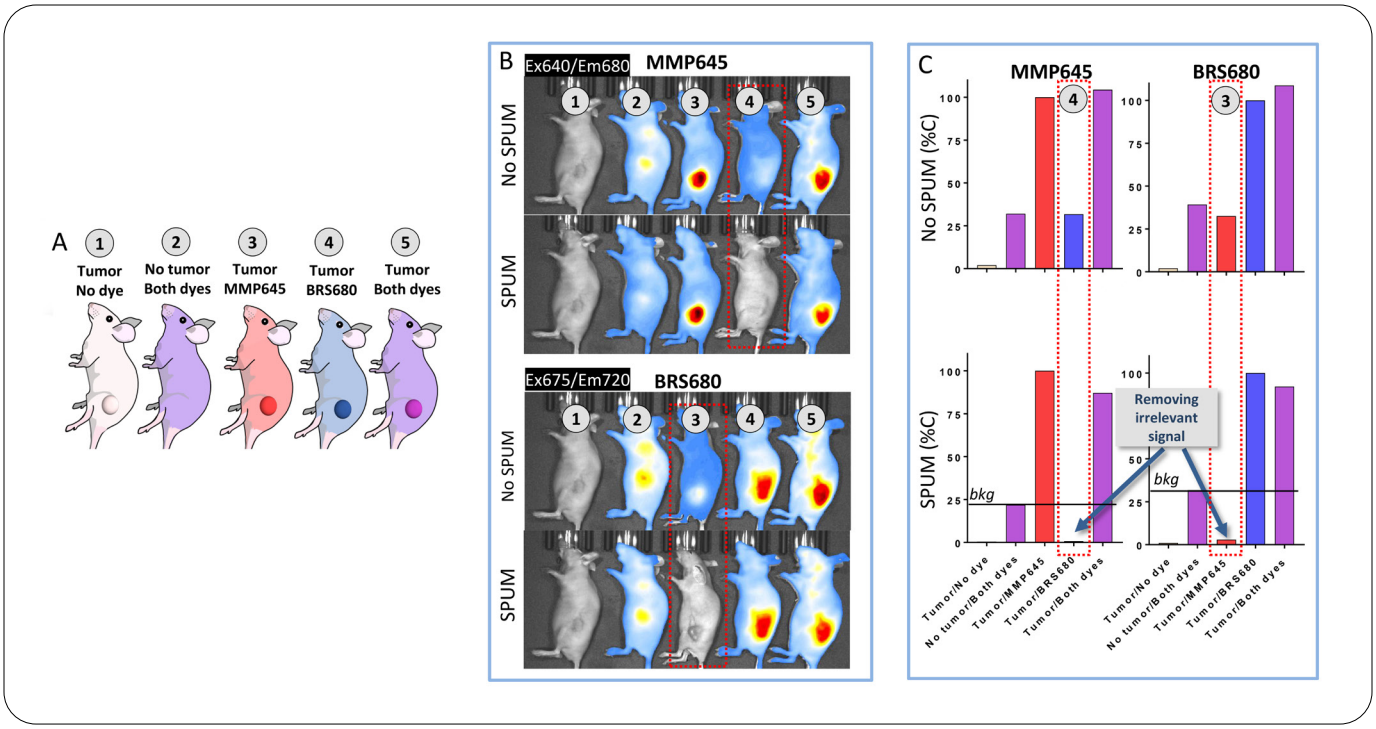


Figure 7. Conventional single ex/em filter pair imaging of s.c. implanted fluorophore mixture (A-G).

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