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Linear fluorescence unmixing in cell biological research

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One of the most important issues in biomedical research microscopy is the analysis of the spatial localization of fluorescently labelled structures. Today, a wide range of available dyes, fluorescent proteins and labelling techniques allows the creation of complex multicolored samples to study intracellular localization. However, analysis of localization and colocalization is often perturbed by a significant overlap of the fluorophores used to label the structures. This problem can be addressed by the use of multichannel fluorescence imaging together with linear unmixing of the image data. This method allows the reliable separation of overlapping fluorescence signals and subsequent accurate and quantitative (co)-localization analysis. In this chapter, we explain the theory of linear unmixing, provide information on how to perform such experiments, and point out technical limitations and potential pitfalls. Finally we demonstrate the benefit of this methodology, employing examples from cell biology and virology.

Keywords fluorescence; linear unmixing; localization; colocalization; fluorescent protein; live cell imaging; cell biology; GFP

1. Introduction

In order to address biocomplexity at the cellular level, experimental approaches are required in which an ever increasing amount of cellular parameters can be quantitatively determined to investigate the distribution, interaction or mobility of proteins or structures. Therefore the need to perform microscopic imaging experiments with many fluorochromes at the same time is constantly increasing. Multicolor fluorescence microscopy offers advanced techniques such as fluorescence recovery after photobleaching (FRAP), fluorescence resonance energy transfer (FRET) and others. One basic prerequisite for the success of these sophisticated experimental approaches is that multiple fluorochromes can be reliably separated and imaged in individual detection channels. However, the spectral properties of the available fluorescent proteins and dyes set an upper limit to the number of fluorochromes that can be detected simultaneously with common microscopic methods. (Multi)-spectral or multichannel fluorescence imaging and linear unmixing extend the capabilities to discriminate distinct fluorophores with highly overlapping emission spectra. Multispectral imaging and linear unmixing is a long established method in earth imaging and remote sensing [1-3]. Compensation for spectral overlap in multicolor flow cytometric analysis [4] is a method very similar to linear unmixing in fluorescence microscopy and is entirely accepted by the scientific community for nearly 20 years [5].

2. Multifluorescence microscopy

As methods like multispectral imaging and linear unmixing only represent tools to answer biological questions in a convenient and accurate way, the experiment as a whole has to be considered before performing these methods. Therefore, some general factors have to be taken into consideration when performing fluorescence live-cell imaging experiments. The biological specimen has to be imaged with a

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sufficient spatial and temporal resolution and images have to be acquired with a good signal-to-noise ratio. In contrast to that, intensity and duration of exposure to excitation light have to be as low as possible and also the number or proportion of labelled molecules in the cells has to be kept low, not to perturb normal cellular health. As an additional difficulty, a selection of dyes or fluorescent proteins as markers for experiments merely by their spectral properties is sometimes impossible. This can be due to biochemical and biological characteristics of these fluorochromes or due to availability to the researcher.

2.1 Conventional multichannel fluorescence microscopy

In conventional multichannel fluorescence imaging, multiple fluorochromes can be used to target specific areas in the specimen. The goal is to choose these fluorochromes in a way that one is able to image each fluorochrome in an individual detection channels. Band pass detection is appropriate to separate the fluorescent signals into the individual detection channels if only minor spectral overlap of the emission and the excitation spectra is present. From a technical point of view, multicolor applications in conventional fluorescence microscopy are dominated by a compromise between narrow excitation and emission filters to reduce crosstalk on the one hand and broadrange/longpass filters to collect enough fluorescence signals and to increase sensitivity on the other hand. Usually large amounts of the emitted fluorescence have to be discarded to ensure reliable separation of at least some combinations of fluorochromes.

Figure 1 schematically displays how to separate fluorochromes using bandpass filters and also depicts the problem of overlapping fluorophores emission spectra.

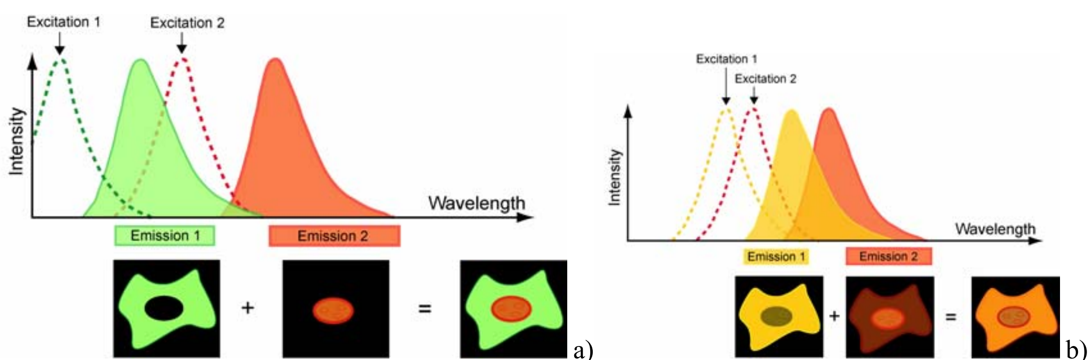

Fig. 1 Theory of fluorochrome separation using bandpass emission filters in conventional multichannel fluorescence microscopy. a) Two fluorochromes (one green and one orange emitting) are successfully separated using two bandpass emission filters, as their emission spectra only show a negligible overlap. The merge of the two schematic channel images generates an image of a cell with green cytoplasm and orange nucleus. b) Two fluorochromes (yellow and orange emitting) with significantly overlapping emission spectra can not be fully separated using two bandpass emission filters. Merging the two channel images leads to an image that lacks pure yellow and orange colors.

A practical example of using bandpass filters is shown in Figure 2. A three-color experiment was conducted by expressing fusion proteins of enhanced cyan fluorescent protein (ECFP), enhanced yellow fluorescent protein (EYFP) and monomeric red fluorescent protein 1 (mRFP1) in HeLa cells. The combination of these three fluorescent proteins (FPs) was chosen for a study examining the nucleo-cytoplasmic shuttling properties of the Human Immunodeficiency Virus (HIV)-1 Rev protein [6]. Although is was possible to separate the three FPs by using narrow excitation and optimized emission bandpass filters, this was at the expense of reduced sensitivity. Exchanging the suboptimal (in terms of sensitivity) bandpass filter for the mRFP1 channel (Em: 610-680 nm) to a filter fitting closer to the mRFP1 spectrum (Em: 570-640 nm), decreased the necessary exposure time from 200 ms to below 25

ms keeping the signal-to-noise ratio constant. However, this resulted in significant crosstalk from EYFP into the mRFP1 channel.

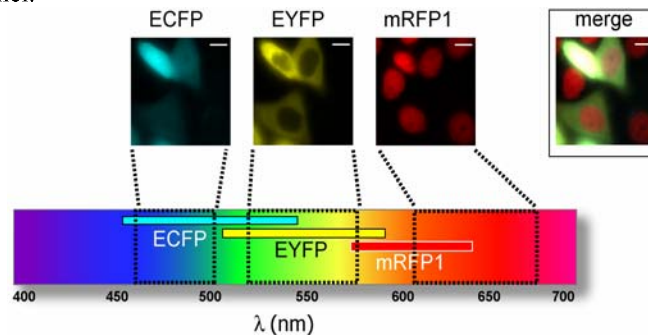

Fig. 2 ECFP-, EYFP- and mRFP1-fusionproteins were expressed in HeLa cells and imaged using a widefield microscope equipped with bandpass filters. The major emission bands for ECFP, YFP and mRFP1 are shown as cyan, yellow and red bars respectively on the optical spectrum. Bandpass filters are denoted with dashed boxes. Bandpass filters were chosen with a safety margin to ensure that no spectral crosstalk. Especially for ECFP and mRFP1 a lot of signal was cut off. Therefore cells had to be exposed to damaging excitation light longer than it would have been necessary with filters matching their spectra closely. Note that ECFP fluorescence is not observed in the EYFP channel because ECFP is not excited by the longer wavelengths of EYFP excitation light.

2.2 Spectral imaging and unmixing microscopy

As each fluorophore has its characteristic spectral profile, this signature can serve as a reference in multicolor approaches. Spectral reference fingerprints can be used in a mathematical procedure to relate the amount of a mixed fluorescence signal to single dyes and thereby to unmix the emission spectrum of a sample of interest. With this technique, no longer an optical separation of signals into channels with non-overlapping bands is attempted but a recording of the complete complex emission signals from multiple fluorophores is carried out. The most essential part of spectral imaging microscopy systems is a spectral dispersion element that separates the incident light into its spectral components.

On the one hand confocal imaging methodology can be used together with gratings and prisms to separate light into spectral components [7-9] (Figure 3a). On the other hand Fourier transform spectroscopy based on an interferometer can be used to distinguish between at least seven different fluorochrome [10-12].

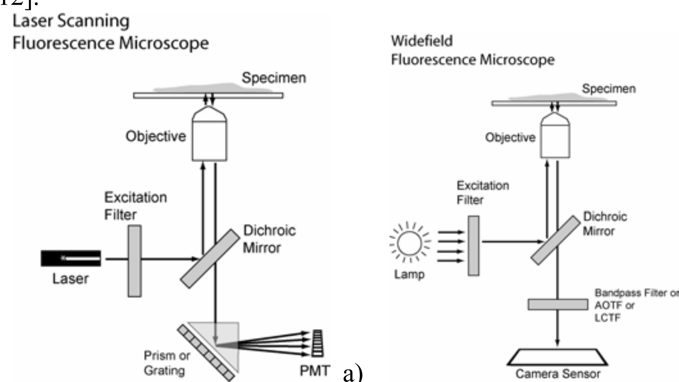

Fig. 3 Microscope systems for multispectral imaging,. a) Grating- (or prism)-based spectral imaging using a laser scanning confocal microscope capable of generating emission fingerprints. b) Filter based spectral (or multichannel) imaging system. The quality of spectral information is dependent on the number of possible channels. If bandpass filters in a filter wheel are used, than the number of positions in the filter wheel limits the number of fluorochromes that can be separated. By using an acousto-optic tuneable filter (AOTF) or a liquid crystal tuneable filter (LCTF)

more flexibility is available. Note that this technique is not restricted to widefield microscopy but can also be applied to confocal or two-photon confocal systems. Note that lightpaths are only schematic.

To be able to unmix spectral data, the relative contribution of each used fluorophore to every single detection unit (band) needs to be available as a reference λ-stack that has to be acquired separately. Fig. 4 depicts how the determination of crosstalk is achieved using a reference λ-stack.

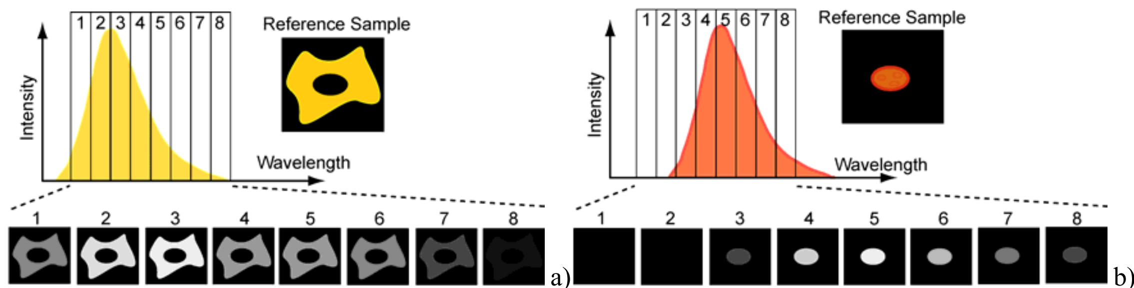

Fig. 4 Theory of emission fingerprinting with reference samples. If two fluorochromes are to be reliably separated (no matter how close their spectra are) then the relative contribution of the fluorochrome to the individual bands (of a certain bandwidth) of the multispectral imaging system is determined. One image is produced of the whole specimen for each band of the detector. a) The yellow fluorochrome contributes most to the bands two to four. b) the orange dye contributes most to the bands four to six.

Having generated emission fingerprints of pure reference samples, the sample of interest is acquired with the same settings. Subsequently the spectral signature is used to digitally separate the fluorescence emissions of the sample of interest by applying a linear unmixing algorithm. From a mathematical point of view, a multifluorescence signal consisting of the fluorescence of n dyes can be understood as an equation system with n unknowns. To solve this linear equation system, n equations describing these variables are required which are delivered by the spectral reference signatures of n dyes [13, 14].

Using this technique, now signals from up to eight FPs (or fluorochromes in general) can be separated.

2.3 Multichannel fluorescence microscopy and linear unmixing

Widefield multichannel microscopy and linear unmixing use the same principles as the spectral imaging methods but from a technical viewpoint, system setups are generally less complex (concerning the spectral separation part at least). Although some restrictions apply, we will focus strongly on this technique in the following sections, as it is the least cost intensive and most straightforward method and therefore available to a broad scientific community. A multichannel imaging setup can be as simple as a conventional fluorescence microscope (Figure 3 b).

The workflow to conduct a successful multichannel imaging experiment with subsequent linear unmixing of n fluorochromes contains the following six steps: 1) Produce n reference samples each with containing a pure fluorochrome . 2) Acquire reference images with at least n channels and n appropriate filter sets. 3) Determine the amount of signal each fluorochrome contributes to the n channels, taking background signal into account. 4) Acquire images of your sample of interest under the same conditions and with the same settings as in step two. 5) Process the image by pixel by pixel with the obtained crosstalk information of step three. 6) Inspect output image for consistency.

Figure 5 displays how the determination of crosstalk for a two-color experiment is achieved. It is crucial to take reference images from pure samples to determine the relative contributions of signal to the channels in the image. By this means the complex spectrum of a fluorochrome is reduced to a fistful of numbers, namely the relative amount of signal this fluorochrome contributes to the individual channels of the image.

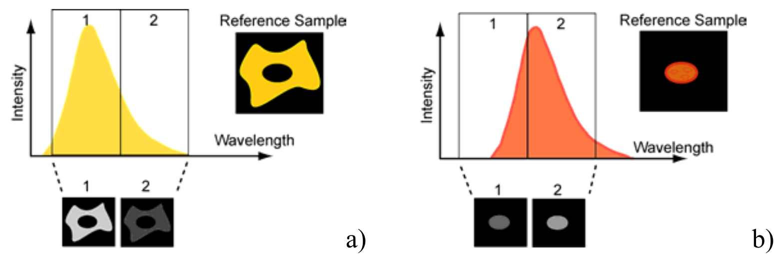

Fig. 5 Theory of multichannel imaging with reference samples. If two fluorochromes are to be reliably separated (no matter how close their spectra are) then at least a two-channel reference image has to be acquired for each fluorochrome to determine the relative contribution of the fluorochrome to both channels. a) The yellow fluorochrome contributes more to channel one than to channel two. b) the orange dye contributes more to channel two than to one.

To get a linear equation system (see section spectral unmixing) that is (at least) just determined and can be solved, the number of bandpasses has to equal the number of dyes used in the experimental setup. The number of dyes that can be unmixed with this technique is therefore strictly limited to the number of bandpass filters of the system used for image acquisition.

3. Applications

In this section we present two examples of how linear unmixing can enhance scientific research. Although the experiments do not represent "high-end" applications with 6 or 7 fluorochromes in one specimen, they may be prototypical for many other applications and problems that arise when multicolor fluorescence microscopy is carried out.

3.1 Linear unmixing applied to stably transfected cell lines

One of the most widely exploited proteins in cell biology is the *Aequorea victoria* green fluorescent protein (GFP) (reviewed in [15]). Although there has been considerable success in developing more and spectrally distinct fluorescent proteins in the past years [16-20], abundance of *A.v.* GFP in the scientific community is still overwhelming. GFP-fusionproteins and cell lines that are either commercially available or circulate in the scientific community, limit conventional multicolor experiments to a few spectrally suitable partners in the blue and the orange/red emission range. Linear unmixing can be of great help overcoming these limitations. Figure 7 shows how a well-know HeLa cell line stably expressing GFP-tagged chromatin [21] can be used in conjunction with CFP-tagged centromere binding proteins.

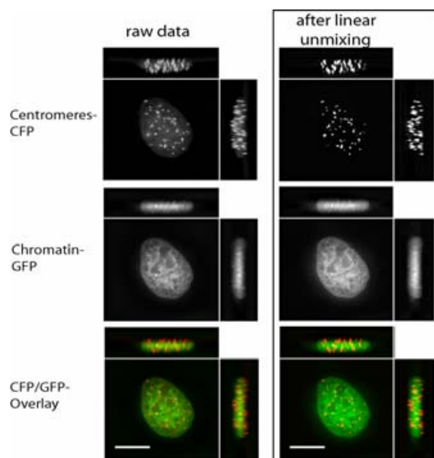

Fig. 6 The nucleus of a HeLa cell with GFP-labelled chromatin and CFP-labelled centromeres is shown. An image z-stack with 30 sections (distance 0.28 µm) was acquired (left column) and linear unmixing with reference samples applied to the whole stack (right column). Linear unmixing removed the diffuse background (representing crosstalk from the GFP) in the CFP channel and added it to the GFP channel that appears slightly brighter after unmixing. Note that this effect is visible also in the side view projections of the z-stack. Filter sets were: BP 436/20 FT 455 BP 480/40 for CFP and BP 475/40 FT 500 BP 530/50 for GFP.

We used a similar approach as shown in Figure 6 to address the influence of the HIV-1 Rev protein on the intracellular localization of a novel cellular factor. A cell line was available expressing a newly identified cellular factor called Risp tagged to GFP. To address the influence of Rev coexpression in living cells on Risp localization, we had to choose from a set of CFP-, GFP- and YFP-containing HIV-1 Rev-fusionproteins (Rev fusions to RFP did not localize properly). Generation of the Risp-GFP cell line was laborious and so the solution was to coexpress the Rev-CFP fusionprotein in the cell line and perform linear unmixing on a image z-stacks of the cells. Finally this approach was successful and confirmed a putative interaction between HIV Rev and the cellular protein Risp [22].

3.2 Removing unwanted fluorescence from images

Investigating the influence of a variety of molecules on cellular health, morphology and metabolism is one of the big challenges of drug research and emerging technologies such as multiparametric fluorescence high-content imaging. Not only the effects of small molecules, proteins or siRNA on cells are tested but also those of complex natural extracts. Plant extracts with a wealth of secondary metabolites are an extremely rich source of candidate drugs. Unfortunately many of these complex natural extracts exhibit a strong fluorescence, often preventing their use together with fluorescence-based assay techniques. We have investigated the effect of Hypericin, one of the major bioactive components of St. John´s Wort (*Hypericum perforatum*) extract and the major source of red fluorescence of alcoholic extracts of St. John´s Wort. We have treated microglial cells for 24 hours with Hypericin, resulting in a strong red fluorescence of the cell that localized to the interior organelles and membrane vesicles of the cells but spared the nucleus. In an effort to prove suitability of linear unmixing for such applications, we chose to label the cytoskeleton with Rhodamin red, a red fluorochrome with an emission maximum very close to that of Hypericin. We applied linear unmixing to the multichannel images and found that a very accurate unmixing of Hypericin and Rhodamine signal was possible (Figure 7).

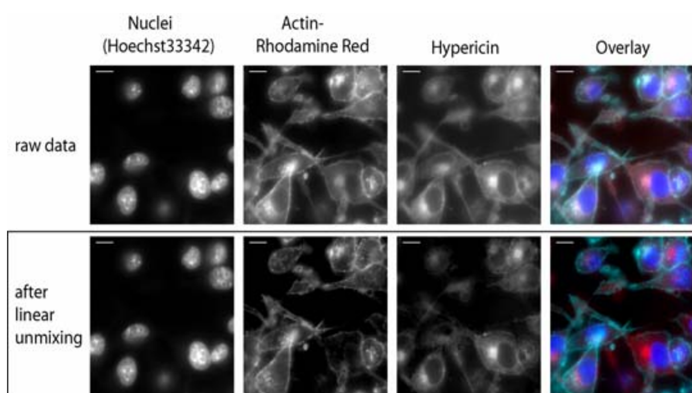

Fig. 7 Separating Rhodamine Red and Hypericin in BV2 microglia cells. Cells were treated for 24 h with Hypericin resulting in unwanted additional red fluorescence in the specimen. Red fluorescence of Hypericin obscures at least partially the Rhodamin red-labelled actin structure. The overlay shows nuclei (pseudocolored in blue), actin (pseudocolored in cyan) and Hypericin (pseudocolored in red). After linear unmixing the localization of Hypericin to the interior organelles of the cell becomes visible. Scale bars: 10 µm.

Detailed evaluations confirmed the successful application of linear unmixing (Figure 8).

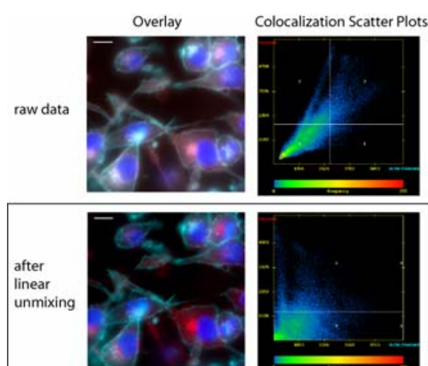

Fig. 8 Colocalization of actin-Rhodamine and Hypericin in BV2 cells before and after unmixing. Scatter plots are shown, displaying Rhodamine signal on the x- and Hypericin signal on the y-axis. Every pixel in the scatter plot represents the corresponding intensity of Rhodamine and Hypericin of a pixel in the image to the left. Before unmixing, increasing signal intensity in one channel corresponds with an increase in the other channel. This is reflected by a “cloud” of pixels with a diagonal orientation from bottom-left to top-right. Such a perfect colocalization is not expected for Hypericin and actin but derives from fluorochrome crosstalk. After linear unmixing this artificial colocalization disappears, presenting a much more reliable localization of actin and Hypericin.

These data provide evidence that fluorescence derived from natural substances could be removed from microscopic images before subjecting the image data to further biological analysis.

4. Pitfalls and troubleshooting

Although spectral imaging itself is an established technique, a number of parameters can be optimized to enhance image quality and/or avoid unmixing-borne artefacts.

4.1 General microscopic setup

It is crucial to ensure that no dust or other particles are present on the fluorescence filter cubes. This will lead to spatially slightly altered signal intensities from one channel image to the other, often not visible to the naked eye. Nevertheless it will result in image artefacts after linear unmixing. Particles that affect all fluorescence channels the same way (e.g. when present on the CCD camera) are less prone to generate artefacts after unmixing but will "only" reduce general image quality.

It is also critical to keep all parameters of the experimental setup for sample acquisition as close as possible to the setup that has been used for acquiring the reference images. Using another microscope objective can significantly alter the emission fingerprint or fluorescence crosstalk of a fluorochrome as objectives can differ greatly in their optical properties (e.g. some objectives are UV-transmissible, others are not). Take also care of using the same type of slide or culture dish, switching from glass to plastic may influence the signal in different channels in a way that is hard to predict.

4.2 Focal planes

Be aware that linear unmixing of several fluorescence channels can only be performed when channel images are acquired at the same focal planes.

When image z-stacks are acquired for subsequent deconvolution of image data, two major points have to be considered. First, to avoid unequal bleaching of the fluorochromes in the specimen, images of every fluorescence channel of a single z-section should be captured before proceeding to the next z-section. Second, if deconvolution is performed before linear unmixing, signal intensities in the output images should match intensities of the input images and must not be scaled differently in any way.

4.3 Pixel shift

Linear unmixing is a pixel based method and is therefore susceptible to errors introduced in the original image not only by noise but also by a pixel-shift in x-, y- or z -direction between the different fluorescence channels. Applying unmixing algorithms to such image data, will generate image artefacts that appear stronger even than in raw data (Figure 9). To avoid such shifts between individual channels of an image, frequently reference structures (e.g. fluorescent beads) should be imaged or shift-free filter sets be used.

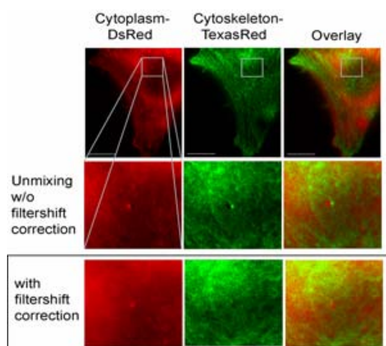

Fig. 9 A HeLa cell is shown that expresses the DsRed fluorescent protein diffusely in the cytoplasm (pseudocolored in red). The cytoskeleton was stained with TexasRed (pseudocolored in green). Linear unmixing with reference samples was successfully applied to the image. However unmixing without previous correction for pixel filtershift leads to an artificial shadow-like effect of spots in the image (see cut-outs in the middle row). Correction for the shift and subsequent linear unmixing reveals the true nature of the structure (same cut-out in bottom row).

4.4 Noise and fluorochrome intensities

As for most other imaging setups, noise is one also of the greatest problems for linear unmixing. In most spectral unmixing software packages, the definition of a homogeneous background noise as an additional fluorophore is possible and can be useful to enhance data quality. Proper definition of background intensity in reference images is also critical. Note that depending on the screen settings, an area containing significant fluorophore signal could in error be selected as background.

If you do not have a proper signal-to-noise ration then linear unmixing will not give satisfactory results. Linear unmixing does not generate signal out of nothing, it just pushes signal to the right channel.

4.5 Autofluorescence and unknown fluorescence sources

It should be taken into consideration that some excitation wavelengths induce significant autofluorescence in biological samples (e.g. UV/blue light in mammalian cells or green light in plant cells). Definition of this autofluorescence as an additional fluorophore can be helpful. As in widefield unmixing microscopy the number of fluorophores usually matches the number of detection channels, the system is just determined and linear unmixing can be performed. If non-determined effects between two or more fluorphores occur, such as e.g. FRET, the calculation of the correct fluorescence crosstalk is not possible and linear unmixing will yield to some extent false results.

4.6 Unmixing without reference samples

In commercially available microscope software packages, algorithms are often implemented to provide unmixing without the need for distinct reference samples. The software automatically looks for spectral information of all components directly in the sample image. An image-analysis algorithm inspects all channels one after the other and tries to identify an area in each channel where no signal is present in all other channels. If that can´t be found, the algorithm takes the area with the lowest signal intensities in the other channels and considers it as an area containing a pure fluorochrome. Applying this method to biological specimen nearly always leads to more or less pronounced artefacts in the unmixed image (Figure 10). This is especially true for widefield microscopy. However this method can be used in some special experimental setups (e.g. mixed cell populations with one fluorochrome per cell population).

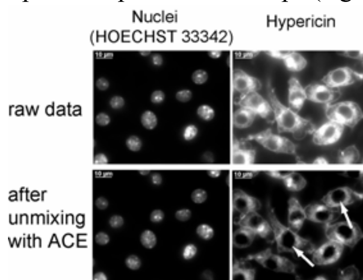

Fig. 10 Unmixing applied to widefield image data without using fluorochrome references. The algorithm tried to identify areas in the channel-images where only one fluorochrome contributes to the signal. As such an area isn´t present (in the widefiel dimage, cytoplasm abov ethe nucleus also contributes to signal in the nuclear area), this resulted in “stamped out” nuclei in the Hypericin channel (two of the nuclei are marked with white arrows), a thing that is immediately understood by users as being “non-biological” and artificial. The wrong comprehension that this kind of data is typical for all linear unmixing methods, contributes to the low acceptance of this technique in the scientific community.

5. Acceptance in the scientific community

No matter how powerful the method of linear unmixing in fluorescence microscopy is, acceptance of it in the scientific community currently seems to be still relatively low.

5.1 Seeing is believing (or not)

Apparently the most important reason why unmixing is not already widely accepted for biological research in the scientific community is that the researcher recognizes any artificial result immediately

when looking at the final image. Researchers usually know their samples and specimen well and see when something is not as it should be. This mostly leads to immediate declaration of a method as producing artificial data and to subsequent rejection of the method. This is in great contrast to flow cytometry. The user will continue using the software compensation until all of his cell populations behave in the scatter plot as they should. He does not see "stamped out" nuclei as shown in Figure 10, even if the effects of under- or overcompensation can be as big or even more pronounced as that (always affecting whole cells not only compartments of cells).

5.2 Time is short

Time is short and most researchers tend to plan more experiments than they could ever conduct. Performing linear unmixing adds additional steps to the workflow of an experiment: production of reference samples, acquiring reference images, calculation of crosstalk and applying linear unmixing algorithms. Many users who do not have a very high pressure for having more colors available might be discouraged and move towards other techniques rather than unmixing. Commercially available software packages already offer help with the workflow for spectral imaging but more developments have to be made. There are a couple of things that could improve in the future. Having dye/fluorochrome databases combined with databases of filter sets, objectives, light sources and sample preparation methods would facilitate using linear unmixing.

6. Conclusion

Multichannel unmixing is an approach that overcomes a number of limitations of common fluorescence microscopy. It allows the use of dyes and fluorescent proteins that could not be discriminated in one experiment so. Especially, demanding biological applications like phenotyping of stem cells, could greatly benefit from such increased performance. The method however opens a variety of new advantages and possibilities that go beyond increasing the number of fluorescent markers that can be used simultaneously. Unmixing strategies can also boost sensitivity, as almost all of the fluorescence that the sample emits can be collected without having to throw away most of the light by using narrow bandpasses. Especially living specimen profit from a reduction in exposure to excitation light and therefore unmixing should always be the methods of choice when performing live cell imaging. Intrinsically, unmixing in fluorescence microscopy does not produce artificially embellished images. If it is applied correctly, quantitative analysis is not only still possible with processed images but can become substantially more informative and reliable. We expect this methodology to become an invaluable tool not only for classical cell biological research but also for emerging technologies like multiparameter high-content image analysis, scanning fluorescence microscopy and others.

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