Blind Unmixing of Images Acquired Using Three and Four PMT Detectors

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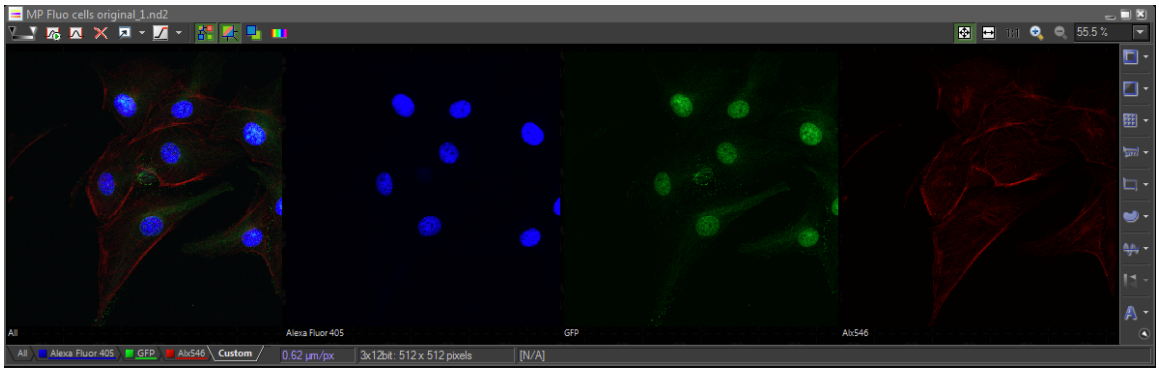
The reason many scientists want spectral imaging capability on their confocal microscope is to minimize, and hopefully eliminate, crosstalk between channels. The A1-DUS spectral detector does an admirable job of this; so good in fact, that unmixed data channels add to the validity of quantitative imaging techniques such as FRET microscopy. However, scientists interested in structural imaging may not always have access to a separate spectral detector.

There is also the special case of multiphoton microscopy. One of multiphoton microscopy's great advantages is the sensitivity gain from not needing to use a pinhole to create thin optical sections. The sensitivity gain results from being able to collect photons scattered anywhere within the full field of view of the objective lens. It is possible to use the A1-DUS spectral detector for multiphoton microscopy, but sensitivity is much greater with the 4-PMT NDD. Significant benefits accrue from being able to unmix multiphoton images. It is challenging to image multiple probes in the same specimen with a multiphoton microscope. It can take tens of seconds to tune the Ti-Sapphire laser between the optimal wavelengths for two, or worse, three fluorescent probes. In addition, one would need to string optical configurations together to combine the images. There is a way around this. The excitation spectra of most fluorescent molecules is not simply double the single photon excitation spectrum. Many fluorescent molecules have high energy two photon transitions (excitation peaks) at wavelengths where single photon transitions at $\lambda/2$ are minor peaks if they exist at all. For many molecules, these peaks will be found between 700nm and 740nm making it possible to excite a number of useful fluorescent molecules together at a single wavelength. Just a few molecules that excite efficiently in multiphoton acquisitions between 700nm and 740nm are DAPI, Hoechst 32258, Cascade Blue, Fluoroscein, Alexa 488, EBFP2, CFP, Mitotracker Red, mCherry, DsRed II, and TagRFP. Unlike in single photon channel series acquisitions, the emissions from many combinations of probes spill over between channels, sometimes in both directions. This would be a serious problem if it were not possible to unmix the images. Fortunately, NIS-Elements includes a powerful spectral unmixing algorithm, blind unmixing, that can applied to any three or four color fluorescence

image or data set. This algorithm can be applied to 3- or 4-color images from the A1-DU-4 or A1-MP-NDD detectors.

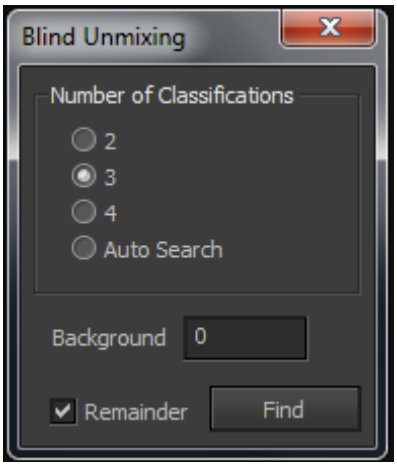
How does blind unmixing work? We know that each fluorescent molecule has its own signature emission spectrum and that spectrum can be very accurately drawn from the distribution of intensities across 32 channels of a DUS spectral detector. We know that these spectra are very repeatable. A spectrum can also be drawn from only three or four points. Its shape will not be known as precisely as one drawn from 32 points, but it will be known well enough to be used for blind unmixing. These simple spectra should be more properly thought of as repeatable intensity distributions between channels. As such, they can be used for unmixing if the spectrum or intensity distribution representing each probe is different enough from the others. As a practical matter, this means that the fluorescent probes should each be associated with a 'color' channel. Combinations of what we usually think of as blue, green, red, and far red probes like DAPI, Bodipy FL, Alexa 568, and Cy5 are good choices. Fluorescent proteins with overlapping spectra but well separated emission peaks like CFP and YFP can also be unmixed. Blind unmixing cannot be successfully applied to closely overlapping fluorescent molecules like GFP and Alexa 488. It should also be understood that blind unmixing results are more qualitative then quantitative. The algorithm first looks at each pixel to find spectral elements or root spectra that the spectra in all pixels can be constructed from. The number of spectral elements that can be extracted from the image is limited to the number of channels in the image. The user can specify a different number of fluorescent probes but the choice is still limited to the number of detector channels. Autofluorescent molecules can be displayed in the unmixed image but they must be counted as probes. The spectral elements extracted from the image are used as reference spectra for linear unmixing. NIS-Elements lets you display the root intensity distributions just as it lets you look at 32-channel spectra.

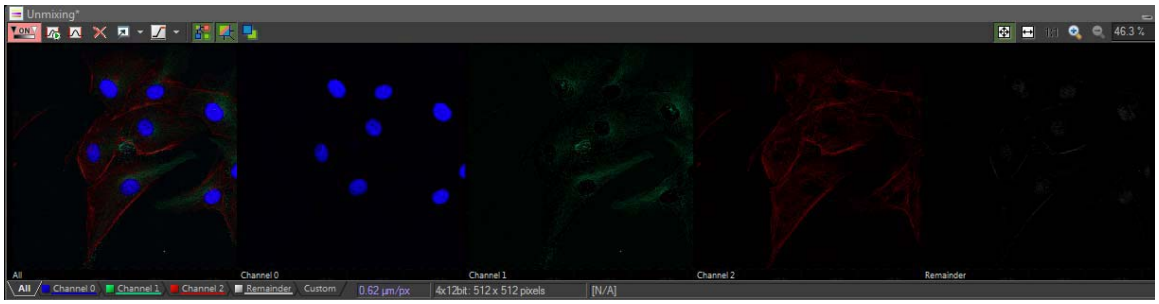
Let's step through a blind unmixing of a three color multiphoton NDD image of a Molecular Probes FluoCells slide. While the combined channels (All) image looks good, DAPI stained nuclei are clearly visible in the green channel and tubulin spills over from the green channel into the red channel. This is clearly unacceptable.



To unmix the image, click on the 'Treat as Spectral' icon  in the Elements tool bar above the image. New channel identification tabs will appear below the image.

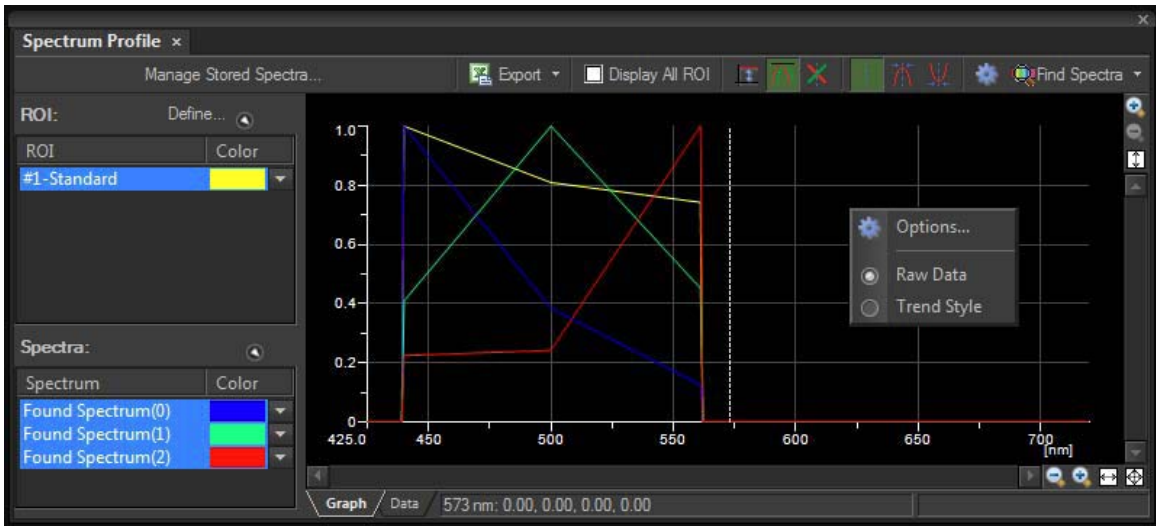
Under 'Image' on the task bar, click on 'Blind Unmixing' in the bottom group of choices. A blind unmixing dialog will open. Enter the number of probes or classifications. Check remainder if you want to be able to see autofluorescence (and other intensities not specifically classified) displayed in the remainder channel. The remainder channel does not count as a classification. Now click the 'Find' button, and the unmixing solution will appear in less than a second.





With remainder checked, the remainder channel will be displayed along with the specifically classified channels. Note that nuclei do not appear in the green channel and tubulin does not appear with actin in the red channel in the unmixed image. Note also how little appears in the remainder channel.

You can display the 'Found Spectra' at any time after you have clicked on 'Treat as Spectral'. With the spectral image open, open 'Spectrum Profile' from 'Visualization Controls'. Click the down arrow and choose the number of spectra you want to display. Right click in the graph area and choose 'Raw Data' to create a line graph. It is often useful to display the average spectrum of the image. The average spectrum is obtained from a full frame ROI and is displayed in yellow in the graph below. The others spectra are displayed in the color assigned to each channel. You can change the display scale by using 'Options'. The scaling of the 'X' axis of the display will be tied to the emission filters in your microscope.



Blind unmixing cannot be run as a real time application because it is a two-part algorithm that requires a full frame image as its starting point. Blind unmixing in combination with the standard detectors of A1 confocal systems brings real spectral unmixing to basic confocal systems and to users of more sophisticated confocal systems who do not need high resolution spectrometers on their microscopes. Note that Blind unmixing can also be used with the 32-channel spectral detector and may prove very useful in separating autofluorescence for which there may not be a good reference spectrum. Blind unmixing also allows multiple fluorescent probes to be imaged in two photon mode in a single scan with the emissions accurately placed in a separate display channel for each probe.