# Spectral Separation of Multifluorescence Labels with the LSM 510 META

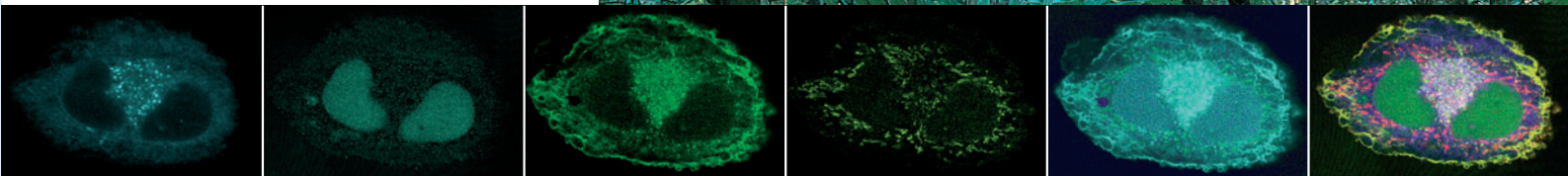*The tropical rain forest displays many hues of green.*

Indians living in the South American rain forest can distinguish between almost 200 hues of green in their multifarious environment. For conventional microscopical detection methods it can be hard even to separate the signals emitted by green and yellow fluorochromes without overlaps.

**Emission Fingerprinting** with the LSM 510 META is a completely new technique for the detection, spectral separation and visualization of multiple fluorescent labels. The technique precisely separates widely overlapping emission spectra and reliably eliminates broad-band autofluorescences. The potential of the LSM 510 META is demonstrated by application of Emission Fingerprinting to a sample tagged with four fluorescent proteins (Cyan FP, Cyan/Green FP, Green FP and Yellow FP).

*Wavelength-coded projections of spectral image stacks of CFP-, CGFP-, GFP- and YFP-labeled cells.*
*Quadruple-labeled cell; wavelength-coded projection of the spectral image stack.*
*Pseudocolor-coded, spectrally unmixed quadruple labeling.*



## Everything simply green?
## Not at all!

ZEISS

# The Problem

Multiple fluorescence labeling is an established method for the simultaneous visualization of different structural or functional features in biomedical samples. The investigation of complex structures and physiological processes requires many different sample parameters to be detected. Therefore there is a great demand for microscopical systems that can simultaneously visualize as many different fluorescent dyes as possible. Inevitably, this means that one has to accept increasingly broader overlaps between emission spectra having typical widths between 50 and 100 nm. Even if a spectral band width of 350 to 700 nm is available for detection, crosstalk between the emission signals will occur as soon as more than three dyes are to be visualized at a time.

In conventional detection systems, a spectral band is selected that detects the strongest possible signal of the desired dye and the weakest possible signal of the spectrally adjacent dyes. It is obvious that this compromise will fail under the requirements outlined above.

The problem aggravates if the samples are to be labeled with fluorescent proteins (XFPs). This labeling method has the advantage that live cells or even entire organisms can express XFPs bound to proteins. Since fluorescent proteins are non-toxic, they allow physiological processes to be visualized in live cells directly (Miteli & Spector, 1997). On the other hand, the spectral variation of the fluorescent proteins available at present is narrower than that of classical fluorochromes. Because of the widely overlapping emission spectra of CFP, GFP and YFP, triple labelings with these XFPs cannot be visualized separately by conventional methods (Fig. 1).
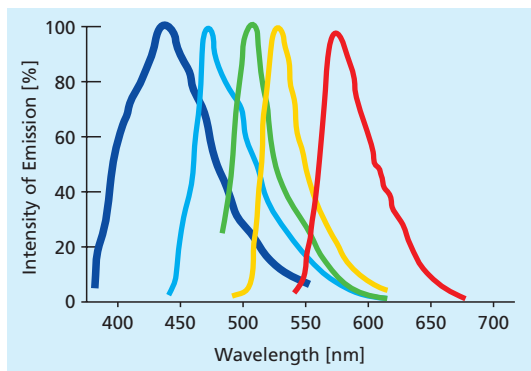


Fig. 1: Overlapping emission spectra of various fluorescent proteins
(Dark blue: BFP, Light blue: CFP, Green: GFP, Yellow: YFP,
Red: DS Red).
Source: www.clontech.com

# The Solution

The LSM 510 META solves the problem by an entirely new approach. It uses a method termed **Emission Fingerprinting**, by which spectrally separated images are obtained in three steps:

**1. Acquisition Lambda-Stacks**
**2. Definition of reference spectra**
**3. Spectral separation
    of the raw data**

This article will describe in detail how the LSM 510 META precisely separates and visualizes the emission signals of dyes with widely or almost completely overlapping emission spectra.

*Fig. 2: LSM 510 META in combination
with the Axiovert 200 M
(side- and baseport configuration),
Axioplan 2 Imaging MOT
and Axioskop FS (with Non Descanned
Detection; NDD) microscopes.*

## Samples and Imaging

**Double labeling** with GFP and FITC: Fixed NIH 3T3 cell culture with cover slip. Cell nuclei labeled with GFP-histone B2, actin filaments labeled with FITC-phalloidine. Sample provided by courtesy of Mary Dickinson of the Caltech Institute, Pasadena, USA (see page 8).

**Quadruple labeling** with CFP, CGFP, GFP and YFP: Fixed HE 393 cell culture with cover slip. The cells were transfected with four different fluorescent proteins labeling different cellular structures (CFP cytoplasm, CGFP nuclei, GFP cell membrane, YFP mitochondria). In addition to the quadruple-labeled samples, four single-labeled ones were available for acquiring the reference spectra. Sample provided by courtesy of A. Miyawaki of the Riken Institute, Wako, Japan (see page 10).

**Imaging:** LSM 510 META UV/VIS confocal laser scanning microscope with spectral detector, release 3.0. Objective lens: 63x/1.4 Oil Plan-Apochromat. Excitation laser lines: 458 nm (70%) and 488 nm (4%). Main dichroic beamsplitter: NT 80/20. Thickness of optical section: 0.8 µm at 543 nm.
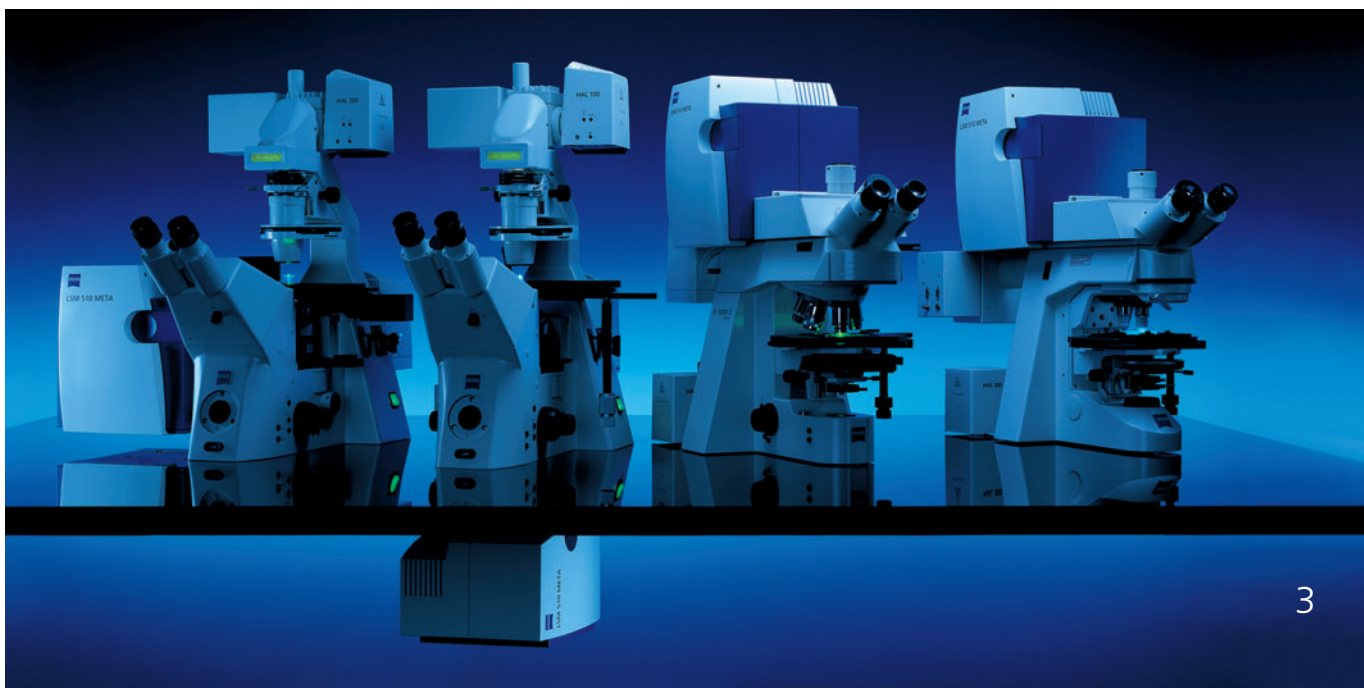
# How Does
# Emission Fingerprinting Work?

**The Principle**

Emission Fingerprinting is a completely novel approach to analyzing multiple fluorescences. Rather than attempting to separate individual spectral bands, this technique detects and spectrally resolves the total fluorescent light emitted by the sample. For the subsequent spectral separation, the technique uses the profile of the spectrum rather than the intensity of a spectral band. This results in pseudo-color-coded multichannel fluorescence images, each of which contains the emission signal of a single channel, i.e. of a single dye. This also applies to fluorochromes having almost completely overlapping emission spectra. The entire process of Emission Fingerprinting consists of three steps:

**1. Acquisition of Lambda-Stacks**

Lambda Stacks are the raw data of Emission Fingerprinting. As a rule, these are three-dimensional image stacks having the coordinates X, Y and lambda ($\lambda$). Each individual image of the stack represents a spectrally defined band of the emission signal, i.e. the information sensed by one element of the META detector (Fig. 3). The spectral bandwidth of a channel is 10.7 nm at minimum. For the investigation of three-dimensional objects or dynamic processes, the acquisition of Lambda Stacks can be combined with that of Z stacks and/or time series.
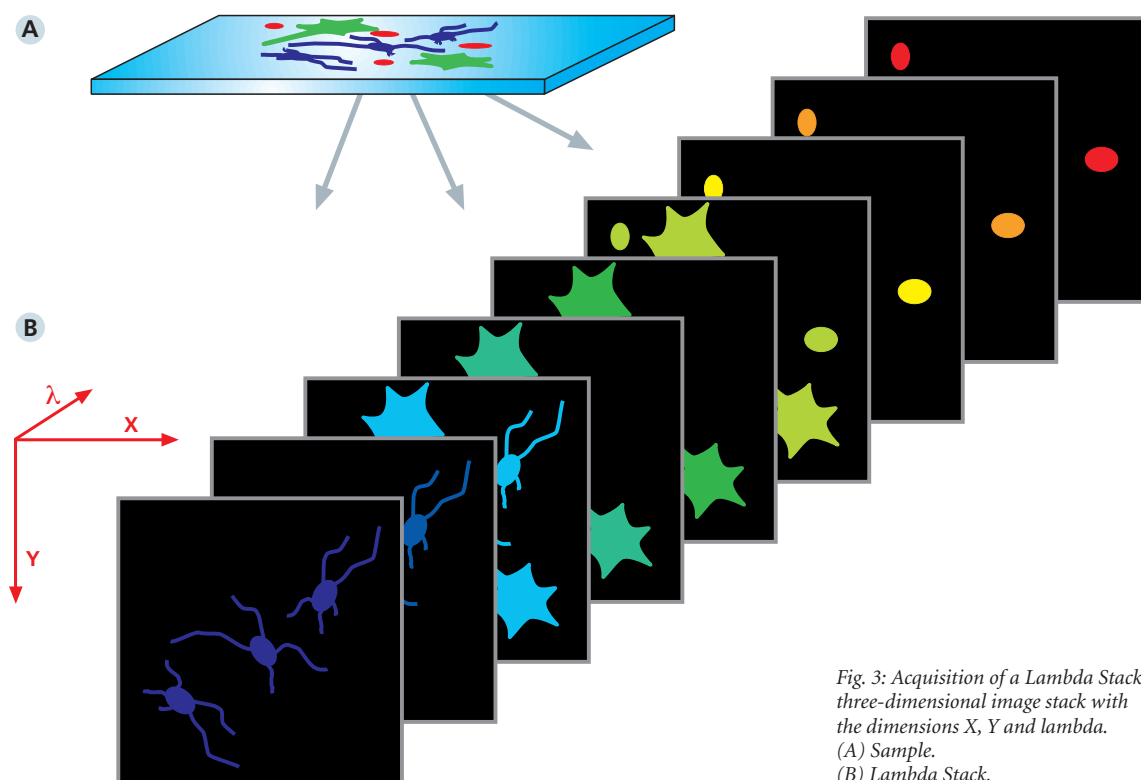


*Fig. 3: Acquisition of a Lambda Stack; three-dimensional image stack with the dimensions X, Y and lambda.*
*(A) Sample.*
*(B) Lambda Stack.*

## 2. Definition of Reference Spectra

Reference spectra are the emission spectra of individual fluorochromes under sample conditions. These spectra are required for the spectral separation of multiple fluorescence labels. Reference spectra can be acquired by either of two methods, depending on the nature of the sample:

• If, in a Lambda Stack, regions can be defined which are labeled by a single dye only, the reference spectra of these dyes can be established directly from the Lambda Stack of the sample. This method is applicable especially if the sample has morphologically distinct structural elements with different tags (Fig. 4).
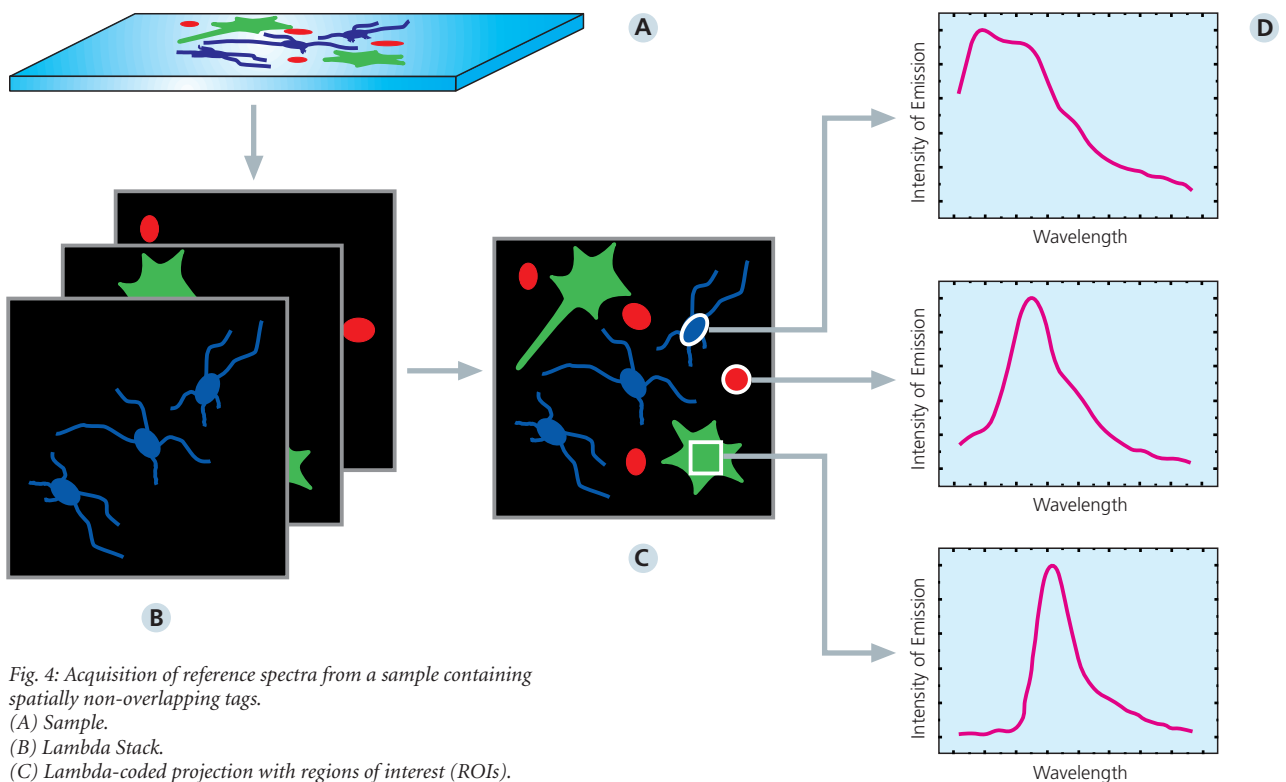


Fig. 4: Acquisition of reference spectra from a sample containing spatially non-overlapping tags.
(A) Sample.
(B) Lambda Stack.
(C) Lambda-coded projection with regions of interest (ROIs).
(D) Spectral signatures of the dyes.

• In samples not containing regions with single tags, reference spectra have to be obtained in another way. This is the case where the labeled structures are either very small or overlap. Also, various tags may be localized in the same subcellular compartment (e.g., proteins that contain a target sequence for the nucleus, marked with different fluorochromes).

Under such conditions, reference spectra can be defined by the acquisition of Lambda Stacks of several samples, each of which has been labeled with a single dye. Each sample thus provides a single reference spectrum; all of them are filed in a spectral database (Fig. 5).
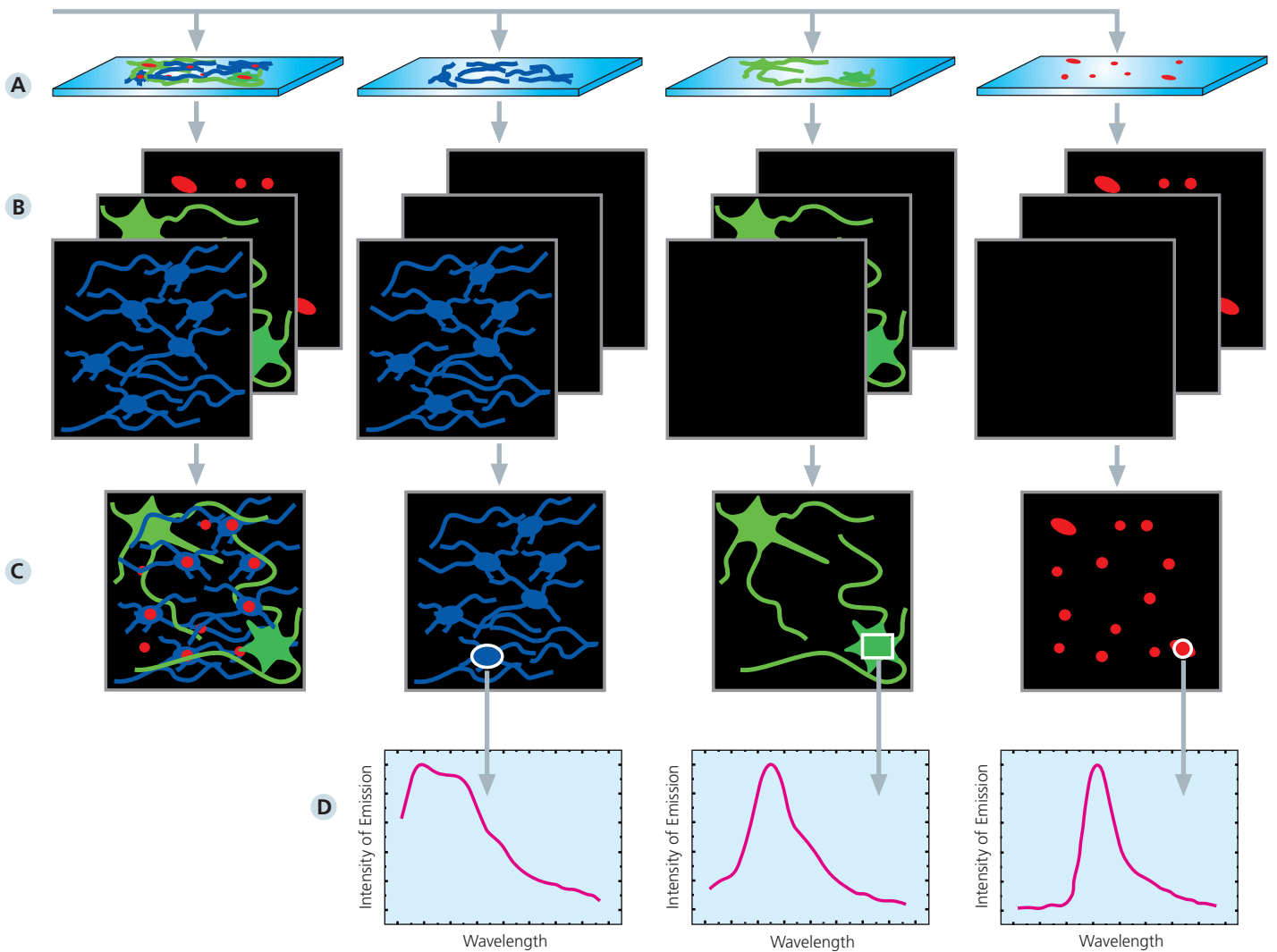
*Fig. 5: Acquisition of reference spectra from a sample containing spatially overlapping tags.*
*(A) Sample and three reference samples.*
*(B) Lambda Stacks.*
*(C) Lambda-coded projections with regions of interest (ROIs).*
*(D) Spectral signatures of the dyes.*

$$S(\lambda)_{sum} = \left[ \text{Intensity}_{dye\,A} \cdot S(\lambda)_{dye\,A} \right] + \left[ \text{Intensity}_{dye\,B} \cdot S(\lambda)_{dye\,B} \right] + \left[ \text{Intensity}_{dye\,C} \cdot S(\lambda)_{dye\,C} \right]$$

## 3. Spectral Separation of the Raw Data

For spectral separation, Lambda Stacks of the sample and the reference spectra of the dyes to be expected in the sample are required. The *Linear Unmixing* software function provided for spectral separation activates a linear algorithm (Landsfort et al., 2001), which computes, for each pixel, the intensities of the emission signal of the dyes used. Consider a pixel representing a sample point at which the three dyes A, B and C with spectra $S(\lambda)_{dye\,A,\,B\,and\,C}$ overlap,

the cumulative spectrum measured there $[S(\lambda)_{sum}]$ can be expressed as shown in the equation above. With the known reference spectra $[S(\lambda)_{dye\,A,\,B\,and\,C}]$, the equation can be solved to find the intensities$_{A,\,B\,and\,C}$. The result is presented by means of a pseudocolor-coded multichannel image, with each channel representing exactly one dye. The examples on the following pages show that the method is also applicable to dyes whose spectra overlap almost completely. Fig. 6 provides an overview of the Emission Fingerprinting procedure.
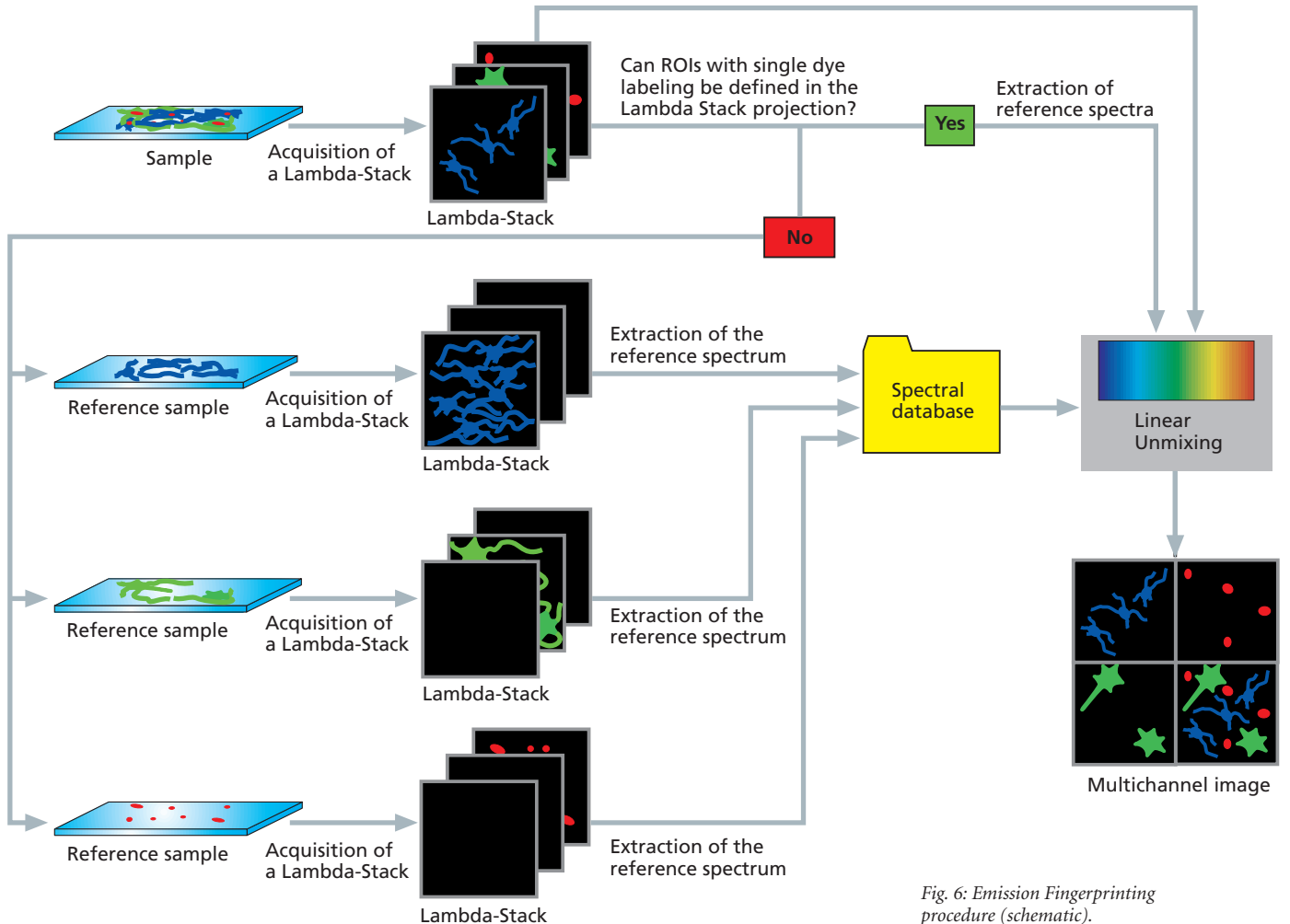


*Fig. 6: Emission Fingerprinting procedure (schematic).*

# Emission Fingerprinting in Practice

Two examples illustrate how widely overlapping emission signals can be separated by means of the Linear Unmixing function.

### GFP and FITC double labeling

Nuclei of the cells were stained with SYTOX Green, whereas the actin filaments were stained with FITC-coupled phalloidine (specimen: M. Dickinson, Biological Imaging Center, Caltech, Pasadena, USA). As both tags mark clearly separate, morphologically distinct structures, it was possible to extract the spectra required for separation directly from the Lambda Stack of the double-labeled sample. In the first step, an eight-channel Lambda Stack was obtained (498-573 nm, spectral bandwidth per channel 10.7 nm) (Fig. 7 A). In the lambda-coded projection (Fig. 7 B), the individual images of the Lambda Stack are color-coded according to their respective wavelengths, and projected in superimposed fashion. This presentation illustrates the problem of spectrally overlapping emission signals. With lambda coding alone, no statement is possible as to which dye is present in which part of the sample. Two regions of interest were selected, with only the cell nucleus selected in one (SYTOX Green labeling, ROI 1, red), and only the actin filaments in the other (FITC labeling, ROI 2, green). The left part of the window shows the mean spectral distribution of the intensities of these ROIs. Either of the two spectra represents the fingerprint of a fluorescent label under sample conditions (the spectral location of the two maxima differs by only 7 nm!). The spectra thus obtained are then subjected to spectral separation. With the *Linear Unmixing* function selected, the software computes a two-channel image (Fig. 7 C), in which either channel contains only the pixels corresponding to the spectral fingerprint of ROI 1 or ROI 2, respectively. The colors used for the ROI frames and spectral curves have been used for coding the respective channels.
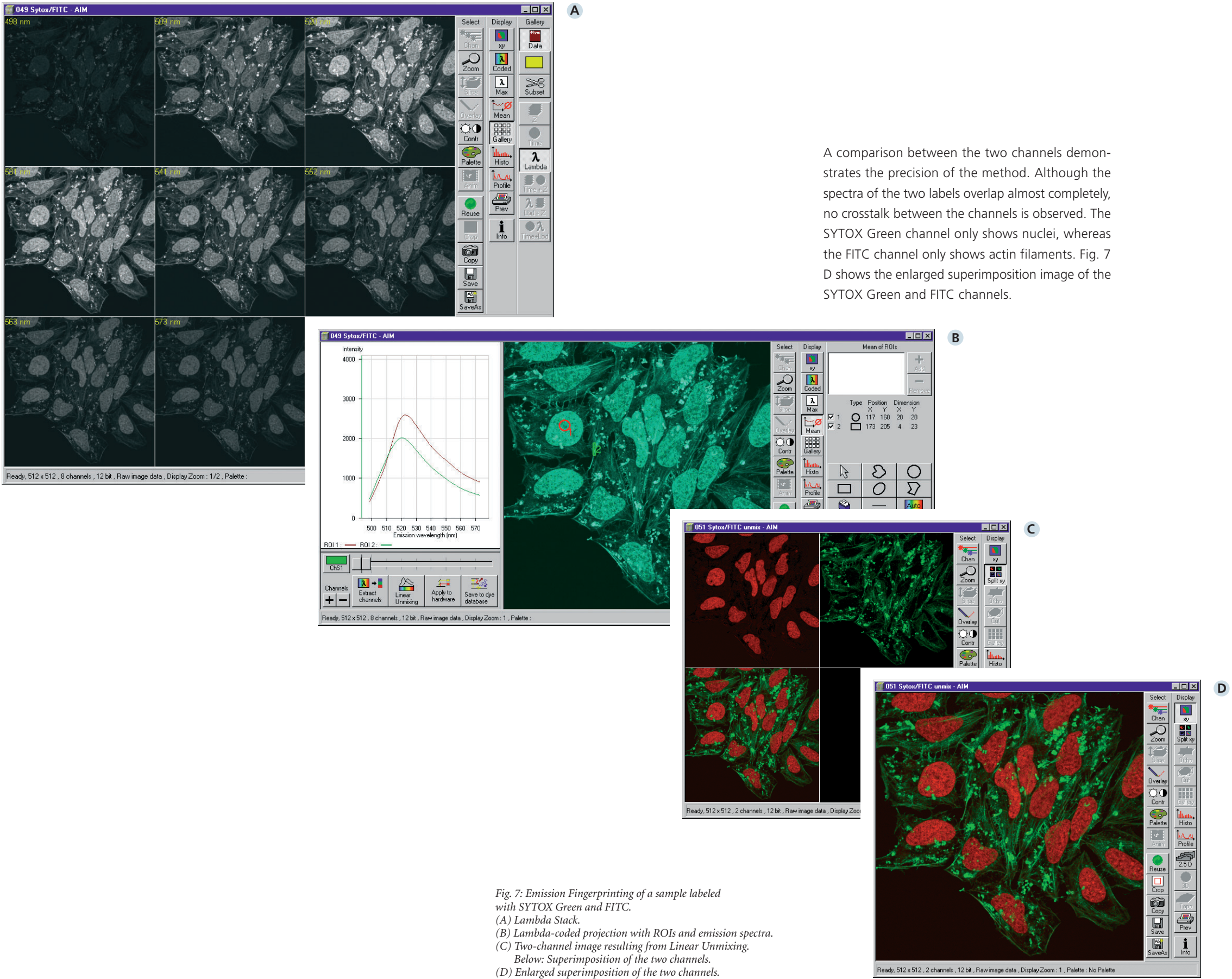
A comparison between the two channels demonstrates the precision of the method. Although the spectra of the two labels overlap almost completely, no crosstalk between the channels is observed. The SYTOX Green channel only shows nuclei, whereas the FITC channel only shows actin filaments. Fig. 7 D shows the enlarged superimposition image of the SYTOX Green and FITC channels.

*Fig. 7: Emission Fingerprinting of a sample labeled with SYTOX Green and FITC.*
*(A) Lambda Stack.*
*(B) Lambda-coded projection with ROIs and emission spectra.*
*(C) Two-channel image resulting from Linear Unmixing.*
*    Below: Superimposition of the two channels.*
*(D) Enlarged superimposition of the two channels.*

## CFP, CGFP, GFP and YFP quadruple labeling

Whereas in the previous example reference spectra were obtained directly from the Lambda Stack, here is an example of the use of a spectral reference database for spectral separation. The sample is a HeLa cell culture in which four different cellular targets were labeled with the fluorescent proteins CFP (cytoplasm), CGFP (nucleus), GFP (cytoplasmic membrane), and YFP (mitochondria) (specimen: A. Miyawaki, Riken, Japan). Given the high degree of *spatial* overlap between the tags, the *Linear Unmixing* procedure required the acquisition of reference spectra from samples labeled with a single

dye each. A Lambda Stack was acquired from each sample (spectral range 471-631 nm, 16 channels of 10.7 nm bandwidth each) (Fig. 8 A). The lambda-coded projections of these stacks (Fig. 8 B) are suitable for defining ROIs from which the reference spectra can be collected (Fig. 8 B,C). With the *Save to dye database* dialog, the spectra can now be filed in a spectral reference database (Fig. 8 D) where they are available for *Linear Unmixing* later.

In the next step, a Lambda Stack of the quadruple-labeled sample is acquired (Fig. 8 E,F). Irrespective of the presentation mode selected - *Gallery* (Fig. 8 E) or *Lambda coded* (Fig. 8 F) - no information about which cellular structures are labeled with which dyes can be extracted here. Even with maximum flexibility
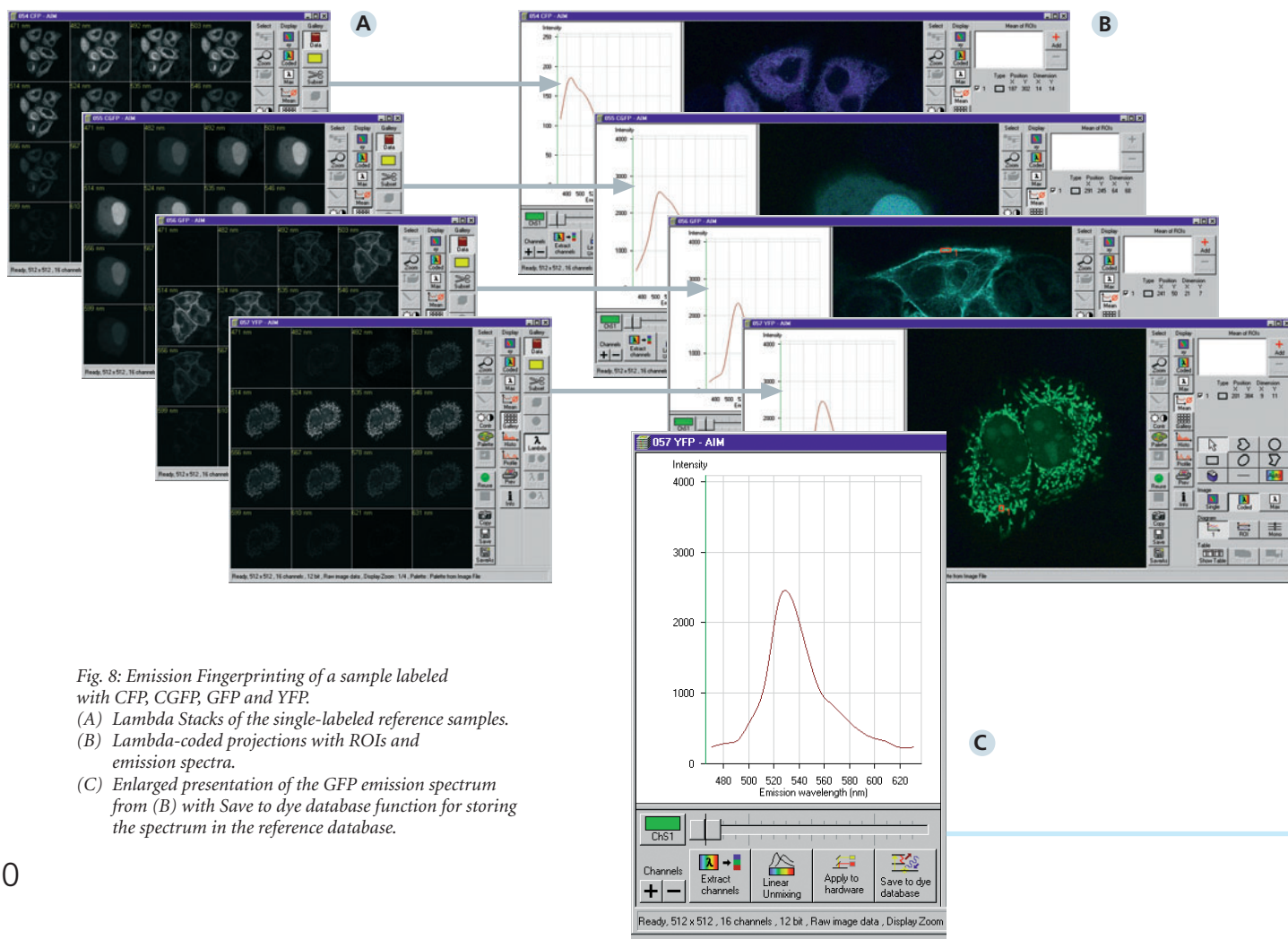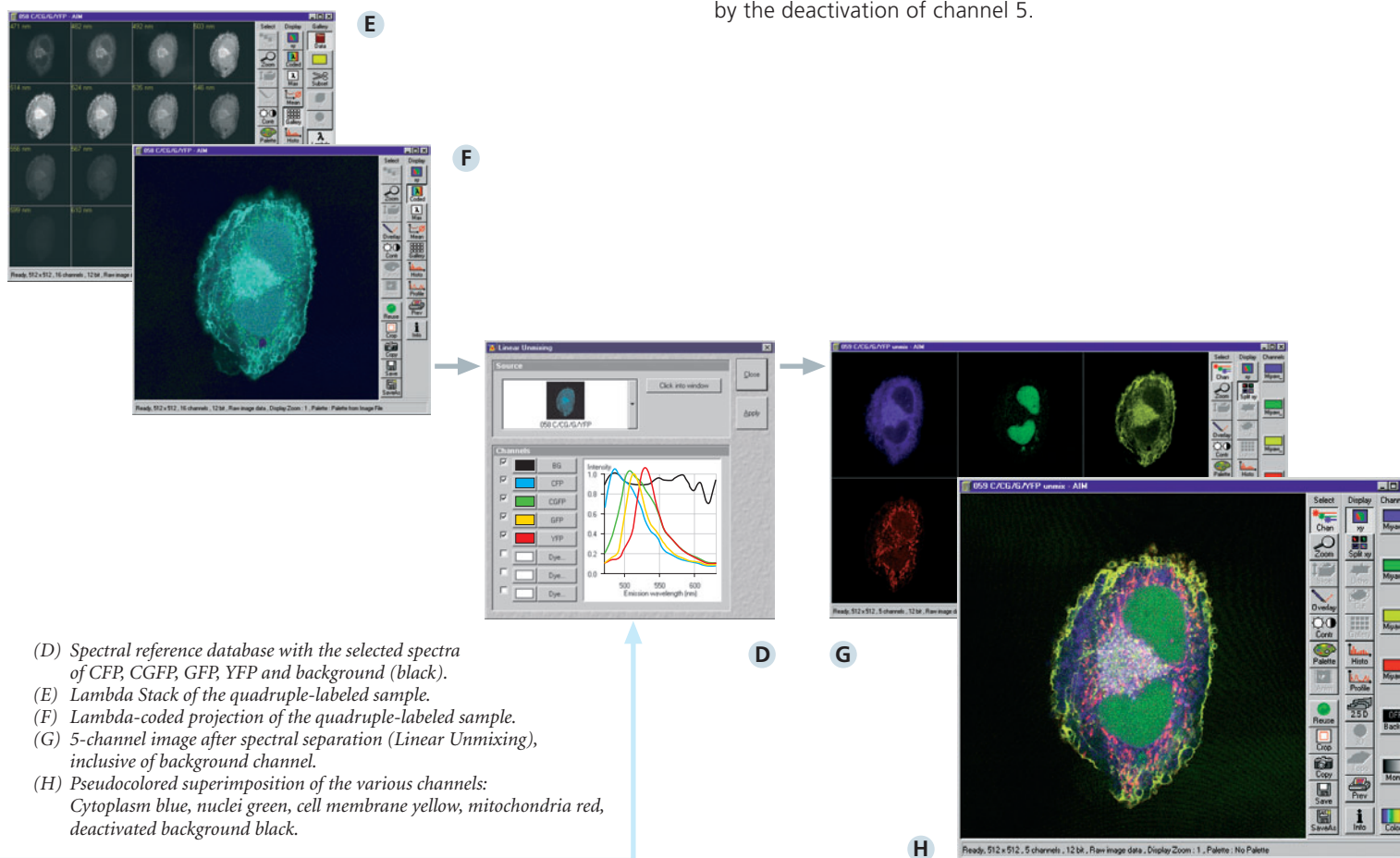


*Fig. 8: Emission Fingerprinting of a sample labeled with CFP, CGFP, GFP and YFP.*
*(A) Lambda Stacks of the single-labeled reference samples.*
*(B) Lambda-coded projections with ROIs and emission spectra.*
*(C) Enlarged presentation of the GFP emission spectrum from (B) with Save to dye database function for storing the spectrum in the reference database.*

in the definition of bandpass filters, the widely overlapping emissions would not allow any gain in information. To optimize the subsequent Linear Unmixing, the spectral distribution of the background signals was also determined and saved it to the database as an additional reference spectrum.

The Lambda Stack of the quadruple-labeled sample is spectrally separated by means of the *Linear Unmixing* dialog in the *Process* menu. Selection of the Lambda Stack to be separated (Fig. 8 D, small window at top left) is followed by loading the previously established reference spectra and the background spectrum from the database. The presentation of the overlapping spectra again illustrates the impossibility of separating the emission signals with bandpass filters. The Linear Unmixing algorithm is started with *Apply* (Fig. 8 D).

The result of Linear Unmixing is a multichannel image in which each channel exactly represents one tag (Fig. 8 G). The first four channels show the emission signals of CFP, CGFP, GFP and YFP (channel arrangement: top row left to right, then bottom row). Channel 5 is the background signal. Channel 6 (shown enlarged in Fig. 8 H) is a superimposition of channels 1 through 5. The various channels are distinguished by freely selected pseudocolors. Only in this way is it possible to distinguish between the differently labeled structures; cytoplasm, nuclei, membrane structures and mitochondria can now be identified. In the channel superimposition, additional background correction was performed by the deactivation of channel 5.

(D) Spectral reference database with the selected spectra of CFP, CGFP, GFP, YFP and background (black).
(E) Lambda Stack of the quadruple-labeled sample.
(F) Lambda-coded projection of the quadruple-labeled sample.
(G) 5-channel image after spectral separation (Linear Unmixing), inclusive of background channel.
(H) Pseudocolored superimposition of the various channels: Cytoplasm blue, nuclei green, cell membrane yellow, mitochondria red, deactivated background black.

# Outlook

The LSM 510 META opens up a new dimension in confocal fluorescence microscopy. Experimental designs such as double labeling with Sytox Green and GFP, whose signal peaks could not be detected separately so far, will become routine. As the example has shown, real quadruple-fluorescence-labeled samples can be unmixed without any problems. The LSM 510 META can unmix the spectral signatures of up to eight fluorochromes. Looking ahead, we can already envisage samples that will exhaust the capabilities of the system and thus stimulate the development of yet another generation of microscopes.

**REFERENCES**

**Miteli, T., Spector, D.L.** (1997) Applications of green fluorescent protein in cell biology and biotechnology. Nature Biotechnol. 15, 961.

**Sawano, A., Miyawaki A.** (2000) Directed evolution of green fluorescent protein by a new versatile PCR strategy for site directed and semi-random mutagenesis. Nucleic Acids Res Aug 15, 28 (16), E 78.

**Landsford, R., Bearman, G. and Fraser, S.E.** (2001) Resolution of multiple green fluorescent protein color variants and dyes using two photon microscopy. Journal of Biomedical Optics 6, 311-318.

**Dickinson, M.E., Bearman, G., Tille, S., Landsford and Fraser, S.E.** (2001) Multi-spectral imaging and Linear Unmixing add a whole new dimension to Laser scanning fluorescence microscopy Bio Techniques 31/6, 1272-1278.

For further details, please contact: