Multifluorescence – The Crosstalk Problem and Its Solution

If a specimen is labeled with more than one fluorochrome, each image channel should only show the emission signal of one of them.

If, in a specimen labeled red and green, part of the green light is detected in the red channel, the phenomenon is known as crosstalk or bleed-through. This may lead to misleading results, especially in colocalization experiments.

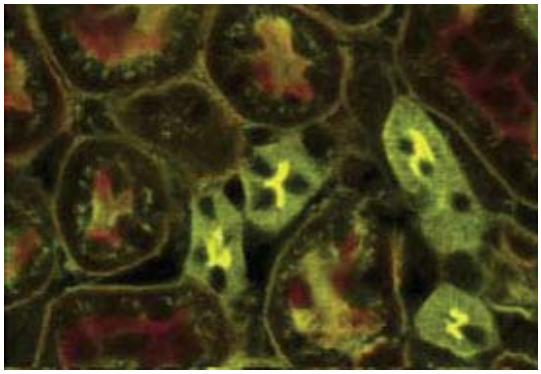
One can distinguish between two kinds of crosstalk: emission and excitation crosstalk.

In a pure emission crosstalk between two fluorochromes A and B, the two emission spectra will overlap, but the laser lines will excite the dyes independently of each other; i.e. there is no overlap of the excitations.
Excitation crosstalk would occur if the laser that excites fluorochrome A also partially excited fluorochrome B.

The problem of emission crosstalk can be solved by sequential excitation and detection (Multitracking) of the fluorochromes. In case of a combination of excitation and emission crosstalk, additional spectral information is needed for separating the emission signals.

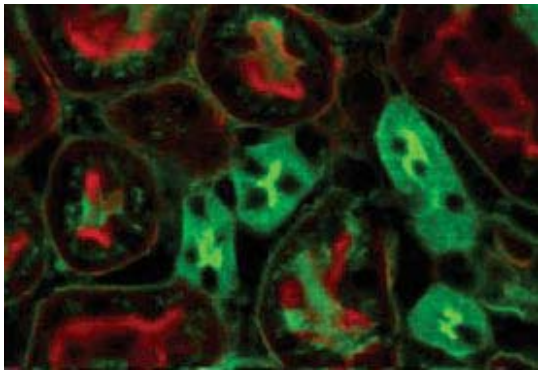
Emission crosstalk

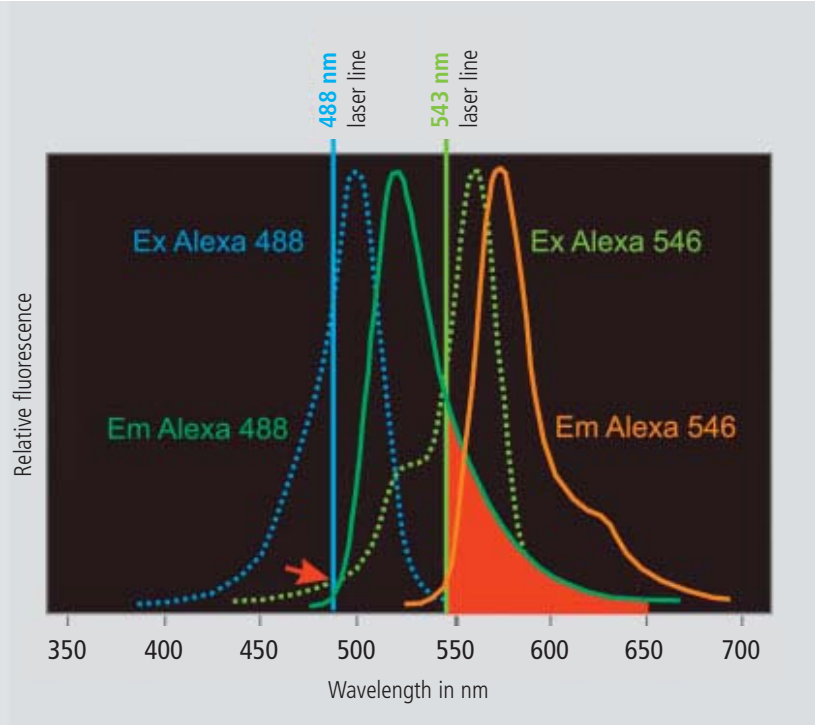
Section through a mouse kidney, double-labeled with Alexa 488 (wheat germ agglutinin) and Alexa 568 (phalloidin). Simultaneous excitation with 488 and 543 nm. The emission of Alexa 488 is detected in both the green (BP 505-530 nm) and red (BP 560-615 nm) channels. Because of this bleed-through, the areas labeled with Alexa 488 appear yellow in the superposition and could be misinterpreted as colocalization with the Alexa 568.



Elimination of emission crosstalk by Multitracking

*If Alexa 488 and 568 are excited and detected sequentially, no green signal is detected in the red channel.
Structures labeled with Alexa 488 appear green in channel superposition.*





Emission crosstalk of Alexa Fluor 488 and 546

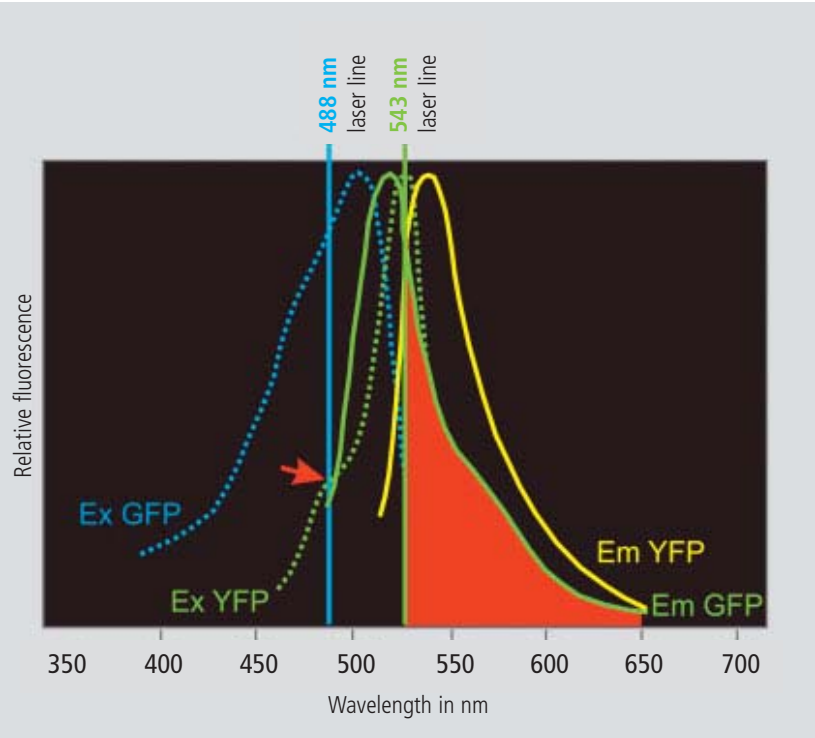
The excitation efficiency of the two fluorochromes is determined by the point of intersection between the laser line used and the excitation spectrum (dotted line).

Accordingly, Alexa Fluor 488 is excited to about 80 %, Alexa Fluor 546 to about 60 %.

At a level of about 5%, the excitation spectrum of Alexa Fluor 546 is also intersected by the 488 nm laser line (arrow). Theoretically, this constitutes excitation crosstalk, as one line excites both markers, but it is inefficient enough to be negligible.

Contrary to this, the emission spectra of the two dyes overlap significantly.

The red area marks the emission crosstalk of Alexa Fluor 488 occurring if Alexa Fluor 546 is detected to the right of the 543 nm laser line.



Combined excitation and emission crosstalk

If GFP is used together with YFP, the emission spectra will overlap considerably.

The red area marks the emission crosstalk between GFP and YFP occurring if YFP is detected to the right of the 514 nm laser line.

In addition, there will be a pronounced excitation crosstalk. The 488 nm line excites not only GFP but also YFP to an efficiency of about 30 % (arrow).

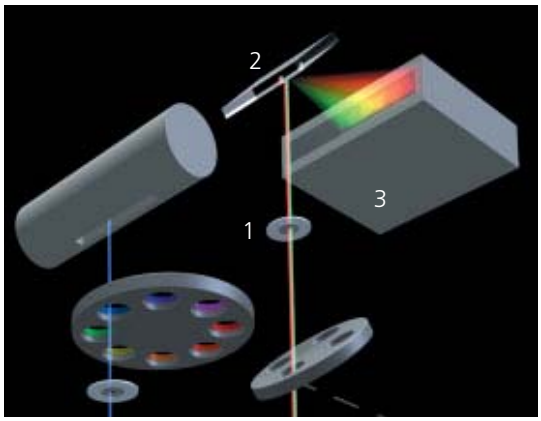
Source: http://home.ncifcrf.gov/ccr/flowcore/welcome.htm; modified

Spectral Imaging

The acquisition of spectral data becomes necessary where the overlapping emission signals of multiple-labeled specimens have to be separated, or where the cellular parameter to be measured is coded by changes of the emission spectrum (e.g., FRET and ratio imaging of ion concentrations).

The LSM 510 META is a system for the fast acquisition of images of high spectral resolution. The hardware enabling this functionality consists of a spectrally dispersive element, a photomultiplier (PMT) with 32 parallel detection channels (META Detector), and special electronic circuitry for detector control and signal amplification.

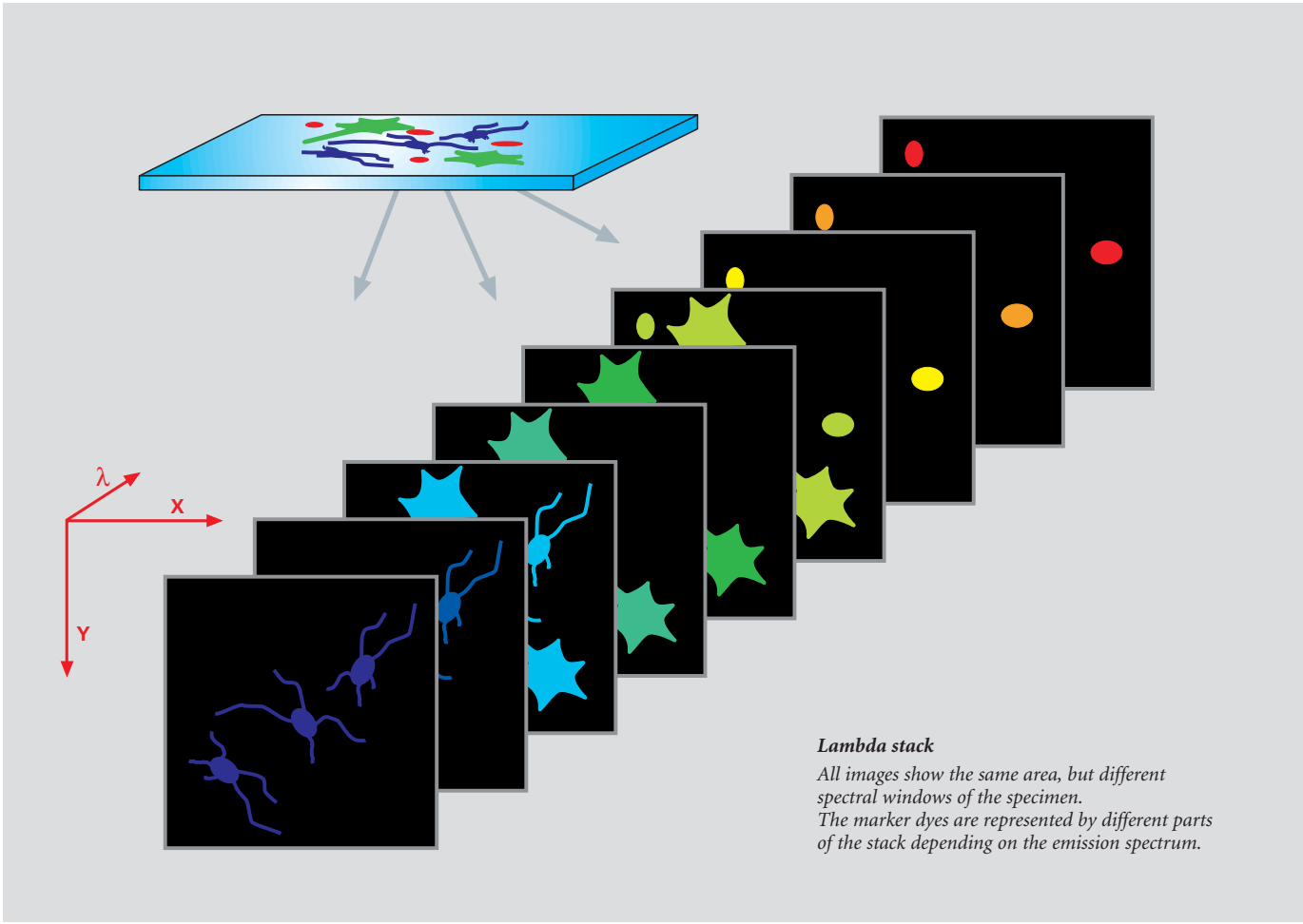
Whereas the beam paths for conventional and META detection are identical on the excitation side, the emission beam for spectral detection, after having passed the pinhole, hits a reflective grating. The grating spreads the beam into a spectrum and projects it onto the surface of the linear detector array. Each of the 32 PMT elements in that array thus registers a different part of the spectrum, each part having a width of 10 nm. The result is a lambda stack of XY images in which each image represents a different spectral window.



META Detector
Part of the beam path in the LSM 510 META scanning head
1 Confocal pinhole
2 Reflective grating for spectral dispersion
3 META Detector with 32 separate PMT elements

The procedure for a lambda stack

1. Select the spectral range in the *Lambda-Mode*
2. Activate the excitation laser lines
3. Carefully control the dynamic range to avoid over- and underexposed pixels *(Range Indicator)*
4. In multiple-labeled specimens, vary the power of the respective laser lines instead of the *Amplifier Gain*, in order to match the signal intensities of the fluorochromes
5. Record the lambda stack
6. Display the data in one of the modes: *Gallery, Single, Slice, Max* or *λ-coded*



Lambda stack
All images show the same area, but different spectral windows of the specimen. The marker dyes are represented by different parts of the stack depending on the emission spectrum.

By connecting adjacent detector elements (binning), the spectral width of the images can be extended. From a lambda stack, the intensity of the signal for each pixel of the image can be extracted as a function of wavelength. These spectral "fingerprints" can easily be obtained for any image area by means of the Mean of *ROI* function. Lambda stacks can be recorded as time series, Z stacks, or as Z stacks versus time. In the last-named case, the result would be a five-dimensional image file with the coordinates, X, Y, Z, lambda and time.

The META Detector is good not only for recording lambda stacks, but also as a channel detector in the conventional mode. By binning the respective detector elements in this mode, the optimum spectral bandwidth can be adjusted for any fluorescent dye.

Spectral Imaging

Emission Fingerprinting

Emission Fingerprinting is a method for the complete separation (unmixing) of overlapping emission spectra. It is used with specimens labeled with more than one fluorescent dye, exhibiting excitation and emission crosstalk.

The typical raw data for Emission Fingerprinting are lambda stacks. The previous chapter described how they are recorded by means of the META Detector. The second step is to define reference spectra for all spectral components contained in the specimen. As a rule, these are dyes internationally used for labeling the specimen. Other possible components are autofluorescent and highly reflecting structures. Autofluorescences, in particular, often have rather broad emission spectra that overlap with the fluorescent markers; this makes them an added source of "impurities" degrading the signals in conventional laser scanning microscopy.

With Emission Fingerprinting, autofluorescences are simply included in the unmixing process. The user can subsequently decide between switching the autofluorescence channel off and using it to obtain structural information possibly contained in the specimen.

The reference spectra can either be loaded from a spectra database, or directly extracted from the lambda stack. For the latter version, the user has two options. One is to define spectra via ROIs. The other uses a statistical method, Automatic Component Extraction (ACE), to find the reference spectra. In either case, the images of the lambda stack must contain structures marked with a single fluorochrome only.

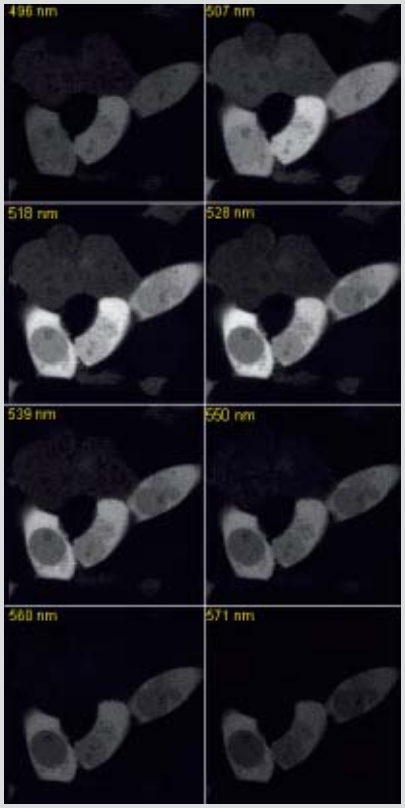
The third step of Emission Fingerprinting is Linear Unmixing, which converts the lambda stack into a multichannel image. Each spectral components of the specimen is then displayed in one channel only. The accuracy of the technique allows the complete unmixing even of such dyes whose spectra have almost identical emission maxima.

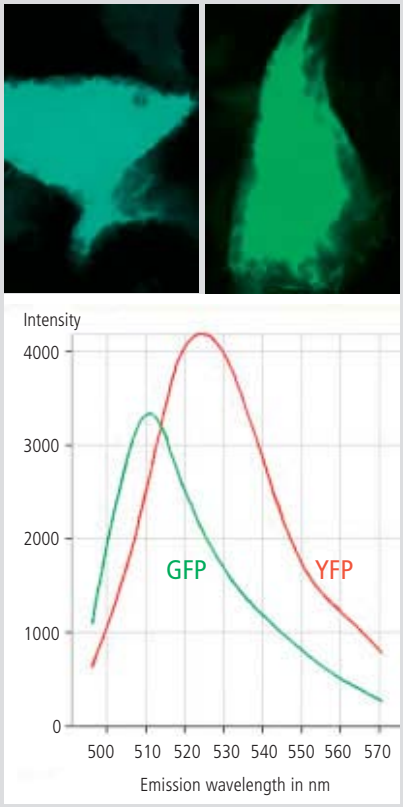
Linear Unmixing

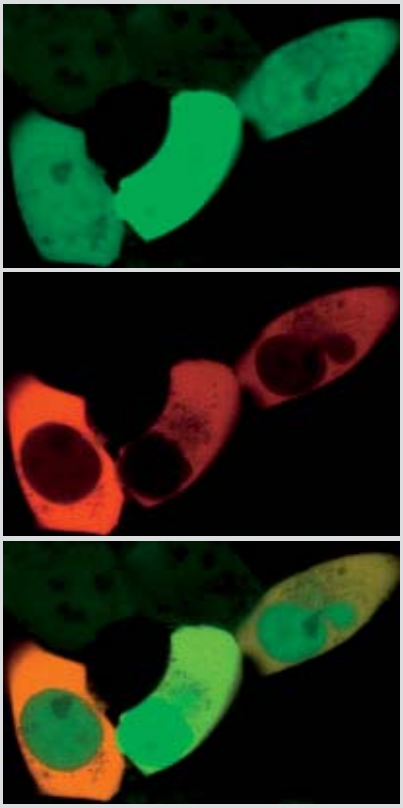
Linear mathematical algorithm for spectral unmixing.
If we regard a pixel of a lambda stack that represents a locus in the specimen where three fluorescent dyes A, B and C with their spectra $S(\lambda)_{dye\ A,\ B\ and\ C}$ overlap, the cumulative spectrum $\Sigma S(\lambda)$ measured can be expressed as

$$\sum S(\lambda) = [intensity \cdot S(\lambda)]_{dye\ A} + [intensity \cdot S(\lambda)]_{dye\ B} + [intensity \cdot S(\lambda)]_{dye\ C}$$

By means of known reference spectra $S(\lambda)_{dye\ A,\ B\ and\ C}$, the equation can be solved for the intensities of the dyes A, B and C, which yields the intensity shares of the three dyes for this pixel. If this calculation is made for each pixel, a quantitatively correct 3-channel image results, in which each channel represents a single dye.







The 3 Steps of Emission Fingerprinting

1 Recording of a lambda stack

The illustration shows an 8-channel image of a cell culture transfected with GFP and YFP. Each image shows the mean wavelength of the channel.

2 Definition of reference spectra

The reference spectra were obtained by means of lambda stacks of cells single-marked with GFP and YFP, respectively. Top: Lambda-coded projections of a cell marked with GFP (left) or YFP (right). Bottom: Reference spectra for GFP (green) and YFP (red).

3 Linear Unmixing

Using the reference spectra from the lambda stack, the *Linear Unmixing* function generates a two-channel image, in which each channel represents only one of the two fluorochromes.
Top: GFP
Center: YFP
Bottom: Both channels superimposed

Spectral Imaging

Channel Unmixing

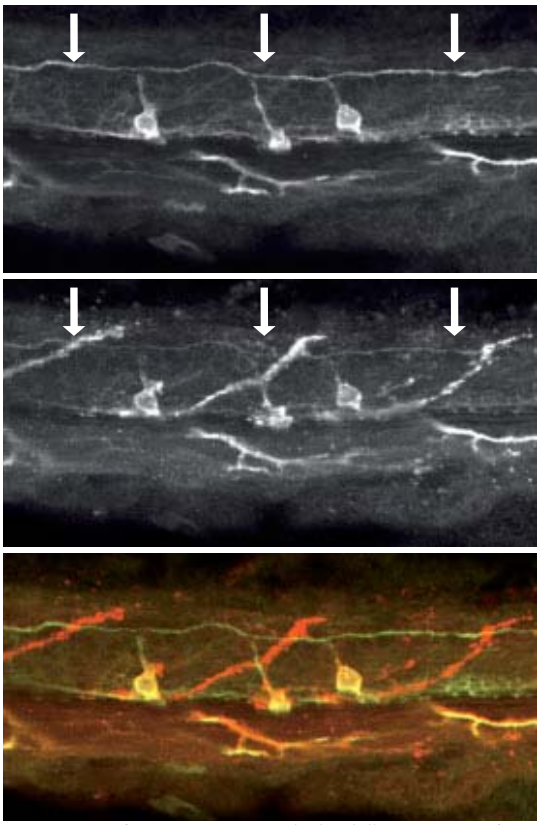
If the emission spectra of fluorescent markers overlap only slightly, the signals can be separated with the *Channel Unmixing* function.

As raw data for unmixing in this case, it is sufficient to have multichannel images in which one of the marker dyes dominates in each channel. Such images can be acquired without the META Detector, i.e. with an LSM PASCAL, LSM 510 or a CCD camera.

Channel Unmixing also allows unmixing based on the excitation behavior of dyes, if the raw data are multichannel images in which the channels differ only by their excitation wavelength.

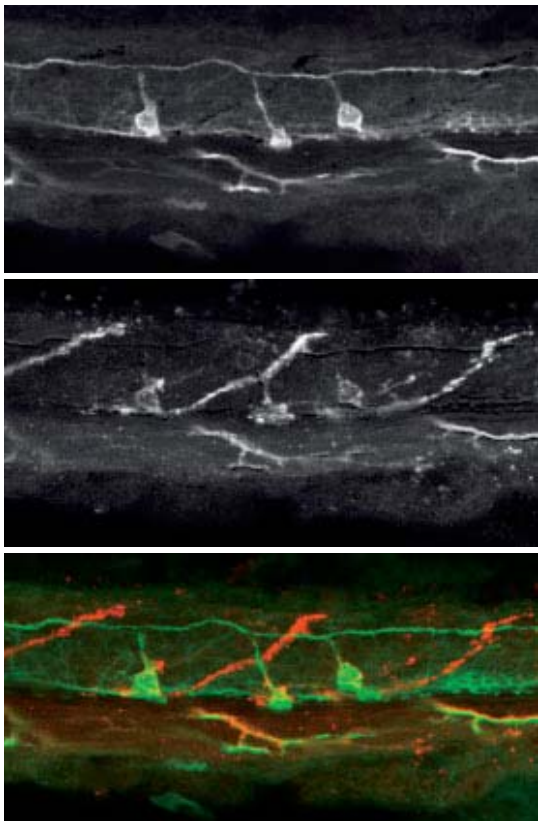
Double labeling of the nervous system of a zebra fish embryo

Two-channel single-track images with emission crosstalk. The nerve labeled with Alexa 488 can be discerned (arrows) in the green (top) and, faintly, in the red channel (center). Bottom: Superposition of the two channels.



Specimen: Prof. M. Bastmeyer, Friedrich Schiller University of Jena, Germany.

The same images after Channel Unmixing. The Alexa 488-positive nerve is visible in the green channel (top) only but vanished from the red one (Center). Bottom: Superposition of the two channels.

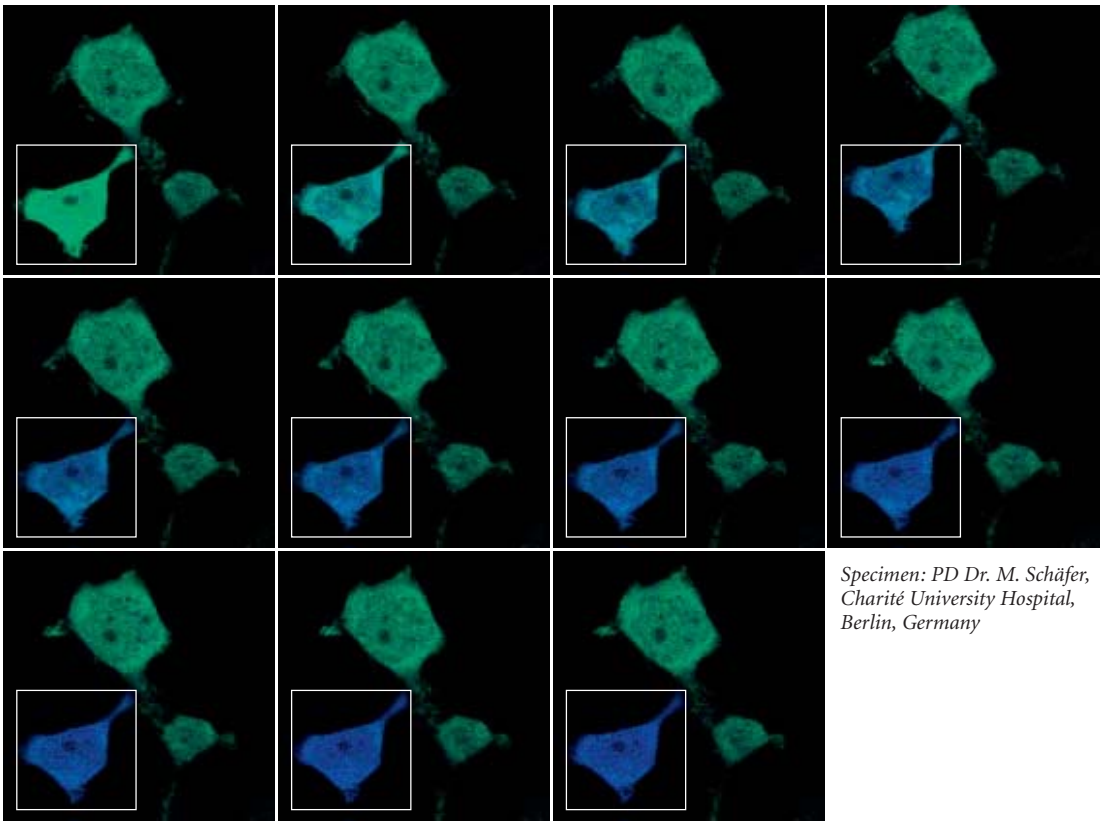


Online Fingerprinting

The functionality of *Online Fingerprinting* can be used to separate overlapping emissions even while a time series is being recorded. This may be of decisive importance where dynamic processes are investigated.

Here, a reference spectrum is assigned to each image channel before image acquisition starts. During the experiment proper, lambda stacks are acquired and immediately unmixed in a background operation. The user sees the unmixed multichannel image during the acquisition of the time series. *Online Fingerprinting* is of advantage especially in spectral FRET experiments and in studies of dynamic processes with fluorescent proteins.

Visualization of FRET by means of acceptor photobleaching
Expression of a FRET-positive protein construct (CFP linker citrin) in HEK 293 cells.
Recording conditions: Simultaneous excitation with 458 and 514 nm. Spectral detection from 462 to 655 nm in Lambda Mode.
Online Fingerprinting and simultaneous display of the two-channel image (CFP blue, citrin green).
During the combined time-&-bleaching series (bleaching region marked), citrin (green channel) as a FRET partner is destroyed by intensive irradiation with 514 nm. The decrease in FRET is visible as an increase in CFP fluorescence (blue channel).



Specimen: PD Dr. M. Schäfer, Charité University Hospital, Berlin, Germany

Spectral Imaging

Excitation Fingerprinting

By means of tunable excitation lasers such as those used in multiphoton systems, it is possible to detect also the excitation spectra of fluorochromes. These can be used for unmixing as an alternative to emission spectra.

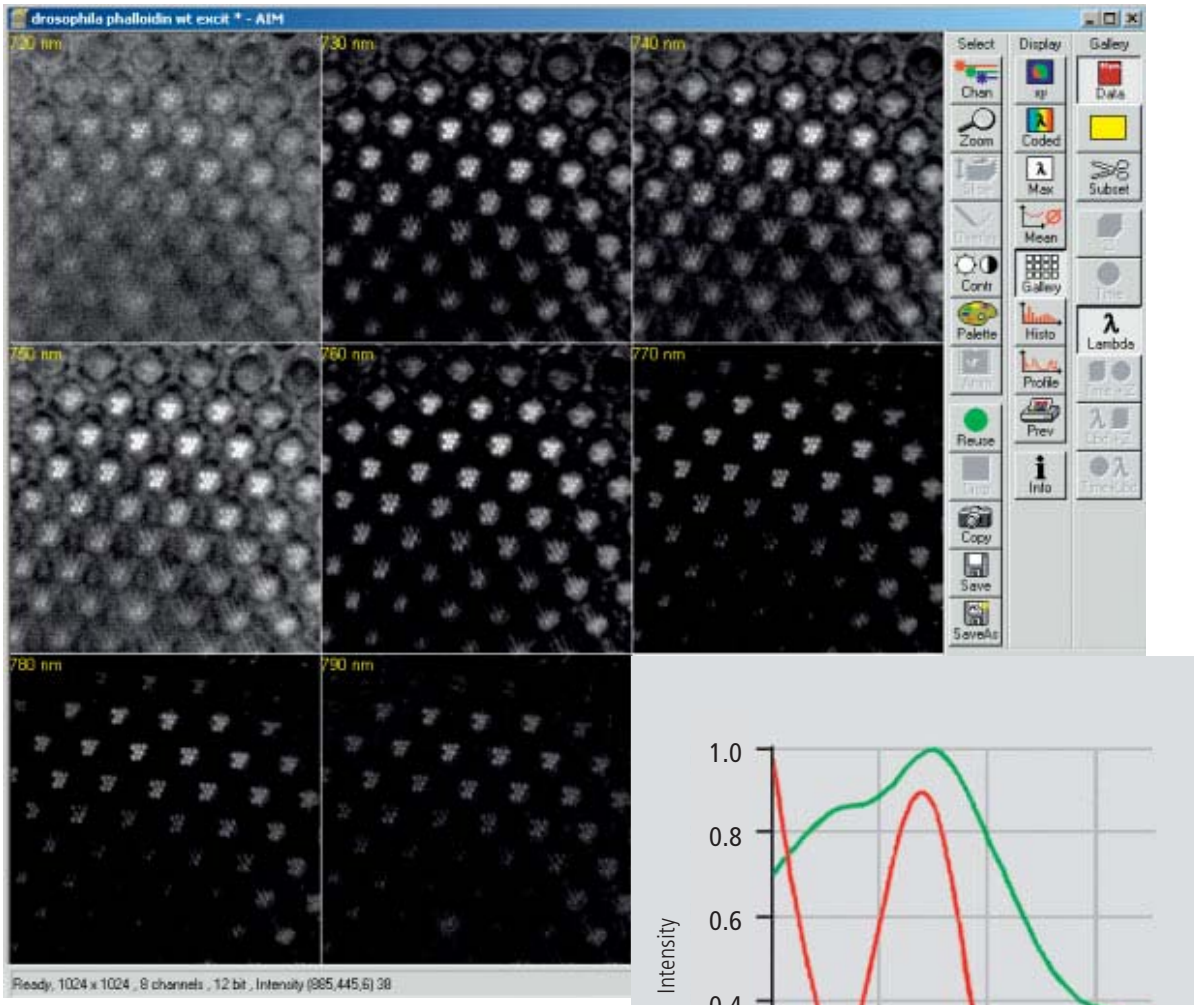
Multiphoton systems are a special class of confocal laser scanning microscopes, distinguished from classical one-photon systems essentially by an additional light source, known as a multiphoton or NLO (non-linear optics) laser.

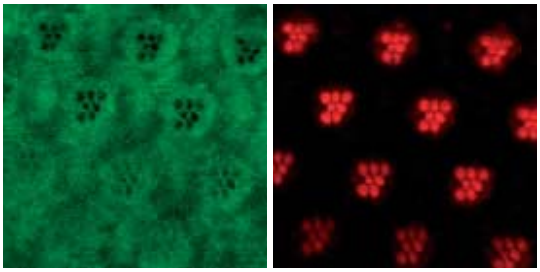
The infrared (IR) light emitted by such lasers can penetrate tissues to greater depths than visible light can. Due to its low phototoxicity, IR light is suitable for long-time observation of live samples. Usually, the emission wavelength of these lasers can be varied continuously to excite the respective fluorochrome used in the multiphoton mode.
In *Excitation Fingerprinting,* this property is used for the acquisition of excitation lambda stacks. For that purpose, the multiphoton laser is controlled by the LSM software to shift its excitation wavelength by a defined interval before every new image. The image stacks thus recorded can be used for the unmixing of spectral components differing by their excitation properties, analogously to the (emission) lambda stacks described before. For more information on multiphoton microscopy, refer to the literature cited on the rear cover.

The procedure for Excitation Fingerprinting

1. Define an excitation lambda stack (wavelength range and interval size) in the *Excitation Fingerprinting* macro
2. Record the excitation lambda stack
3. Define the reference spectra via single-labeled specimen regions, single-labeled reference samples, or by using the *ACE* function
4. Run *Linear Unmixing*

Excitation Fingerprinting separates widely overlapping emission signals by their excitation spectra.





Retina of a Drosophila fly, labeled for actin (Alexa Fluor 586 phalloidin); autofluorescence and emission signal can be cleanly separated by Emission Fingerprinting.

Specimen: PD Dr. O. Baumann, University of Potsdam, Germany