Chapter 5

Clearing Up the Signal: Spectral Imaging and Linear Unmixing in Fluorescence Microscopy

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Abstract

The ongoing progress in fluorescence labeling and in microscope instrumentation allows the generation and the imaging of complex biological samples that contain increasing numbers of fluorophores. For the correct quantitative analysis of datasets with multiple fluorescence channels, it is essential that the signals of the different fluorophores are reliably separated. Due to the width of fluorescence spectra, this cannot always be achieved using the fluorescence filters in the microscope. In such cases spectral imaging of the fluorescence data and subsequent linear unmixing allows the separation even of highly overlapping fluorophores into pure signals. In this chapter, the problems of fluorescence cross talk are defined, the concept of spectral imaging and separation by linear unmixing is described, and an overview of the microscope types suitable for spectral imaging are given.

Key words Spectral imaging, Linear unmixing, Image analysis, Fluorescence cross talk, Multichannel imaging

1 Introduction

The introduction of fluorescent dyes for microscopy and their combination with immunochemistry provided important stimuli for light microscopy after decades of relative stagnation in regard to new developments. The possibility to exclusively visualize highly specific intracellular structures in distinctive colors against a dark background has changed our visual perception as well as our understanding of cellular mechanisms. The replacement of photographic film by highly sensitive monochromatic CCD cameras that generate digital images that can be easily merged into dramatic multicolor images helped to establish fluorescence light microscopy as the method of choice for cell biological imaging. Fluorescence imaging was also ideally suited for a groundbreaking new technology, confocal microscopy, which emerged as a powerful tool for biological imaging at the end of the 1980s and allowed the generation of highly resolved three-dimensional datasets of

Stephen W. Paddock (ed.), *Confocal Microscopy: Methods and Protocols*, Methods in Molecular Biology, vol. 1075,
DOI 10.1007/978-1-60761-847-8_5, © Springer Science+Business Media New York 2014

biological samples. The cloning of genes for autocatalytic fluorescent proteins in the middle of the 1990s allowed the observation of living structures in fluorescence with unprecedented ease and revolutionized the field of in vivo imaging, as acknowledged in the 2008 Nobel Prize for Chemistry awarded to O. Shimomura, M. Chalfie, and R. Tsien.

These days, the availability of an elaborate palette of fluorescent dyes extending even beyond the visible spectrum, an almost equally well-distributed range of fluorescent proteins and instruments capable of simultaneously acquiring dozens of spectral image channels provide us with an unprecedented richness in labeling possibilities [1–4]. This however also highlights inherent limitations in the specificity of fluorescence signals. Even though commonly used fluorophores seem to possess very distinct color signatures to the eye of the observer, their true spectral distributions are wide and significantly overlapping with the spectra of other fluorescent dyes. Some combinations of fluorophores, as well as a higher number of labels within one sample, will result in signals that cannot reliably be separated. Incomplete separation however makes quantitative analysis or the study of localization impossible.

Recently the analysis of spectral datasets and the signal separation by linear unmixing to overcome these problems have become widely used. Spectral imaging was initially used for spectral karyotyping [5] and subsequently combined with linear unmixing for immunohistochemistry [6]. It generated much interest after being applied to two-photon microscopy [7] and subsequently to confocal microscopy [8].

In this chapter we are going to define the problems inherent in fluorophore cross talk and cross-excitation and we are going to highlight the instrumentation and the processing methods that allow the reliable separation of multiple fluorescence signals.

2 Fluorescence Cross Talk and Cross-Excitation

Fluorescence at the molecular level consists of the ability of a molecule to absorb the energy of a photon and to subsequently reemit a photon of less energy. Only photons within a certain energy range can be absorbed and the fluorescence emission can only happen within a second defined energy range. The wavelength of a photon is inversely proportional to its energy level, so that this relation can also be described inside the color spectrum of light, with longer (i.e., “red”) wavelengths corresponding to lower energy levels. The difference between the wavelength at which a fluorophore is most efficiently excited and the wavelength at which most emission photons are generated is referred to as the Stokes’ shift. In reality fluorescence phenomena are far from monochromatic. The wavelength maxima are embedded in much wider excitation and

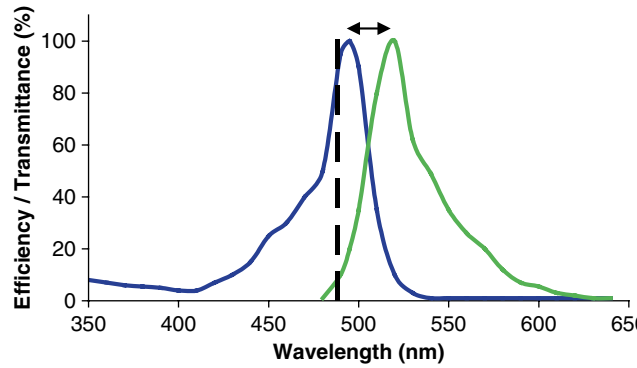

Fig. 1 Excitation profile (*blue line*) and emission profile (*green line*) of Fluorescein isothiocyanate (FITC) excited with a 488 nm argon laser (*broken line*). Stokes shift is illustrated by the *double arrow*. The long slopes of the excitation spectrum to the blue and of the emission spectrum to the red are clearly visible

emission spectra which represent the efficiency of excitation at a certain wavelength and for the emission side the likeliness of a photon being generated at a certain wavelength. Most fluorophores have near mirror image symmetry between their excitation and emission spectra, with the excitation spectra extending to significantly shorter wavelengths than the excitation maximum and with emission wavelengths tailing far to the red of the emission maximum (Fig. 1). Even for fluorophores with fairly defined spectra, each spectrum can cover around 100 nm, meaning that, inside our spectrum of visible light from 350 to 700 nm, most fluorophores are excitable or detectable over a significant range [9].

Fluorophore cross talk can be defined as the overlap of the emission spectra of two different fluorophores. In practice it means that, depending on the spectral region chosen for detection, the signal will consist of contributions from both fluorophores if both are excited at the same time (Fig. 2). Fluorophore cross-excitation describes the phenomenon of simultaneous excitation of two fluorophores due to the fact that at the excitation maximum of one of them, the other can often be excited with significant efficiency. Sometimes, both phenomena are jointly referred to as fluorophore cross talk, but for a more thorough understanding, it is useful to separate them. Another frequently used term is bleed-through.

Specificity problems in samples with several fluorescence stains may be compounded by the fact that, depending on their efficiency in absorbing and also in emitting photons, some fluorophores are significantly brighter than others. Combinations of “dim” and “bright” fluorophores may cause problems in reliably identifying the signals of the “dim” dyes. The same problem can arise even if both fluorophores are equally bright, but their relative concentrations in the sample are significantly different.

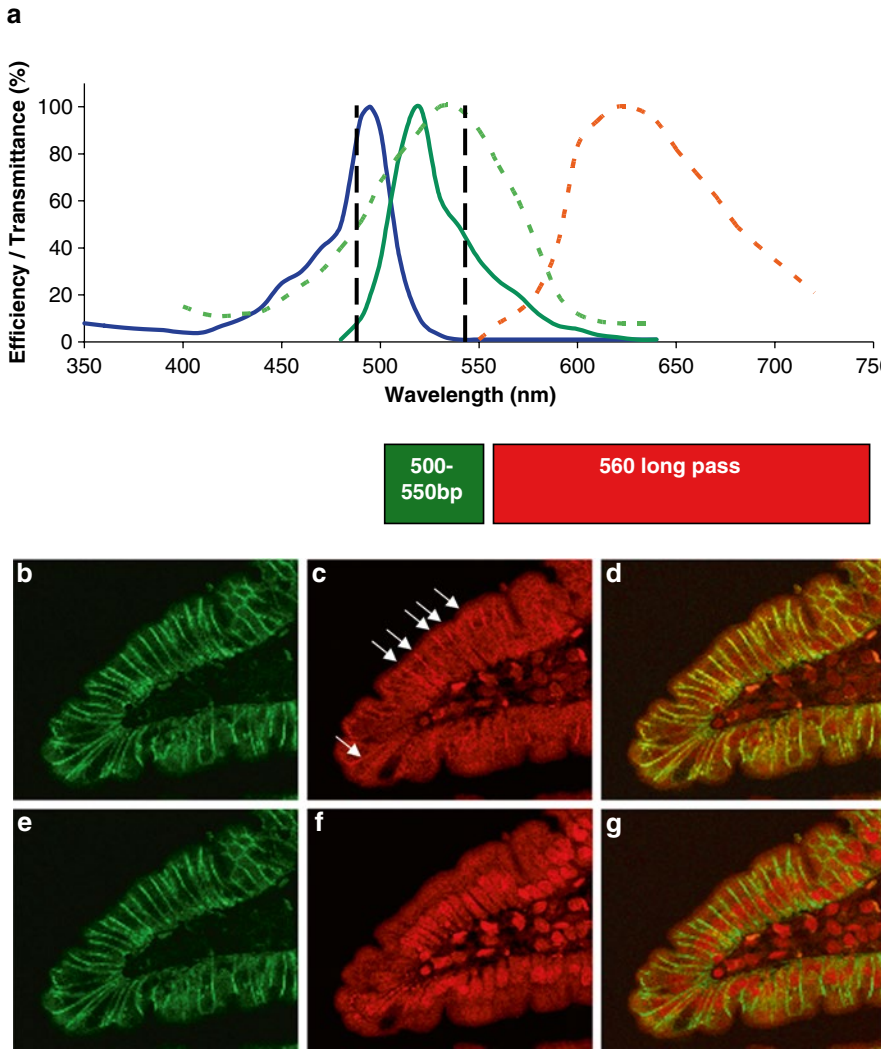

Fig. 2 (**a**) Excitation and emission spectra of FITC (*solid lines*) and propidium iodide (*broken lines*) showing the laser lines at 488 nm and 543 nm (*black*). The emission filter sets routinely used are shown below the spectra and the level of FITC emission which bleeds through into the red channel during simultaneous data collection using a 560 long pass emission filter is blocked in red. (**b**–**g**) Confocal images of a gut section labeled with anti-glucose transporter 5 and a secondary FITC antibody (*green*) and the nuclear stain propidium iodide (*red*). (**b**)–(**d**) are imaged simultaneously and (**e**)–(**g**) are imaged sequentially. FITC emission is shown in *green* (**b**, **e**, 488 nm excitation, 505–550 band pass emission) and propidium iodide emission is shown in *red* (**c**, **f**, 543 nm excitation, 560 long pass emission), (**d**, **g**) Composite images of the green and red channels. The bleed through of FITC emission into the red channel during simultaneous collection is highlighted by the *arrows* in (**c**) and consequently alters the color of the *green* localization in (**d**)

Methods to insure a reliable identification of the fluorescence signals in a sample containing several labels are therefore a fundamental requirement for the analysis of fluorescence microscopy images.

Even though the wide spectra of fluorophores are indeed prone to problems of cross talk and cross-excitation, specific recognition of fluorescence microscopy signals has been successfully

achieved for most standard applications. The use of properly configured fluorescence filtersets, i.e., the ensemble of excitation filter, dichroic mirror, and emission filter, achieves a reliable separation of the signals of the most commonly used fluorophore combinations. This separation is possible because the characteristics of most fluorophore spectra are the following:

- Excitation spectrum: Long tail to the shorter ("blue") wavelengths, rapid drop after the excitation maximum.
- Emission spectrum: Rapid rise to the emission maximum, long tail to the longer "red" wavelengths.

In a pair of two partially overlapping fluorophores A and B (with fluorophore B having spectra more shifted to the red relative to fluorophore A), B is thus likely to get cross-excited at the excitation maximum of A. However, in the shorter wavelengths of A's emission spectrum, there will be no contribution from B, as fluorophore B only starts emitting shortly before its own emission maximum. Fluorophore A can therefore be separated reliably from B through the use of a band-pass emission filter that collects fluorescence only from that part of A's emission spectrum that does not overlap with B's emission. In fluorophore B's emission range, especially around its own emission maximum, there will be significant overlap with emission from fluorophore A. However, as fluorophore A's excitation spectrum drops rapidly after its excitation maximum, B can be excited at its own excitation maximum without any simultaneous excitation of A. Emission overlap thus becomes irrelevant as A is not emitting and B can be imaged specifically by using an excitation band-pass filter that does not overlap with A's excitation spectrum.

This is the most commonly used solution for the imaging of standard fluorophore combinations in fluorescence microscopy. It is robust and its main disadvantage, the incomplete collection of fluorescence emission due to the use of band-pass emission filters, can be minimized by the choice of fluorophores that are bright and that are spectrally as separated as possible.

This solution becomes less applicable under several conditions:

1. In the presence of higher numbers of fluorophores.
2. In the presence of fluorophores that are not matched to existing filtersets.
3. In time-limited (and exposure-limited) situations like in vivo imaging.

Karyotyping using multicolor fluorescent in situ hybridization (FisH) probes is a good example of the first case. To reliably identify all chromosomes, a high number of fluorescence signatures are needed. Specificity for such samples was initially achieved through the use of highly restrictive filter combinations, at the cost of signal efficiency [10, 11].

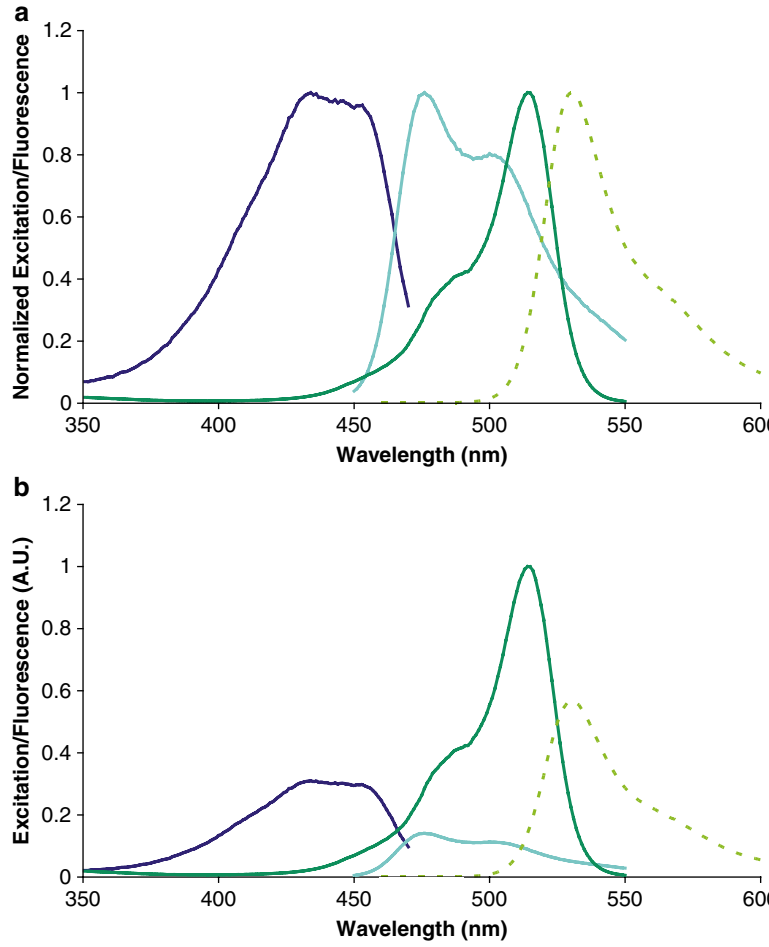

Fig. 3 Excitation and emission spectra of two frequently combined fluorescent proteins, Enhanced Cyan Fluorescent Protein (ECFP, excitation: *blue*, emission: *cyan*) and Enhanced Yellow Fluorescent Protein (EYFP, excitation: *green*, emission: *yellow*). The normalized spectra (**a**) show the significant amount of overlap of the ECFP emission with the EYFP emission. Without normalization (**b**) it becomes clear that EYFP is the significantly brighter fluorophore due to its higher absorption and emission efficiency. YFP can also be excited with light suitable for ECFP excitation, and can provide a significant fluorescence signal that overlaps with the second half of the ECFP emission, so that a band-pass filter is needed to insure specific detection

The second case can be encountered when dealing with genetically encoded protein-tags. The currently available fluorescent proteins offer a wide spectral distribution [1, 2, 12], but their spectra are often not matched to existing filter combinations and due to their complex properties (brightness, pH stability, oligomerization) they can not readily be "mixed and matched" solely on their spectral properties. Viable combinations often present significant problems with fluorophore cross talk (Fig. 3).

The use of specific filtersets for multichannel fluorescence imaging normally requires sequential acquisition of the different channels. The more channels there are to be acquired, the longer the whole acquisition process takes. During in vivo experiments, the observed process may be so fast that the next image of a time-series already needs to be taken when the acquisition of all channels is not even finished. Also, a living sample may change even while the different fluorescence channels are being recorded, so that the information in the channels is not completely matched.

Confocal microscopes generally have more than one fluorescence detection channel and therefore could be used for more time-efficient parallel acquisition of several channels. If the aim is however to separate fluorescence signals clearly into detection channels confocals can be operated in sequential imaging modes with the same problem of accumulating acquisition times. Simultaneous excitation of several dyes would immediately lead to problems with fluorophore cross talk and it is normally avoided by offering the possibility of imaging modes like "multitracking," "sequential imaging," etc.

The cases described above make it clear that standard fluorescence imaging approaches cannot provide solutions for some of the current experimental requirements of biological imaging.

Recent instrument developments and the implementation of image processing approaches originally established in remote sensing do however provide solutions, allowing the clear separation even of highly overlapping fluorescence signals.

3 The Concept of Spectral Imaging and Linear Unmixing

In remote sensing, multiband images taken by satellites represent the same geographical region in distinct spectral channels of the visible and also the invisible (and radar) spectrum. The signals of different types of vegetation and geological formations show a characteristic spectral distribution and are in this aspect similar to the emission spectra of fluorophores.

For the analysis of such multiband images, approaches have been developed that allow a clear assignment of distinct spectral signatures to specific ground features, even though such signatures are not specific for one image channel, but distributed over many image channels (called image bands) and significantly overlapping with one another [13, 14]. In the last years some of the analysis methods established in remote sensing for multiband data have also been applied for multichannel fluorescence microscopy datasets [7, 8].

Three approaches were tested for the analysis of fluorescence microscopy data:

- Supervised classification analysis.
- Primary component analysis.
- Linear unmixing.

The first two methods are classification-based. Such classification approaches have been used for some time in spectral karyotyping using multicolor FisH [5] where the labeled chromosomes have only one characteristic signature. Classification techniques do however not work for colocalized fluorophore signals such as can be found in tissues or cells. It has been shown that the third method, linear unmixing, is the one best suited to analyze mixed contributions to a pixel, as would be the case for colocalizing labels [6–8].

4 Linear Unmixing

Fluorescence signals can be described as a linear mixture of contributions coming from the fluorophores present in the observed volume. The concentration of the fluorophores in the observed spot determines their contribution to the total signal. If one looks only at parts of the total signal that would correspond to different fluorescence image channels, the relative contribution of the fluorophores to a channel will vary according to the distribution of their emission spectra, even though the concentration of the fluorophore is the same for all channels.

As a linear equation the contribution of fluorophores to an image channel can be expressed in the following way:

$$S(\lambda) = A_1 \times \mathrm{Fluo1}(\lambda) + A_2 \times \mathrm{Fluo2}(\lambda) + A_3 \times \mathrm{Fluo3}(\lambda)\ldots \quad (1)$$

where S represents the total detected signal for every channel λ, Fluo$X(\lambda)$ represents the spectral contribution of the fluorophores to every channel, and A_x represents the abundances (i.e., concentrations) of the fluorophores in the measured spot.

More generally, this can be expressed as:

$$S(\lambda) = \sum A_i \times R_i(\lambda) \quad (2)$$

or

$$S = A \times R \quad (3)$$

where R represents the reference emission spectra of the fluorophores [8].

If the reference spectra R for all contributing fluorophores are known, the abundances A can be calculated from the measured signal S. The process through which this can be achieved is called linear unmixing. It calculates the contribution values that most closely

match the detected signals in the channels. A least square fitting approach minimizes the square difference between the calculated and measured values with the following set of differential equations:

$$\frac{\partial \sum_j \left\{ S(\lambda_j) - \sum_i A_i R_i(\lambda_j) \right\}^2}{\partial A_i} = 0 \tag{4}$$

where j represents the number of detection channels and i the number of fluorophores.

The linear equations are usually solved with the singular value decomposition (SVD) method [6, 15], so that after the calculation of the weighing matrix (A), clear representations of the separated fluorophores can be created. The separated fluorophore signals can then be displayed as distinct image channels without contributions from any other label in the sample. The intensity distribution of the signals in each position is preserved as the total signal is redistributed into the specific fluorescence channels, but not altered. It can therefore be analyzed quantitatively.

5 Requirements for the Linear Unmixing of Spectral Datasets

5.1 Reference Spectra

To be able to calculate the fluorophore contributions, linear unmixing requires knowledge of the reference spectra for the fluorophores present in the sample (Fig. 4). For maximum accuracy, such reference spectra are best taken from samples containing only the fluorophore of interest. They can also be taken from mixed samples, if specific regions only contain the signal of interest, but this contains a risk of introducing contaminating contributions from other fluorophores.

For instruments with one detector, or with several detectors that are spectrally calibrated to each other, reference spectra can be saved in a spectral database and can be reused whenever needed. Reference spectra normally have to be taken under exactly the same detection conditions as the sample so if the spectral information is sampled by several detectors which have independent settings from each other (e.g., for gain or the spectral detection range) the reference spectra have to be acquired for every session. The characteristics and strengths of different instrument designs will be discussed in a later section of the chapter.

5.2 Channel Number

For a successful separation of overlapping signals, the number of spectral detection channels has to be equal to, or greater than the number of fluorophores present in the sample. Only then can the linear equations that represent the channels be solved for all the unknowns (i.e., fluorophore contributions). In the case of fewer channels than fluorophores, the equation system is *underdetermined* and a unique solution of the equations is therefore not possible.

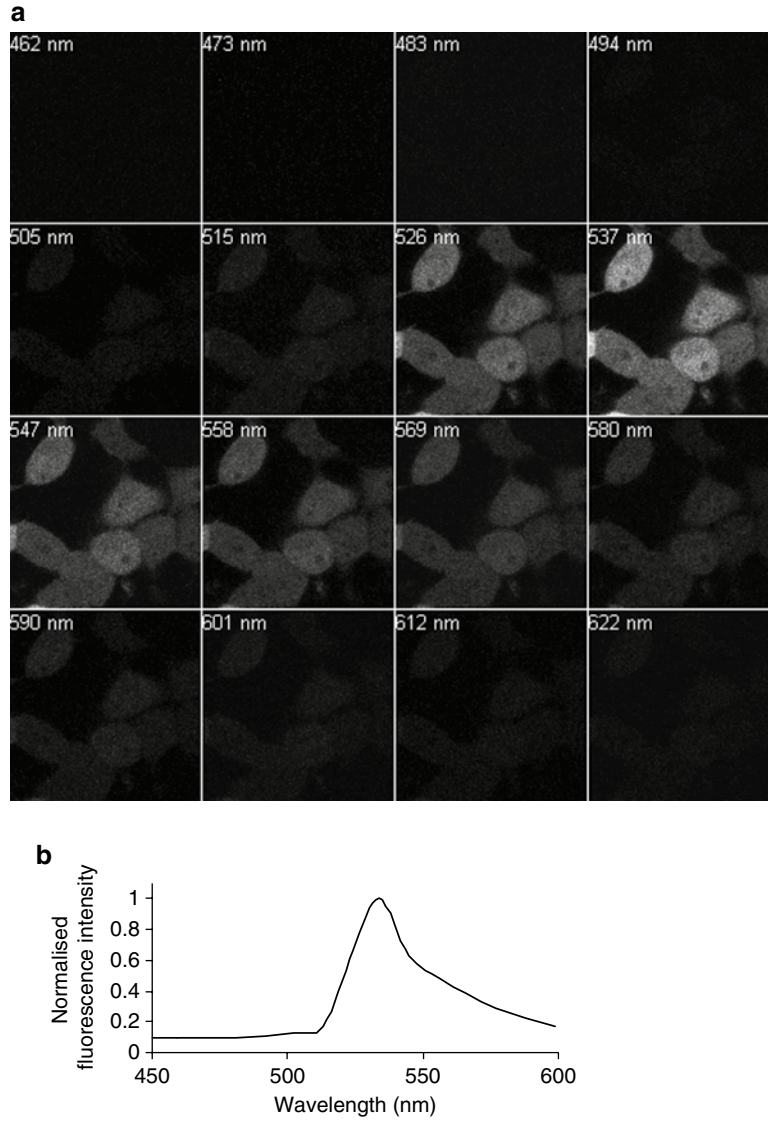

Fig. 4 Gallery of 16 YFP emission images spanning 456–628 nm in bins of 10.7 nm of 293T cells transfected with a plasmid expressing YFP transiently throughout the cell. YFP was excited using a 514 nm laser with a HFT 458/514 main dichroic, and a Plan-Apochromat 63×/1.4 oil DIC objective. The wavelength data on each image represents the center point of each 10.7 nm bin. A spectral curve for YFP emission (**b**) covering the range of the wavelength scan can be generated from the proportions of YFP fluorescence in the 16 bins

6 Related Methods to Linear Unmixing

The separation of fluorescence microscopy spectral datasets can also be achieved with methods other than linear unmixing. In very simple cases, like unidirectional bleed-through of one channel into the other, a simple subtraction of the known contribution to the second

channel can be used to separate the signals (subtractive compensation). This approach is for example used in simple variants of FRET measurements by sensitized emission [16] and in some of the first methods for simultaneous fluorescent protein detection [17].

In approaches like Automatic Dye Separation [18], Automatic Component Extraction (ACE) and Blind Source Separation [19], spectral separation can be achieved without the knowledge of reference spectra. Such approaches are especially useful for spectral data that are acquired with several detectors that are not spectrally calibrated to each other as they avoid the need to take a new set of references whenever settings are modified. An important requirement for this approach however is the presence in the dataset of significant amounts of "pure" areas containing the signature of only one of the fluorophores of interest. This information is necessary for the extraction of robust reference spectra and in cases of too abundantly co-localized fluorescence signals these methods fail.

7 Microscopes for Spectral Imaging

Spectral imaging for subsequent linear unmixing can in principle be performed on any fluorescence microscope that is capable of generating a multichannel image [20–22].

In the last years there have however been many technical developments in microscopy that specifically provide greater spectral flexibility and that are especially suited for spectral data analysis. A short overview of different instrument solutions shall therefore be given (*see* also Table 1).

Table 1
Overview of different commercial microscope setups for spectral imaging

Acquisition mode	Overdetermined ($N_c \gg N_f$)	Determined ($N_c \approx N_f$)
Parallel	Zeiss LSM 710/780 (N_c: 32–34)[a] Nikon A1 (N_c: 32)	Leica SP5/SP8-AOBS[a] Olympus FluoView 1000[a] Zeiss LSM 710 (N_c: 3)[a] Beamsplitter setups Confocal microscopes
**Sequential	Leica SP5/SP8-AOBS[a] Leica SPE Olympus FluoView 1000[a] Zeiss LSM 710/780 (N_c: 3)[a] Zeiss LSM 700 SpectraCube (FTS) Nuance (LCTF detection) Optical Insights Spectral-DV	Widefield microscopes w. filterwheels/ filtercubes

N_c number of detection channels, N_f number of fluorophores
[a]Configuration with 32 channel PMT array
**Sequential: λ-series into a single detector. Parallel: multiple detectors (2–5)

7.1 Confocal Microscopy

The importance of spectral flexibility beyond what is possible through the use of filterwheel combinations is illustrated by the fact that all current high-end confocals of the major confocal microscope providers contain solutions for spectral imaging. It is important to understand in this context that the term "spectral confocal microscope" describes a design that offers spectral flexibility for the detection and therefore an easy way of generating spectral datasets. Linear unmixing is however a data-processing step and its implementation inside the microscope software varies significantly between different companies. Some offer for example spectral separation during the acquisition and others only as a postprocessing step.

Spectral single beam scanning confocal microscopes separate the information contained in the de-scanned emission light into its spectral components by using either a prism (Leica confocal microscopes) or a diffractive grating (Zeiss, Nikon, Olympus confocal microscopes) or a blazed holographic grating (Zeiss 710). The spectral detection flexibility is then given either by projecting the entire spectrum on a fixed array of 32 photomultiplier tubes (PMTs) (Zeiss, Nikon) or by reflecting the light on fewer and separate PMTs whose spectral detection range is controlled by blocking sliders in front of the PMTs and where unused spectral information is passed on to the next PMT in the optical arrangement (Leica, Olympus).

In the case of the 32 element PMT arrays, the information is directly available as a spectrum of up to 32 points with a spectral resolution of approx. 9–11 nm (Fig. 4). The signals of two or four PMT elements can also be "binned" into a single channel to increase the collected signal per channel at the cost of spectral resolution (reduced to ~20–40 nm). To emulate the effect of a band-pass filter, virtual channels can be created by collecting all the information inside a spectral detector by combining the information of all PMT elements that cover that range.

If the information is sampled onto separate PMTs with variable slits in front of them, the main function for the spectral PMTs is to offer the possibility to freely optimize the band-pass characteristics of all detection channels. The spectral information consists of significantly fewer channels and the appearance of the spectrum may be altered by different bandwidths and gain settings of the detectors, but the channels can also be used for linear unmixing. On confocal microscopes of this design type, the spectrometer appearance of PMT array signals can be emulated by sequentially modifying the detection range of a single PMT by shifting the slit in front of it. This allows the readout of very defined spectra, but it can only be done in sequence, not instantaneously.

The advantage of PMT arrays lies in their defined spectral calibration which permits the use of existing libraries of reference spectra for linear unmixing. The advantage of separate detectors is the flexibility in their ranges and gain settings that can accommodate strong intensity peaks in parts of the spectrum without saturating the signal.

As the beam containing the fluorescence information needs to be dispersed into its spectral components for the different PMT detectors, all spectral detection systems can only work with a single confocal pinhole in front of the dispersive element and not with multiple pinholes in front of the individual detectors. As the thickness of an optical section is wavelength-dependent, this should be taken in consideration when working over a big range of the spectrum. Normally, however, spectral imaging has its use in the separation of closely spaced, overlapping signals that are not strongly affected by this consideration.

Although most current "spectral" confocal microscopes are based on a prism or grating as the dispersive element, a recent design by Zeiss also deserves mention as it provides some spectral flexibility in a different way. In the LSM 710 confocal microscope, a variable secondary dichroic (VSD) beamsplitter allows to choose the separation wavelength between the two available detection channels freely. This is not the same as the completely free spectral definition of detection channels possible in fully "spectral" confocals, but it allows an optimization of the channel properties that serves as a good basis for subsequent unmixing. Also, sequential spectral series can be acquired in a similar way to the one mentioned above for single PMTs.

Dedicated spectral imaging solutions also exist for widefield microscopy. These special solutions either provide multiple channels at the same time in the form of split images or they allow the sequential detection of the spectral information.

One of the first methods to generate spectral microscopy data uses an interferometer coupled to a microscope for Fourier Transform Spectroscopy (FTS). This technique was used initially for spectral karyotyping [5] and subsequently in conjunction with linear unmixing for the separation of seven fluorescence signals in a fixed tissue section [6].

The DualView series of imagesplitters (Optical Insights) offers two- and four-channel solutions where images corresponding to different regions of the spectrum are projected onto different areas of the camera CCD chip. Using the same camera to simultaneously image two or four images inevitably causes a corresponding loss in spatial resolution. Therefore other solutions exist that project two spectral images on different cameras. This requires an acquisition set-up that can simultaneously read out multiple cameras. The Spectral DualView uses a grating to generate true spectral information along a line of the image field. A two-dimensional spectral image is then generated sequentially by moving the sample line by line through the detection area.

The Nuance Multispectral Imaging System (Cambridge Research & Instrumentation, Inc.) uses a Liquid Crystal Tunable Filter (LCTF) in front of the camera to sequentially generate a series of spectral images of fluorescence or transmission signals that can then be separated into their contributions.

Spectral separation can also be achieved using the different distributions of the excitation spectra of fluorophores. Using different excitation wavelengths and detecting with a wide emission window to efficiently collect the signals generates image channels that can be processed easily with the same procedures as data with different emission channels [22]. Accordingly spectral separation can be achieved by tuning the wavelength of a two-photon light-source and thus generating "excitation fingerprint" datasets that are then used for unmixing. For confocal imaging, the recently introduced white-light lasers in conjunction with freely definable acousto-optical beam splitter (AOBS) configurations also allow the full use of the excitation spectra for spectral analysis and imaging.

The combination of different excitation and emission settings for unmixing significantly increases the number of information-bearing channels even on systems with only few changeable components on the excitation and emission side [22, 23]. Incorporating excitation variations also significantly helps with blind spectral decomposition approaches that work without predefined reference spectra [19, 23].

8 Method Limitations and Optimization Approaches

As with any methodology, spectral imaging and linear unmixing is affected by different parameters that need to be optimized for the best possible results. The amount of overlap of the contributing spectra influences how reliably different signals can be distinguished. Datasets with a strong spectral overlap will be more affected by noise on the image data. This may result in noisy image channels or in the incomplete separation of different fluorophores. In such cases the signal to noise ratio needs to be improved by averaging or longer integration. In detection systems that allow the definition of the spectral channels by the user, the channels should be configured to detect the maximum difference between the spectra, as this directly influences the separation of the data [22, 24]. If the fluorophores in the sample can be chosen during the sample preparation, the combinations that provide the most difference should be favored rather than relying on the ability of the unmixing software post-acquisition.

For linear unmixing of a spectral dataset, all contributing spectra have to be defined correctly. The absence of a contributing spectrum, or the use of incorrect spectra can cause mis-assignments of the signals that will lead to a misrepresentation of the fluorophore distributions in the image. If there is an autofluorescent background, it has to be considered as an extra fluorophore, otherwise it will lead to false results. The quality of an unmixed spectral dataset can in some software packages be assessed by visualizing the residual values after unmixing, i.e., the difference between the

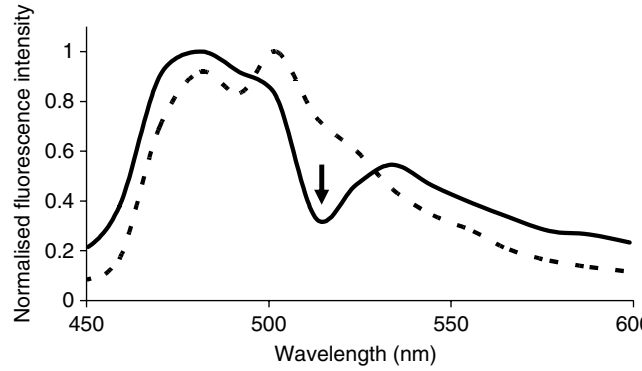

Fig. 5 Spectral profiles of CFP emission generated from similar wavelength scans to those in Fig. 4a. The profile generated using 458 nm laser excitation and a HFT458 nm main dichroic is shown with a *broken line* whilst the equivalent profile using a HFT458/514 nm main dichroic is shown in a *solid line* and illustrates the drop in fluorescence intensity at 514 nm as the HFT458/514 main dichroic reflects this wavelength (*arrow*)

calculated fit and the data values. Significant amounts of residuals indicate a separation problem due to noise or spectral overlap or due to wrong or incomplete spectral information.

It is very important to take into account that the change of optical components like the beamsplitter will significantly affect the apparent detected spectra. The replacement of a single dichroic with a double dichroic can completely alter the spectra due to the additional dip in transmission and will therefore require the collection of its own set of reference spectra (Fig. 5). These problems are alleviated in confocal microscopes by the use of Acousto-Optical Beamsplitters (AOBS, Leica) that only deflect the selected excitation laser wavelengths and by the use of high incidence beamsplitters (Zeiss, Nikon) that also have very steep and defined reflection characteristics.

Depending on the optimization functions incorporated in the unmixing routines, the number of spectra selected as potential contributors may also affect the outcome. Generally only the spectra present in the sample should be selected for the unmixing step. To improve the results, unmixing routines can use non-negativity constraints (i.e., there can be no negative fluorophore contribution to a signal) and iterative end-member ejections (i.e., the removal of spectra with no or negative contributions to the pixel from the calculation and the repetition of the unmixing step with fewer spectra) [25].

The number of channels into which a spectral dataset is split may also affect the quality of the separated result. Since the prerequisite for spectral separation is that the number of channels equals or exceeds the number of fluorophores in the sample, an equal

number of channels is in principle sufficient. The advantage is that signals are stronger since they are collected into fewer channels and that less signal-independent detector noise is accumulated [22, 24]. On the down-side, the effect of channel positioning for optimal separation is stronger when collecting fewer channels. A significantly over-sampled signal (as is acquired by 32 channel PMT array detectors) is less prone to optimization effects by the position of the channels. Additionally, the signal on PMT array detectors can be configured to be collected into fewer and wider bins by combining the input of several neighboring PMTs.

9 Applications for Spectral Imaging

9.1 Multilabeling

As the linear unmixing of spectral imaging data provides signal specificity its main applications are experiments that contain multiple signals like multi-fluorophore Fluorescence in situ Hybridization (FisH) as used in spectral karyotyping [5] or the simultaneous staining of multiple tissue markers [6].

9.2 Autofluorescence Removal

Sample autofluorescence is a persistent problem of fluorescence microscopy when working in tissues, especially in plants [26, 27]. It often has a wide distribution and can therefore not be separated from the signals of interest through the use of band-pass filters. Also, in signal strength it may completely mask a weakly expressed fluorescent protein [28]. It can however in many cases be spectrally defined and then treated as an additional signal in the unmixing process. This allows for an efficient removal of the autofluorescence signal from the image channels, which only then can be utilized for quantitative colocalization studies [29].

9.3 Time-Lapse Imaging

Since time limitations are not relevant in fixed samples, the problems of fluorophore overlap are generally caused by the high number of fluorophore signals that cannot be separated reliably even by sequential imaging of the channels. Due to the time constraints in in vivo imaging significant overlap problems can already be encountered with significantly fewer fluorophores due to simultaneous imaging or if the choice of fluorescent tags is limited to pairs that have very similar spectral characteristics, as is the case for many fluorescent proteins [30], Fig. 6.

9.4 Fluorescence Resonance Energy Transfer

Fluorescence Resonance Energy Transfer (FRET) is a powerful tool for molecular interaction studies and generally involves the use of a matched pair of fluorophores (FRET pair) respectively as donor and acceptor of the transferred energy. A prerequisite for the pairing of two fluorophores for FRET is significant spectral overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor. For many FRET pairs this required

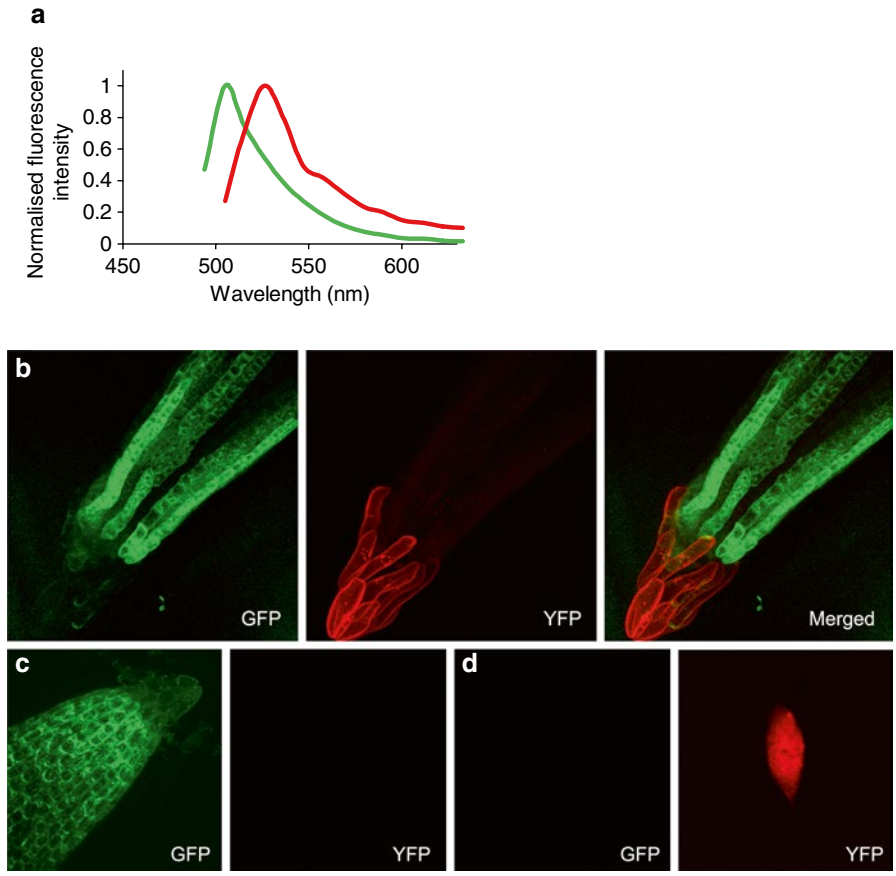

Fig. 6 GFP (*green*) and YFP (*red*) emission spectral profiles taken from wavelength scans of an *Arabidopsis thaliana* mGFP5 enhancer-trap line and a histone-YFP transformed *Arabidopsis thaliana* seedling root respectively (**a**). GFP and YFP were excited using a 488 nm laser with a HFT488 main dichroic. The wavelength scans covered 490–630 nm in bins of 10.7 nm using a plan-Apochromat 63×/1.4 oil objective. A wavelength scan of an *Arabidopsis thaliana* seedling root can be spectrally unmixed to reveal GFP (*green*) and YFP (*red*) expression (**b**) using the curves shown in (**a**). The mean wavelength images in (**b**) were captured in three dimensions (*x*, *y*, *z*) and are shown as 3D projections of the spectrally separated fluorochromes. To verify the spectral separation a wavelength scan of an *Arabidopsis thaliana* mGFP5 enhancer-trap seedling was unmixed using the GFP and YFP spectral curves (**c**) and correct assignment of the GFP fluorescence (*green*) to the GFP channel is shown (YFP channel is *blank*). Also a wavelength scan of an *Arabidopsis thaliana* seedling transformed with histone-YFP was unmixed using the GFP and YFP spectral curves (**d**) and correct assignment of the YFP fluorescence (*red*) to the YFP channel is shown (GFP channel is *blank*)

spectral proximity leads as well to significant problems in fluorophore cross talk and cross-excitation that make a quantitative analysis problematic. Spectral imaging and linear unmixing methods have in the last years been used to correct for the channel contaminations in acceptor photobleaching methods [28, 31–34], Fig. 7, as well as in methods for the detection of ratiometric signals and sensitized emission [35–38]. It is especially important for FRET

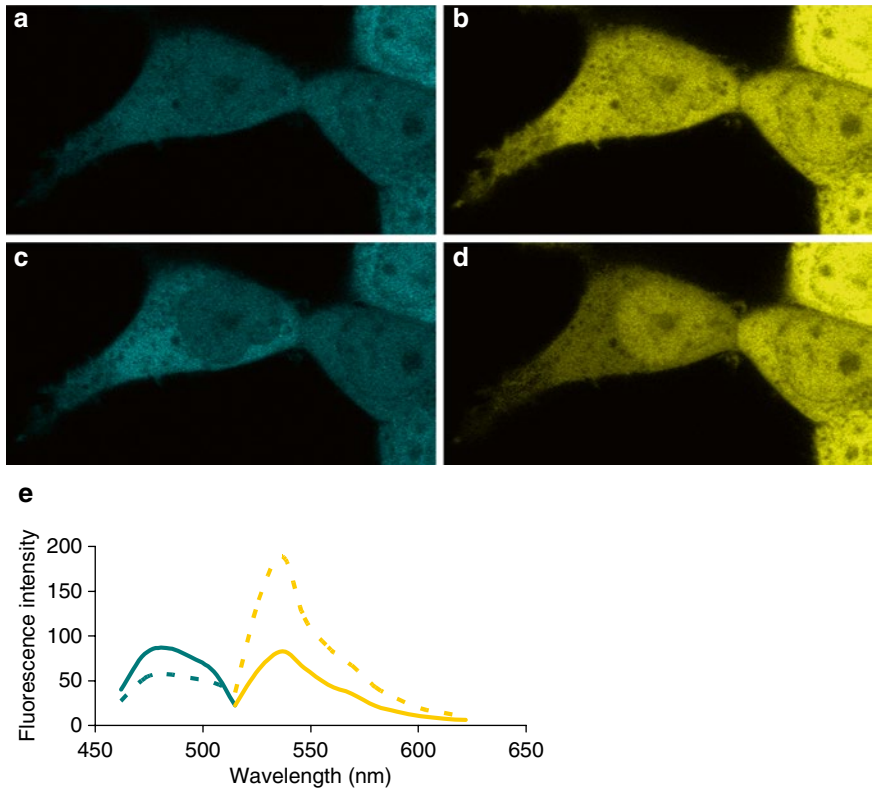

Fig. 7 293T cells transfected with a plasmid expressing tandem linked CFP and YFP. CFP and YFP were excited with 458 nm and 514 nm lasers respectively using a HFT458/514 main dichroic and plan-apochromat 63×/1.4 oil DIC objective. A wavelength scan was taken between 456 and 628 nm in bins of 10.7 nm. Data were spectrally unmixed using curves similar to those shown in Figs. 5.4b and 5.5. Images of unmixed CFP and YFP prior to acceptor (YFP) photo bleaching are shown in (**a**, **b**) and after acceptor photo bleaching in (**c**, **d**). The cytoplasmic bleach region is outlined in (**b**). After bleaching YFP using the 514 nm laser at 100 % transmission the intensity of the YFP in the cytoplasm decreases (**d**, **e** *broken yellow line* cf. *solid yellow line*) and the intensity of the CFP increases (**c**, **e** *broken blue line* cf. *solid blue line*)

pairs with high efficiency that unfortunately coincides with poor separation [33, 39].

The possibility to combine multiple markers and probes for multiparameter imaging [40, 41] increases the need for reliable signal separation. This has been demonstrated by the use of two fluorescent protein-based FRET pairs that can be excited by a single excitation wavelength and whose FRET interactions can be resolved by linear unmixing [38].

10 Conclusions

Commercially available spectral imaging and linear unmixing software has solved the problems of spectral bleed through encountered in multicolor confocal laser scanning as well as in widefield

microscopy. The user can capitalize on the increased numbers of fluorochromes available, use them simultaneously in a single experiment and be confident that mixed fluorescent samples are separated cleanly and clearly into their respective detection channels. Spectral unmixing can bring increased sensitivity over conventional optical filter systems because the entire emission spectra is collected and it also has the advantage of allowing fluorochromes whose spectra do not fit with conventional optical filters to be used.

Spectral unmixing can not only be used to identify and remove the interference from autofluorescence, which is especially problematic in plants, but also to analyze the autofluorescence spectra and study the development of the underlying molecules in situ. Spectrally separated autofluorescence can also be used as a "background" image for the sample on which to superimpose the specific probe fluorescence. Users can also verify their fluorescent signal by checking the spectra obtained from their sample with well documented, previously published spectra and identify any spectral shifts due to different cellular environments. Furthermore, spectral imaging enhances the visualization of molecular interactions or conformational changes detected by FRET and increases the number of FRET pairs available to include the more spectrally overlapping high efficiency FRET pairs. All of the above advances are possible in 4 dimensions (x, y, z, t) with the added benefit of multicolor analysis allowing dynamic live cell imaging of multiple fast cellular processes.

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