

Quantitative Spectral Unmixing of Fluorescence Signals

The biggest limitation in fluorescence microscopy continues to be the spectral overlap among the ever-growing variety of available fluorophores. Techniques that address this limitation, such as spectral imaging combined with spectral unmixing, are attracting a lot of attention. Even so, the quantitative signal extraction of overlapping fluorescence signals utilizing filters and algorithms has been at best a complex process, and many researchers have been disappointed with the inexact results of linear unmixing. This technical note describes how linear and blind unmixing of numerous overlapping signals with the MAG Biosystems™ SpecEM™ system and Mélange™ software is both quantitative and easy to perform.

OVERLAPPING FLUORESCENCE SIGNALS

With the rapid advances being made in molecular cloning methods and fusing of cDNA with fluorescent protein variants, fluorescent labeling in live cells of nearly any protein is now possible.¹ Experiments have moved beyond using a single fluorophore to the incorporation of multiple fluorophores in a single imaging experiment. Traditionally, this has been done by using a carefully selected set of dyes that have nonoverlapping spectral emissions, as well as optical filters that can be changed or cycled through to reveal different fluorescent emissions.

Many of the most useful fluorescent proteins (e.g., CFP, GFP, and YFP), however, have strongly overlapping emission spectra that make them very difficult to separate via such filtering methods. Additionally, the spectra of the fluorophores of interest are often masked by a specimen's autofluorescence. In these cases, much of the valuable emission fluorescence data is discarded in attempts to achieve reliable separation of the fluorophores.¹ Even when there isn't severe overlapping and background autofluorescence, various dynamics within the cell structure will alter the collected emission spectra in unpredictable ways. In practice, the spectra of fluorophores in an intracellular context are almost always different than those measured *in vitro* in solution.²

To date, the best way to overcome these limitations is spectral unmixing. The SpecEM spectral imaging system from MAG Biosystems permits the collection of full fluorescence spectral information at each pixel in an image. In a single sample, multiple fluorescent dyes attached to different structures can be accurately and completely acquired.

LINEAR AND BLIND UNMIXING ALGORITHMS

Linear unmixing algorithms determine the contribution of different fluorochromes to the total signal and redistribute the intensity values for each color channel, thereby isolating signals of interest from the emission of the other fluorochromes. To accomplish this, the spectral properties of the individual fluorochromes need to be known. In current practice, the published curves from the fluorochrome manufacturer are often used.

For example, if a sample is stained with Molecular Probes' Alexa Fluor® 555 and Texas Red®, then there will be (assuming no autofluorescence) some combination of the two emissions at each pixel in the fluorescence image. To accurately utilize this data, the quantity of each fluorophore at each pixel must be determined. Using the published fluorescence emission curves for Alexa Fluor 555 and Texas Red, the amount of each probe in the measured spectrum can be determined at each pixel via an unmixing algorithm (see **Figure 1**).

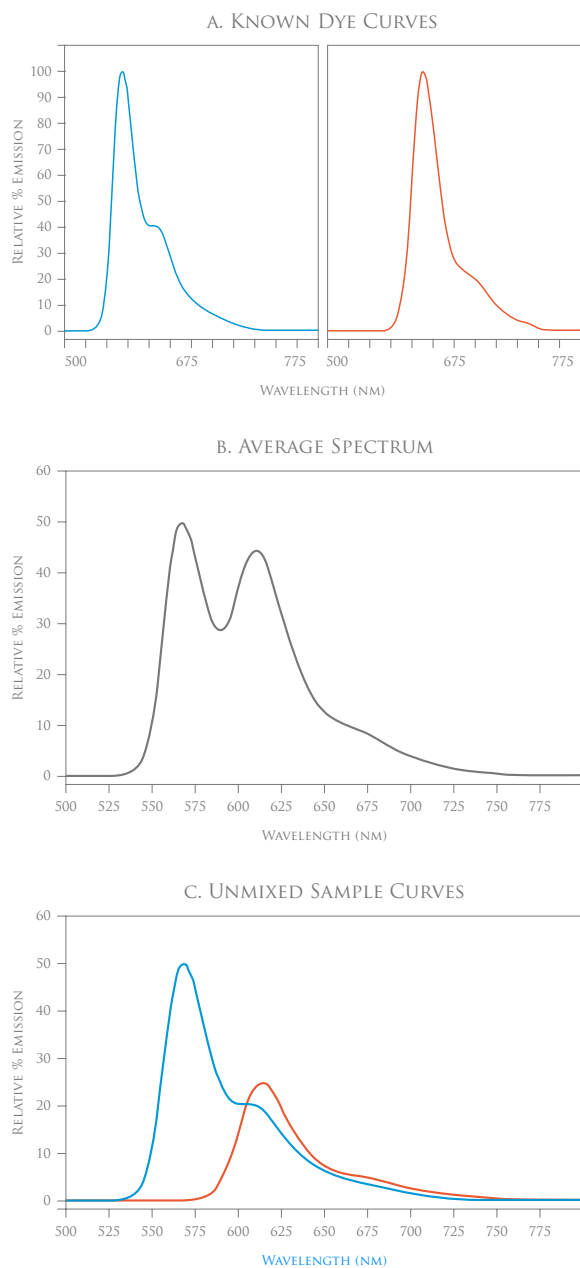


Figure 1.

[A] Unmixing of control-standard Alexa Fluor 555 and Texas Red dyes reveals emission spectra from sample conditions.

[B] Average spectrum of experimental sample.

[C] Unmixed spectra from sample showing quantitative fluorophore contributions.

Obviously, linear spectral unmixing is quantitative only when every component spectrum can be measured separately. In biological applications, however, it is not easy to obtain pure component spectra to be extracted from cellular fluorescence because the spectra of fluorophores in the intracellular environment are almost always different from those measured in solutions *in vitro*. Moreover, specimens often have significant intrinsic fluorescent components (i.e., autofluorescence) whose precise spectra are unknown, which generates inaccuracies when estimating contributions of each probe in the fluorescence data obtained.²

Autofluorescence tends to have a broad emission curve that makes it difficult to find a wavelength region where only the emission of the fluorophore of interest is present. If significant autofluorescence exists within a cell, then blind spectral unmixing algorithms can be used to determine the contribution of the unknown spectra. Utilizing such algorithms in MAG Biosystems' Mélange software (which comes with the SpecEM system), researchers are able to quantitatively calculate the fluorescent-labeled signal and separate it from the autofluorescence of the sample.

APPLICATIONS FOR FLUORESCENCE UNMIXING

As the number of available live-cell fluorescence imaging techniques continues to increase, correct spectral unmixing offers an unprecedented tool that will prove invaluable for a wide variety of bio-imaging applications.

Spectral FRET

In order to obtain information regarding inter- or intramolecular distances between cellular components, many researchers are turning to FRET (Förster resonance energy transfer) imaging. One of the greatest barriers to achieving quantitative FRET results is the spectral overlap between the donor and the acceptor emission spectra. Filter-based imaging techniques are unable to distinguish whether the signal reaching the detector is from FRET or from the bleedthrough of the donor emission into the FRET channel. Spectral imaging coupled with spectral unmixing, however, allows researchers to quantitatively separate the FRET emission from donor bleedthrough, resulting in more quantitative, accurate FRET data.

Autofluorescence removal

Many applications are now using specimens from whole tissues or organisms that exhibit large amounts of autofluorescence. The autofluorescence is attributable to the inherent chemical composition of cells and tissues, in which components such as NADH and riboflavin and flavin coenzymes have fluorescent emissions.³ This has presented a challenge in fluorescence microscopy, as the broad range of emission from autofluorescence can mask actual labeled fluorophores in samples. With the advent of spectral imaging, the problem can now be overcome by utilizing the powerful technique of spectral unmixing, where the emission of the autofluorescence can be extracted from that of the actual signal. This enables researchers to quantitatively image data from even highly autofluorescent tissue samples.

CONCLUSIONS

By collecting nearly all the fluorescence emitted without compromising discrimination between distinct fluorescent molecules, spectral imaging combined with quantitative unmixing is able to overcome many of the limitations in fluorescence microscopy. This solution enables the identification of specific fluorochrome emissions within the total signal and, by properly redistributing the intensity, allows researchers to restore a clear signal for each color channel, unaffected by overlapping signals. The original, full-spectrum image information is all that is needed to perform these calculations, reliably yielding quantitative data without any alteration or embellishment of the original images.

References

1. Zimmermann, T., J. Rietdorf, and R. Pepperkok. Spectral imaging and its applications in live cell microscopy. *FEBS Letters* **546**, 87–92 (2003).
2. Shirakawa and Miyazaki. Blind spectral decomposition of single-cell fluorescence by parallel factor analysis. *Biophysical Journal* **86**, 1739–52 (2004).
3. Aubin J.E. Autofluorescence of viable cultured mammalian cells. *J Histochem. Cytochem.* 1979 Jan; 27(1): 36–43.



www.photomet.com

info@photomet.com

USA 520.889.9933

Asia Pacific +65.6841.2094

France +33.1.60.86.03.65

Germany +49.89.660.779.3

Japan +81.3.5639.2731

UK +44.1628.890858