Using Spectral Unmixing to Separate Multiple Interfering Fluorescent Signals

Abstract

Conventional fluoresce imaging uses a single excitation/emission (ex/em) filter pair to detect fluorescent signals for image generation. However, this approach is less effective when there are two or more spectrally overlapping fluorophores present in the system. To address this issue, the Living Image® software on an IVIS® imaging system provides a spectral unmixing (SPUM) tool for identifying and quantifying each fluorescence source. As fluorescent probes are designed to visualize bio-physiological functions, SPUM imaging allows users to simultaneously obtain multiplex fluorescent functional readouts and thus help to achieve the three Rs standard in animal research, i.e.: Refine, Reduce and Replace. The ability to visualize multiplex biological functions also greatly improves scientific interpretation, and the high-quality images can be used for presentation and publication.

The first step of SPUM analysis requires establishing an acquisition sequence of ex/em filter combinations for the algorithm to identify each fluorescent source. It is important to design a sequence whose spectral range encompasses all responsible fluorescent sources. The second step is to use the sequence to acquire a representative image dataset and generate a working spectral library for all fluorescence sources. The established acquisition sequence and spectral library can then be saved for acquisition and analysis of future datasets. A common application of SPUM is to remove the interfering background fluorescence from the true target fluorescence signals of interest. This technical note demonstrates how to use SPUM to identify and separate interfering fluorescent signals. It starts with a theoretical discussion of the origins of typical spectral interference and some practical advice on proper study design then moves to the technical details for acquisition sequence setup and image dataset generation. For library generation, this technical note outlines how to use different SPUM methods to identify and separate multiplex fluorescence signals. At the end of the note, we will demonstrate the advantage of using SPUM for quantitative analysis of multi-component fluorescence images.
**SPUM Overview**

Living Image SPUM tool is an effective way to isolate and quantify each individual fluorescence sources when multiple spectrally-overlapping fluorophores are present in the system. 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). 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 Imaging provides four methods for source isolation: Guided, Manual, Library and Automatic (Table 1). In most cases, we recommend the Guided method for this purpose. 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. Details on each methods will be discussed in the Spectral Unmixing Acquisition section.

![Figure 1. SPUM analysis workflow.](image)

**Table 1. SPUM methods for library generation.**

<table>
<thead>
<tr>
<th>Method</th>
<th>10-mouse Manifold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guided (Recommended)</td>
<td>A convenient way to compute each component’s spectrum. This method assumes that each component is only mixed with tissue auto-fluorescence (TAF).</td>
</tr>
<tr>
<td>Manual</td>
<td>Full control to define TAF and each fluorescent component’s spectral properties.</td>
</tr>
<tr>
<td>Library</td>
<td>Use previously established spectral library for consistent, accurate results.</td>
</tr>
<tr>
<td>Automatic</td>
<td>Providing a quick way to explore SPUM datasets, given sufficient signals present for each component.</td>
</tr>
</tbody>
</table>
Before designing how a proper multiplex imaging study is discussed, common sources of fluorescence interference must be reviewed. There are two major types of interfering fluorescence signals. The first type is caused by intrinsic sources such as skin, muscle and soft tissues that can produce ubiquitous, low level auto-fluorescence across most of the visible, red and far-red light wavelengths (400-720 nm). Another major intrinsic source is food, since rodent chow pellets commonly contain alfalfa which has strong fluorescence in the spectral range of 650-720 nm (Figure 2A). When using fluorescent agents in this particular range, chow interference strongly affects the accuracy of imaging results. The second type of interference is that introduced by investigators when multiplexing two or more closely related fluorescent agents with considerable overlap in their spectra (Figure 2B).

The good news is that the impact of interfering fluorescence sources can be prevented or greatly reduced by proper study design. Although low grade tissue autofluorescence is ubiquitous and unavoidable, we can certainly prevent food alfalfa interference by feeding animals with alfalfa-free diet prior to and throughout the course of study, especially for studies involving imaging the stomach/abdominal region in the 650-720 nm range. In addition, by choosing fluorophores with sufficient spectral separation, we can also minimize interference in multiplex imaging, making it possible to visualize each fluorescent component by using only their optimal ex/em filter pairs. Nevertheless, in situations where the spectral overlap of two signals is very likely, SPUM is a great tool for identifying and isolating each target fluorescent source for visualization and analysis.

**Figure 2.** Sources of fluorescence interference.
Figure 3 illustrates the challenge of interference by fluorescence from chow alfalfa when attempting to image BombesinRSense 680 (BRS680) in animals. The fluorescent emission spectra of regular and alfalfa-free food pellets together with the spectrum of far-red BRS680 were determined using a 605 nm excitation filter (Figure 3A). It is evident that the alfalfa in regular mouse chow has considerable fluorescence emission in the same range as BRS680, while the alfalfa-free chow produces much less interfering fluorescence. To demonstrate how the conventional single filter pair imaging approach fails to visualize BR680 in the GI region, BRS680 was subcutaneously injected into two ventral sites (chest and abdomen) in mice fed either regular chow or alfalfa-free chow. Figure 3B shows that the alfalfa has strong ex 640/em 680 fluorescent signals in regular chow, and thus it is not surprising that alfalfa-containing chow shows strong interfering fluorescence in the GI/abdomen region when imaging the animals using the same filter pair, while alfalfa-free diet avoided this issue. Later in the note this particular simple model will be used to demonstrate the effectiveness of Spectral Unmixing to isolate target fluorescence signals.
For a fluorescent imaging study that involves multiple overlapping signal sources, it is paramount to design a proper, representative pilot study for acquisition sequence determination and library generation. A good study design is the key to a robust acquisition sequence and an accurate spectral library for future analysis. A proper design should consider the known properties of fluorescent agents (wavelength, PK, etc.) as well as their route of administration (local or systemic). In particular, a good design should allow user to easily define the following input regions for SPUM: a reference region that contains only the tissue auto-fluorescence (TAF); and for each target fluorophore Xᵢ, a region that contain the target fluorescence Xᵢ plus TAF (Xᵢ + TAF). These regions are required when using the recommended Guided SPUM method to calculate the pure spectrum Xᵢ for the library. In a sense, the algorithm subtracts the TAF signals from the mixed (Xᵢ + TAF) signals to obtain the pure Xᵢ signals: (Xᵢ + TAF) - TAF.

Figure 4 illustrates two design examples when a single fluorescent source X₁ is used on mice with known food alfalfa interference in the guts. The first example (#1) is suitable for locally administrated X₁ (e.g. subcutaneously). In this case, the injection site can be easily identified as X₁ + TAF and the gut region as the Alfalfa + TAF region. A reference TAF region can be chosen in an area away from the injection site and gut. In the second example (#2), the tumor-targeting fluorescent agent X₂ is delivered systemically (e.g. intravenously). Thus the tumor can be used as the (X₂ + TAF) region. Of note, this pilot study will need to include a second mouse that is not injected with X₂ in order to define the TAF and (Alfalfa + TAF) regions.

Figure 4. Proper pilot study design for generating a SPUM library.
Design a Pilot Study to Generate a Working SPUM Library continued

Figure 5 illustrates a typical pilot study design when two fluorescent agents $X_1$ and $X_2$ are systemically administered (i.e. iv injection) to target tumor. For studies involving multiple systemic fluorescent sources, the use of alfalfa-free food is strongly recommended to simplify the design. In this case, a minimum of three tumor-bearing mice (left three mice in Figure 5) were used to identify the following required regions for SPUM: a non-injected mouse to provide TAF, a second mouse injected with $X_1$ to provide $(X_1 + $TAF$)$ and a third mouse injected with $X_2$ to provide $(X_2 + $TAF$)$. As the IVIS system has a 5-mouse capacity for planar imaging, including two additional mice that can be used for library validation is recommended. Both validation mice are injected with $X_1 + X_2$ (right two mice in Figure 5). 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. As shown in this example, the $X_2$ also detects its native targets in the pancreatic region.
This section outlines the use of a simple model (illustrated in Figure 3) to guide you through the SPUM process. The model involves a locally injected fluorescent agent, BombesinRSense® 680 (BRS680) in the presence of both ubiquitous tissue autofluorescence (TAF) and food alfalfa interference. 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. We recommend using the Imaging Wizard, which can guide you through the SPUM acquisition process (Figure 6). 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 PerkinElmer 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.
User-defined Acquisition Sequence for SPUM Analysis

Beside using the Wizard, the user can also define an imaging sequence from the ground up. Since this option gives the user full control to customize each step in the sequence, the user need to have prior knowledge of the sources present in the system. It is best-suited for studies involving custom-designed fluorescent agents which are currently not included in the built-in fluorescent agent library.

The following are some important considerations when configuring each imaging step:

1. Click “Image Setup” and enter desired filter combinations in the Sequence Editor (Figure 7). You can specify exposure time, binning, F/stop and ex/em filters for each step in the control panel. For example, you can select “Auto” exposure time, Binning: 4, F/Stop: 2, Excitation Filter: 605, and Emission Filter: 660, then click “Add” to include the step in the sequence.

2. Autoexposure is recommended. However, If you are not using autoexposure, determine in advance the optimal exposure time, binning, and F/stop for your subject using the specific excitation and emission filters for your particular fluorescent agent to achieve 600-60,000 counts without pixel saturation. Using specific, defined parameters for each step could speed up the whole acquisition process.

3. Repeat to compile all desired ex/em steps into the sequence. For each imaging step, 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 >40 nm separating the two. This is the reason why we did not include the filter pair ex640/em660.

4. After completing sequence setup, click on the disk icon to save it and the click on “Acquire Sequence” to execute the acquisition process.

Figure 7. Setting up a multi-step sequence for image acquisition.
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 8). On the "Units" drop-down menu, select "Radiant Efficiency" or "Counts" and review the data. Make sure there is no overexposure image in the series. There are four methods for SPUM: Guided, Manual, Library, and Automatic. The Guided option is the recommended method. 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. Table 2 summarizes the features of each method.

![Figure 8. Loaded sequence dataset and SPUM analysis methods.](image)

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantage</th>
<th>Feature</th>
<th>Suitable for Spectral Library Generation?</th>
</tr>
</thead>
</table>
| Guided (Recommended) | A convenient way to compute each component's spectrum. This method assumes each component is only mixed a single TAF. | 1. User-defined background TAF spectrum.  
2. Compute pure spectrum of each fluorescent component by TAF subtraction.  
Both methods require prior knowledge of each component's location. Also, in most cases, the guide method is sufficient for studies with a single TAF source. | Yes.  
The Guided method is a simple way to establish Spectral Library using positive and negative control subjects. |
| Manual          | Full control to define tissue auto-fluorescence (TAF) and compute each fluorescent component's spectral properties. | Works best when analyzing longitudinal image data acquired using the same filter sequence. | N/A (Using libraries generated by Manual or Guided methods). |
| Library         | Use previously established spectral library for consistent, accurate results. |                                                                 |                                            |
| Automatic       | Providing a quick way to explore SPUM datasets, given sufficient signal present for each component. | The PCA tool can be used to identify potential fluorescent components in an image. | Not 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 8).

2. Switch display units to radiant efficiency. Select “Guided” from the Method dropdown menu and click “Start Unmixing” (Figure 8). 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 9).

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 “BRS680” and “Food” in this example (Figure 9).

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

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 10).

Guided SPUM Library Generation (Recommended)

Figure 9. Guided SPUM method.

Figure 10. SPUM results using the Guided mode.
The Manual SPUM option is more complex, requiring more steps, but gives the user full control to identify and separate each fluorescent component in the image.

The overall procedures are similar to the Guided option.

To begin:

1. Select Manual from the Method dropdown menu (Figure 8). 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 11).

2. Click on the Pen Tool, and use your computer mouse to mark each component. First use the green color to mark tissue autofluorescence (TissueAF). Click “Add” and use red color to mark the area known to have both TissueAF and BRS680 signals (TissueAF+BRS680). This step is not necessary when the image sequence is acquired using the Wizard. Repeat this step to add and mark the blue TissueAF+Food signals in the abdomen. It may be helpful to uncheck the Overview button and scroll through the individual filter sets to find the location of the fluorescent signals, and brightness can be adjusted here. The pen tool can be used on overview or individual filter pairs. You may name them accordingly (Figure 11).

3. Launch the Compute Pure Spectrum interface to define one marked spectra as a mixed spectrum (TissueAF+BRS680) and the second marked spectra as a known signature (TissueAF) to be subtracted from the first (Figure 12). Rename the new spectrum accordingly (BRS680 only). Click “Apply” to add the new “pure” component to the main list. Repeat this step to calculate the pure spectra for the other known source (Food only). Click “close” after you complete this step.
5. Once both AF680 only and Food only spectra have been created, they can be applied back to the original ImageCube to unmix both AF680 and Food fluorescence as separate components (Figure 13). Leave mixed spectra unchecked but make sure the correct components to be unmixed are marked with the check box, and click Unmix to retrieve the results.

6. Once the unmixing has been verified, the components can be saved as a Spectral Library and applied to different imaging sessions only if they used an identical acquisition sequence (Figure 14). The library should be saved in the default LI SPUM database directory (~Living Image\database\SPUM), as the tool will only recall it there.

7. Alternatively, if previously saved Spectral Library components are available, they can be imported into the Unmixing Wizard as a new component by clicking on the green + icon, choosing “Import” from the Pen Tool dropdown menu, and loading a Spectral Library file (Figure 15). This feature is very useful for correcting tissue auto-fluorescence at locations difficult to draw on. In such case, the user can import a previously established auto-fluorescence spectrum as long as it is generated under the same imaging settings (filter sequence, mouse strain, etc.).
The Automatic Spectral Unmixing method compares intensity changes throughout the field of view that have resulted from the filter scan against the spectral profiles of fluorophores available in the software’s built-in spectral library. These known fluorophores can also be chosen via a dropdown menu, and if not present, the unknown option can be chosen. Up to four component signatures may be selected. If the number of spectral components is unknown, the Principle Component Analysis (PCA) can be activated where a statistical breakdown of the spectra will be displayed and a proposed number of components given.

To use Automatic SPUM:

1. If the number of components is unknown, use the Draw Mask tool to select the region for PCA analysis (Figure 16).
2. Click the “PCA” button and the suggest number(#) of components will be provided in a new window. In this example, the PCA analysis suggests there are 3 fluorescent components (e.g. TissueAF, Food, and BR680) present in the selected region as the explained variance reach more than 99% (Figure 17).
3. In the main window, select Photograph and use the threshold tool to map both mice for SPUM. Also, from the PCA analysis, we know there are three components in the image and we know two of them are background signals. Make sure both Background Signal boxes are checked for the tissue autofluorescence and food signals. The third component can be listed as “Unknown” in this example. If needed, you can add any additional components to the list using the green “+” button (Figure 18).

![Figure 16. Select region for PCA analysis](image)

![Figure 17. Results of PCA analysis.](image)

![Figure 18. Setting up components for Automatic SPUM.](image)
4. Once all components have been defined, click Finish to view the unmixed results (Figure 19).

5. In this example, all three components are automatically labeled as UMX1, UMX2, UMX3 by default. By comparing their spectra, you can rename them to Autofluorescence, Food and BR5680 accordingly.

6. Or alternatively, you can manually search the built-in library and select the correct fluorophores during the component setup step (Figure 20).

Of Note, the Automatic SPUM method works best when the fluorophores are in the built-in library. Although the Automatic SPUM results and the resolved spectra for each component can be saved for later use, this practice is not recommended especially when using novel or custom fluorophores that are not validated in the built-in library. In such situations, it is recommended to use the Manual or Guide methods for more accurate, user-defined results.

Figure 19. Automatic SPUM results.

Figure 20. Select fluorophore for built-in library.
Using a Saved SPUM Library

The Spectral Libraries generated by the Manual and Guided methods can be called using the Library method. The Library method is ideal for spectral unmixing of longitudinal imaging datasets. The Library spectral unmixing method will call a saved *.csv file to unmix the currently loaded sequence. Of note, in order to achieve consistent SPUM results, it is critical to use a matching spectral profile specific to the particular study design (same filter sequence, same subject orientation, etc.).

To use the Library mode:

1. Select the Library option from the dropdown menu and click Start unmixing (Figure 8). The Load Spectrum Library window will appear for you to select the library to be applied to the image series (Figure 21).

2. Check the “show qualified only” option to eliminate choices that do not pertain to the acquired sequence. It is checked by default. Please note that Spectral Libraries can only be applied to image series acquired using the same acquisition sequence as they were originally defined.

3. Once the correct Spectral Library has been found, highlight the name in blue and click on “Apply”. The sequence will be automatically unmixed using the methodology saved in the Spectrum Library file and results will be displayed (Figure 22).

Figure 21. Loading a saved library for SPUM.

Figure 22. SPUM results using Library mode.
How to Review SPUM Results

Reviewing Spectral Unmixing Results

The unmixed spectral images will appear as several Component image panels (one for each fluorophore unmixed) and a pseudocolor-coded Composite image which includes all fluorophores. Any image can be double-clicked to show in a separate window for analysis or image capturing. 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 the Region of Interest (ROI) tools for quantification (Figure 23, top panel), while the Composite image will allow for alterations of the pseudocolor levels for a better visualization of each fluorescent component (Figure 23, bottom panel).

Figure 23. SPUM imaging results can be used for: 1. quantitative analysis in the Component view (top), and 2. producing a multi-component pseudocolor image in the Composite view (bottom).
As most biological investigations require systemic administration of multiple fluorescent agents, it’s highly recommend using alfalfa-free mouse food pellets to prevent possible interference in the 640-680 nm range and adherance to the study design recommendation shown here and in Figure 5. This example demonstrates the effectiveness of SPUM when imaging two systemically administrated, tumor-targeting fluorescent agents: MMPsense 645 (MMP645) and BombesinRSense 680 (BRS680) (Figure 24A). 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 24B, No SPUM), and we saw considerable cross-talk signal in mouse #3 and #4. After applying SPUM, we were able to separate both fluorescent signals, and more importantly, we were able to quantify them individually (Figure 24C). 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 24. Conventional single ex/em filter pair imaging of s.c. implanted fluorophore mixture (A-G)
EXAMPLE: SPUM Analysis for Local Injections and Chow Signal

The Challenge of Using Single Filter Pair Imaging to Distinguish Closely Related Fluorophores

Another example showing SPUM’s advantage to not only remove intrinsic background signals but also to separate two spectrally overlapping fluorescence agents, VivoTag 645 and VivoTag 680, that introduce considerable cross-interference. In this model, a gradient mixture of VivoTag 645 and VivoTag 680 was subcutaneously introduced on nu/nu mice fed with alfalfa food. Eight sites, A to H, were injected with increasing VivoTag 645 levels (0 to 100 pmol) and decreasing VivoTag 680 levels (100 to 0 pmol) as shown in Table 3. As reference, this study also included pure VivoTag 645, 680 and an alfalfa food pellet in the field of view. Therefore, we have four components (VivoTag 645, 680, alfalfa and TissueAF) that have overlapping spectra in this range. It is rather challenging to visualize individual components using the conventional single ex/em filter pair approach (Figure 25A and 25B). In particular, besides intrinsic autofluorescence and alfalfa food fluorescence, strong introduced interference is seen between VivoTag 645 and 680 fluorophores (arrows), making quantification inaccurate in all ranges of fluorophore amounts (Figure 25C).

Table 3. Emission spectra and mixture gradient injected at 8 different sites (A to H).

<table>
<thead>
<tr>
<th>Site</th>
<th>VivoTag 645 (pmol)</th>
<th>VivoTag 680 (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>1.56</td>
<td>50</td>
</tr>
<tr>
<td>C</td>
<td>3.13</td>
<td>25</td>
</tr>
<tr>
<td>D</td>
<td>6.25</td>
<td>12.5</td>
</tr>
<tr>
<td>E</td>
<td>12.5</td>
<td>6.25</td>
</tr>
<tr>
<td>F</td>
<td>25</td>
<td>3.13</td>
</tr>
<tr>
<td>G</td>
<td>50</td>
<td>1.56</td>
</tr>
<tr>
<td>H</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 25. Conventional single ex/em filter pair imaging of s.c. implanted fluorophore mixture (A-G).
Using SPUM to Identify and Quantify Closely Related Fluorescence Signals

SPUM can readily identify all four fluorescent components present in this example: TissueAF, Alfalfa, VivoTag 645 and VivoTag 680. Following the instructions provided in the previous sections, a comprehensive imaging sequence was designed. As shown in Figure 26A, the sequence covered all sources in this spectral range. Applying SPUM on this dataset yielded pure spectra for each component (Figure 26B). From there, it was possible to quantify the individual fluorescence signals respectively and a composite image of all four components was generated (Figure 26C). The SPUM results were easily validated as all reference sources (pure VivoTag 645, 680 and Food Alfalfa) were correctly identified and separated. More importantly, the quantitative analysis of VivoTag 645 and VivoTag 680 showed much improved linearity and accuracy (Figure 26D; arrows) as compared to the unmanipulated monochromatic data (Figure 25C).

Figure 26. Using SPUM to separate the fluorophore mixture image shown in Figure 25.
Discussion

This technical note provided an overview of the spectral unmixing (SPUM) tool of the IVIS Living Image software with detailed instructions on how to design your fluorescent imaging study accordingly to avoid or reduce fluorescence interference from various sources. This is especially important if you plan to use fluorophores in the range of 650-720 nm where food alfalfa fluorescent can cause strong interference. Of course, this intrinsic fluorescence can be easily prevented by maintaining your animals on alfalfa-free diet prior to, and during, the course of the imaging study. Nevertheless, there will be situations when two spectrally close-related fluorophores need to be used and considerable fluorescent interference could be introduced. Using SPUM, it is not only possible to deconvolute the pure fluorescent signals for each fluorophore, but most importantly, the resolved spectral signature of each fluorescent component can be saved for later use on future longitudinal data, making repeated measurement on the same subjects more accurate and meaningful.

In particular, for training purposes, we demonstrated how to manually design an acquisition sequence for SPUM. You can also take advantage of the Imaging Wizard and Autoexposure setting if the fluorophores are already included in the built-in spectral library. 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. For questions on how to use the Imaging Wizard, please see the respective quick reference guides associated with these workflows. You can also find information pertaining to the use of these features in the Spectral Unmixing Wizard Setup reference guide.

Also discussed in detail are the four methods for SPUM: Guided, Manual, Library, and Automatic. To establish a Spectral Library, it’s recommended the users to take advantage of the Guided method and design a pilot imaging study accordingly with proper negative control subjects to define the background fluorescence and positive subjects to define target fluorophore signal. The Manual method provides full control for the user to define regions of interest and methods to compute the pure spectrum for each fluorescent component. Both Manual and Guide methods are suitable for generating Spectral Library, which can then be used in the Library modes for repeated SPUM analyses on longitudinal data. Please note that a Spectral Library can only be applied to an image series acquired with the same filter combination sequences and fluorophores as during its creation. The Automatic mode provides a quick way to review a SPUM dataset, and the PCA tool can be used to identify fluorophores present in the image.

With proper study design, SPUM is a powerful tool for analyzing multi-component fluorescence images and produces quantitative results. As fluorescent probes are designed to non-invasively visualize bio-physiological functions, SPUM imaging allows users to investigate multiplex biological functions in living animals.

References


