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Spectral Imaging and Linear Unmixing in Light Microscopy

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Abstract Fluorescence microscopy is an essential tool for modern biological research. The wide range of available fluorophores and labeling techniques allows the creation of increasingly complex multicolored samples. A reliable separation of the different fluorescence labels is required for analysis and quantitation, but it is complicated by the significant overlap of the emission spectra. This problem can be addressed on the acquisition and the processing side by the use of spectral imaging in conjunction with linear unmixing of the image data. This method allows the reliable separation of even strongly overlapping fluorescence signals and has become an important tool in colocalization and in FRET studies. In this chapter, the microscope techniques available for spectral imaging are presented and the theory of linear unmixing is explained. Possible limitations as well as approaches for image optimization are discussed to help to realize the full potential of this novel method. Biological applications that can be improved by spectral imaging and linear unmixing are presented.

Keywords Spectral imaging · Linear unmixing · Fluorescent proteins · FRET

List of Abbreviations

CFP	Cyan fluorescent protein
EGFP	Enhanced GFP
EYFP	Enhanced YFP
FRET	Fluorescence resonance energy transfer
FTS	Fourier transform spectroscopy
GFP	Green fluorescent protein
SNR	Signal to noise ratio
YFP	Yellow fluorescent protein

1 Introduction

Fluorescence microscopy is an essential tool for modern biological research, especially in cellular and molecular biology. In its methods it is a continuously expanding field. New fluorophores are introduced [1, 2] and more and more spectral variants of fluorescent proteins are made available as markers [3–5].

Multichannel fluorescence imaging makes use of this diversity of available markers to visualize different aspects of the same specimen with specific fluorescent labels. In this way the localization of several cellular proteins can be compared during processes like cell division [6] or secretion [7]. More and more channels contain complex information and quantitative analysis is required on these data. A quantitative approach depends on exact data. On closer observation, there are however inherent problems with multichannel fluorescence images for this. Fluorescence signals may not be completely separated in the different channels and an unambiguous identification (and quantitation) may therefore not be possible. This is an important consideration especially when the amounts of the different labels are very different or when the choice of available labels is limited as it is the case for fluorescent proteins.

What is required are techniques to reliably analyze complex multichannel data. Recently, processing methods developed for multiband satellite images have successfully been applied also to the analysis of microscope images [8, 9]. By this linear unmixing of multichannel data, "clean" representations of the fluorophores in the sample can be created and be quantitatively analyzed.

In this chapter, the problems of multichannel fluorescence imaging are explained and methods to correct the image data are presented. Important parameters of the methods as well as possible limitations are discussed and applications for these techniques are shown.

2 The Problem: Crosstalk

Upon excitation with light of appropriate wavelengths, fluorophores will reemit some of the acquired energy as fluorescence. The emitted fluorescence

light is distributed over a wide spectral range according to the chemical properties of the fluorophore. This characteristic emission wavelength distribution is the emission spectrum of the fluorophore (Fig. 1A). The emission spectra of most fluorophores share two characteristics. Their onset in the shorter wavelength range is clearly defined by a steep increase towards the emission peak. The decrease of emission contributions towards the longer wavelengths is much less steep. A fluorophore emission spectrum therefore has a long emission tail towards the red that covers up to 100 nm of spectral range behind the peak emission. It contains a significant amount of the total emission of a fluorophore.

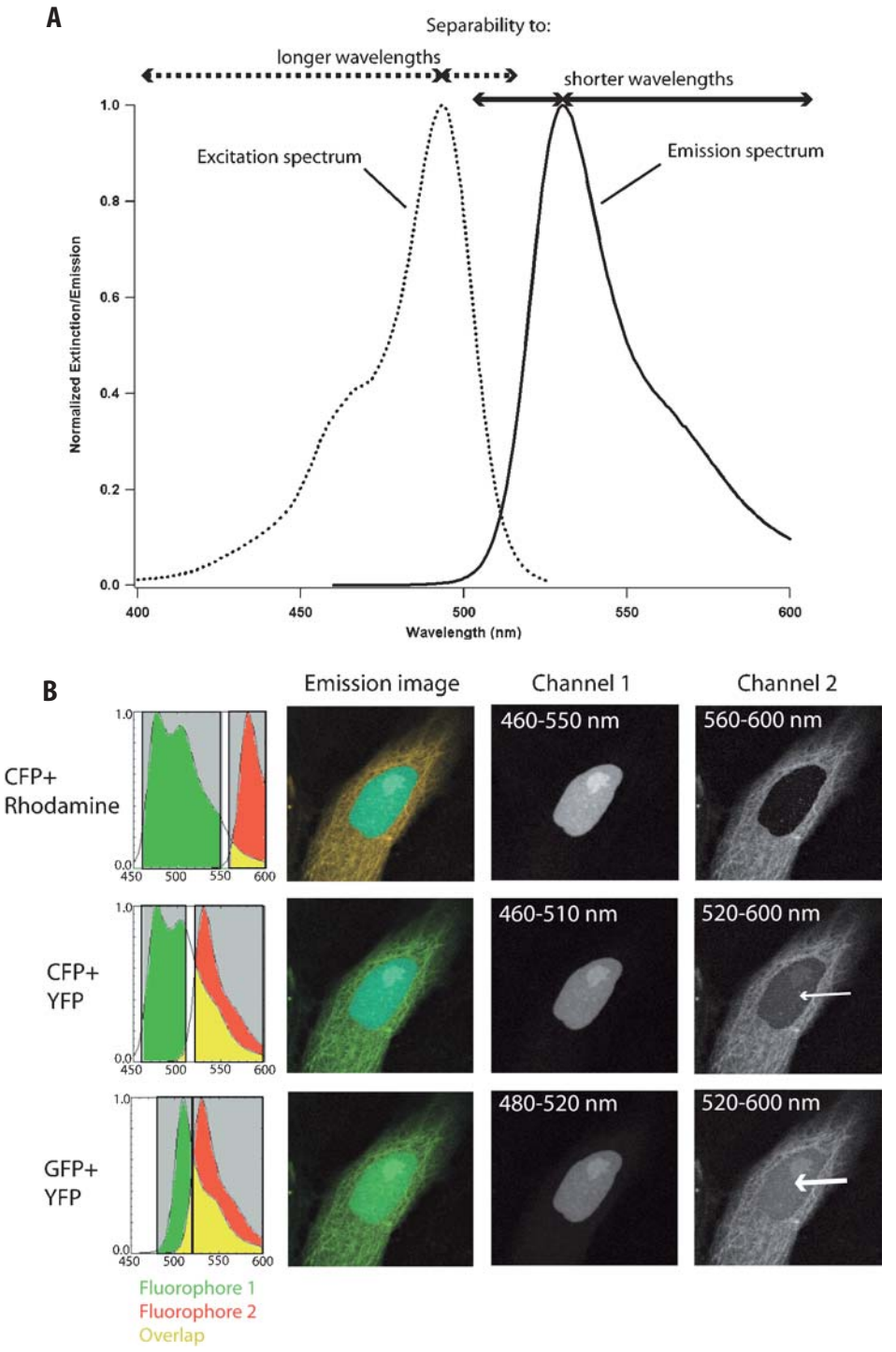
The overlap of different fluorophore spectra due to their wide emission range is an inherent problem of multichannel fluorescence imaging (Fig. 1B). In the absence of additional signals, a fluorophore can be detected through a longpass filter that covers its whole emission spectrum. If additional fluorophores are present, a bandpass filter around the emission peak is required to constrict the spectral detection range and to thus reduce the crosstalk.

While looking into the microscope, the problem may not be so severe. The fluorophores have distinct color hues that are discernible by the eye. We can thus distinguish between a green GFP signal and the more yellowish autofluorescence that may also be there. This is not the case for most of the detection devices used for fluorescence imaging. The spectral information (color) is lost and only intensity information is preserved. Like in a black and white photograph, it is then not possible anymore to draw conclusions about the real color of an object. Accordingly it is hard to assign a structure to a specific fluorescence label (Fig. 1B). This is especially important in cases where labels may colocalize.

For many standard fluorescence imaging applications the problem of crosstalk between fluorophores can be easily overcome. The perceived 'brightness' (amount of emitted photons) of a fluorophore signal in an image channel is determined by two factors: the spectral range detected by the emission filter *and* the efficiency of fluorophore excitation. This efficiency is determined by the absorption spectrum of the fluorophore (Fig. 1A) and the wavelength of the light used. Therefore even a fluorophore having a significant overlap in the emission with another fluorescent label may not produce significant crosstalk if the excitation wavelength is chosen properly. As the excitation efficiency of a fluorophore steeply decreases behind its excitation peak, fluorophores of longer wavelength properties can usually be specifically excited at their excitation maximum without co-exciting a fluorescent label of shorter wavelength (Fig. 1A).

In standard fluorescence imaging, two complementary mechanisms are thus used to separate multiple fluorescent labels reliably.

– A bandpass filter around the emission maximum separates the generated emission of a fluorophore from contributions of co-excited labels of longer wavelength.


A
Separability to:
longer wavelengths
shorter wavelengths
Excitation spectrum
Emission spectrum
Normalized Extinction/Emission
Wavelength (nm)
B
Emission image
Channel 1
Channel 2
CFP+
Rhodamine
460-550 nm
560-600 nm
CFP+
YFP
460-510 nm
520-600 nm
GFP+
YFP
480-520 nm
520-600 nm
Fluorophore 1
Fluorophore 2
Overlap

– Light at the excitation maximum of one fluorophore does not usually further excite shorter wavelength fluorescent labels. Thus, no crosstalk is generated by them.

This approach works well with the standard labels used in fluorescence imaging (e.g. DAPI, FITC, TRITC, Cy5 or equivalent labels). There are however drawbacks and limitations:

– The acquisition of the channels is sequential. Only one excitation can be used at the same time to avoid crosstalk.
– A bandpass emission filter specific for a certain fluorophore detects only approximately 50% of the available photons of this fluorophore. The rest is rejected because they are outside the spectral range of the filter.
– The approach does not work for fluorophores with highly overlapping spectra or with unusual Stokes shifts (spectral distance between excitation and emission).

This translates into limitations in speed (sequential imaging), sensitivity (detected photons) and labeling (available fluorophores).

In advanced imaging applications, and especially in in-vivo imaging, these limitations are very significant.

– Sequential imaging means more time is required for the acquisition of a single timepoint and temporal resolution is impaired. In addition, the fluorescence channels themselves may be mismatched if a fast movement occurs between the channel acquisitions.
– The amount of available fluorophore in the sample may be very small. In such cases sensitivity in the detection is required.
– The choice of spectral variants of fluorescent proteins for in-vivo imaging is still very limited. Those that work best have considerable spectral overlap that cannot be separated by using specific filtersets. Many of the available in-vivo dyes have spectral characteristics that are not easily matched to standard filtersets.

Methods that accurately correct channel crosstalk instead of just avoiding it by sequential imaging are a possibility to overcome these limitations.

Fig. 1 **A** Excitation and emission spectra of fluorophores. On the excitation side, fluorophores are separable from longer wavelength fluorophores because of the steep decrease of their absorption properties towards the red. Inversely, the emission spectrum is clearly limited towards the blue, allowing separation from shorter wavelength dyes. **B** Examples of increasing crosstalk of fluorophore emissions. CFP and rhodamine are almost completely separated in their emission and no crosstalk is perceived when they are imaged through suitable filters. CFP+YFP and GFP+YFP show increasing amounts of crosstalk between their emissions (indicated in *arrows of increasing size*). Different emission spectra were assigned to the same two-channel data set by computer simulation

3 Solutions

Multichannel fluorescence imaging of fluorophores with overlapping spectra is confronted with similar problems as the very different field of remote sensing. Multiband images taken by satellites contain different aspects of the same scene. These image bands do not contain clearly separated information about objects in the image; this information is instead distributed over several or all of the image channels. The information for a certain type of object does however have a certain characteristic distribution over the image bands, a 'signature'. For years, methods have been applied in the remote sensing field to extract specific object information by using the known signatures of objects and resolving their amount of contribution to the total signal of the multiband dataset [10].

The object signature of multiband satellite images is quite similar to the representation of a fluorescence emission spectrum in multiple filtersets. Recently the same approach used in satellite imaging has also been applied to the imaging of multiple fluorophores [8, 9, 11].

Three analysis methods are commonly used in satellite imaging and have been tested for multichannel microscopy [8]:

- Supervised classification analysis
- Primary component analysis
- Linear unmixing

The first two methods are classification based. Classification analysis of spectral data has for some time now been used in spectral karyotyping [12] where objects (in this case chromosomes) have only one characteristic signature. Those approaches are however not suitable for the quantitative analysis of samples with possibly colocalized labels as is the case for immunostainings or GFP preparations of tissues or cells. Linear unmixing is the method best suited to analyze mixed contributions to a pixel, as is the case for colocalized labels [8, 9].

3.1 Linear Unmixing

3.1.1 Principle

Linear unmixing is based on the assumption that the total detected signal S for every channel λ can be expressed as a linear combination of the contributing fluorophores FluoX:

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

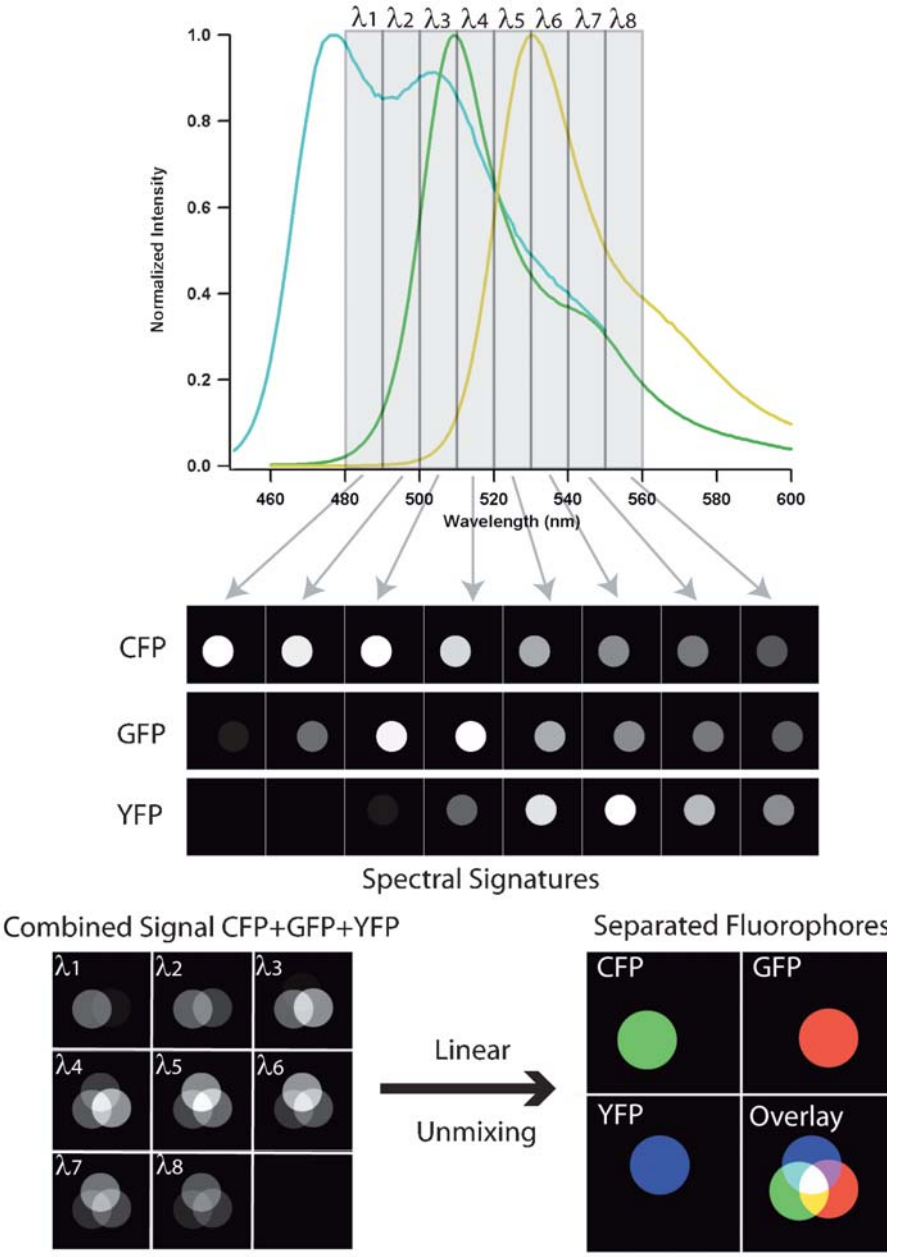


Fig. 2 Spectral imaging of fluorescence signals. Contributions of CFP, GFP and YFP to eight successive spectral channels are shown. The distribution of emission signal to the channels is a direct representation of the fluorophore emission spectrum and constitutes a spectral signature. With linear unmixing using these spectral signatures as reference, even combined and mixed signals can be clearly separated into the fluorophores that contribute to the total signal

A_x represents the amount of contribution by a specific fluorophore.
More generally this can be expressed as

$$S(\lambda) = \sum A_1 \times R_i(\lambda) \tag{2}$$

or

$$S = A \times R \tag{3}$$

where R represents the reference emission spectra of the fluorophores [11].

With the signal S detected and the reference emission spectra R known, the contributions A of the fluorophores in the sample are determined by calculating contribution values that most closely match the detected signals in the channels. This can be done by a least-square fitting approach that minimizes the square difference between calculated and measured values with the following set of differential equations:

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

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 method [9, 13]. After the calculation of the weighing matrix A, clear representations of the separated fluorophores can be created (Figs. 2 and 3).

For the linear unmixing of spectral data, several criteria have to be met (see Table 1):

- The number of spectral detection channels must be at least equal to the number of fluorophores in the sample. If this is not the case, multiple solutions are possible and no unique result can be attained for spectral separation.
- All fluorophores present in the sample have to be considered for the unmixing calculation. If this is not done, the results will inevitably be false! The unmixing calculation is however not affected by taking into account fluorophore spectra in addition to the ones present in the sample. Zero contri-

Table 1 Requirements for correct results in linear unmixing

Unmixing parameter	$<N_{fluorophores}$	$=N_{fluorophores}$	$>N_{fluorophores}$
Detection channels	No unique solution (underdetermined)	Unique solution (determined)	Unique solution (overdetermined)
Factors in the unmixing equation	False result	Correct result	Correct result

$N_{fluorophores}$ stands for the number of fluorescent labels present in the sample.

bution values will be assigned to them. In the case of background contributions (e.g. autofluorescence), these also have to be defined spectrally and be treated as additional spectra.

It has to be considered that linear unmixing is performed on measured microscope data. Accordingly the values used for the unmixing calculations unavoidably include measurement errors that depend on the measurement conditions. By using a fitting approach (least squares, see above) valid results can be obtained even with such deviations and an estimate of the quality of the fit is possible (for overdetermined but not for determined equation systems). Factors influencing the quality of linear unmixing will be discussed in the section 'relevant parameters'.

Not all cases that are possible for equation systems in linear algebra have to be considered for measured data. As a fluorophore is either present or absent in the sample, negative contribution values are not possible and can be excluded from the available solutions. By using constrained unmixing algorithms, the calculated fluorophore contributions are made to sum to unity to avoid leftover values. This facilitates comparisons between separated fluorophores and keeps the unmixed data in close relation to the measured values.

In addition to the linear unmixing method described above, alternative approaches for the spectral unmixing of microscopic data have also been implemented, especially for datasets consisting of only few spectral channels [14]. These approaches are based on the correlation of the intensity values of a pixel in different image channels (as can be visualized in scatterplots similar to the ones used in cytofluorimetry). The unmixing is achieved by finding the distribution angles of the desired fluorophores in the scatterplot and by orthogonalizing them into separate channels ("stretching" them onto different axes of the plot). The method in principle does not require a priori information about the spectra because the main distributions can be found by line fitting. This only works reliably however only if significant amounts of the labels are present without colocalization.

3.1.2 Methods

3.1.2.1 Unmixing on the Emission Side

Spectral imaging has been implemented in several ways on widefield or confocal microscopes. Some of the solutions require specific instrumentation, but the method is also very generally applicable since any multi-channel fluorescence image can be considered as a series of spectral images. On a standard widefield fluorescence microscope, spectral separation of overlapping fluorophores was shown to be improved by determining and correcting for the crossover of individual fluorophores into different filter sets [15, 16]. Fourier

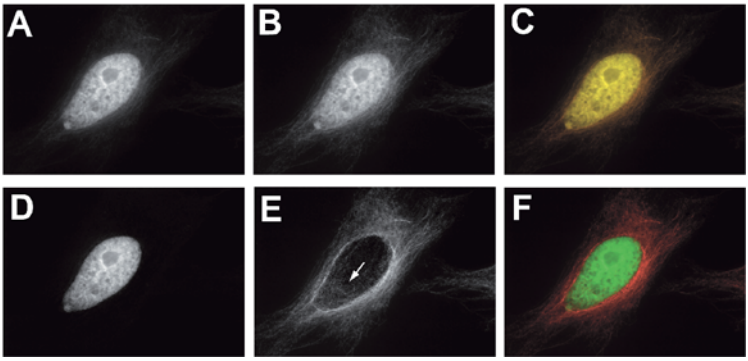


Fig. 3A–F Application example for spectral imaging and linear unmixing. Excitation based unmixing of HeLa cells containing Histone-EGFP and Alexa 488 stained microtubules. The overlap of the two emission spectra is significant as the emission peaks (EGFP: 508 nm, Alexa 488:515 nm) are only 7 nm apart. The sample was imaged on a widefield microscope with just one emission filter (530/50) and beamsplitter (500) under two different monochromator excitations using autoexposures for the channels: **A** image taken with 470±7 nm excitation; **B** image taken with 490±7 nm excitation; **C** overlay image of the two channels without linear unmixing. The crosstalk of the SFP-labeled nucleus is clearly visible as yellow; **D–F** images of the cells after linear excitation unmixing: D – Histone-GFP; E – Alexa 488 labeled microtubules. Even the microtubules on top of the nucleus are detected in this widefield image; (*arrow*) F – overlay image of the unmixed fluorophores

transform spectroscopy (FTS) has been implemented by coupling an interferometer to a widefield microscope to obtain detailed spectral information at every position in a sample [9, 17]. Initially just used with classification algorithms to do spectral karyotyping [12], the applications were subsequently extended to microscope data with colocalized labels. Analyzing FTS image data with linear un-mixing using singular value decomposition, up to seven fluorophores were simultaneously imaged and distinguished [9]. The same linear unmixing approach was then also applied to spectral data obtained with two-photon confocal microscopy and a liquid crystal tunable filter to separate up to four FP variants [8]. These approaches acquire the spectral data sequentially as a series of images (λ-stack). This is time-consuming and usually requires several minutes to acquire one λ-stack. The methods further suffer from fluorophore specific photobleaching rates which become most significant in sequential image acquisition. This poses serious problems for imaging living samples where the localization of the FPs can rapidly change during data acquisition and photobleaching may cause unpredictable effects on cell physiology. These problems with sequential data acquisition can be overcome by acquiring the spectral information into parallel detection channels. This has now been implemented on commercial confocal microscopes such as the LSM510 Meta from Zeiss which uses a grating for the spectral dispersion of the signal onto a multidetector array [11, 13]. Flexible parallel acquisition of spectral image data has also been implemented on prism based spectral confocal micro-

Table 2 Overview of the hardware capabilities ofdifferent microscope setups for spectral imaging

Acquisition mode	Overdetermined ($N_c \gg N_f$)	Determined ($N_c \approx N_f$)
Parallel	Zeiss LSM 510 Meta[a]	Leica SP/SP2/SP2-AOBS[b] Bio-Rad Rainbow[b] Beamsplitter setups Confocal microscopes
Sequential	Zeiss LSM 510 Meta[a] Leica SP/SP2/SP2-AOBS[b] Bio-Rad Rainbow[b] LCTF detection SpectraCube (FTS)	Widefield microscopes w. Filterwheels/filtercubes

[a] The Zeiss Meta detector can read out 8 of its 32 channels at the same time. To read out all 32 channels, 4 sequential acquisitions have to be made.
[b] Sequential: λ-series into a single detector. Parallel: Multiple detectors (3–4).
N_c: number of detection channels.
N_f: number of fluorophores.

scopes, the Leica AOBS and SP systems [14]; see also [18]. More recently, a filter-based spectral confocal approach was also introduced (Bio-Rad Rainbow)

It has become apparent that linear unmixing of microscope data can be realized on a wide variety of microscopic setups. This is because in many cases only a few channels ($N_{channels} \geq N_{fluorophores}$) are required for spectral imaging and unmixing. The number of fluorophores in a typical biological sample is very often limited to two or three. These may however be strongly overlapping as is the case for FPs and therefore require unmixing. An overview of systems and their capabilities is given in Table 2.

3.1.2.2 Unmixing on the Excitation Side

The range of possible approaches for linear unmixing can be significantly extended by looking at the second characteristic property of fluorophores, their excitation spectra. As for emission spectra, linear unmixing can also be applied based on the fluorophores' excitation spectra. Instead of exciting at one wavelength and collecting the emitted fluorescence into spectrally different detection channels, it is possible to excite sequentially at different wavelengths and detect the respective fluorescence with one detector only (Fig. 3). The total emission can be collected into just one detector so that the SNR for the acquired data is very high. The data quality is an important parameter for linear unmixing (see section 'relevant parameters'). Data acquired in this way can then be analyzed with the same algorithm used for emission based unmixing (see section 'linear unmixing'). The excitation-based implementation of spectral un-

mixing should be ideal on set-ups equipped with excitation sources that allow fast switching of the excitation light.

Excitation-based unmixing is being used in commercially available setups for widefield as well as for two-photon imaging. In contrast to the parallel detection possibility for emission unmixing, excitation unmixing is unavoidably based on sequentially acquired data.

As the number of fluorophores separable by linear unmixing is limited by the number of channels available for analysis (see Table 1), the combination of excitation and emission unmixing even increases the number of fluorophores that can be distinguished in a sample.

The quality of the unmixed data depends directly on the acquisition settings and the quality of the spectral data. It is therefore important to understand thoroughly the parameters affecting spectral imaging and linear unmixing to be able to judge the strengths and weaknesses of the diverse acquisition possibilities.

3.1.3 Relevant Parameters

Since spectral imaging is an established method in the field of satellite imaging and remote sensing, factors affecting efficiency have already been thoroughly considered [10]. The method is predominantly limited by factors such as image background or detector noise, but the appropriate selection of the number and bandwidth of the detection channels with respect to the overlap of the fluorophores to be distinguished also plays an important role [19, 20]. These limitations become most critical when imaging living specimens, where the signals of interest are usually weak and photodamage by intense irradiation has to be avoided.

3.1.3.1 Reference Spectra

For correct unmixing it is essential to use proper reference spectra. This is best done by measuring reference samples that contain only one of the fluorophores. In cases where it is certain that the signals are not colocalized in the regions used as references, samples with multiple labels could also be taken. This approach does however bear the risk of taking spectra with slight contributions of other fluorophores as references. In the unmixed image, this would result in assignment errors that would make any analysis of colocalization impossible.

For the processing of spectral data, it is advisable to take only the reference spectra of the fluorophores contained in the sample for the unmixing calculation. Inclusion of unnecessary reference spectra may degrade the resulting image because noise and other aberrations contained in the spectral data would result in a higher unmixing error under these conditions.

Reducing the number of reference spectra is one approach to optimize unmixing. In methods like iterative endmember ejection, spectra that are clearly not present in a pixel (negative contribution) are not used in the unmixing calculation.

3.1.3.2 Background

Removing any signal not originating from the fluorophores to be analyzed by background subtraction is an essential prerequisite for the linear unmixing analysis [9]. As with ratiometric measurements [21], failure to correct the background properly leads to significant intensity-dependent artifacts in the processed images. However, in cases of spectrally homogeneous background, there is the option to treat the background as a further fluorophore and thus separate it from the specimen specific fluorescent signals by linear unmixing.

3.1.3.3 Noise

Since linear unmixing is a pixel-based method, it is susceptible to errors introduced into the original images or the reference spectra by the Poisson noise of the fluorescence light itself and the detector readout noise. These sources of noise become important at low light levels in live specimen imaging where usually the illumination light and exposure time have to be kept at a minimum to preserve the physiological integrity of the sample. Computer simulations show that in the absence of noise, unmixing efficiencies are independent of the number of detection channels used (Fig. 3B). However, in the presence of detector readout noise, the errors in the processed image increase relative with the number of detection channels (Fig. 4A,B). For a constant detector readout noise, the detector signal to noise ratio (dSNR, does not include other sources of noise) will decrease according to 1/n (n=number of detection channels) for each detector. This decrease of the dSNR is only partially compensated by the noise averaging effect that occurs due to the increased number of detection channels (improvement according to $n^{-1/2}$; see [22]). Consequently, the dSNR decreases according to $n^{-1/2}$ and thus sampling the spectral information into few detection channels with broad bandwidth should result in superior quality of the unmixed data compared to sampling into a large number of detection channels with narrow bandwidths. This observation is relevant for measurements with high detector readout noise or with low signals. The total SNR (all noise types) of a measurement is affected by the number of detection channels proportional to the contribution of detection noise to the total noise.

Detection noise is signal independent and therefore additional detectors will increase the total noise of a measurement. This is not the case for the Poisson distributed noise of the emission light (photon shot noise), a significant factor for weak fluorescence signals. It is not significantly affected by the number of

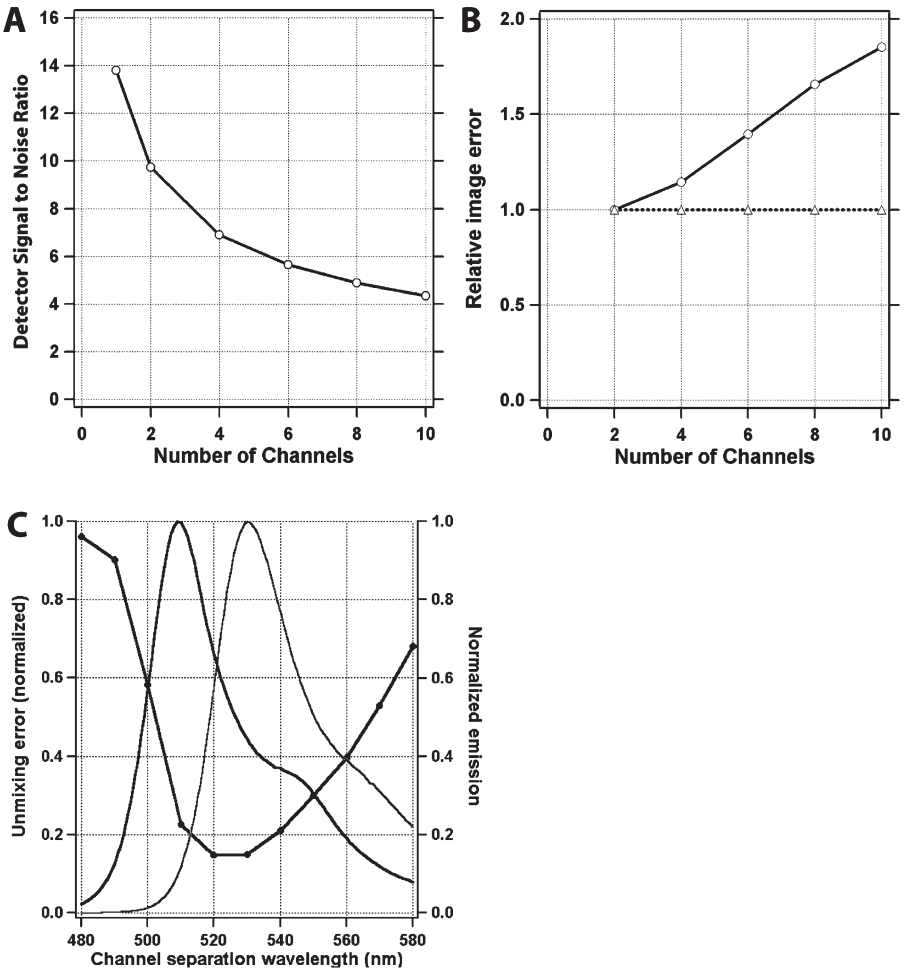


Fig. 4A–C Examples of factors influencing the efficiency of spectral unmixing. The simulation data were created and processed with routines written in Interactive Data Language (Research Systems, Inc.): **A** decrease of the detector signal to noise ratio in dependence of the number of channels used for sampling the spectral information; **B** dependence of unmixing efficiency on the number of detection channels in the absence and presence of noise. The relative increase of the unmixing error is independent of the actual noise level. Image error values in the graph are normalized to the image error obtained for two channel unmixing. *Solid line with circles*: relative image error in the presence of detector noise. *Broken line with triangles*: relative image error without noise; **C** influence of detection channel characteristics on the unmixing exemplified for two detection channels. A GFP signal was simulated and unmixed against YFP with detector noise added. Moving the border between the two detection channels to different wavelengths demonstrates the existence of an optimal position corresponding to a minimal unmixing error. The spectra for GFP (*dark gray*) and YFP (*light gray*) are superimposed on the plot (*right axis*)

detection channels acquiring the total signal. This is due to the fact that the standard deviation of the Poisson noise depends on the intensity of the signal. With this signal-dependent noise, less signal per channel also means less noise in that channel. Although the photon shot noise SNR ($N/\sqrt{N}$ for N photons) is lower for weaker signals per channel, the total photon noise of all channels remains constant ($SNR = \frac{\sum N_j}{\sqrt{\sum (\sqrt{N_j})^2}}$ for j channels). Thus results obtained with few or many channels are in this aspect similar.

3.1.3.4 Detector Channel Arrangements and Spectral Overlap of the Fluorophores

In contrast to significantly overdetermined systems, the filter settings for setups with approximately as many channels as fluorophores should be chosen carefully for optimal results [19, 20]. An optimized arrangement of the detector channels to obtain the least unmixing error can be determined according to the spectra of the fluorophores (Fig. 4C). This is most easily demonstrated with two partially overlapping fluorophore spectra (a,b) detected with two channels. The optimized solution corresponds to a maximal separability, found by calculating the endmember separability with the SVD method (see above) for the diverse filter setups. Alternatively, it can also be found using a 'figure of merit' as a measure of the efficiency in the use of emitted photons [19]. For most fluorophore combinations, the optimal cutoff between two channels is located at the intersection of the falling slope of the first with the rising slope of the second fluorophore spectrum.

However, even with optimized channel settings, the unmixing efficiency is directly affected by the amount of spectral overlap between the fluorophores. In the absence of noise, the degree of overlap does not affect unmixing efficiency. In the presence of noise the errors of the unmixed data increase with the spectral overlap of the fluorophores. One way to overcome this is by over-sampling, which has been nicely exploited on a widefield microscope by applying sequential imaging with detection filter arrangements partially overlapping in sequential acquisitions [23].

In the case of significantly overdetermined setups (non-overlapping), the separability (and therefore the quality of the unmixed image) is solely determined by the amount of overlap between the used fluorophore spectra.

3.1.3.5 Number of Channels

Both determined (n channels=n fluorophores) as well as overdetermined systems (n channels>n fluorophores) can be used for spectral imaging and linear unmixing [19, 20]. Are there differences in performance?

As shown above, readout noise increases with the number of detection channels used. In cases of significant readout noise, this affects the quality of the unmixing. On the other hand, an estimate of the error of the fit is possible for overdetermined, but not for determined systems by comparing the fit with the raw data of each channel.

Whereas the positioning of the detection channels is very relevant for determined setups (see above), significantly overdetermined systems are less susceptible to this influence.

The following conclusions can thus be drawn:

The best unmixing results are obtained with few and wide channels as this gives higher signals per channel and minimizes possible readout noise problems. If the gains of the channels can be set independently, the separability of the signals can be enhanced in this way. Such an approach does however require fine tuning of the settings, as only optimized settings will give an improved result. Not properly chosen settings will give inferior results.

Overdetermined systems may not inherently give better results, but can be used without the fine tuning of filter settings.

3.2 Subtraction

In some cases of overlapping fluorophores, a straightforward subtraction approach, as used in many formulas for sensitized emission detection (see below), can be used instead of linear unmixing. Here, the contribution of the first fluorophore A into the detection channel for the second fluorophore B is determined and expressed as a normalized value R_A:

$$R_A = \frac{A_2}{A_1} \tag{5}$$

To get the second fluorophore without contribution of the first, a simple subtraction is performed using the information of the first channel:

$$B = Ch_2 - (R_A \times Ch_1) \tag{6}$$

This approach works also for fluorescent proteins [24]. It also works for more than two fluorophores (see below).

There are however limitations to this technique. It requires at least one spectral channel to contain one fluorophore without any contribution of the other:

$$R_x(\lambda) - \sum_i R_i(\lambda) = 0, \quad x \in \{1 \ldots i\} \tag{7}$$

That channel is used for the subtraction. In cases of mixed contributions in all channels it is not applicable! In cases of multiple fluorophores this condition

has to be met in the beginning and again after each subtraction of a fluorophore. The subtraction technique is therefore limited to only a subset of the cases that can be solved by linear unmixing. In practice this means that not all fluorophore combinations can be used and that more restrictive filter settings have to be used that do not sample all of the available signal.

A second shortcoming of the subtraction technique is that while bleed-through is corrected, the additional signal contained in other channels is not further utilized. In linear unmixing, the total signal of all channels is distributed onto the fluorophores according to their contributions. The unmixed signals can therefore be significantly brighter than their representation in the single spectral channels. With the subtraction method, this is not possible.

The susceptibility of the subtraction technique to noise artifacts is equal to the linear unmixing technique.

In comparison to the subtraction method, linear unmixing is more flexible, uses more of the available information and is thus the approach best suited to process data with overlapping fluorophores.

4 Applications

Almost any fluorescence microscope can be used for spectral imaging and the data can be stored as image files. But how to perform the linear unmixing? By now, unmixing tools are provided in the software of several confocal microscopes (Zeiss, Leica, BioRad). They are now also available for some widefield microscopes (Olympus BioSystems). In addition it is possible to implement linear unmixing easily in any image processing package or as an ImageJ plugin. Appendix A contains a numerical recipe for simple linear unmixing that can be used in any software.

With the processing tools available, spectral imaging and linear unmixing can be applied to many forms of biological imaging.

4.1 Timelapse Imaging

Because of the highly dynamic nature of processes in living samples, it is essential for in vivo co-localization experiments or ratiometric methods to gather information of two or more fluorescent molecules at the same time. With GFP-tagged proteins this is usually difficult to achieve due to the lack of spectral variants that can be excited simultaneously but do not bleed through significantly in the emission channels. Further complications arise when the need to resolve fast processes, such as the movement of membrane transport carriers [25], precludes the use of time consuming sequential image acquisition which can overcome bleed through problems with GFP variants to some extent [26]. For these problems, spectral imaging offers the possibility to excite two spec-

trally similar fluorophores, for example EGFP and EYFP, with just one wavelength (e.g. 488 nm) and detect them simultaneously without significant losses of the emitted fluorescence. In contrast to common methods involving glass filters to separate the emitted light from distinct fluorophores, spectral imaging collects almost all of the fluorescence emitted, which is critical for work with living samples. Thus spectral imaging, even with only two fluorophores being involved, should always be the method of choice when working with living samples.

4.2 Fluorescence Resonance Energy Transfer

A further application of spectral imaging is in FRET microscopy, which is an important tool for imaging the dynamics of cellular processes like protein-protein interactions or post-translational modifications [27]. However, an efficient FRET signal requires significant overlap between the emission spectrum of the donor and the excitation spectrum of acceptor fluorophore. This requirement is inevitably accompanied by a significant overlap of the emission spectra of the donor and acceptor and complicates the determination of FRET efficiencies [27, 28]. As spectral imaging is well suited to separate even highly overlapping donor and acceptor emissions, FRET imaging with already established donor acceptor pairs can be facilitated [13] and applied to previously unused FP-based donor acceptor pairs with increased FRET efficiencies due to the increased spectral overlap of the donor emission and acceptor excitation [18].

FRET in living samples is very often observed with methods that detect and quantify the sensitized emission of the acceptor. In the most common methods, the channel crosstalk of the measurement is corrected by subtraction of image channels [27] (see section 'Subtraction'). It is easily possible to replace this step with linear unmixing. As linear unmixing covers also cases of strongly overlapping fluorophores, the method can thus be extended and less restrictive filter settings that collect more of the signal can be used (contributions of both fluorophores to both channels are allowed).

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Appendix

Numerical Recipe for Linear Unmixing

To make the principle of linear unmixing more easily understandable, the solution for a two channel/two fluorophore situation is shown.

$Ch_{x,y}$ represent the signals in detection channels x and y, and A_x, B_x and A_y, B_y the normalized contributions of *FluoA* or *FluoB* to channels x and y as they are known from the spectral signatures of the fluorescent proteins.

Deduction:

$$Ch_x = A_x\,FluoA + B_x\,FluoB$$

$$Ch_y = A_y\,FluoA + B_y\,FluoB$$

$$Q = \frac{Ch_x}{Ch_y} \Rightarrow Ch_x = QCh_y \Rightarrow$$

$$A_x\,FluoA + B_x\,FluoB = Q(A_y\,FluoA + B_y\,FluoB)$$

$$A_x\,FluoA + B_x\,FluoB = QA_y\,FluoA + QB_y\,FluoB$$

$$A_x\,FluoA - QA_y\,FluoA = QB_x\,FluoB - B_x\,FluoB$$

$$FluoA(A_x - QA_y) = FluoB(QB_y - B_x)$$

$$\frac{FluoA}{FluoB} = \frac{QB_y - B_x}{A_x - QA_y}$$

Application:
In order to determine the fluorescence emitted by each of two individual fluorophores (*FluoA*, *FluoB*) in co-localization or FRET experiments, only four equations have to be applied for every image pixel i:

$$Q(i) = \frac{Ch_x(i)}{Ch_y(i)} \tag{A1}$$

$$R(i) = \frac{FluoA(i)}{FluoB(i)} = \frac{B_yQ(i) - B_x}{A_x - A_yQ(i)} \tag{A2}$$

The total signal $S(i) = \sum_{k=1}^{n} Ch_x(i)$ as the sum of all channels can then be divided into the contributions of FluoA and FluoB by

$$\text{FluoA}\,(i) = \frac{S\,(i)}{1 = \dfrac{1}{R\,(i)}} \tag{A3}$$

$$\text{FluoB}\,(i) = \frac{S\,(i)}{1 + R\,(i)} \tag{A4}$$

or computationally more simply as

$$\text{FluoB}\,(i) = \frac{S\,(i)}{1 + R\,(i)} \tag{A3'}$$

$$\text{FluoA}\,(i) = S\,(i) - \text{FluoB}\,(i) \tag{A4'}$$