

Colocalization of fluorescent markers in confocal microscope images of plant cells

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Published online 20 March 2008; doi:10.1038/nprot.2008.31

This protocol describes the steps needed to perform quantitative statistical colocalization on two-color confocal images, specifically of plant cells. The procedure includes a calibration test to check the chromatic alignment of the confocal microscope. A software tool is provided to calculate the Pearson and Spearman correlation coefficients ('Pearson–Spearman correlation colocalization' ImageJ plug-in) across regions of interest within the image. Steps are included to help the user practice using the software. The result is a quantitative estimate of the amount of colocalization in the images. Manual masking takes about 1–15 min per image, depending on the detail required, and calculating the correlation coefficients is almost instantaneous. Examples of suitable dyes for such two-color colocalization include Oregon Green or Alexa Fluor 488 dyes in the green range (excited with 488-nm laser line) and Alexa Fluor 555 dye in the red range (excited with 543-nm laser line).

INTRODUCTION

For a number of years, colocalization of fluorescent markers in images has been used to identify colocalized molecular structures, such as proteins. Typically, two different proteins of interest with fluorescent probes that emit different wavelengths of light are selected. These two emission spectra are recorded in different channels of a digital image; so, for example, brightness of one of the fluorescent markers might be recorded in the red channel and the other in the green channel.

At its most basic, colocalization analysis involves a qualitative judgment by a researcher as to the level of co-occurrence of these fluorescing proteins. This is typically achieved by manual inspection of the resultant images, usually by looking for the presence of a 'mixed color'—yellow in the case of colocalization in the red and green channels (see Fig. 1 for some examples). However, there are several problems associated with manual observation of colocalization. Manual measurements are inherently subjective, and observers' opinions on the level of co-occurrence might vary widely; this is especially true if color blindness is a factor. Display technologies also vary and can have a nonlinear response to image color—markers that appear to be highly correlated when displayed on one piece of equipment may look only weakly related on another. Regardless of the display used, signals can be highly correlated and yet do not produce a visible 'mixed color', if one signal is much weaker than the other, for example¹. These problems are compounded by production of the image on different media—a printed image in a publication may represent colors differently to the image displayed on a monitor. It can also be problematic selecting which images from a large set to use as examples in publications; quantified results can often better represent result sets than exemplar images alone.

Increasingly, techniques are being developed that aim to quantify, at least relatively, the level of colocalization between fluorescently labeled molecular species. Sometimes this can be as simplistic as displaying and manually examining the intensity profile along a transect through part of the image. However, these data can be

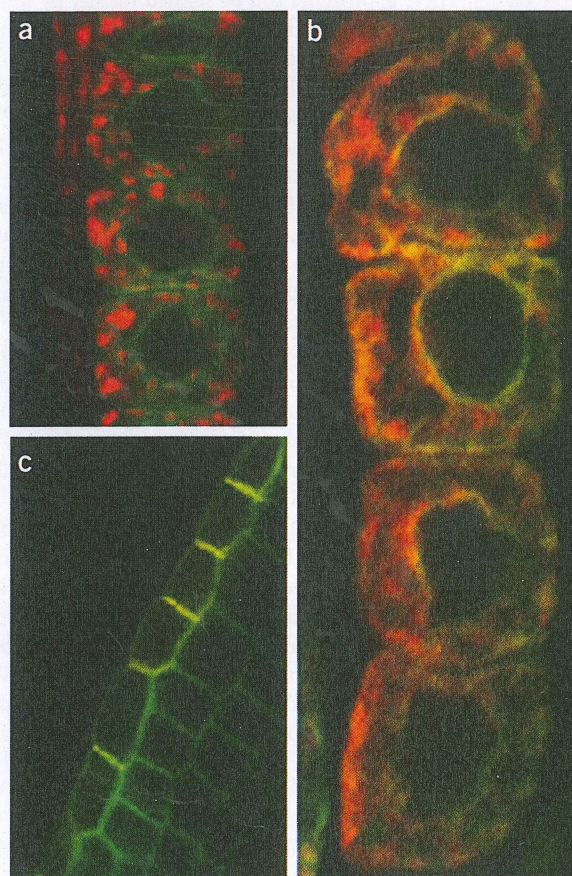


Figure 1 | Examples of image outputs from confocal colocalization experiments. (a) Endoplasmic reticulum marker AXR4-green fluorescent protein (GFP) (green) and Golgi marker γ -Cop (red)⁸. Poor colocalization. (b) Endoplasmic reticulum markers AXR4-GFP (green) and BIP (red)⁸. Good colocalization. (c) Plasma membrane markers GFP-LTI6a (green) and N-YFP-AUX1 (red)¹⁹. Good colocalization in plasma membrane in lateral root cap cells.

difficult to interpret, especially if it crosses a complex image such as that of a plant cell. Such structures can have many substructures of differing intensities packed into a small area, pushed against the margin by a large vacuole, for example. Interpreting such image data manually can generate anomalous results. Calculating colocalization across areas of an image provides more robust results, and statistically led approaches offer an automatic solution. They are often based on measuring a form of correlation between the signal levels present in the image channels, each of which represents the relative brightness of a particular fluorescent label². Other methods additionally use probabilistic models to be able to make a certainty judgment about the existence of colocalization³. Alternative approaches look for a co-occurrence or overlapping of the signals⁴ or the patches derived from these signals⁵, or they are based on protein densities within regions⁶. Regardless of the details of the method used, statistical approaches to colocalization produce quantitative data that represent a complete data set, rather than the one or two example images usually presented when qualitative judgments of colocalization are made.

There has been interest recently in developing 3D approaches to colocalization. Although most of the light captured by a z-section taken on a confocal microscope originates from the plane of interest, some extra light is added from the neighboring planes. This appears out of focus, as determined by the point-spread function. There is therefore a danger that 2D colocalization methods might base calculations on signals that in fact originate from outside the plane of interest. 3D methods perform colocalization from volume reconstructions, thereby reducing this problem by considering all available planes. These approaches can be refined further by using rotational stages to produce accurate 3D models⁷. However, it is still important to consider 2D colocalization, as discussed in this work, as it remains a very common method, and the basic principles extend to 3D. But it is as well to be aware of its limitations.

In what follows, we describe the steps needed and issues to be faced during any attempt to quantify fluorescent colocalization in plant cells. We then consider the process used in previously published work⁸ in more detail, where colocalization in plant cells was quantified. We provide a protocol for its execution using a plug-in module provided for the widely used, public-domain image analysis environment ImageJ⁹. We additionally describe a sensible calibration test for taking confocal colocalization images and highlight common pitfalls during the image-capture process. We will demonstrate the output of this technique on the examples in **Figure 1**, which were produced using fluorescent proteins and immunofluorescent techniques, and also compare some results with common existing plug-ins to confirm functionality.

It is true that the existing ImageJ colocalization plug-ins offer various levels of functionality and features for quantifying colocalization. Two good examples are the JACoP plug-in², which implements a number of different methods including calculating Pearson correlations, and the Colocalization Finder (<http://rsb.info.nih.gov/ij/plugins/colocalization-finder.html>), which offers an interactive scatter plot. However, existing methods do not calculate Spearman coefficients as standard and none offer as much flexibility in region masking as the proposed software.

Calibration for colocalization

When any measurements are made from images, it is important to consider the quality and parameters of the equipment that

produced them. Full calibration of confocal microscopes is no trivial task¹⁰, but at least one test should be completed to assess the consistency of the images produced by the confocal system before attempting colocalization; the spectral registration of the system should be confirmed to be of suitable accuracy. Chromatic aberration is an effect in which different wavelengths of light are diffracted by differing amounts¹¹ within the confocal microscope. The effect can be demonstrated by passing white light through a prism and seeing a rainbow of colors emerge, and it can result in red light appearing at a different position in the image plane than, for example, the position of green light. This is clearly a problem for colocalization studies, as truly colocalized specimens may not appear so in the final image. Chromatic aberration can be caused by scanner alignment problems if multiple scans must be made for each color band (especially during volume scans), or by the sample itself moving between scans, or by optical effects such as refraction at the coverslip or within the optics of the microscope itself. To ensure that the two-colored specimen responses are accurately aligned, truly colocalized markers in calibration targets, for example, microspheres stained with multiple fluorescent dyes, can be imaged and the alignment of the different wavelengths of emission spectra examined.

Zoom level and resolution can also influence colocalization measures. Clearly, using a perfect infinite zoom lens would show that no physical structures are actually truly colocalized, as they cannot occupy exactly the same space. It is desirable, however, to consider structures that are sufficiently close to be colocalized. How 'sufficiently close' is defined will depend on the optical resolution of the imaging system and the biological effects of interest. As the zoom level and imaging scan resolution are decreased, structures that are further apart in reality appear colocated in the image as they are closer together than the smallest distance that can be resolved by the imaging system. In other words, decreasing these factors increases the chance of seeing colocalization where there is in fact none. A further situation that must be avoided is having fluorescent markers with emission or excitation spectra that are too close together. If one marker produces a signal change in both channels, then clearly these channels will be correlated. This is called 'cross talk' and can be avoided by choosing spectra that are separated as widely as possible and using a narrow detection band for the emitted light. It can also be tested for by activating only one channel at a time—any signal in the other channel indicates cross talk.

Full details of these issues and other calibration tests can be found in existing literature^{2,10}. In what follows, we assume that the reader is confident that the imaging system is producing images suitable for colocalization tests. Note that to produce absolute quantitative measures can be problematic in confocal microscopy^{12,13}. However, this is less of an issue in colocalization studies, as the final result is usually a unit-free relative estimate of the amount of colocalization of signals in the image, and not an absolute value in real-world units. Further practical steps for ensuring quality in colocalization studies can be found in the literature¹⁴. What is key is that the user understands the issues involved in the generation of fluorescent confocal images and the impact these factors may have on their colocalization research.

Image storage and compression

It is not just the microscopy optics that can degrade the quality of the image. In many laboratories, image capture and analysis are

separate activities; images will be acquired and stored for later analysis. It should be noted that the file format chosen for image storage can also affect localization (and other) measures. Most domestic and some commercial image file formats compress the images they represent to reduce storage requirements. Compression may be lossless (such as TIFF LZW), in which case the original data can be reconstructed from the compressed version, or lossy (such as JPEG), in which case the image is permanently changed by conversion to the file format. Care must be taken to ensure that no, or only lossless, compression is performed during image storage.

Figure 2 illustrates the effect that compressing an image with JPEG compression can have on the quality of the information stored. These compression techniques degrade data in ways that the eye is poor at perceiving, such as the spatial resolution of color. Therefore, the image may appear the same to the naked eye, but the underlying data can be heavily distorted. As it is the color information that is normally corrupted the most, these issues are of particular significance in colocalization studies.

Segmenting colocalization images

Once images have been captured, some way is needed to determine the area over which the measurements are to be made. Though it is possible that colocalization measures are needed over the whole image, this is often not the case. Attention might be restricted to a particular organ, cell type or even an individual cell. The process of selecting these regions of interest is called segmentation. Segmentation can be done automatically, semiautomatically or entirely manually. There is a trade-off between the approaches in terms of accuracy and speed. Interactive methods are an increasingly common compromise. Here, for example, a user may be required to specify some key points on a segment boundary. The software can then identify a contour incorporating the points that follow, for example, an edge of a structure¹⁵. These methods are less subjective and faster than manual methods, whilst being more reliable than fully automatic methods often are. Additionally, simple thresholding might be employed, where signal values below a fixed level are ignored; this approach can be used to eliminate areas of the image that are likely to contain background noise.

Whatever the method used, segmentation produces a binary (black and white) mask image with the same dimensions as the input image. Black pixels indicate areas of the image that are not of interest, and white pixels indicate the areas we are interested in. Colocalization calculations will only be made on pixels that have a corresponding white pixel in the mask image. In what follows, we use a combination of a manually created mask image and ImageJ selections to define regions of interest. The mask, if required, is simply created in a paint program. It could be replaced by an automatically generated mask involving the automatic segmentation of the image, and this is an area people with access to image analysis expertise may wish to pursue. Areas can also be defined by creating selections in ImageJ. Whichever method is used, these regions of interest allow colocalization analysis to be carried out only on specific structures or areas that are important for the experiment. It also allows areas of, for example, over-saturated pixels to be omitted from analysis; sometimes over-saturation in some image areas is required to generate enough illumination from the darker areas of interest. Masking allows attention to be focused only on significant areas. It is particularly suited for plant cell images, as structures such as particular subcellular components, for

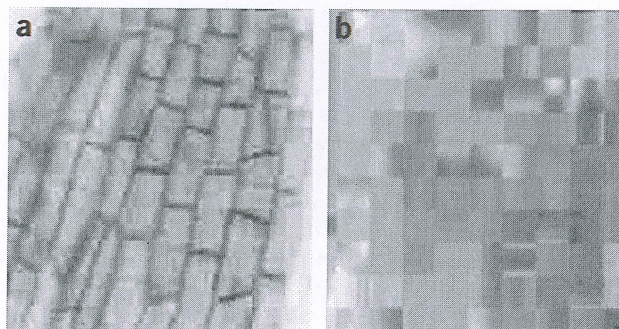


Figure 2 | A medium-quality JPEG image, decomposed into intensity (left) and hue (right) channels. Note how the intensity channel on the left appears to represent a reasonable amount of information; however, the hue channel on the right, which represents the color information, is recorded with much lower detail.

example, vacuoles, might need to be omitted from analysis because of low signal response⁸ or because they are not of biological interest in a particular investigation.

Statistical colocalization measures

In what follows, we assume, following previously published work⁸, that a measure is required of colocalization between the red and green channels of a confocal image, examples of which are presented in Figure 1.

The values recorded in the red and green channels of the image can be thought of as a set of n pairs of numbers (x_i, y_i) ; the problem is to decide whether the pairs are related in some way, for example, when one value in the pair increases, does the other value increase too? Statistical methods exist with which this kind of relationship can be quantified. A common method relies upon Pearson's correlation coefficient:

$$\rho_P = \frac{\sum x_i y_i - \frac{1}{n} \sum x \sum y}{\sqrt{(\sum x_i^2 - \frac{1}{n} (\sum x_i)^2) (\sum y_i^2 - \frac{1}{n} (\sum y_i)^2)}}$$

This method makes assumptions about the data that might not always hold in fluorescent confocal microscopy. In particular, it can only detect linear relationships between the two sets of data, and linearity is something that cannot be guaranteed in images, as the brightness of the recorded data may not increase linearly with protein concentration, for instance. Alternative statistical correlation methods are available, which do not place such rigid constraints on the data. One alternative is Spearman's rank correlation coefficient:

$$\rho_S = 1 - 6 \sum \frac{d_i^2}{n(n^2 - 1)}$$

where d_i is the difference between the rank positions in the (x_i, y_i) data pair. Rank ordering the data means sorting a channel into ascending order and attributing a value to each data point indicating its position in this sorted list. Tied values are given the arithmetic mean value of their combined rank, and after the tie, rank ordering continues as if there has been no tie in the data. This rank ordering method is insensitive to any monotonic transformation of the data. The use of both Pearson and Spearman statistics is employed here to give two quantitative outputs per image—both are calculated automatically with the supplied plug-in software.

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Both tests produce values in the range $[-1, 1]$, 0 indicating that there is no discernable correlation and -1 and $+1$ meaning strong negative and positive correlations, respectively. The importance of tied data should be considered, as it is likely that many data points will be tied. This is because, with a data range of 256 values (as in a common 8-bit image channel), even an image patch of size 50×50 pixels might be expected to contain 10 data points at each intensity level, if image values are uniformly distributed. Data that are clustered around certain intensities will generate many more ties. The above Spearman calculation has a longer form, based on the Pearson equation but using ranked data, which can be used with heavily tied data which might occur over large image areas. In practice, on the presented examples this does not alter the results. However, both long and short forms are calculated by the plugin for completeness.

Care must be taken in attributing significance to the results of these computations. Though high absolute values indicate a high probability of colocalization, it is possible to obtain quite high values by chance. Lookup tables can provide statistically significant thresholds for the results of the tests, which are dependant on the number of samples used. These tests give a *P*-value for any

correlation coefficient. A *P*-value represents the probability that the result was generated by chance, so lower *P*-values are more significant. These statistical differences may not have any biological significance, however, and so should be treated with care. Also, if we are using pixel values that are drawn from neighboring pixels, the values cannot be thought of as statistically independent, as they are likely to be drawn from the same portion of material. Therefore, additional work must be exercised to produce reliable *P*-values. Permutation tests can be performed to generate these *P*-values, taking into account the spatial distribution of the signals, variations of which have been used in previous colocalization work³. For this work, reporting the correlation coefficients along with supporting scatter plots and images is considered quantitative enough to enable readers to make their own judgments about the level of colocalization, although readers are directed to the previously mentioned literature for more details about the statistical significance tests³.

Data from the image are only entered into the calculations above if at least one of the channels at that data point has an intensity value above that which might be considered image noise. This value is user controlled via the ImageJ plug-in.

MATERIALS EQUIPMENT

- This protocol does not require specific equipment; any two-channel confocal microscope that can resolve the two markers separately, and any PC that is capable of running the software is acceptable. More details on example equipment can be found in existing papers¹⁶ and a full account in the corresponding *Nature Protocols* article¹⁷
- Leica SP2 confocal microscope. We expect that this method can be performed on any two-channel microscope
- Microbeads stained with multiple fluorescent dyes. The diameter of the beads should ideally be comparable to the resolution of colocalization that is required, and the fluorescent properties should be as similar as possible to the probes used in the actual experiment. For example, Molecular Probes TetraSpeck Fluorescent Microspheres. Premounted slides of beads can be purchased (Molecular Probes TetraSpeck Fluorescent Microspheres Size kit (T14792)) or it is possible to create your own samples. Typically these beads are stained with four fluorescent dyes at 365 nm excitation/430 nm emission (blue), 505/515 nm (green), 560/580 nm (orange), 660/680 nm (dark red)
- Fluorescent dyes/proteins—with as widely separated emission/excitation spectra as possible. The exact choice of dye is governed by the laser lines that are available. There are many dyes that can be used for excitation with Argon laser line 488 such as Oregon Green, Alexa 488, FITC. The choice of dye in the red range are Alexa 543, Alexa 555 and TRITC
- PC that can run ImageJ (development machine was a 2.4 GHz machine, with 2Gb RAM running Windows XP, although minimum requirements are lower—see the ImageJ website below for details)
- ImageJ public domain image processing software <http://rsb.info.nih.gov/ij/>. Version 1.39c or later version
- GNU Image Manipulation Program, <http://www.gimp.org>, open source (optional)

PROCEDURE

General settings of the confocal microscope

- 1] Set the pinhole size to 1 airy unit (AU). Generally, this gives the highest image quality versus brightness trade-off. Reducing the pinhole size can increase xy resolution at the cost of reduced signal intensity, and conversely increasing the pinhole size above 1 AU will increase the signal intensity at the cost of reduced resolution, as light is then collected from the neighboring planes¹⁸. A setting of 1 AU represents a compromise. Deviations from this value should be biologically and technically justified.
- 2] Set the averaging level of the image acquisition. The calibration test in Steps 7–16 should be run with the level of averaging that will be used in the final experiment. Scan averaging has the advantage of being more resistant to noise effects, but increases undesirable side effects such as photobleaching; a trade-off is required.
- 3] Make sure that the laser is warmed up, as power can vary significantly in this period (you should allow at least 30 min).
- 4] Choose the bit depth of your image. This determines the number of gray levels that can be differentiated in the image; 256 levels for 8 bit and 4,096 levels for 12 bit. For almost all scenarios, 8-bit is recommended, assuming that the responses from the probes produce light of roughly the same intensity distribution that can be recorded comfortably within the 256 gray-level range. The accompanying plug-in is designed to work with 8-bit images.
- 5] Set the laser power and gain controls. Use the over- and under-lookup tables to make sure pixels of interest are not over- or underexposed. Make sure the dynamic range falls within the recordable range, for example, for 8-bit images, the full range is 0–255; however, it is best to allow a safety net, so something like 40–190 is a safer range to work with, as this prevents signal clipping. Note that areas of the image that are not of interest can be over- or underexposed, as these areas can be

masked out in the later stages of analysis. This may be necessary to make the significant areas of the image fall inside the ideal exposure range. Be aware of effects such as photobleaching (resulting in decreased emission signal strength over time) and sample death that will occur more frequently with increased laser power. If photobleaching is a problem, choosing a more photostable marker or lowering the laser power can help¹³.

6| Zoom and image resolution are important—think about how close things need to be before they should be considered colocalized. Set up the microscope to use an optimal resolution for your study—scanning at too high a resolution may be counterproductive, as bleaching and time-delay effects caused by an increased scan time may occur. Zooming too far can also lead to oversampling, in which case a point source is collected by more than two adjacent pixels on the detector. The Nyquist sampling theorem reveals that the image of a single point source should be collected by two pixels on the detector for the image to be a faithful reproduction². Collecting a point source image on more than two pixels causes a lowering of the signal intensity with no increase in the quality of the data gained.

Calibration of confocal microscope • TIMING 1+ h, dependant on whether corrective steps are needed

7| If you are not confident that your confocal microscope is adequately well calibrated, at least the following steps should be carried out to test for chromatic shift (more details in the literature¹³). Acquire some microbeads stained with multiple fluorescent dyes. These test targets therefore have perfectly collocated dyes. Suitable examples include Molecular Probes TetraSpeck Fluorescent Microspheres.

8| Set up the confocal microscope as for the experimental setup, if you have not already done so (Steps 1–6).

9| Ensure that the laser(s) have warmed up for a sufficient time (at least 30 min).

10| Place a slide of microspheres ready for imaging. Ideally, these should be mounted in the same medium and on the same slides as will be used for experimental work.

11| The microspheres should mostly be on the same plane if the premounted spheres are used, although this is not essential. Adjust the z-section of microscope such that the center of most of the spheres is being imaged, if possible.

12| Take several images of the spheres using the same settings as will be used in the actual experiment. Normally, this will involve using two different wavelengths to excite two of the fluorescent dyes on the beads, and the emitted light will be collected and saved in different color channels of an image.

13| For each image taken, load the image in the microscope manufacturer's software and view the images, overlaying the color channels to produce a composite image. Zoom in on several beads in turn across the image plane. If red and green channels have been used, then a truly colocalized bead should produce a yellow bead. A chromatically shifted system will produce more of an 8-shaped bead image, with the central overlapping region being yellow and the nonoverlapping regions being green and red (see Fig. 3). It is worth noting that most of the error can be in the axial direction, so taking volume images and examining an axial yz or xz section can be advised—although, for 2D work, this presents less of an issue.

14| Quantify this aberration by measuring the distance between the centers of the red and green circular projections in the image. This distance, averaged across sufficiently many bead images, will give an indication of the average level of chromatic shift. If this distance is large enough to affect the resolution of colocalization required in the actual experiment, then correction may be necessary. An alternative method is to calculate the correlation coefficients using the Pearson–Spearman correlation (PSC) colocalization plug-in (Steps 27A–32). An unshifted image here should produce very high correlation coefficients (typically $r \gg 0.9$), although noisy images may produce less.

15| If there is significant chromatic shift, the following may be altered and the test reperformed from Step 10: (i) Try cleaning the lens as per manufacturer's instructions. (ii) An objective with a higher numerical aperture and/or less chromatic aberration effects should produce less chromatic shift. (iii) The mounting medium can have a significant effect, as its physical properties may alter the amount of refraction when light enters and leaves the sample slide. Investigate the possibility of changing this. (iv) Confocal scanning mode: simultaneous scanning (detecting all fluorophore emissions simultaneously) can reduce chromatic shift due to less need to move mechanical components. However, this scanning mode can lead to increased risk of cross talk between channels, so increased care

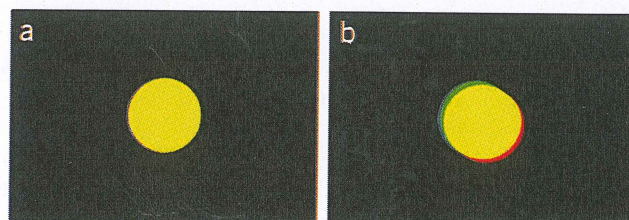


Figure 3 | Schematic images of an aligned (left) and chromatically shifted (right) image of a calibration bead.

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must be taken¹¹. Some improvement may be seen by switching from volume sequential to 2d-section or line sequential scanning modes, as volume sequential scanning can introduce significant shifts in the z-direction¹¹.

16| Save these calibration images for future reference, in an uncompressed image format such as TIFF.

▲ CRITICAL STEP To ensure reliability, Steps 18–24 should be replicated with several samples.

■ PAUSE POINT Can leave the microscope now as long as the settings are not changed. Remember to switch on the laser for a similar period of time when the protocol is continued.

Preparation of sample ● **TIMING** Largely dependant on the type of preparation used. Time to optimize cross-talk reduction (Step 20): ~1 h

17| Consult existing protocol¹⁷ for details.

18| *Consistent staining.* It is important that any dye in the sample is distributed in a uniform manner within the structures of interest; patchy distribution that will lead to erroneous colocalization results may be produced.

19| Choose sufficiently separated fluorescent excitation/emission spectra, otherwise cross talk will show up as inaccurately high correlation values.

20| Check for cross talk. Cross talk can be detected by selectively exciting each marker in turn, and looking for a signal response in the closed channel. A response in a closed channel could mean either the excitation or emission detection spectra overlap. Detecting which spectra it is requires observation of either differentially marked samples with one marker each or observation of particular locations of the sample that are known to harbor only one of the marker types, for example, cell walls versus cell nucleus. To remedy a cross-talk problem, the laser power could be reduced as much as possible, the excitation wavelengths separated further and the detection bandwidth for each marker can also be narrowed.

Image samples ● **TIMING** 30 min laser warm-up period + time to image each sample

21| Make sure the laser is warmed up. Allow at least 30 min to warm up before taking measurements¹⁰.

22| Take the images. Refer to existing protocol¹⁷ for details. Take several images of each sample to ensure reliability.

23| Store the images in uncompressed format, with microscope data file. Microscope manufacturer's software usually stores the images uncompressed with the data file in a database. You may later need to export them as a common, nonproprietary format, for example, TIFF to use them in ImageJ.

24| *Backup data.* Copy all the data to another device.

■ PAUSE POINT All further work is carried out on recorded images and so is not time critical.

Performing colocalization analysis ● **TIMING** Steps 25–30: 1–20 min per image depending on the presence and complexity of a mask

25| Close ImageJ if it is open.

26| Install the PSC colocalization ImageJ plug-in by copying the file from the download location (it is available from <http://www.cpiib.ac.uk/~afrench/coloc.html>) into the ImageJ\Plugins folder. Start ImageJ. The tool should now be available from the ImageJ Plug-ins menu.

27| The plug-in expects the input image to contain the data from the separate fluorophore emission in the red and green channels, and an optional mask in the blue channel. The mask channel has nonzero values where we are interested in the data in the first two channels, and is black elsewhere. Initially, this third channel should be completely empty (all black) in a conventional red/green colocalization image. There are two protocol options below dependant on whether a subselection (option B) is required or whether the whole image is to be processed (option A) (see Fig. 4).

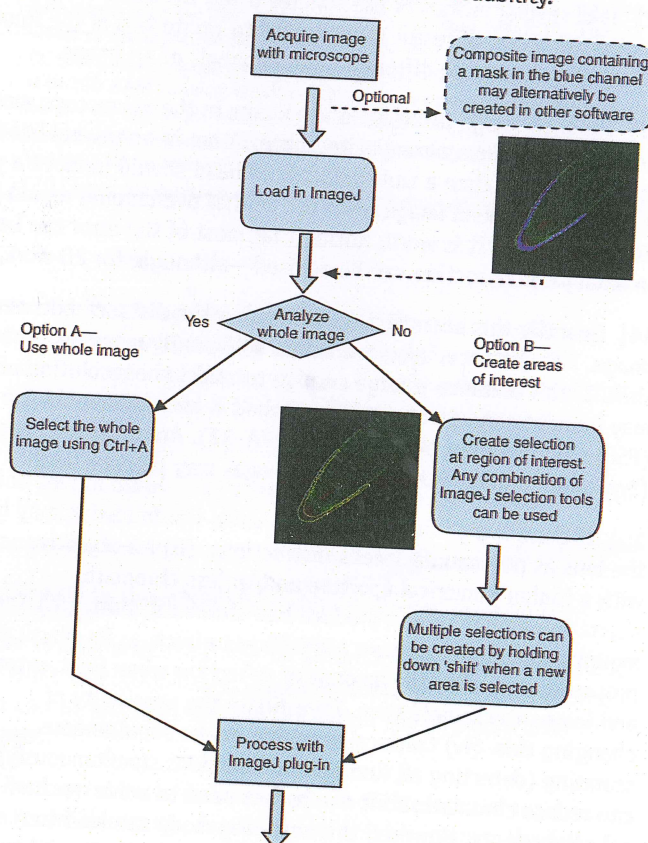


Figure 4 | Flowchart of the mask/selection creation process.

(A) Calculating correlations over whole image

- (i) Load the experimental image in ImageJ.
- (ii) Make the image window active by clicking on the title bar.
- (iii) Press Ctrl+A to select the entire image. When activated, the plug-in will then calculate correlations across the whole image.

(B) Calculating correlations over subselections

- (i) Load your image in ImageJ.
- (ii) By default, a confocal image will normally have data in the red and green channels, and a blank blue channel.
- (iii) Use selection tools to mark areas of the image that you wish to analyze. Any selection tool in ImageJ can be used. A useful tool is the Selection Brush. This is found by right clicking the Elliptical Selection Tool (second button from the left) and selecting Selection Brush Tool. Double-clicking the tool icon allows the diameter of the brush to be set. This can be used to 'paint' selections onto the image. Patches can be expanded or reduced depending on whether the user begins a stroke inside or outside a boundary. To create additional, unconnected selection regions, shift-left click in the new area. The free-hand selection tool is also useful. In fact, any combination of tools can be used. Remember to shift-left click to add a new region to the existing regions, rather than start a new selection.

You have now indicated the regions of the image that are used for correlation calculations. If there exists a mask in the blue channel, only pixels with nonzero values in the blue channel that are also within the ImageJ selection areas will be counted (see Fig. 5). If no blue mask is present, only the ImageJ selections will be used to define regions.

▲ CRITICAL STEP Note that creating a mask manually is a subjective process, and so care should be taken when implementing it. It is particularly important that all areas of biological interest are included in the mask. If the user selects only areas that seem to show high levels of colocalization, then clearly the statistics will be skewed to show this. The mask images should be available to support published material so that this can be evaluated by reviewers.

▲ CRITICAL STEP Data in the blue channel, if any, are used as an optional mask—only pixels with nonzero values in the blue channel and within an ImageJ selection are used in calculations. If required, this type of mask can be created in any painting package that allows the user to draw in only one channel. It is then very easy to 'paint' a mask onto the image using drawing tools. This is an easy way to distribute an image with an attached mask. The advantage of this method is that it is possible to create this image, with a mask in the blue channel, in a number of different painting or image processing applications outside of ImageJ, some of which offer very flexible mask creation options, as standard painting tools can be used to paint only in the blue channel. The free, open source GNU Image Manipulation Program offers such a feature; by locking the red and green channels, the user is able to manipulate the values in the blue channel in a flexible and intuitive way, or paste in a binary mask image. Similar approaches can be used to mask out saturated pixels at this stage (see Table 1 for details). If this is desired, the user may save the finished image as, for example, an uncompressed TIFF incorporating the mask in the blue channel and import into ImageJ before the following steps. If no blue mask is required, then the software will work using the ImageJ selections alone. See Figure 6 for an example of masking options.

? TROUBLESHOOTING

28| Select the plug-in PSC colocalization from the ImageJ->Plugins menu.

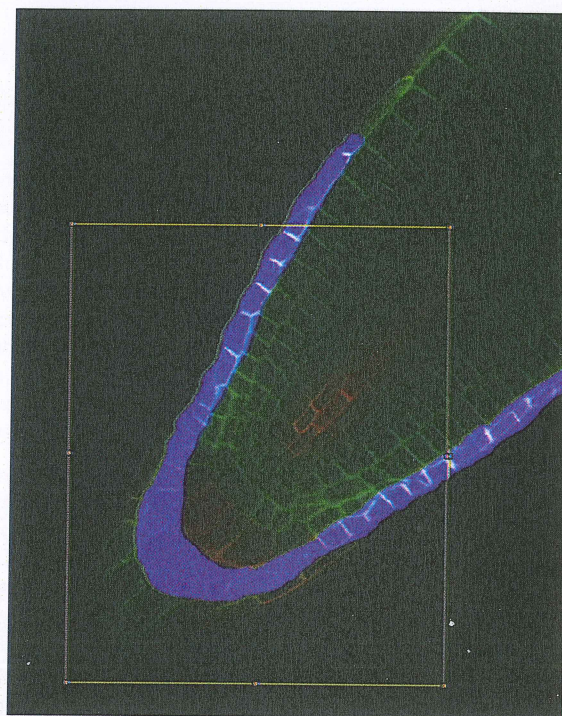


Figure 5 | An image with a blue channel mask and an ImageJ selection. Only those pixels covered by the blue mask and inside the ImageJ selection will be counted.

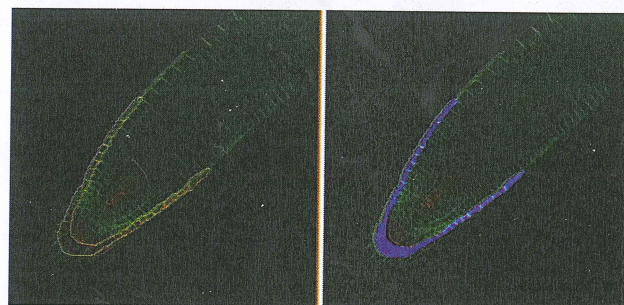


Figure 6 | Two types of masks. Left: ImageJ selection overlaid on the experimental image (Step 27B(iii)). Right: an example of blue channel mask. In this case, the blue mask will give the same results as the ImageJ selection, as the same region is masked.

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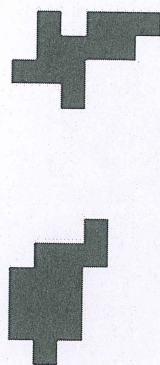


Figure 7 | Using this technique, pixels saturated in the red channel have been excluded from the blue mask.

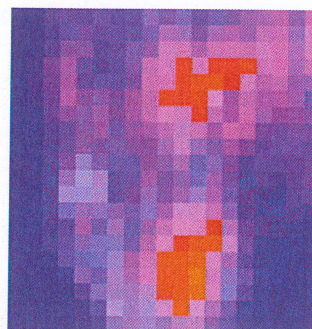


Figure 8 | Composite image as a result of including **Figure 7** as a mask in a red-green image. Note that the saturated red pixels will not be included in the calculation.

- 29| Select a threshold level, under which pixel values are considered noise and not analyzed. This prevents low-level image acquisition noise from distorting the statistics. To turn this off and count all pixels, set this value to 0; the default level is 40.
- 30| The plug-in will calculate Pearson and Spearman coefficients across the selected areas of the image, and if a blue mask exists, only masked pixels within the selection will be calculated.

31| A scatter plot is created. The Pearson product-moment correlation coefficient is a measure of the strength of linear relationship between the two signals. The Spearman rank correlation coefficient does not require that the relationship between the signals is linear; it is a measure of how well any monotonic function between the variables can describe the relationship. Therefore, it is possible that the Spearman coefficient will be large when the Pearson coefficient is smaller, when the data do not appear as a straight line on the scatter plot. Spearman coefficient is therefore a useful measure of how well two signals are correlated when they might not be following a linear relationship.

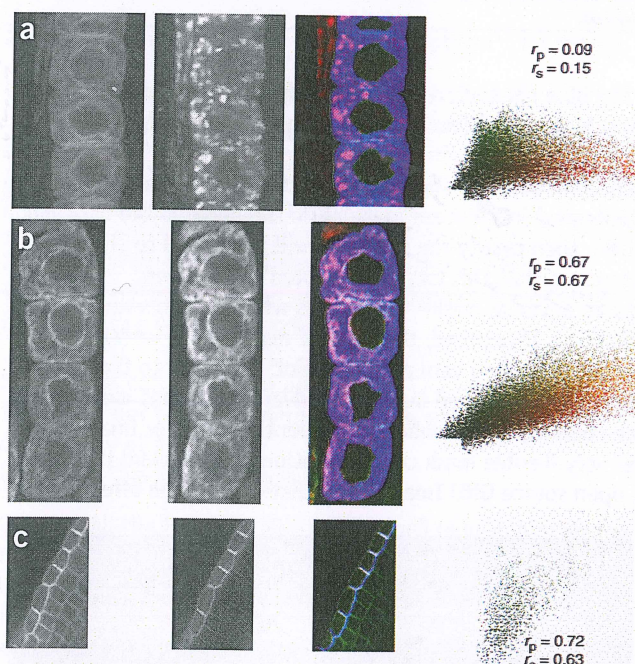


Figure 9 | Columns from left to right: green channel, red channel, combined image with overlaid mask in blue channel, resulting in scatter plot of intensities across the two channels. See **Figure 1** for red-green overlaid image without the mask. The Pearson correlation coefficient r_p and Spearman correlation coefficient r_s are indicated on the scatter plots. (a) Endoplasmic reticulum marker AXR4-GFP (green channel) and Golgi marker γ -Cop (red channel)⁸. Poor colocalization. (b) Endoplasmic reticulum markers AXR4-GFP (green channel) and BIP (red channel)⁸. Good colocalization. (c) Plasma membrane markers GFP-LTI6a (green channel) and N-YFP-AUX1 (red channel)¹⁹. Good colocalization in plasma membrane in lateral root cap cells.

32| Report coefficients of correlation and present scatter plots and overlaid images. These can also be saved for future use. See ANTICIPATED RESULTS for examples.

33| Repeat the analysis protocol from Step 28 for each image that requires analysis. For replicate images, it may be suitable to use the same selection, which could be saved using ImageJ's File->Save as->Selection... command. If the sample has moved at all, then a new mask will likely need to be created. It is important that the same biologically significant areas are masked for each replicate.

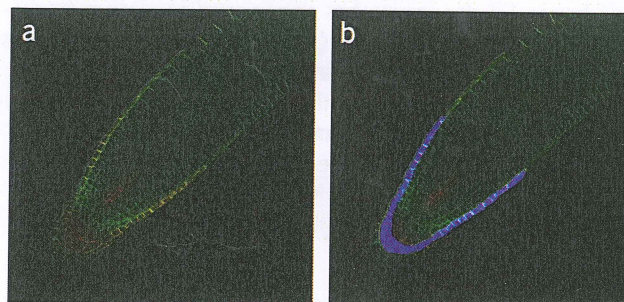


Figure 10 | Test image. (a) Unmasked and (b) masked versions of the image.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1** (see also **Figs. 7** and **8**).

TABLE 1 | Troubleshooting table.

Step	Problem	Solution
27	There are saturated pixels that need removing from the computation (Fig. 7)	A mask can be created to discount pixels with a value of 255 from the computations. In ImageJ, use Image->Color->RGB Split to divide the color image into separate channels. In the channel with saturated pixels, select the window, then select Image->Adjust->Threshold... Move both sliders to the right end as far as possible—now only pixels with a value of 255 should be selected. Press Apply, and close the threshold controls. If necessary, use Ctrl-shift-I to invert the image to make the saturated areas black, and areas of interest white. This new image is a mask that can be used in the blue channel to mask out saturated pixels. See below for how to use this image. Multiple channel masks can be combined into one mask using ImageJ's Image Calculator functions
	Combining a black-and-white image into the blue channel of a sample image to use as a mask (Fig. 8)	Load the red/green image in ImageJ, followed by the black-and-white mask image—only white areas will be included in the calculations. Select Image->Color->RGB Merge... For the red and green channels, choose the original red/green image file name, and for the blue channel, choose the name of the black-and-white mask file. The resulting composite image is ready to be used with the PSC plug-in. Before running, remember to select the areas of the image to process using ImageJ selection tools (Ctrl-A selects the whole image)

ANTICIPATED RESULTS

Figure 9 shows the separate red/green channels from the images in **Figure 1**, the mask image as created by Step 27 and the scatter plot created in Step 31. Coefficients of correlations are presented over the scatter plots. The range is from -1 , a strong negative correlation, to $+1$, a strong positive correlation. The closer to zero a coefficient is, the weaker the correlation and hence the less evidence there is for colocalization.

In addition to the example above, where functionality overlaps, the new software has been compared to the Pearson coefficient as calculated by some common existing ImageJ plug-ins, to ensure consistency (**Table 2**). **Figure 10** illustrates the test image used.

As the Spearman coefficient is not available in existing colocalization plug-ins as far as these authors are aware, the new software was compared with a manual calculation of Spearman carried out in a spreadsheet. The test image used, **Figure 9c**, produced identical Pearson and Spearman coefficient results to the plug-in for both masked and unmasked versions of the data.

TABLE 2 | Comparison of Pearson coefficient calculations of **Figure 10** from various ImageJ plug-ins.

	Unmasked image	Masked image
Author's plug-in—PSC		
JACoP ²	0.671	0.760
Manders coefficients ^a	0.671	N/A
Intensity correlation analysis ²⁰	0.671	0.760
Image correlationJ ²¹	0.671	0.760
	0.670	N/A

^ahttp://www.uhnresearch.ca/facilities/wcif/software/Plugins/Manders_Coefficients.html

ACKNOWLEDGMENTS We acknowledge EPSRC and BBSRC CIBS programme funding to CPiB and funding from the Belgian Federal IUAPVI programme to M.J.B. and R.S. as part of the BARN consortium.

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1. North, A.J. Seeing is believing? A beginner's guide to practical pitfalls in image acquisition. *J. Cell Biol.*, **172**, 9–18 (2006).
2. Bolte, S. & Cordelières, F.P. A guided tour into subcellular colocalization in light microscopy. *J. Microsc.*, **224**, 213–232 (2006).

3. Costes, S.V., Daelemans, D., Cho, E.H., Dobbin, Z. & Pavlakis, G. Automatic and quantitative measurement of protein–protein colocalization in live cells. *Biophys. J.*, **86**, 3993–4003 (2004).
4. Manders, E.M.M., Stap, J., Brakenhoff, G.J., Van Driel, R. & Aten, J.A. Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. *J. Cell Sci.*, **103**, 857–862 (1992).
5. Lachmanovich, E. *et al.* Co-localization analysis of complex formation among membrane proteins by computerized fluorescence microscopy: application to immunofluorescence co-patching studies. *J. Microsc.*, **212**, 122–131 (2003).

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6. Glynn, M.W. & McAllister, A.K. Immunocytochemistry and quantification of protein colocalization in cultured neurons. *Nat. Protoc.*, **1**, 1287–1296 (2006).
7. Heintzmann, R. & Cremer, C. Axial tomographic confocal fluorescence microscopy. *J. Microsc.*, **206**, 7–23 (2002).
8. Dharmasiri, S. *et al.* AXR4 is required for localization of the auxin influx facilitator AUX1. *Science*, **312**, 1218–1220 (2006).
9. Abramoff, M.D., Magelhaes, P.J. & Ram, S.J. Image processing with ImageJ. *Biophotonics Int.*, **11**, 36–42 (2004).
10. Zucker, R.M. & Price, O. Evaluation of confocal microscopy system performance. *Cytometry*, **44**, 273–294 (2001).
11. Manders, E.M.M. Chromatic shift in multicolour confocal microscopy. *J. Microsc.*, **185**, 321–328 (1997).
12. Pawley, J. The 39 steps: a cautionary tale of quantitative 3-D fluorescence microscopy. *Biotechniques*, **28**, 884–888 (2000).
13. Pawley, J. *Handbook of Confocal Microscopy* 3rd edn (Springer Science, New York, 2006).
14. Smallcombe, A. Multicolor imaging: the important question of co-localization. *Biotechniques*, **30**, 1240–1245 (2001).
15. Kass, M., Witkin, A. & Terzopolous, D. Snakes: active contour models. *Int. J. Comp. Vis.*, **1**, 321–331 (1988).
16. Friml, J., Benkova, E., Mayer, U., Palme, K. & Muster, G. Automated whole mount localisation techniques for plant seedlings. *Plant J.*, **34**, 115–124 (2003).
17. Sauer, M., Paciorek, T., Benkova, E. & Friml, J. Immunocytochemical techniques for whole-mount *in-situ* protein localization in plants. *Nat. Protoc.*, **1**, 98–103 (2006).
18. Centonze, V. & Pawley, J. Tutorial on practical confocal microscopy and use of the confocal test specimen. In *Handbook of Biological Confocal Microscopy* 3rd edn. (ed. Pawley J.) 627–649 (Springer Science and Business Media, New York, 2006).
19. Swarup, R. *et al.* Structure–function analysis of the presumptive *Arabidopsis* auxin permease AUX1. *Plant Cell*, **16**, 3069–3083 (2004).
20. Li, Q. *et al.* A Syntaxin 1, G_{α_q} and N-type calcium channel complex at a presynaptic nerve terminal: analyses by quantitative immunocolocalization. *J. Neurosci.*, **24**, 4070–4081 (2004).
21. Chinga, G. & Syverud, K. Quantification of paper mass distributions within local picking areas. *Nordic Pulp Paper Res. J.*, **22**, 441–446 (2007).