Quantification of colocalization and cross-talk based on spectral angles

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Summary

Common methods for quantification of colocalization in fluorescence microscopy typically require cross-talk free images or images where cross-talk has been eliminated by image processing, as they are based on intensity thresholding. Quantification of colocalization includes not only calculating a global measure of the degree of colocalization within an image, but also a classification of each image pixel as showing colocalized signals or not. In this paper, we present a novel, automated method for quantification of colocalization and classification of image pixels. The method, referred to as SpecDec, is based on an algorithm for spectral decomposition of multispectral data borrowed from the field of remote sensing. Pixels are classified based on hue rather than intensity. The hue distribution is presented as a histogram created by a series of steps that compensate for the quantization noise always present in digital image data, and classification rules are thereafter based on the shape of the angle histogram. Detection of colocalized signals is thus only dependent on the hue, making it possible to classify also low-intensity objects, and decoupling image segmentation from detection of colocalization. Cross-talk will show up as shifts of the peaks of the histogram, and thus a shift of the classification rules, making the method essentially insensitive to cross-talk. Cross-talk, or bleed-through, is the incomplete separation of fluorescence emission from different fluorochromes at image capture. Fluorescence emission intended to be associated with a particular wavelengths may also give rise to detected intensities at other wavelength. This is caused either by fluorescence emission spectra having components outside the main intended wavelength range and/or by incomplete spectral separation of the different detected wavelengths. Stable methods for suppression of cross-talk are dependent on image capturing techniques and hardware settings. Hardware solutions for avoiding cross-talk are typically expensive and have to be adapted to the specific image capturing apparatus.

The first methods for quantification of colocalization based on pattern recognition techniques were developed in the beginning of the 1990s (Manders et al., 1992). Soon after, the same authors (Manders et al., 1993) presented the first colocalization coefficients that are still in frequent use. These methods, as well as their modifications, are based on intensity thresholding and are therefore more or less user dependent or require pre-processing (Smallcombe, 2001; Lachmanovich et al., 2003; Kreft et al., 2004; Landmann & Marbet, 2004; Rubio et al., 2004; Fricker et al. 2006; Zinchuk et al., 2007; Adler et al., 2008). A fully automated method for quantification of colocalization was presented by Costes et al. (2004) with the caveat that images are free from cross-talk. Carlsson & Mossberg (1992) presented a method for partial removal of cross-talk. However, remaining cross-talk still causes problems for pixel classification. A different approach was introduced by Li et al. (2004), where image channel correlation is observed rather than individual pixel intensities. The major drawback of this method is its inability to localize in the same area or very near each other in the final image due to their close proximity within the microscopic structure. This is known as colocalization. Colocalization is particularly important for revealing information on how and where biomolecules such as proteins and protein complexes interact within a cell, as well as in which sub-cellular compartments they are present.

Background

Highly specific staining methods and fluorescent biological markers emitting light at different wavelengths together with fluorescence microscopy allow for detailed studies of the spatial distribution and localization of biomolecules. The source of two different emission signals can often be physically located
colocalization in the image. This was solved by an object-based method proposed by Jaskolski et al. (2005). In general, object-based methods are not desired in modern microscopy because of their speed, especially when it comes to multi-dimensional image data. Most of these methods, as well as other relevant methods were recently reviewed and compared by Bolte & Cordelières (2006) and Comeau et al. (2006).

One of the main ideas of the presented spectral decomposition method, or SpecDec, is to exclude any kind of segmentation, especially thresholding, from pre-processing, as objects of interest may have very varying intensity values. All pixels, including dark background pixels, are classified as colocalized or not, and no threshold as to what are object or background pixels is needed. Image segmentation defining objects or regions of interest as well as image background may be addressed in later processing steps. Some pre-processing is often necessary. However, the accuracy of the final measure of colocalization is strongly dependent on the quality of the input images (Adler et al., 2008). We also recommend, that if available, some standard techniques such as deconvolution (Landmann, 2002), similar scaling of intensities in all image channels and removal of chromatic shift (Manders, 1997; Kozubek & Matula, 2000) should be applied to the image during pre-processing as these factors all have impact on the final quantification of colocalization. It is also important to strive for image data with a large dynamic range and similar scaling in all image channels already at image acquisition.

When analysing light coming from an imaged position, the light has a certain distribution in wavelength. In digital images, each pixel is associated with such an imaged position, which means that each pixel is associated with a certain wavelength distribution. When analysing spectral components of an image, it is common to define one or several predetermined wavelength intervals, within which the intensity is determined. If a complete spectrum is of interest, the wavelength intervals are typically selected border to border, in order to cover the entire range. In fluorescence microscopy, it is common to record two (or more) greyscale images corresponding to intensities in, e.g. the red and green wavelength regions, respectively, where each greyscale image is referred to as an image channel. If we use a 1D linear image representation (independent of the true image dimensionality), each pixel has a spatial position \( i \), where \( i \) ranges from 1 to the number of pixels in the image. Each pixel can then be described by a pixel vector \( p_i \), associated with at least two values representing intensities within a respective image channel. For a fluorescence microscopy image with two channels, e.g. one representing intensities in the red wavelength interval, and on in the green, then \( p_i = (R_i, G_i) \), i.e. \( R_i \) and \( G_i \) are the red and green intensity measures, respectively, of \( p_i \). This is the general nomenclature used in the following brief description of previously published methods for quantification of colocalization. The novel SpecDec method proposed by us is described in detail in the ‘Methods’ section.

Global measures of colocalization

Pearson’s correlation coefficient and overlap coefficient. The first historical approach used for quantification of colocalization was Pearson’s correlation coefficient (1), describing the degree of overlap, or correlation, between two patterns (Manders et al., 1992).

\[
 r^p = \frac{\sum_i(R_i - \bar{R})(G_i - \bar{G})}{\sqrt{\sum_i(R_i - \bar{R})^2 \sum_i(G_i - \bar{G})^2}}
\]

where \( \bar{R} \) and \( \bar{G} \) are the average values of \( R_i \) and \( G_i \), respectively.

In Pearson’s correlation, the average pixel intensity values are subtracted from the original intensity values, resulting in a coefficient ranging from \(-1\) to \(1\). A value of 1 indicates perfect correlation and \(-1\) represents negative correlation, i.e. completely contrasting patterns. This makes the Pearson’s correlation coefficient difficult to interpret for values close to zero, and a new coefficient defined as the overlap coefficient (2), with a value ranging from 0 to 1, was suggested by Manders et al. (1993).

\[
 r = \frac{\sum_i R_i G_i}{\sqrt{\sum_i R_i^2 \sum_i G_i^2}}
\]

A disadvantage of the overlap coefficient is the strong influence of the ratio of the number of objects in each of the components. Pearson’s correlation as well as the overlap coefficient only provide a global measure of colocalization, and no pixel classification. Nevertheless, van Steensel et al. (1996) show how Pearson’s correlation coefficient can be used for analysis of colocalization. The method is not sensitive to colour shift between channels, but limited to detection of colocalization that appears as punctuate patterns having higher intensities in both channels.

Threshold based quantification of colocalization and pixel classification

If each pixel vector \( p_i \) of a two-colour image is plotted in a space spanned by the two intensity elements a conventional scatter-plot is obtained. A scatter-plot of Fig. 1A is shown in Fig. 1B. Single-coloured pixels are distributed along the axes, whereas colocalized pixels are distributed closer to the diagonal of the scatter-plot, and cross-talk (as well as noise) is seen as deviations of the single-coloured vectors from the axes. Detection of colocalized pixels (pixel classification) as well as quantification of colocalization is often based on a division of this 2D space into different regions representing pure red, pure green and colocalized pixels.

Manders’ colocalization coefficients. A biologically meaningful set of colocalization coefficients were presented by Manders
Fig. 1. (A) An artificial image of three objects; one red, one with red–green colocalization (slightly shifted towards red), and one green and Gaussian white noise (zero-mean and $\sigma = 5$). (B) Scatter-plot of A. Red and green pixels are located along the axes, whereas colocalized pixels are located closer to the diagonal. (C) Angle histogram of A. Clusters of pixels with similar hue cannot be seen due quantization noise. (D) The same angle histogram after weighting. Analysis cannot be performed on this histogram either. (E) The angle histogram shown in C after weighting and quantization noise compensation. The red, colocalized and green pixels are seen as clustered peaks in the angle histogram. (F) An artificial image made up of one pixel of each possible combination of red and green intensity of an 8-bit image. This image should, in theory, result in a flat angle histogram as every hue is equally frequent. (G) The principles for compensation of quantization noise. The contribution from pixel vector $p_i = (R_i, G_i)$, can be thought of as a square, and the square is smeared among the angle bins crossing the square. The shaded area corresponds to the contribution from $p_i$ to bin $j$. (H) Without weighting and compensation for quantization noise, the resulting angle histogram contains false peaks. (I) Peaks are still present when weighting is applied. (J) The peaks disappear after compensation for quantization noise.
et al. (1993). To cancel out the dependence of the ratio of the number of objects present in the overlap coefficient, the colocalization coefficient is divided into two different coefficients (3). The coefficients are not dependent on the relative intensities of the signals and have later become well known under the name of Manders’ colocalization coefficients.

\[
M^M_R = \frac{\sum_i R_i G_i > T_R}{\sum_i R_i}, \quad M^M_G = \frac{\sum_i G_i R_i > T_G}{\sum_i G_i}
\]

(3)

where \(T_R\) and \(T_G\) are intensity thresholds in the red and green channels, respectively, defining the level of background noise. In the ideal case, the images are free from background noise and \(T_R = T_G = 0\). This is however usually not the case, and the choice of thresholds, which is manual, will have a great impact on the colocalization coefficients. The coefficients are also very sensitive to the presence of cross-talk in the image data.

Costes’ automatic thresholding. Manual intensity thresholding typically based on visual inspection of images often leads to inconsistent results biased by the user. Costes presents an automated method for thresholding (Costes et al., 2004) by taking into account the amount of correlation in different regions of a scatter-plot of the image data. An automatic threshold search is done along a line whose slope \(a\) and intercept \(b\) are obtained by a linear least square fit over all pixels in the image, i.e. \(G_i = aR_i + b\). The automated threshold search starts with two intensity thresholds, \(T_R\) and \(T_G = aT_R + b\), that are applied simultaneously to the red and green channels, respectively, starting with \(T_R\) equal to the maximum intensity of the image. \(T_R\) is reduced step by step, and Pearson’s correlation coefficient \(r^2\) is calculated for all pixels with intensities below \(T_R\) and \(T_G\) in the red and green channel, respectively. The algorithm continues reducing \(T_R\) until \(r^2\) becomes negative. Colocalization coefficients are thereafter calculated using Manders’ coefficients (3), with \(T_R\) and \(T_G\) obtained by Costes’ method. As thresholds are parallel to the axes of the scatter-plot, this method requires images free from cross-talk.

It is worth noting here that Costes’ method also includes a step for comparing the derived measure of colocalization with a randomized image, providing a valuable measure of confidence. This type of step has not yet been added to the method presented in this paper.

Quantification of colocalization and pixel classification based on spectral angle

Conical selection of area of colocalization. As mentioned above, most well-known methods for determination of colocalization using scatter-plots are based on intensity thresholding with thresholds parallel to the axes of the scatter-plot. In Peñarrubia et al. (2005), the importance of relative measures of red and green signal is brought up, i.e. that data pairs with very different signal intensities should not be considered as colocalized. It is therefore suggested that ratios, represented by conical regions in the scatter-plot, provide a better measure of colocalization. The basic step of this method is selection of threshold levels for both channels either manually or based on an analysis of the histogram of the red and green channel. A conical area of colocalization is thereafter selected to improve the result of intensity thresholding. The angle of the cone is varied until the Pearson’s correlation satisfies requirements similar to Costes’ automatic threshold search. As manual steps are included in the analysis, it is affected by user bias.

Spectral decomposition. An image consisting of two colour channels, e.g. red and a green, showing objects that are partially overlapping can be thought of as a collection of pixel samples from a colour spectrum varying from red to orange to yellow to yellowish green to green. This corresponds to the hue component in HSI (Hue, Saturation, Intensity) colour model (Gonzalez & Woods, 2008), where colour and intensity information is decoupled. Finding colocalization will then become a matter of classifying the pixels as belonging to a certain part of this spectrum, independent of pixel intensity. In remote sensing, where images are often multispectral, i.e. consisting of a large number of colour channels, this is referred to as spectral decomposition (Kruse et al., 1993). The method can be applied directly to fluorescence microscopy data, and has proven to be powerful in classification of pixels in images containing as many as 16 colour channels (Göransson et al., 2009).

Here we introduce SpecDec, a new automated thresholding method with angled thresholds that provides pixel classification, quantification of colocalization, as well as automated compensation for crosstalk. It is important to remember that any quantification of colocalization will be limited not only by the choice of algorithm, but also by the image-acquisition procedures, the optical system, and the reliability and quality of the biological markers applied. An excellent review of these factors is provided by Bolte and Cordelières (2006).

Methods

The spectral colour, or hue of a pixel can be described by the angular deviation of the pixel vector \(p_i\) from the red intensity axes of a scatter-plot, i.e. the angle describing the hue of pixel vector \(p_i\), is given by \(\alpha(p_i) = \arctan \left( \frac{G_i}{R_i} \right)\), as shown in Fig. 1B. An angle histogram is then simply a histogram where each bin represents a given angle interval. The SpecDec method consists of two steps. First, an angle histogram, describing the distribution of pixel angles, is created. Thereafter, pixel classification rules are defined by the shape of the angle
histogram, and colocalization coefficients are calculated. The classification rules will automatically compensate for cross-talk; however, the angle histogram may also be used independently for quantification of cross-talk. Images can then be cross-talk compensated, and subsequently subject to one of the aforementioned cross-talk sensitive methods for quantification of colocalization. Before the angle histogram is created, the scatter-plot is shifted by subtracting the minimal intensity values in all channels separately, reducing intensity offset. This is necessary as an intensity offset may distort the angle histogram.

Creating an angle histogram

If creating an angle histogram from an image that contains one pixel of every possible combination of red and green intensities, one would, in the analogue case, expect to get a flat angle histogram. However, if the intensity values are discrete, as for the image seen in Fig. 1F, with intensities ranging from 0 to 255, the angle histogram will be disturbed by so called quantization noise, creating false peaks, as seen in Fig. 1H. The peak at $45^\circ$ appears because as many as 255 pixel vectors $p_i$ (of length $>0$) have the same discrete intensity value in both red and green, i.e. $R_i = G_i$, contributing to the bin at $45^\circ$ whereas a pixel vector with $R_i = 255$, $G_i = 254$ has a unique angle. This effect will be seen for any choice of bin size that preserves intensity information, where a single bin $j$ contains counts from all angles $\alpha(p_i) \in [\gamma_{j-1}, \gamma_j]$, where angles $\gamma_{j-1}$ and $\gamma_j$ define the range of bin $j$.

Quantization noise appears due to the fact that the intensity value $R_i$ of pixel vector $p_i$ actually represents any of the analogue real numbers ranging from $R_i$ to $R_i + 1$. E.g. intensity value 0 corresponds to the quantization of any real number between 0 and 1, and intensity value 255 corresponds to any real number between 255 and 256. The same is true for $G_i$. The analogue contribution to vector $p_i$ can then be represented by a square, as illustrated in Fig. 1G, and $p_i$ should contribute to all bins $j$ partly or completely covering the range of angles between the lower-right and upper-left corners of $p_i$.

If the angle bins of the histogram are large, and the values of $R_i$ and $G_i$, are high, $p_i$ will only contribute to one angle bin. For small bins and/or small values of $R_i$ and $G_i$, the square representing $p_i$ will be covered by more than one angle bin. To mimic the analogue contribution of $p_i$ to the angle histogram the contribution of $p_i$ is divided among all angle bins crossing the square. A reasonable assumption is that the probability distribution of analogue values within each square is constant, and the contribution to each angle bin is therefore assumed to be proportional to the area of the square covered by the angle bin. If each pixel with maximal intensity, in one of the channels is given its own bin, the number of bins in a non-linear histogram is $K = 2 \cdot 2^N$, where $N$ is the number of bits for intensity quantization. A single bin $j \in [1, K]$ is associated with the area between angles $\gamma_{j-1}$ and $\gamma_j$ defined as:

$$
\gamma_j = \begin{cases} 
\arctan \frac{x}{\sqrt{2}}, & j \in [0, \frac{K}{2}] \\
90^\circ - \gamma_{K-j}, & j \in [\frac{K}{2} + 1, K]
\end{cases}
$$

Then, the contribution $c_{i,j}$ of $p_i$ to bin $j$ is calculated as the definite integral of:

$$
c_{i,j} = \int_{R_i}^{R_i+1} \left( \max \left( G_i, \min \left( G_i + 1, x \tan \gamma_j \right) \right) - \min \left( G_i + 1, \max \left( G_i, x \tan \gamma_{j-1} \right) \right) \right) dx, \quad j \in \left[ 1, \frac{K}{2} \right]
$$

The contribution to bins $j = K/2 + 1, \ldots, K$ are, due to symmetry, equal to contribution values of angle bin $K-j+1$, or $c_{i,j} = c_{i,K-j+1}$, if $R_i$ and $G_i$ are swapped in Eq. 5. All definite integrals $c_{i,j}$ are pre-calculated and stored in an adequate look-up table for fast and easy retrieval.

In order to avoid a large impact from pixels having low intensity (typically dark background pixels), the contribution from each pixel to the histogram is weighted by the length of the pixel vector $p_i$ defined by its infinity norm. The total value $h$ of bin $j$ of the angle histogram then becomes:

$$
h_j = \sum_i \max(R_i, G_i) \cdot c_{i,j}
$$

The result of vector length weighting of the angle histogram in Fig. 1H is shown in Fig. 1I. If both vector length weighting and quantization noise compensation is employed, the almost flat angle histogram shown in Fig. 1J is achieved, as desired. Without the above described transformation, the image in Fig. 1A will give rise to the angle histogram shown in Fig. 1C, which does not show any clear clusters as it is disturbed by quantization noise. Vector length weighting only will give the result shown in Fig. 1D, with no obvious improvement of histogram shape. When also compensating for quantization noise, three clusters of pixels of similar hue are clearly visible as peaks in the histogram in Fig. 1E, making any statistical treatment of the histogram easier to perform.

Angle histogram-based pixel classification and quantification of colocalization

Once the angle histogram is created, the statistics of the histogram is used to proceed with the analysis. An angle interval corresponding to, e.g., a red, a green and a colocalized pixel class is defined. In the histogram of Fig. 2A, it can be seen that above a certain background level, mainly caused by low-intensity pixels representing background, most of the pixels are gathered in a few angle intervals.

The classification is initialized with classification rules determined by the angles midway between red ($\alpha_{R0} = 0^\circ$),
Fig. 2. (A) Angle histogram of 1(A) after weighting and quantization noise compensation. Classification rules are set up by detecting the local maximum within each of the red, colocalized and green interval of the angle histogram (represented by red, yellow and green lines, respectively), and thereafter placing classification rules midway between these maxima, illustrated by dashed blue lines in Fig. 2A. The final classification rule between red and yellow, or colocalized pixels, is determined by the angle $A_{R} = \alpha_{R} + \alpha_{Y}$, and between green and colocalized pixels by $A_{G} = \alpha_{G} + \alpha_{Y}$, illustrated by dashed blue lines in Fig. 2A. The classification rules are also shown as lines in the scatter-plot of Fig. 2B. Applying the classification rules defined above to Fig. 1A, will give the classification result shown in Fig. 2C. As can be seen, all pixels, including non-zero background pixels, are assigned a class.

In the image analysis described so far, classification is only made into classes having particular specified spectral properties, independent of intensity. This means that even pixels with low intensity, e.g. general background pixels are classified according to their spectral properties. In additional processing steps other criteria, such as intensity or spatial properties, can be used for further detection of objects and background. One approach is to evaluate the intensities, or length, of the vectors and discard short pixel vectors as background. Figure 2D shows the result of vector length thresholding of the classification result from Fig. 2C. The different objects of interest are now nicely outlined.

Image segmentation as a post-processing step opens the possibility to customize an automated search using a priori information on structures and morphologies, such as linear elements of the cytoskeleton and punctate compartments such as vesicles, in the colocalized data. Thus, the fact that the pixels belong to the same class becomes more important than their actual intensities. As an illustrative example, objects of Fig. 2C may also be defined based on a cluster analysis of the classification result. Clusters of pixels of the same class are identified and pixels falling outside the clusters are reclassified as background. This can, e.g. be achieved by applying a $3 \times 3$ opening filter (i.e. binary erosion followed by dilation, Gonzalez & Woods, 2008) to each of the three classes (described as binary images). The remaining pixels, shown in Fig. 2E outline the objects of interest, without the use of any intensity threshold. This approach may however fail if the background noise is not white.

If intensity thresholding had been applied directly to the image in order to detect colocalized object (such as with Costes’ or Manders’ method), the less correct classification result shown in Fig. 2F would be achieved.

The quantification of colocalization is usually only of interest for the objects in the image, and not for the background regions, and therefore the quantification of colocalization is based on objects only. Of course one can chose to classify all image pixels as objects, and then get a global measure of colocalization. If a simple intensity threshold $T$, e.g. derived by automated thresholding, is applied to define objects,
colocalization coefficients will be given by

\[
M^\text{SpecDec}_R = \frac{\sum_i R_i | p_i | p_i \geq T \land A_R \geq \alpha_R \lor C_R \leq \alpha_C}{\sum_i R_i | p_i | p_i \geq T \land A_R \geq \alpha_R},
\]

\[
M^\text{SpecDec}_G = \frac{\sum_i G_i | p_i | p_i \geq T \land A_G \geq \alpha_G}{\sum_i G_i | p_i | p_i \geq T \land A_G \geq \alpha_G} \tag{7}
\]

The red colocalization coefficient is the fraction of sums of intensities from the red image channel. The nominator sums all pixel vectors within the colocalized sector of the scatter-plot, and the denominator sums all pixel vectors within the colocalized or red sector of the scatter-plot, and vice versa for the green colocalization coefficient. If intensity thresholding is included, all pixel vectors must also have a length greater than the background threshold T.

**Angle histogram-based quantification of cross-talk**

When defining angle intervals corresponding to the ‘pure’ elements, information about the actual cross-talk present in the data can be obtained. In the example of red, green and colocalized pixels, one can see that the representative angle of the ‘pure’ colours is not situated in immediate vicinity of a respective axis of the scatter-plot. This means that a pixel showing only red emitted light still contributes to the green intensity and vice versa. In other words, the larger the angle difference between the pixel vector and the corresponding scatter-plot axis, the larger is the cross-talk. The cross-talk from the green channel to the red is equal to \(\tan(\alpha_R)\), where \(\alpha_R \leq 45^\circ\) and from red to green is equal to \(\tan(90^\circ - \alpha_G)\), where \(\alpha_G \geq 45^\circ\). By expressing the colour channels of the image as linear combinations of these respective representative angle vectors, colour channels compensated for cross-talk are produced (Carlsson & Mossberg, 1992).

\[
G_{\text{comp}} = \max(0, G - R \cdot \tan(\alpha_R)),
\]

\[
R_{\text{comp}} = \max(0, R - G \cdot \tan(90^\circ - \alpha_G)) \tag{8}
\]

This means that the classification is performed as a composite step of a cross-talk removal step. If a scatter-plot is created using the pixel vectors as expressed in terms of the representative angle vectors of the ‘pure’ colours, such a scatter-plot will be essentially free from cross-talk and colocalization can be detected and quantified using, e.g. Manders’ or Costes’ method (3).

**Experiments and results**

A set of test images composed of Gaussian shaped objects with added Gaussian white noise (zero-mean and varying standard deviation) was created. Objects in the red and green colour channels were either completely overlapping (creating colocalized objects), or not overlapping at all. All images were 8-bit and the standard deviation of the noise was varied in intensity from 1 to 15. Cross-talk and red-shifted colocalization was simulated by adding or subtracting fractions of the two colour channels from one another. The test images were used to compare stability and robustness of different methods for quantification of colocalization. Real image data, as described below, was also included in the evaluation of the method. All described algorithms were implemented in MATLAB (The MathWorks, Inc., Natick, MA, USA), and are available from the authors on request for research purposes.

**Simple ‘yellow’ colocalization**

The stability and robustness of the SpecDec was compared to quantification of colocalization achieved by Manders’ and Costes’ methods. Manders’, Costes’ and the SpecDec should all result in the same colocalization coefficients on test images without noise. As Manders’ as well as the SpecDec method have the option of including an intensity threshold defining background and objects, the same background threshold, either \(T = 50\) or \(T = 5\), was used for Manders’ and SpecDec. Also, in this comparative study, objects were pure red and green (apart from noise) as neither Manders’ nor Costes’ methods can compensate for cross-talk. Test images were recreated 40 times for each level of noise to calculate the variance of the coefficients. A typical test image (noise \(\mu = 0, \sigma = 10\)) is shown in Fig. 3A. Using Costes’ automatic thresholding, resulting in the dashed blue thresholds illustrated in the scatter-plot of Fig. 3E, some pixels will end up in the wrong class, as shown in 3B. Using the SpecDec method, the angle histogram of 3F is created and local maxima are detected at the red, yellow and green lines, respectively, and classification rules are positioned at the dashed blue lines. These angles are also shown in the scatter-plot of Fig. 3G, and the resulting classification mask at \(T = 50\) is shown in Fig. 3C. Figure 3D illustrates the stability of the colocalization coefficients at increasing levels of noise. Only red–green colocalization coefficients are plotted as red–green and green–red coefficients will behave equally for this test image. All three methods start out with red–green colocalization coefficients \(M_R = 0.5\) at 0 noise. As the level of noise increases, Manders’ method as well as Costes’ method will underestimate the colocalization at \(T = 50\) for Manders’, whereas SpecDec will give a comparably correct estimate of colocalization also at high levels of noise. If decreasing the object/background threshold to \(T = 5\), Manders’ method will instead over-estimate the colocalization, while SpecDec is still stably quantifying the colocalization to \(M_R = 0.5\).

The SpecDec method was also tested on real image data to evaluate its capability to classify pixels. The image of Fig. 4A shows a small part of an image with cultured cells where mutated and wild type mitochondrial DNA (mtDNA) has been detected by Padlock/rolling circle amplification (Jahangir Tafrechi et al., 2007). The number of red (mutant), green
Fig. 3. Comparison of stability and robustness for different methods for quantification of colocalization using an artificial image containing one red, one green and one colocalized object, and increasing amounts of Gaussian noise. (A) A typical image with noise $\sigma = 10$, and examples of results using Costes’ (B) and SpecDec (C) method. (D) The colocalization coefficients vary as the level of noise increases. Each image was recreated 40 times, providing a measure of variance. Manders’ method was tested using two different, fixed intensity thresholds ($T = 5$ and $T = 50$). Costes’ automated thresholds were re-calculated for each new image, the example for image A illustrated in (E). The angular thresholds, or classification rules for SpecDec were also re-calculated for each new image, the example for image A illustrated by an angle histogram (F) and classification rules in the scatter plot (G). As the number of red and green objects is the same, and the noise is increased for both channels, SpecDec will result in the same colocalization coefficient independent of background threshold.

(wild type) and colocalized (mixed) signals provide information on mtDNA mutation load and segregation patterns. The cell nucleus is visualized by DAPI stain (blue). The angle histogram of Fig. 4B shows three distinct peaks representing red, green and colocalized pixels, and classification rules as dashed blue lines, also included in the scatter-plot of Fig. 4C. If applied without a background threshold ($T = 0$), the result of Fig. 4D is obtained. The red and green signals are detected correctly, and the image background is dominated by yellow-coloured background noise. If a background threshold of $T = 20$ is applied, the more clear result shown in Fig. 4E is obtained. For comparison, applying Manders’ method
Fig. 4. Example on real image data. (A) Original image of wild type and mutated mtDNA detected by padlock-probing and red or green detection probes. (B) Angle histogram with peaks representing red, colocalized and green pixels and classification rules as defined by SpecDec. (C) The corresponding scatter plot. (D) Result of SpecDec without intensity thresholding ($T = 0$). It is clear that most of the colocalization is found in the noise of the image background. (E) Result of SpecDec followed by thresholding at $T = 20$ removes almost all yellow pixels. (F) The result using Manders’ method with $T = 0$. Almost all pixels are classified as colocalized, including all clearly single-coloured signals. (G) The result using Manders’ method with $T = 20$. The result is very similar to that achieved with SpecDec, however very sensitive to the choice of $T$. (H) Costes’ method fails as the automated thresholding is affected by the image background. (I) The result of Costes’ method. (J) Objects may be detected independent of classification. Here, objects are enhanced by filtering of a maximum intensity projection of the red and the green channel, and individual objects are defined as local maxima. (K) Objects detected and classified using the information in D. (L) Zoom in on K.
without background threshold \((T = 0)\) will give the result shown in Fig. 4F, where almost all image pixels are classified as colocalized as the relative red and green signal intensities are not taken into consideration. The result will improve at a background threshold of \(T = 20\), as shown in Fig. 4G. It is however important to note that the final result is strongly dependent on the manual choice of background threshold. Costes’ automated threshold detection will fail on this image due to the background variation as seen in the scatter-plot of Fig. 4H and the classification result of Fig. 4I.

The SpecDec method provides the option to decouple pixel classification and object detection. Fig. 4J shows a combined red and green channel (by maximum intensity projection) followed by object enhancement by a Laplace-type filter. Objects are thereafter defined as local maxima in the filtered image. Once objects are detected, the intensity and threshold independent classification result of Fig. 4D is called to decide the class of each detected object. Object classes are represented by red, green and yellow circles overlaid on the input image in Fig. 4K, and a zoomed region in Fig. 4L. Separating object detection from pixel classification provides a stable way of quantifying the number of objects of each type independent of intensity thresholding.

**Unequal number of red, green and colocalized objects**

A second test of robustness and stability was run on an image where the number of red and green objects differed. Figure 5A shows an example image with four red, one green and one colocalized object. The level of random Gaussian noise was increased from \(\sigma = 0\) to \(\sigma = 15\), and each image was re-created 40 times to get a measure of variance of the colocalization coefficients. Manders’ method and SpecDec provide a correct quantification of colocalization in images without noise, but Costes’ method will under-estimate the green colocalization due to premature stopping, as shown in Fig. 5B. Manders’ method and SpecDec will however behave in a similar way, although SpecDec is slightly less sensitive to noise. Examples of results at \(\sigma = 10\) and corresponding colocalization coefficients are shown in Fig. 5C–E.

The presented SpecDec method will unfortunately fail (in its fully automated form) on images where only a small number of pixels are colocalized as the cluster representing the colocalized pixels will be hidden in the noise in the angle histogram. To be able to do a correct pixel classification on this type of images, our suggestion is applying the algorithm on a training image containing a few colocalized object(s), as well as red and green objects. Classification rules extracted from the angle histogram of the training image can then be applied to the full image. Training the classifier on a sample image may actually be a good option also in other situations, e.g. when quantifying colocalization in a large number of images acquired under similar imaging conditions.

**Cross-talk**

Lastly, quantification of colocalization and pixel classification was tested on images with cross-talk. Cross-talk was simulated by adding the red channel multiplied by a factor representing the degree of cross-talk to the green channel of an image and adding Gaussian white noise. Figure 6A shows an image with Gaussian white noise \((\mu = 0, \sigma = 10)\) and 25% of the red intensity (Fig. 6B) added to the green channel (Fig. 6C). The angle histogram of Fig. 6D shows how the red peak has been shifted from \(0^\circ\) to approximately \(18^\circ\) due to cross-talk and noise. If no noise was present, the peak should be located at \(14^\circ\), corresponding to 25% cross-talk. Due to noise, 3.5% cross-talk is also detected from the green to the red channel. For higher values of noise \((\sigma > 5)\), cross-talk is overestimated by up to 10%. Slightly over-estimated cross-talk does not affect pixel classification significantly, as can be seen in Fig. 6E and L.

Classification rules represented by dashed blue lines are found by automated analysis of the shape of the angle histogram, and the resulting pixel classification provided by SpecDec is shown in Fig. 6E. Applying Costes’ method to the same image will lead to incorrect classification of all red objects as colocalized due to cross-talk, as shown in Fig. 6F. Also Manders’ method will fail as it cannot handle images with cross-talk, as seen in Fig. 6G. Using Eq. 10, cross-talk is removed, resulting in the image shown in Fig. 6H, with compensated channels shown in Fig. 6I and J. The red peak of the new angle histogram has now been shifted to zero, as seen in Fig. 6K. Note that the \(y\)-scaling of the angle histogram is always 0–1, and the large red and green peaks representing ‘pure’ coloured pixels suppress the yellow peak. Classification rules can however still be extracted successfully, and SpecDec will give the result shown in Fig. 6L, which is essentially equal to the result before compensation for cross-talk. After cross-talk compensation, Costes’ method provides a greatly improved pixel classification, detecting all red objects correctly as seen in Fig. 6M. Also Manders’ method will classify the objects correctly after cross-talk compensation (Fig. 6N). Basing the detection of peaks in the angle histogram on a search starting from initial reference angles of \(0^\circ\) and \(90^\circ\) for red and green staining, respectively, and \(45^\circ\) for colocalization, the algorithm will only be able to quantify cross-talk smaller than 41%, which correspond to the angle of \(22.5^\circ\). If cross-talk is greater than 40%, or even than 30% if Gaussian white noise cannot be neglected, we suggest using methods for cross-talk suppression described by Patwardhan and Manders (1996), as well as Demondolx and Davoust (1997), first, and then applying the method proposed in this paper for complete cross-talk compensation.

To conclude, intensity thresholding does not take the relative red to green intensity of a pixel into account. Therefore dark pixels may be misclassified as single coloured, as seen in Fig. 2F, and bright pixels may be misclassified as colocalized, as
Fig. 5. Comparison of stability and robustness when the number of red and green objects differs. (A) A typical input image with four red, one green, and one colocalized object (noise $\sigma = 10$). All images were re-created 40 times for evaluation of variance. (B) Colocalization coefficients at increasing levels of noise. (C–E) show examples of results at noise with $\sigma = 10$. Manders’ as well as SpecDec produce fairly correct results, whereas Costes’ method will over-estimate red, and under-estimate green colocalization.

seen in Fig. 6G. When spectral angles are used as classification rules, pixels are rarely misclassified in this manner as all pixels within a class have the same range of relative red to green intensity.

**Conclusion and discussion**

This paper describes a novel method, referred to as SpecDec, for automated classification of pixels as being colocalized or not based on spectral information and classification rules derived from an angle histogram. The angle histogram is created in such a way that quantization noise is reduced, and the final pixel classification provides a stable method for quantification of colocalization as well as cross-talk. As the degree of cross-talk can be quantified in a fully automated manner, SpecDec can also be used for automated cross-talk reduction.

Several tests of robustness and stability of SpecDec were performed on artificial images designed to mimic real fluorescence microscopy data. SpecDec proved to be more robust and stable when it comes to quantification of colocalization in images free from cross-talk as compared to previous methods presented by Manders and Costes. If we put robustness and stability on side, quantification of colocalization using Manders’ method is strongly dependent
Fig. 6. Pixel classification in the presence of cross-talk. (A) A typical input image with four red, one green and one colocalized object, and red–green cross-talk created by adding 25% of the red channel to the green channel (noise $\sigma = 10$). (B) The red channel of A. (C) The green channel of A (cross-talk is seen as weak signals at the positions of the red objects). The angle histogram in (D) clearly shows that the red peak is shifted towards green. The highest peaks of the red and green cluster are detected at 18° and 88°, making the cross-talk measure over-estimated by 4° and 2°, or 7.5% and 3.5%, respectively. The classification result using SpecDec is shown in E. Costes’ method (F) as well as Manders’ method (G) will fail and classify most red object pixels as colocalized. Using the angle histogram created by SpecDec to quantify the amount of cross-talk, the input-image can be cross-talk compensated, as shown in (H). (I) The red channel of A. (J) The green channel of A (cross-talk is no longer present). The angle histogram in (K) shows that the red peak is shifted back to 0°. The result of SpecDec (L) is very similar to the result before cross-talk compensation whereas Costes’ (M) as well as Manders’ (N) methods result in more correct pixel classifications after cross-talk compensation.

on the manual choice of background, making it very sensitive to user bias. Although fully automated and independent of the user, Costes’ automatic thresholding failed to quantify colocalization correctly in images where the numbers of red and green objects differ. Neither Manders’ nor Costes’ method produced correct pixel classification in images with cross-talk. Pixel classification using SpecDec is independent of cross-talk, and could therefore produce correct classification results also in the presence of cross-talk. We also show that if the degree of cross-talk is quantified using SpecDec, cross-talk can be reduced in an automated manner. Applying Manders’ or Costes’ method to the cross-talk compensated images will then give satisfactory results.

Before an angle histogram is created, it is necessary to reduce the background intensity as an intensity offset may lead to a distorted angle histogram resulting in inaccurate or even
wrong classification. In the presented version of the method, intensity offset is reduced by subtracting the minimal intensity values in all channels separately, although the pre-processing techniques suggested in the Introduction may be sufficient to reduce the offset error. Subtracting a given percentile of the intensity distribution will probably be more stable, however, at the risk of losing some of the low-intensity information. For comparative studies, offset reduction should be the same for all image data. The dynamic range of the image may also affect the angle histogram, and we strongly recommend that one should try to optimize this already at image capture.

In the presented SpecDec method, images with two colour channels are assumed, that is, the pixel vectors have two elements. However, SpecDec is also applicable on pixel vectors of higher dimensionality. For instance, if biological markers of three different wavelengths are available, three colour channels corresponding to one wavelength each can be obtained and considered as a set of pixel vectors having three elements. In such a case, the angle histogram produced by SpecDec will also acquire a higher dimensionality. If the pixel vector has three elements, the angle histogram will be a two-dimensional histogram. Such a histogram may be visualized on a spherical surface, with the axes corresponding to the ‘pure’ colours for each wavelength being directed in three linearly independent directions. Definitions of angle intervals for pixel classification will then also be performed in two dimensions, and the borders between pixel classes will be borders on a two-dimensional surface. This can of course be generalized into any number n of pixel vector elements, which gives angle histograms, classification rules and borders in (n – 1) dimensions. Visualizing scatter-plots and angle histograms with more than three dimensions becomes complex, but the corresponding mathematical operations are indeed possible to perform.

We have also assumed that three classes, plus a background class, are typically used. However, if only colocalization is of interest, there is no need to define angle intervals corresponding to the pure colours. All pixels falling outside the colocalization angle interval can be neglected or treated as background. In some cases, there may also be more than one type of colocalization present. It can be of interest to distinguish between colocalization with different relative concentration. For instance, if two red biological markers are colocalized with one green marker, such situation may be of interest to distinguish from a case where one marker of each colour is present. In such a case, an additional angle interval discriminating between yellow and orange should be defined from the angle histogram.

In the current version of SpecDec, classification rules are defined by first detecting the largest peak for each of the red, green and yellow interval of the angle histogram, and thereafter placing classification rules midway between each pair of neighbouring peaks. Many interesting thresholding techniques have been developed for regular greyscale images (Sahoo et al., 1988), and these methods as well as different histogram smoothing techniques are currently tested to further improve the stability and robustness of the SpecDec method. Especially when it comes to quantification of cross-talk, stability can be improved by more advanced methods for analysis of the angle histogram.

As SpecDec performs pixel classification and quantification based on an angle histogram, it is independent of the spatial and time resolution of the image data, and thus directly applicable to 3D images or time-sequences. The algorithm is also expandable to 12-bit images, simply requiring a larger look-up table for the removal of quantization noise.

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