The "why" and "how" of spectral unmixing.

In vivo fluorescence imaging displays a very broad utility and has become a well-established modality for functional imaging in small animals. Nevertheless, fluorescence imaging is often perceived as difficult and cumbersome, especially in comparison with bioluminescence imaging. Spectral unmixing is by far the best technology tool to make fluorescence imaging more sensitive, specific, and more reliable.

Advantages of spectral unmixing

Increasing signal-to-noise through removal of autofluorescence

Sensitivity is always limited by the signal-to-noise ratio! Even for bioluminescent imaging where the background noise is extremely weak, the noise eventually becomes too high to detect the signal. In fluorescence imaging the background noise, called autofluorescence, is unfortunately very high and generates a challenge for sensitive detection. The background intensity however is wavelength dependent and autofluorescence is much lower in the near-infrared (NIR) region (Figure 1). Nevertheless, also in the case of NIR imaging, signal detection is limited by the background noise.

An additional problem for *in vivo* fluorescence imaging is the strong fluorescence of chlorophyll (Figure 1, middle image, upper row). Since common rodent diets contain chlorophyll, mainly from the Alfalfa component of the food, it is important to feed animals with an Alfalfa-, or chlorophyll-free diet. Alternatively, the chlorophyll signal can be separated out with the help of spectral unmixing (see Figure 3).

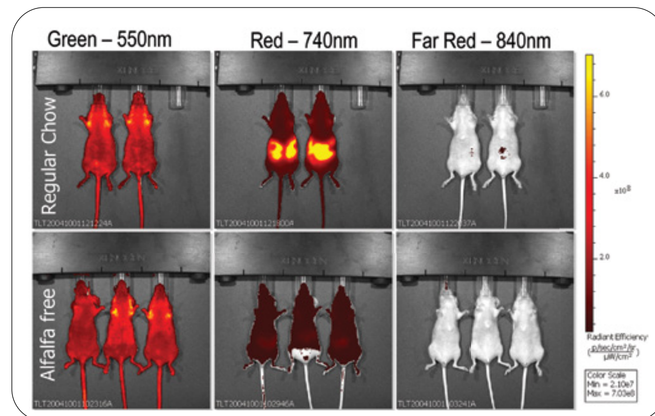

Figure 1: Fluorescence images of wild type (wt) mice show the autofluorescence background at different wavelengths. Mice labeled "Alfalfa free" were fed with a chlorophyll-free diet (AIN-76A, Research Diets) for at least two weeks before imaging.

Spectral unmixing helps to separate the autofluorescence background from the fluorescent signal, thereby increasing the signal-to-noise ratio and enhancing sensitivity of detection. In practical terms, it is almost impossible to generate signals with a good signal-to-noise in the lower part of the spectrum up to 650 nm without spectral unmixing, and it is also very beneficial – and sometimes even necessary - in the NIR range.

An impressive example of signal-to-noise (S/N) improvement through spectral unmixing is shown in figure 2. A weak dihydroethidium (DHE) signal in the brain was imaged with a series of 20 nm bandpass filters. The S/N ratios in those images range from 0.4/1 to 0.7/1, which means the autofluorescence background signal is about twice as high as the DHE signal. However, the spectra of DHE and autofluorescence are different, and after spectral unmixing the S/N increased to 22/1 which also made the DHE signal clearly visible in the unmixed image.

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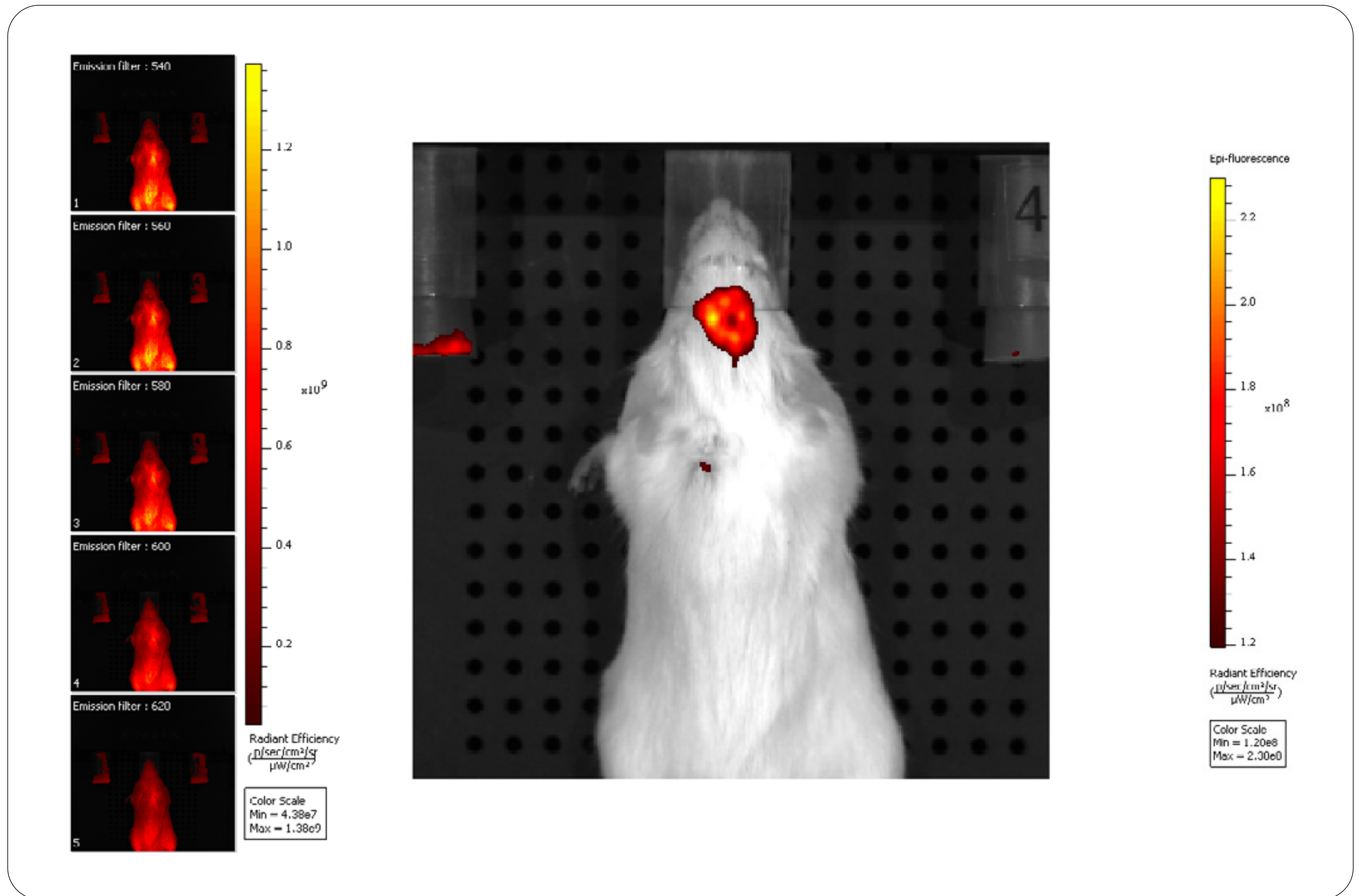

Figure 2: Spectral Unmixing of a dihydroethidium (DHE) signal from autofluorescence background. DHE is a superoxide indicator and was injected in a mouse bearing a glioma in the brain. The left panel shows the raw images of a spectral imaging sequence with five 20 nm wide fluorescence emission filters, ranging from 540 to 620 nm. The image on the right shows the DHE signal after spectral unmixing.

Autofluorescence removal also improves the quantitation of fluorescent signals. Simple background subtraction (e.g. with the help of a background ROI) does also help the quantitation, but it assumes that the autofluorescence intensity is equal at the background and measurement area. While the spectrum of the autofluorescence is basically homogeneous throughout the mouse area, the intensity is not. Therefore, removal of the autofluorescence by spectral unmixing results in better quantitation.

Separation of multiple signals

With deeper insight to biological processes, it is inevitable that models become more complex, and a simultaneous readout of multiple parameters are necessary for a better understanding. This also holds true for the acquisition of imaging data. While it may often be useful to merge functional optical imaging data with structural data from µCT, MRI or ultrasound, it often is even more beneficial to combine multiple functional readouts that are acquired simultaneously by fluorescence and/or luminescence imaging.

The ‘classical’ combination of a single luminescent and a single fluorescent signal is just the beginning. A multitude of fluorescent probes are available commercially, or can be easily generated by fluorescent labeling of proteins (e.g., antibodies), DNA/RNA, nanoparticles etc. Since the recommended wavelength range for *in vivo* imaging should be above 600 nm, it is often difficult to use multiple fluorophores that are very far apart (Δ > 100 nm) and spectral unmixing is the only possibility to avoid crosstalk between the fluorophores. Genuinely separating various fluorophores by spectral unmixing is the only way to allow true multiplexing of fluorescence readouts. Of course, these readouts can be additionally combined with luminescent data. Recent developments even allow for the separation of deep tissue bioluminescent signals by spectral unmixing [1].

Figure 3: Spectral unmixing of two activatable probes (IVISense™ Pan Cathepsin 680 and IVISense MMP 750 FAST) in a mouse model of orthotopic breast cancer. Raw data was acquired with 20 nm bandpass emission filters (720, 740, 760, 780, 800, 820, 840). The 680 and 750 probe signals, as well as the food background (chlorophyll) and autofluorescence (not shown) were unmixed using the CPS algorithm.

Typical emission spectra of fluorophores, with the exception of quantum dots, show a rapid rise in intensity followed by a slow decline resulting in a long 'tail' of the spectrum. Therefore, when multiplexing several fluorophores, it is often relatively easy to get 'clean' data for the dye with the shortest wavelength, but the rest of the dyes suffer from 'bleed through' of signals with shorter wavelengths (Figure 3). The opposite is true for the excitation spectra of common fluorophores, and this is important when performing excitation scanning and unmixing, as it is possible with the IVIS© optical systems and the Living Image© software.

In figure 3 it seems that the food background is well separated from the IVISense '750' and IVISense '680' dyes. However, as is apparent from the spectral plot, the "chlorophyll" signal and the signal of the '680' dye are strongly overlapping, and a complete separation is not possible. In this case, both signals are also spatially separated and that gives the impression of a complete separation.

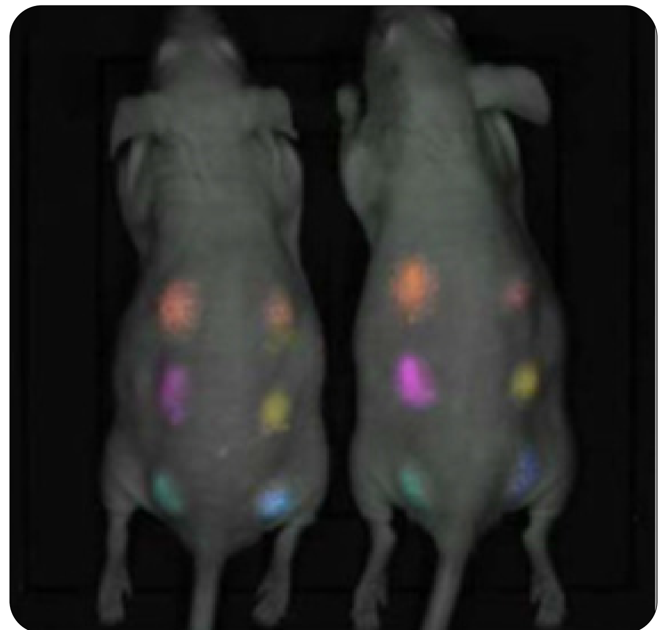

Figure 4: Spectral unmixing of 6 different fluorescent proteins injected subcutaneously in mice. Shown is the composite image with each signal displayed in a different color. From [4].

The spectral unmixing algorithms that Revvity is using in the Living Image© software, don’t really limit the number of fluorophores that can be unmixed. The fluorescent filter selection would limit the number to about 20 fluorophores. However, for good spectral resolution there should be about 40 nm separation between dyes and for *in vivo* imaging the dyes should be emitting above 600 nm to avoid strong tissue absorption. This limits the ‘meaningful’ number of dyes to about 7 or 8 at maximum. An excellent example is shown in figure 4. While the spectral separation isn’t always at the desirable 40 nm, the spectral unmixing algorithms improve the separation of 6 different fluorescent proteins (+ autofluorescence) to an impressive degree.

Spectral unmixing technology

How does spectral unmixing work?

Since fluorescent light emissions combine linearly, mixtures of signals can be mathematically disentangled (or unmixed), as long as the spectrum of the desired signal(s) and that of the autofluorescence or other components are known or can be deduced from the data, yielding images that reflect the abundance of each component isolated from the others [2].

Linear spectral unmixing is a mathematical method used to decompose a source spectrum into a set of pure spectral signatures, called endmembers. The result of the unmixing is a measure of the fractions of the individual endmember, called abundances. This technology is used in many areas aside from biology (e.g. chemistry and geology) and the different algorithms used are discussed elsewhere [3].

The challenge of spectral unmixing *in vivo*

In order to determine the abundances of the endmembers, the pure spectral signatures must be known. However, the spectra as measured in the living animal may differ considerably from published spectra or *in vitro* measurements due to environmental effects (e.g., pH, ionic milieu) and from the combined influence of light scattering and absorbance due to such constituents as collagen, melanin, hemoglobin, and red blood cells.

That differences between *in vitro* and *in vivo* spectra can be very drastic, is impressively shown in figure 5. The upper graph shows the *in vitro* spectra of six different luciferases and mutants thereof. All spectra have maxima below 600 nm, typically around a wavelength of 560 nm. The insert shows that there are second maxima for a few luciferases around 620 nm, but at a much lower intensity. In a dramatic difference, the *in vivo* spectra of all luciferases, except *Renilla* luciferase, show maxima at 620 nm, while the signals below 600 nm are strongly absorbed by the tissue.

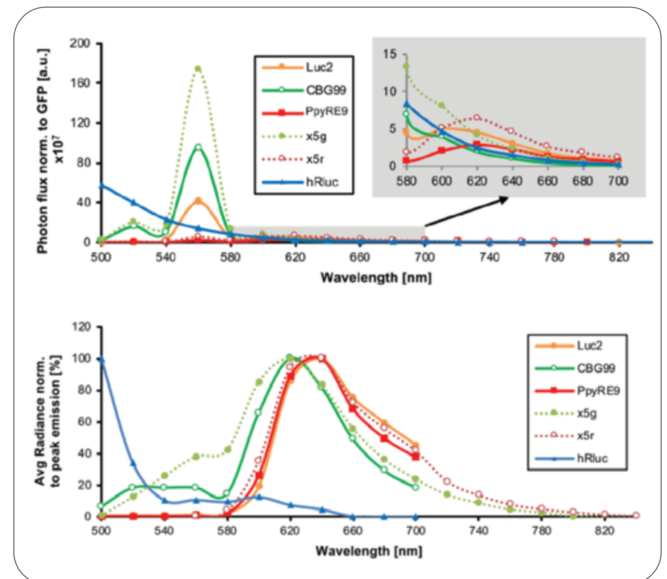

Figure 5: *In vitro* (above) and *in vivo* (below) spectra of different luciferases. From [5].

This shows that the actual spectra used for the spectral unmixing algorithms must be measured *IN VIVO*! However, the problem is that except for the autofluorescence signal (e.g., in a control animal), *in vivo* all spectra can only be measured in a ‘mixed’ version, combined with the universal autofluorescence background spectrum.

Value of the ‘compute pure spectrum’ technology *for in vivo* spectral unmixing

This is where the spectral unmixing technology implemented in Revvity’s Living Image© software exhibits its unique capabilities. The approach taken by the patented ‘Compute Pure Spectrum’ (CPS) technology is to measure the mixed spectra emitted from the surface of the experimental animal, in addition to the ‘pure’ autofluorescence spectra and then calculate the apparent ‘real’ spectrum of the endmembers of interest [2]. This calculated spectrum is then used to provide the unmixing algorithms with a correct estimate of all components, allowing for accurate quantitation of the abundance for each endmember.

The functionality of this approach is demonstrated in figure 6. It is easy to visualize that the mixed, blue spectrum can be separated into the green tissue autofluorescence and the red IVISense 750 spectra. A detailed description of the technology and its application can be found in the technical note “Using Spectral Unmixing to Separate Multiple Interfering Fluorescent Signals”.

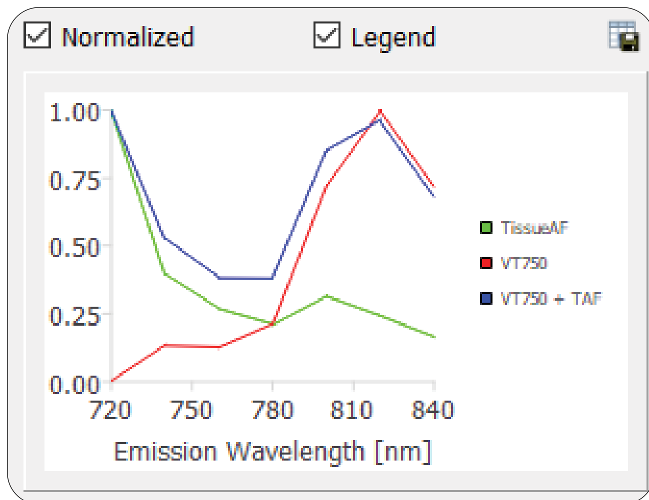

Figure 6: Spectral plot from the Living Image software showing the spectrum of tissue autofluorescence (green), the mixed spectrum of IVISense 750 and tissue autofluorescence (blue) and the calculated pure IVISense 750 spectrum (red).

The essence of fluorescence imaging *in vivo*

A large array of narrow banded fluorescence filters is the prerequisite to scan spectra of fluorescent dyes and luminescent signals. Furthermore, spectral unmixing is an essential component of fluorescence imaging, improving sensitivity, quantitation and multiplexing. The spectra of the different signals must be known in order to separate them by spectral unmixing and due to environmental effect and tissue absorption the spectra have to be determined *in vivo*.

The compute pure spectra technology incorporated in the Living Image software is uniquely suited for calculating the pure signal spectra from the mixed *in vivo* spectra that cannot be measured in a pure form except for the autofluorescence.

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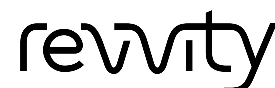

Revvity
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com

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