VOLUME 3
APRIL
2002

G.I.T.

1/02
PP. 8–11

REPRINT

SPECIAL EDITION OF G.I.T. LABORATORY JOURNAL · BIOFORUM INTERNATIONAL

IMAGING & MICROSCOPY

RESEARCH • DEVELOPMENT • PRODUCTION

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GIT VERLAG
A Wiley Company
D-64220 Darmstadt
www.gitverlag.com

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Emission Fingerprinting with the LSM 510 META

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Multifluorescence microscopy is an established and widely used technique to visualize and discriminate between multiple structural and functional elements in biological samples ranging from single cells to whole organisms. Traditionally, the differentiation of multiple fluorochromes introduced into a probe is accomplished by assigning a spectral band to each fluorescent species. This is commonly realized by using a set of optical bandpass filters to select the individual emissions. However, even in simple systems with two or three fluorochromes, crosstalk between emission channels may limit the ability to distinguish the signals with confidence (Fig. 1). Such overlap of emissions requires, in many cases, the selection of narrow bands within the emission spectra for detection. As a result, a significant part of the light to be detected is discarded and the intensities of the signals of interest are considerably reduced. Overall, only a small number of fluorochrome combinations is available for efficient separation with optical bandpass filters.

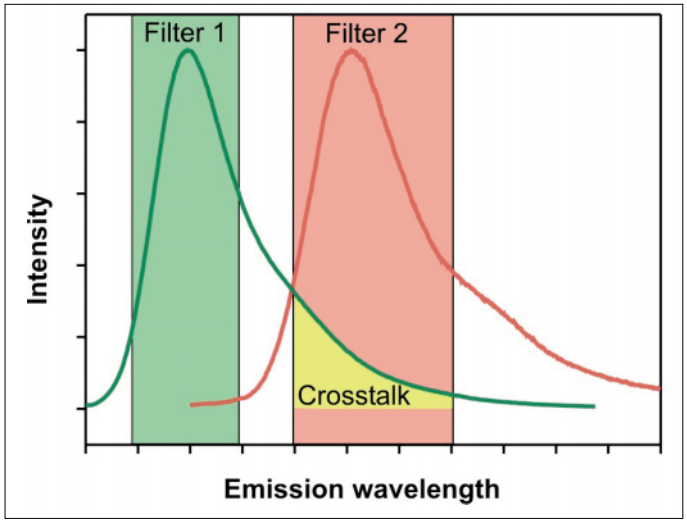
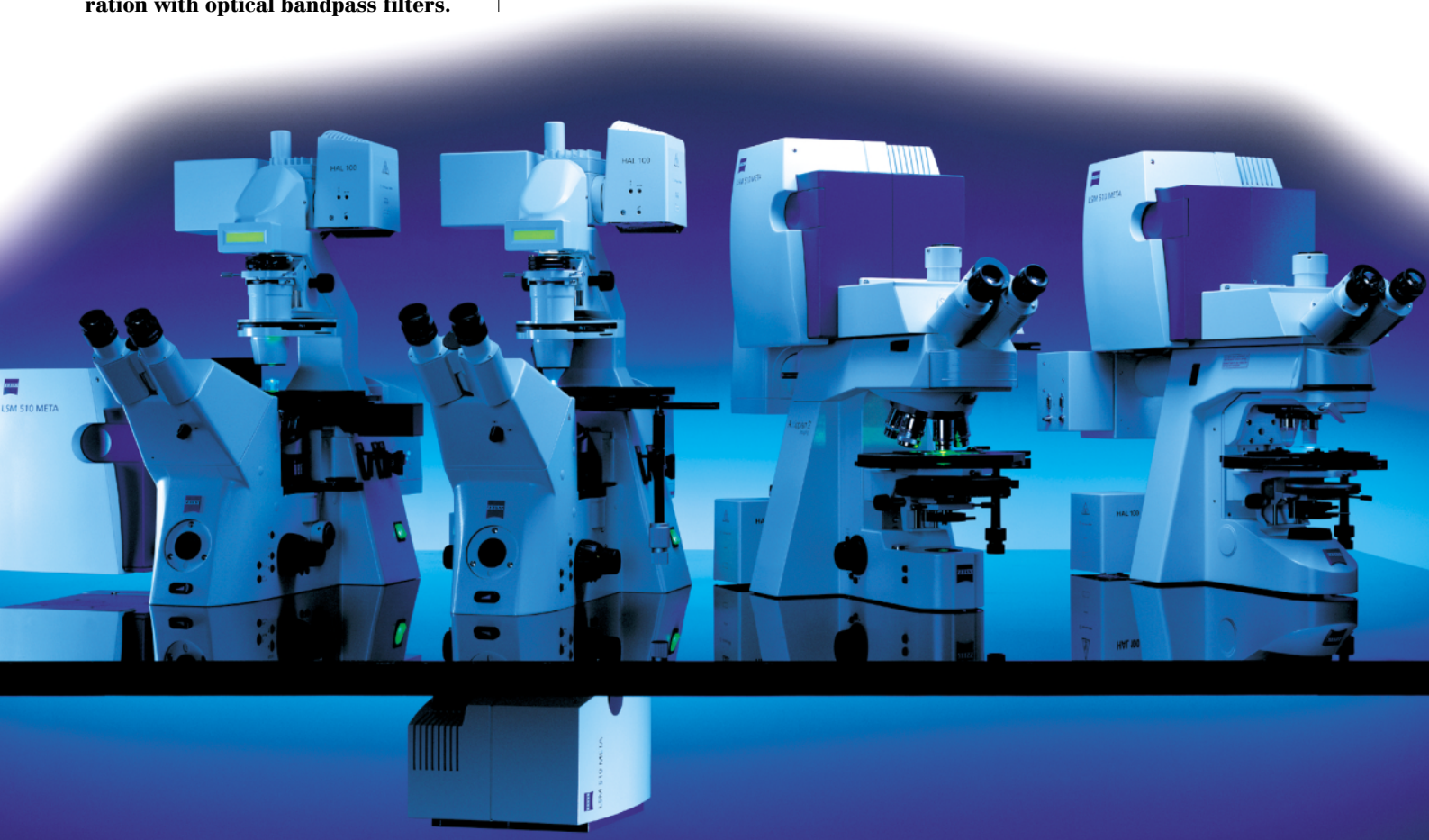


Fig.1: Selection of emission signals using traditional bandpass filter technology. Emission crosstalk between two fluorophores, represented by green and red emission spectra, is indicated by the yellow area. Shaded areas symbolize bandpass filters used to separate the signals.

Multitracking for crosstalk reduction

In recent years, confocal laser scanning microscopy helped to overcome some of these limitations. One solution originally introduced with the LSM 510 from Carl Zeiss is Multitracking, a method that uses frame-wise or extremely fast line-wise switching between excitation laser lines. Alternate scans are used to avoid the simultaneous excitation of and, hence, emission from the fluorophores. This approach is ideal for those applications in which fluorophore combinations differ with respect to their excitation profiles such as Fluorecein- and Rhodamine-type dyes. However, it is not



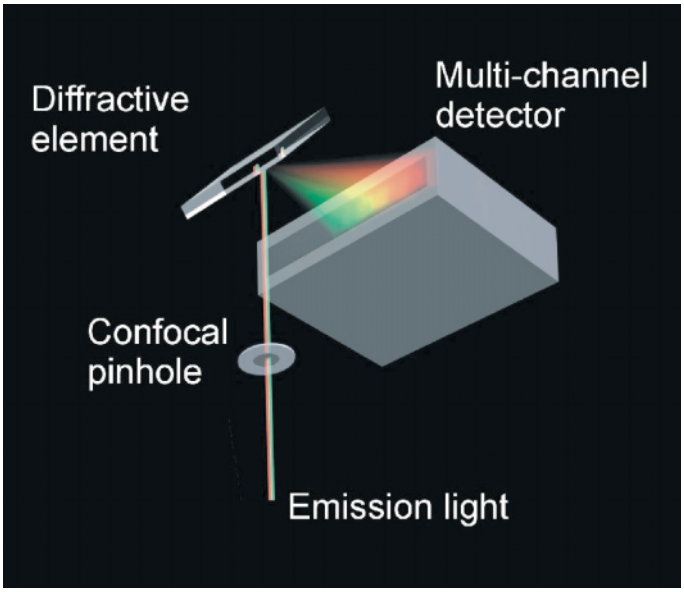


Fig. 2: Schematic representation of the META detection module of the LSM 510 META.

suited to separate multiple emissions excited by a single laser line and not applicable to multi-photon microscopy.

Emission Fingerprinting – the innovative approach

A completely novel approach realized in the new LSM 510 META overcomes this dilemma and opens up a plethora of new experimental designs by allowing to separate even signals with extremely overlapping emission profiles. The new technique combines innovative confocal detector technology with intelligent processing in a method called Emission Fingerprinting. This technique no longer attempts to optically separate signals into channels defined by non-overlapping spectral bands, but is based on the initial recording of the complete complex emission signals originating from the labeled sample. Using an optical diffractive element, the system splits the fluorescent light that has passed the confocal pinhole into its spectral components. These are projected onto a multi-channel detector consisting of 32 photo-multiplier elements collecting photons across the whole visible spectrum (Fig. 2). Parallel recording of the signals detected by these simultaneously illuminated elements results in a Lambda Stack (or a series thereof) representing the complete spectral distribution of the fluorescence signals for every point of the confocal microscopic image.

Knowledge of these spectral signatures is then used for digital separation of the fluorescence emissions. This is based on linear comparisons of the spectral emission profiles with reference spectra characterizing the individual fluorochromes present in the sample. Reference spectra may either be derived from singly labeled control specimens and stored in a spectra database or directly taken from the experimental sample by selecting Regions of Interest. Since all functions needed for this process are implemented the LSM 510 META software, it is accomplished via a simple 3-step procedure (Fig. 3):

1. Acquire a Lambda Stack.
2. Select Reference Spectra characterizing the spectral emission properties of up to 8 fluorophores in the sample.
3. Start unmixing the signals by the click of a button.

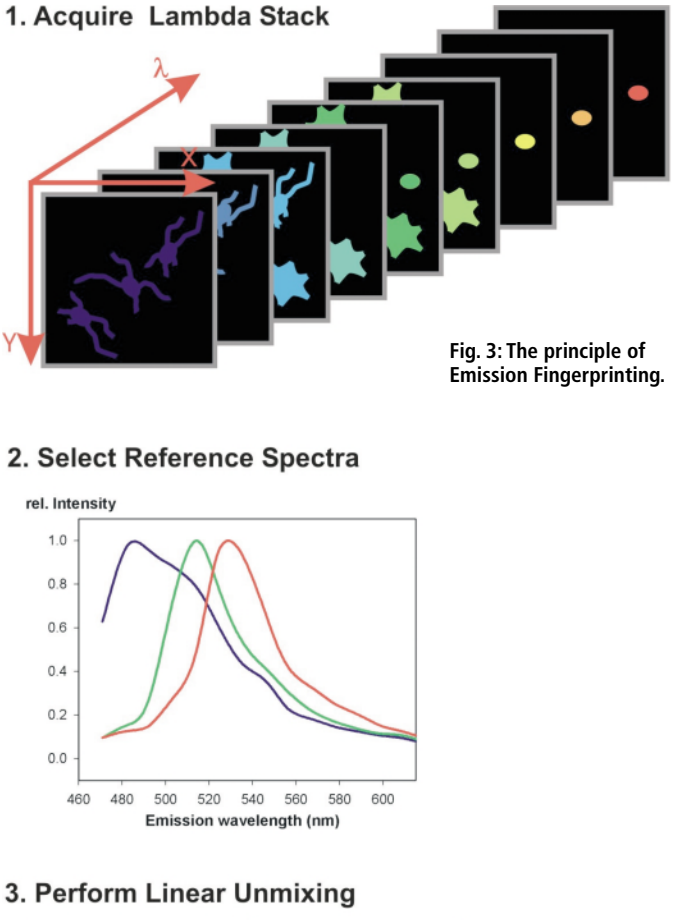


Fig. 3: The principle of Emission Fingerprinting.

The result is a multi-channel image with every channel representing the quantitative distribution of an individual fluorochrome for every voxel in the image. Since the design of the META detection module permits sampling of emissions over the whole visible spectrum, any fluorophore emissions in this range may be collected by electronic activation of the corresponding detector elements. Electronic selection not only guarantees stable recording, but also eliminates the need to sequentially step through individual bands to obtain a Lambda Stack. This reduces the total exposure to the exciting light and minimizes the detrimental effects of phototoxicity and photobleaching. Following this approach, experimental designs that have so far been out of reach can now be managed routinely. For instance, samples labeled with SYTOX® Green and FITC, two fluorochromes with nearly identical emission profiles, emission peaks only about 7 nm apart and so far impossible to discern, may now be separated with confidence (Fig. 4). Likewise, fluorescent proteins, today commonly used as non-toxic tags of proteins of interest, may be used in, so far, unrealistic combinations. As depicted in an example provided by Drs. Miyawaki and Hirano from the RIKEN Institute in Wako, Japan, Emission Fingerprinting of CFP, CGFP, GFP and YFP, all expressed in different sub-compartments of a single cell, results in clear signal separation despite heavy spectral and spatial overlap (Fig. 5).

Ideal for FRET and ion imaging

Applying the Emission Fingerprinting approach furthermore successfully eliminates broadband background- and

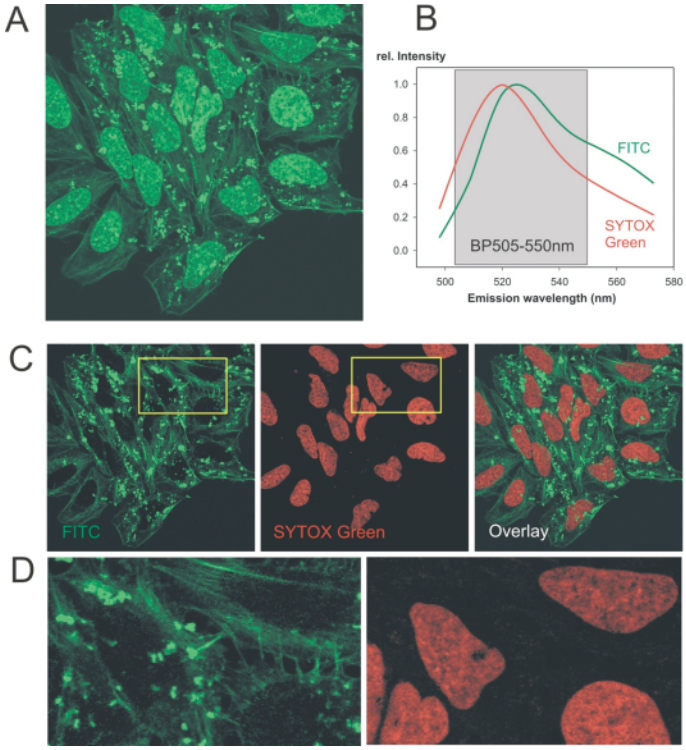


Fig. 4: Emission Fingerprinting with the LSM 510 META applied to cultured cells labeled with SYTOX Green (nuclei) and FITC-coupled Phalloidin (actin filaments). A: Sample as imaged through a 505-550nm bandpass filter. B: Emission spectra of SYTOX Green and FITC recorded with the LSM 510 META. C: Result of Linear Unmixing using the Reference Spectra depicted in B. D: Enlarged portions of the images shown in D. (Drs. M. Dickinson, S. Fraser, Caltech, Pasadena, USA).

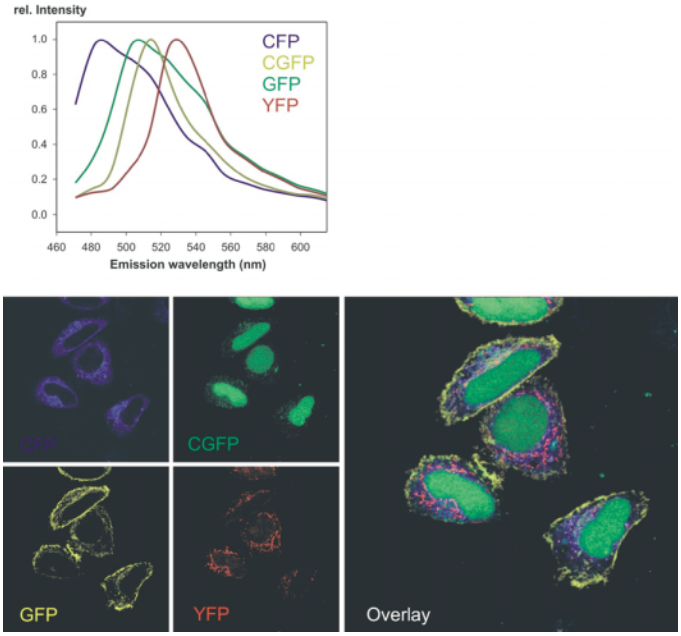


Fig. 5: Emission Fingerprinting performed on cells simultaneously expressing fluorescent proteins CFP (blue), CGFP (green), GFP (yellow), and YFP (red) in endoplasmic reticulum, nuclei, plasma membranes, and mitochondria, respectively, results in clear signal separation (Drs. A. Miyawaki and Hirano, RIKEN, Wako, Japan).

autofluorescence that often restricts or even prevents the analysis of biological samples. Another exciting field of applications relates to the imaging of fluorescence resonance energy transfer (FRET) and probes whose emission profiles change in an environmentally sensitive manner, such as exploited with many ion sensitive probes (Indo-1, SNARF, etc.). Signals from both, synthetic ion sensing emission ratio dyes and the recently developed fluorescent protein-based FRET sensors are composed of two spectrally overlapping components representing the ion-free and ion-bound states or the FRET donor and acceptor, respectively. Using conventional filter-based technology, signal crosstalk between the detection channels defined on the basis of bandpass filters negatively affects the dynamic range of the detection system. This, ultimately, contributes to defining the detection limits in either of these cases. As with the multifluorescence approaches discussed above, Emission Fingerprinting eliminates the need to discard photons containing useful information. Instead Emission Fingerprinting first registers the complete signals derived from the fluorophores or fluorophore states and then distributes these into separate channels making them accessible for subsequent analysis.

Easily upgraded

As implemented in the LSM 510 META, the META detection module replaces one of the conventional detectors and can be used in combination with the other on-board single detectors, all with individually positionable and adjustable pinholes for optimum signal yield and image quality. Combined with z-series and time series collection, spectral imaging using the META detector expands the dimensions available to confocal microscopy. Thus, it is ideally suited to provide detailed information on the spatio-temporal relationships of structures and molecules of interest. Furthermore, binning of multiple detector elements on the META detector into a single output channel may be used to define a spectral detection band of choice. Already installed LSM 510 systems may be upgraded with the new detector module, making the new technology accessible to existing LSM 510 users.

Conclusion

The LSM 510 META with integrated Emission Fingerprinting functions is a new powerful tool for multicolor imaging. The results obtained with this system show that many dyes until now considered impossible to be separated may be used in combination, greatly extending the number of choices and experimental strategies available to the scientist in bio-medical research. Overall, this solution opens new doors for experimental designs in confocal and multi-photon microscopy that in the past have been locked due to a lack of flexibility and technology.

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