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Colocalization Analysis in Fluorescence Microscopy

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Abstract

The measurement of colocalization requires images of two fluorophores that are aligned, with no cross talk, and that the intensities remain within the response range of the microscope. Quantitation depends upon differentiating between the presence and absence of fluorescence, and measurements should be made within biologically relevant regions of interest. Co-occurrence can be measured simply by area and with the M1 and M2 coefficients, and should be compared to random distributions. Correlation analysis should use the Pearson and Spearman coefficients, which need to be measured by replicate based noise corrected correlation to eliminate errors arising from differences in image quality. Ideally, both co-occurrence and correlation should be reported.

Key words: colocalization, co-occurrence, correlation, Pearson correlation coefficient, RBNCC

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1. Introduction

The degree of colocalization is a description of the relative distribution of two fluorophores [1]. Measurements can be made by comparing populations of pixels in images of both fluorophores. It is also possible to compare populations of objects, rather than pixels, which falls outside the scope of this chapter. The term colocalization is widely used and actually covers two quite different questions:

1. Are the fluorophores in the same place (co-occurrence)?
2. Is there a relationship between their intensities (correlation)?

When describing patterns of distribution it is important to clarify whether co-occurrence or correlation is meant, or to report both [2]. It is also important to fully describe all image processing and stay within ethical limits [3].

Co-occurrence reports on the overlap between the fluorophores, measuring either the fraction of a region of interest (ROI) containing both fluorophores or, separately for each fluorophore, the fraction of the total fluorescence found in common areas, the M1 and M2 coefficients. Correlation examines the relationship between the intensities of the fluorophores, using the Pearson, Spearman and ICQ coefficients [4,5,6]. Complete co-occurrence in the absence of correlation is possible and a strong correlation among co-occurring pixels can be found even when co-occurrence is rare.

It is important to make measurements in biologically relevant regions of interest. If a pair of fluorophores is restricted to the nucleus, then reporting the co-occurrence over the whole cell is less informative. Similarly, if the distribution is restricted to the nucleus and is random, a correlation measurement covering the whole cell, and therefore including areas devoid of fluorescence, will be inappropriately high, since including a mass of pixels with low, but matched, intensities increases the measured correlation [4]. This raises the question of how to define ROIs; using a third fluorophore is one possibility.

An additional prerequisite for analysis is to differentiate between areas containing and areas devoid of fluorophore, which is usually achieved by intensity thresholding [7,8].

The accuracy of any measurement inevitably reflects the quality of the original data, in this instance the digital images. An overlap between the intensity range in background pixels and that of the pixels containing fluorophores makes intensity thresholding less reliable. Similarly, correlation measurements made between two sets of images of the same cell will differ if the quality of the images differs. This raises two important issues: (1) how to assess and standardize image quality and (2) whether a correction for measurements made from less than perfect images is possible.

The measurement of colocalization can usefully be divided into two parts: (1) the acquisition of the images and (2) the measurement of colocalization.

2. Materials

Prepare slides with the relevant cells or tissue and fluorophores. All experimental groups should be processed concurrently, if possible, to ensure that differences in colocalization cannot be attributed to the experimental conditions and not to variations in protocols or reagents.

2.1 Slides

1. Uniformly fluorescent slide, e.g. 92001 autofluorescent plastic slides from Chroma Inc., (Bellows Fall, VT, USA); (*see Note 1*).
2. Fluorescent microspheres with wide emission range covering that of fluorophore 1 and fluorophore 2 (Invitrogen Inc., Carlsbad, CA, USA).
3. Blank, specimen without either fluorophore.
4. Specimen with fluorophore 1 only.
5. Specimen with fluorophore 2 only.
6. Specimen with fluorophores 1 and 2.

2.2 Imaging Systems

Two channels, one for each fluorophore, are required. Consecutive image acquisition is preferable. The following imaging systems meet these requirements:

1. Confocal microscope.
2. Multiphoton microscope.
3. Structured illumination microscope.
4. Widefield microscope (*see Note 2*).
5. Total internal reflection fluorescence microscope.
6. Stimulated emission depletion microscope.

2.3 Software

For co-occurrence and correlation, the following software packages may be used:

1. ImageJ (open source) with plugins Colocalization_Finder, Image Correlator, JACoP and MBF.
2. Axiovision (Zeiss MicroImaging, Oberkochen, Germany).
3. CoLocalizer Pro (Colocalization Research Software) (*see Note 3*)
4. Huygens (SVI, Hilversum, The Netherlands).
5. ImagePro (Media Cybernetics, Bethesda, MD, USA).
6. Imaris (Bitplane, Zurich, Switzerland).
7. MetaMorph (Molecular Devices, Sunnyvale, CA, USA).
8. Slidebook (3I, Intelligent Imaging Innovations, Denver, CO, USA).
9. Volocity (Perkin Elmer, Waltham, MA, USA).

For replicate based noise corrected correlation (RBNCC):

1. Huygens (SVI, Hilversum, The Netherlands).

3. Methods

3.1 Choice of Fluorophores and Filters

1. Examine the excitation and emission spectra of the selected fluorophores (for instance, see <http://www.spectra.arizona.edu>; www.invitrogen.com, <https://www.micro-shop.zeiss.com/?l=en&p=us&f=f>), and make sure that, at least theoretically, the images formed in each channel will contain only photons from one fluorophore when using the chosen illumination (**Fig. 1**).
2. Check that the filters inside the microscope are appropriate for the selected fluorophores. Details of the filters should be obtainable either from the filter or microscope manufacturer.

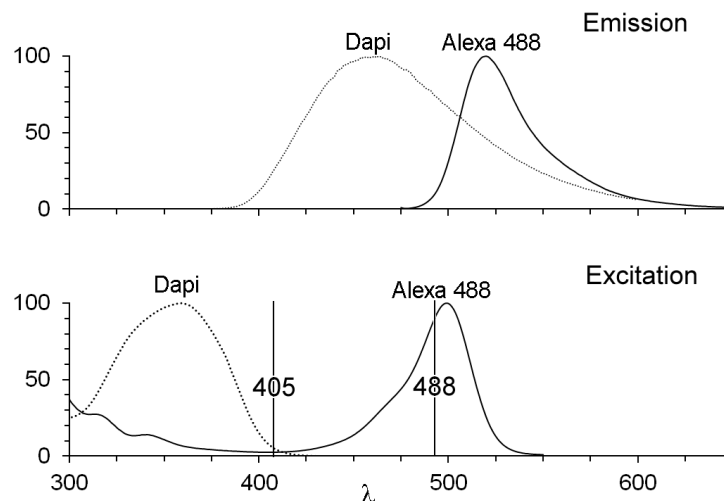


Fig. 1. The excitation and emission spectra of DAPI (4',6-diamidino-2-phenylindole) and Alexa Fluor-488 with excitation at 405 and 488 nm. Published spectra can be used to predict which combinations of fluorophores will be free from cross talk. The spectra suggest that the 405 nm excitation is not optimal for exciting DAPI and that 405nm can weakly excite Alexa Fluor-488. Whether both fluorophores would be excited in practice by 405 nm must be checked experimentally using the test slides with single fluorophores. It appears unlikely that the 488 nm line that is optimal for Alexa Fluor-488 would excite DAPI as well. The emission spectra show considerable overlap between DAPI and Alexa Fluor-488, due to the very long tail in the DAPI emission. This overlap would be a problem if the 405 and 488nm excitation lines were used concurrently, and the two images therefore need to be obtained sequentially using each excitation line individually. In practice it is always best to test the control slides, since the environment *in vivo* or *in vitro* differs from that used to obtain the published spectra. Consideration needs to be given to how light is handled within the microscope and to the properties of the emission filters and dichroics.

3.2 Microscope Setup

1. Check for even illumination. Record images from the uniformly fluorescent test slide. View images with a false colour rather than a grey or monochromatic look up table (LUT). If the illumination is not uniform, the light source(s) should be checked and adjusted (see **Note 4**).

2. Check the alignment of the images. Use the test slide with fluorescent microspheres and acquire an image in each channel. Overlay the images (using for instance ImageJ) and check that they are precisely aligned. If the images are not aligned, there are optical filter sets whose alignment is optimised (for instance available from Chroma Inc., or Semrock Inc., Rochester, NY). It is also possible to align images after acquisition. Make sure that objectives with minimal chromatic aberration are used. If there is chromatic aberration the alignment may differ across the imaging area.
3. Optimise image acquisition settings. Image the two slides containing single fluorophores, and establish the range of settings for acquiring images that fall within the response range of the system. Specifically, for an 8 bit image, no pixels should have values of 0 or 255. This is best seen with an over-range false colour LUT. Record the gain, offset, exposure time etc. for each fluorophore.
4. Check for autofluorescence. Record images showing areas with the specimen and areas outside the specimen using the settings from step 3 in Subheading 3.2 with the unlabelled specimen. If no structure is apparent, then autofluorescence is not a problem. If structure is apparent, then either the sample preparation or the selection of fluorophores need to be changed. (see **Note 5**).
5. Optimize the illumination intensity. Co-occurrence and correlation analyses assume a linear relationship between the number of molecules of the fluorophore and the fluorescence (see **Note 6**). Test for overexcitation by acquiring an image, reducing the illumination, and acquiring a second image. Compare the two images: in the absence of saturation there will be a linear relationship between the intensities of homologous pixels in the two images. This relationship can be seen using a scattergram (for instance using the ImageJ “image correlator” plugin). Reduce the illumination to avoid saturation.
6. Check for potential cross talk and bleedthrough. The most basic requirement is that each of the images used to measure colocalization shows only the emission from a single fluorophore. This may not be the case if the excitation wavelength chosen for one fluorophore also excites the other fluorophore, and the range of wavelengths detected in each channel overlap with this range. This is checked by imaging the two slides with only a single fluorophore, while recording images in both channels using the settings from step 3 in Subheading 3.2. For each fluorophore, signal should be present in only one of the two channels.

3.3 Image Acquisition

1. Establish criteria for selecting or rejecting cells for analysis (see **Note 7**).
2. Blind the microscope operator to the experimental origin of the specimen if possible. This will eliminate any operator bias.
3. Make sure that the specimen has the same staining pattern throughout the slide by moving the stage and observing multiple fields of view.
4. Use the range of settings established in step 3 in Subheading 3.2, and acquire images of the specimens. Adjust the gain and offset of the detectors or the

exposure time of a camera to maintain the intensities within the response range of the detectors/camera.

5. Acquire images using the settings from step 3 in Subheading 3.2. Adjust the number of images averaged and the scan speed or, if using a wide field system, the exposure time until the images do not appear pixellated (**Fig. 2**) (see **Note 8**).
6. Select cells from the specimen by randomly moving the stage and image the cell nearest to the centre of the field of view if it meets the criteria established in step 1 in Subheading 3.3.
7. Acquire pairs of images. Replicates of each image are necessary for correlation analysis using RBNCC.

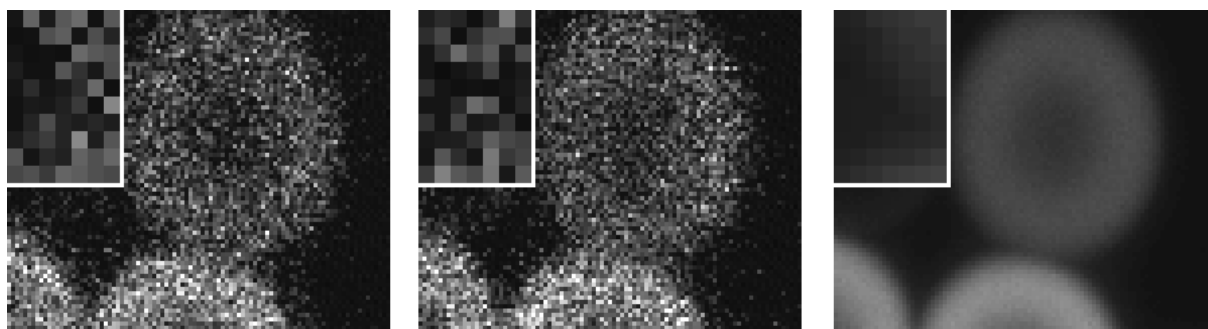


Fig. 2. Image Quality. Three images of the same specimen (6 μm fluorescent microspheres) are shown. The image on the *right* has been averaged over 64 scans, while the centre and *left* images were obtained from single scans. Note that although the single scan images appear pixellated, the number of pixels in all the images is the same. The magnified area shown in the *top left* of each panel demonstrates that there is considerable variability between the images, indicating poor image quality.

3.4 Display of Colocalization

1. Overlay the images, using for instance the Image Color Merge channels in ImageJ.
2. Insert one image in the blue layer and the second image in yellow by inserting it in both the red and green layers, giving grey or white in areas of similar intensity (see **Notes 9 and 10**).
3. A binary display showing colocalized pixels is available in the ImageJ plugin “Colocalization_Finder”.
4. Scattergrams showing the relationship between the intensities in pair of images are widely available, including the “Image Correlator” plugin in ImageJ (**Fig. 3**).

3.5 Measurement of Colocalization

There is a substantial number of similar commercial software packages available for measuring fluorophore colocalization (see Subheading 2.3). User preference and available funds are suggested as issues determining the choice of the appropriate software package.

3.5.1. Thresholding and selection of a ROI

1. Threshold each channel to differentiate between the presence and absence of fluorescence. Simple thresholding tools are widely available, for instance Image/Adjust/Threshold in ImageJ.
2. Individual cells should be analysed separately and, ideally, the operator should remain unaware of the experimental origin of the images being analysed.
3. Select biologically relevant areas for measurement of colocalization: for instance, the entire cell, the plasma membrane, the nucleus, the cytoplasm etc. ROIs can be created manually using the freehand selection, by thresholding, or from combinations of other ROIs. For instance the cytoplasm can be obtained from the difference between the ROIs of an entire cell and its nucleus. ROIs may also be defined by thresholding images of additional fluorophores (see **Notes 11 and 12**).

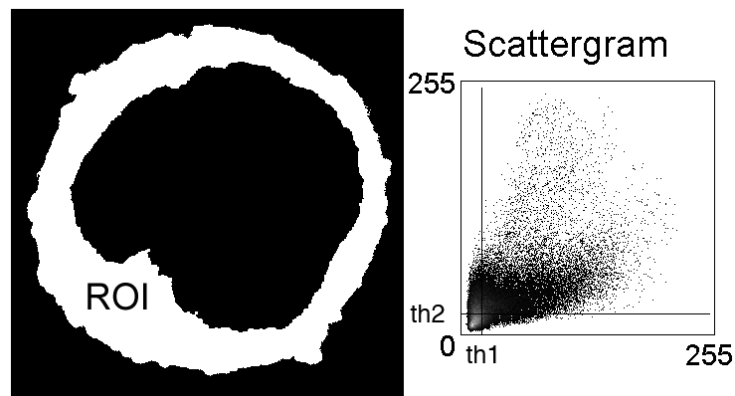


Fig 3. Scattergram presenting the overall relationship between the intensities of homologous pixels. For each pixel the intensities in the two channel are used as the coordinates for the scattergram, and the value at that location in the scattergram indicates the incidence of that combination. A perfect correlation would appear as a *straight line* passing through the origin (if the offset of the detector are accurately set). The slope is not important, since it reflects the gain setting for the two channels, but linearity and the spread are relevant. To select pixels above background, i. e. those with the fluorophore present, a threshold is set for each channel (th1 and th2).

3.5.2 Co-occurrence Analysis of Colocalization

Co-occurrence in its simplest form measures what fraction of the pixels in a ROI contains both fluorophores. The M1 and M2 coefficients indicate what fraction of the total fluorescence is found in common areas.

1. Use software to measure the common area as well as the M1 and M2 coefficients. An outline of the steps involved in co-occurrence measurements is shown in **Fig. 4** and could be implemented in any image analysis package.
2. Compare the size of the area where the fluorophores are co-occurring with the area likely to be occupied if the fluorophores were distributed randomly. The area occupied by the binary images, R_{fraction} and G_{fraction} , can be used to obtain the expected occupancy: $= R_{\text{fraction}} * G_{\text{fraction}}$. If each fluorophore occupied 50% of the ROI, then a random common distribution might be expected to be around 25%.

Other randomization schemes are available within JACoP and MBF, which are large that can be added to the ImageJ image analysis software.

3.5.3. Correlation Analysis of Colocalization

Correlation considers whether there is a relationship between the intensities within a ROI. The Pearson correlation coefficient is available within almost all commercial software and three ImageJ plugins (JACoP, MBF, Cocalization finder), the ICQ coefficient and Spearman rank coefficients are less widely available. We do not recommend the Mander's Overlap coefficient (see **Notes 13 and 14**).

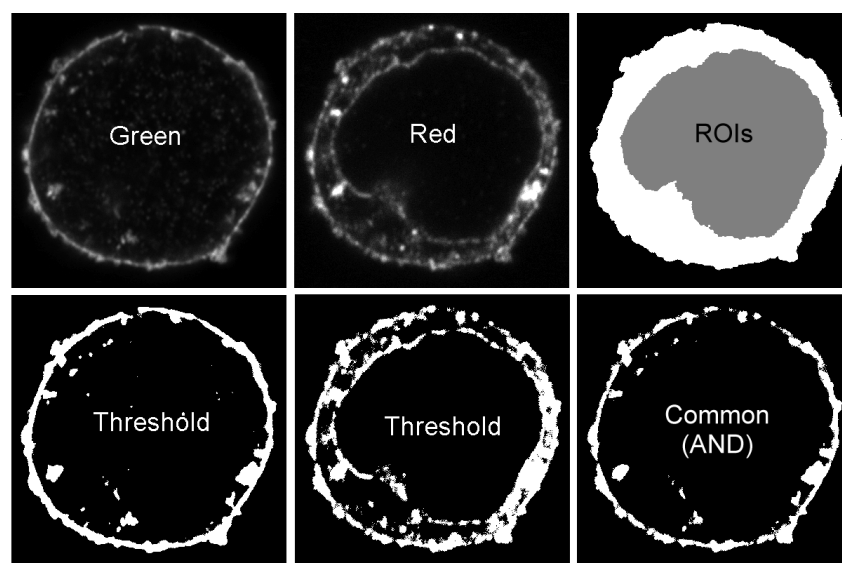


Fig. 4. Selection of a ROI and measurement of co-occurrence. Two channels (*Red* and *Green*) are shown and each channel is thresholded to differentiate between the presence and absence of fluorescence, producing a pair of binary images. The two binary images are combined, using a logical AND operation, to reveal common areas containing both fluorophores. The ROIs shown in the *top right* panel, the whole cell, and the nucleus, could be combined to select the cytoplasm. The ROIs might be selected manually, from the individual channels, from a combination of the channels, or from a third image of another fluorophore. Other biologically relevant ROIs might be the plasma and nuclear membranes. Co-occurrence by area can be measured for the whole cell by expressing the area of the combined thresholded image (areas in common) as fraction of the area of the cell. Areas of binary images are obtained by measuring the mean intensity of the binary image. Co-occurrence by intensity (M1 and M2) requires the sum of the intensity for each fluorophore within the ROI and the sum of the intensity within the common area within the ROI.

3.5.4 Replicate Based Noise Corrected Correlation Analysis of Colocalization

It is unfortunate that the measured correlation between two fluorophores depends upon the quality of both images and not solely on the underlying relationship between the fluorophores. Since we want a measure of colocalization that only depends on the underlying relationship, the contribution of image noise must be eliminated. The trick is to utilize the measure of quality outlined in Subheading 3.2 as a correction to the measured correlation (**Fig. 5**).

1. Threshold the individual images manually from intensity distribution histograms manually or use the automated approach described by Costes et al. [8] or those available within ImageJ (see **Note 15**).
2. Determine the quality by measuring the Pearson correlation coefficient between the two replicate images, A1 with A2 and B1 and B2 (see **Note 16**).
3. Combine the two self correlation coefficients to obtain a correction factor.
4. Make the initial colocalization measurement by measuring Pearson correlation coefficients between A1- B1, A1-B2, A2-B1 and A2-B2. (see **Note 17**).
5. Calculate the arithmetic mean of the four between fluorophore correlations.
6. Use the correction factor to adjust the measured mean correlation coefficient and obtain a Pearson correlation coefficient that is independent of the quality of the images (see **Note 18**).

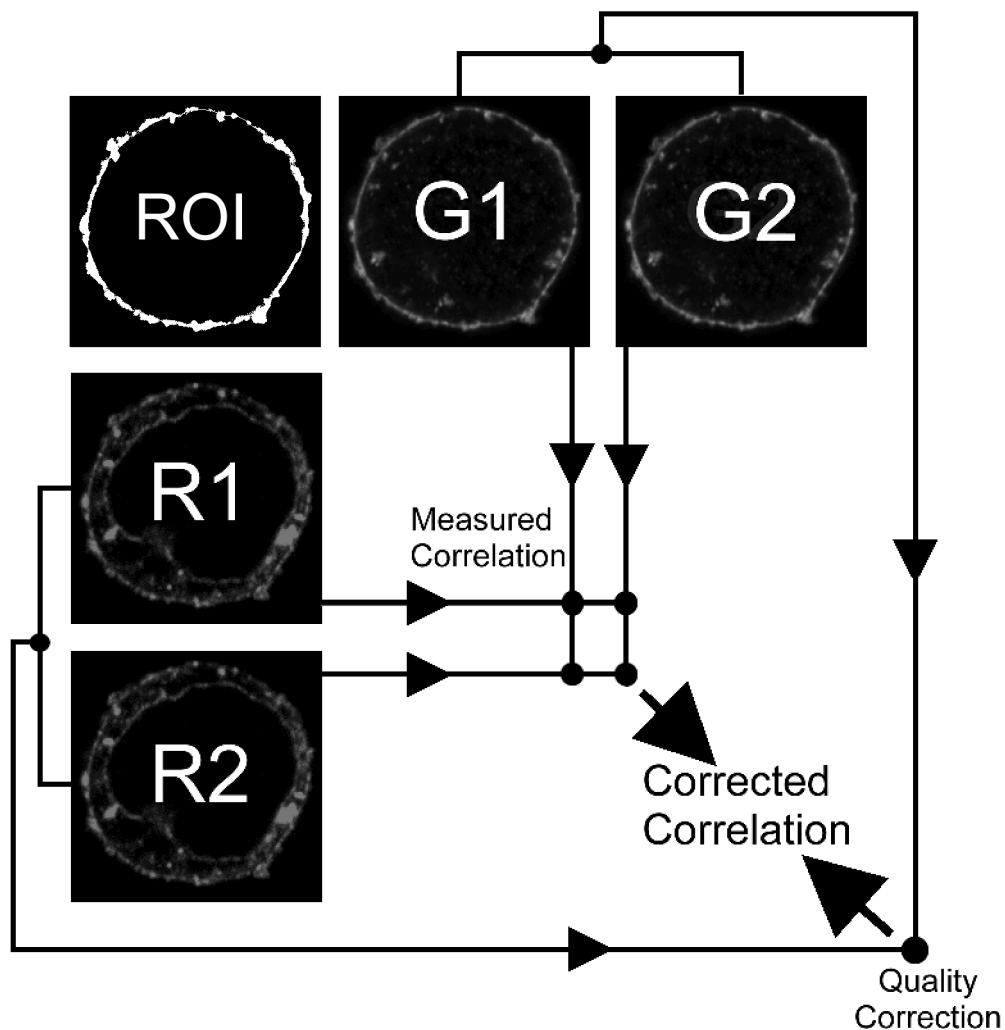


Fig. 5. Replicate Based Noise Corrected Correlation - Measurements of correlation that are independent of image quality. The schematic shows two images for each fluorophore (R1 & R2 and G1 and G2) and there are therefore four separate measurements of the red-green correlation. The measured correlation is taken to be the arithmetic mean of the four individual red-green correlations. Comparisons between the two sets of replicate images are used to assess image quality and the quality measurements for the two fluorophores are combined into a correction factor that is applied to the measured correlation between the fluorophores, producing a correlation between the fluorophores that is not underestimated due to the presence of image noise.

4. Notes

1. A uniformly fluorescent slide can be prepared with a layer of concentrated fluorophore sealed between a slide and coverslip.
2. If a widefield system is used for colocalization analysis, it should be complemented with image deconvolution. A widefield image contains the sum of the fluorescence from in focus and out of focus planes, so fluorophores in the nucleus would appear to colocalize with a fluorophore in the cytoplasm above or below the nucleus. Image deconvolution uses knowledge of the optical system to return the fluorescence to its point of origin and a widefield Z series becomes a series of in focus slices—the fluorophores in the nucleus and cytoplasm will no longer colocalize. Deconvolution also improves the quality of confocal images.
3. Although this software makes provision for using the co-occurrence and correlation coefficients discussed in this chapter, it uses undocumented image processing routines and should therefore be treated with caution.
4. If the illumination is uneven it may be possible to use only the central part or another uniformly illuminated subset of the imaging area. It is also possible to correct for uneven illumination using the image of the uniform sample; areas of the image that are poorly illuminated need to be scaled up and areas with above average illumination need to be scaled down. The rescaling can be achieved by dividing the images of the fluorophores by the image from the uniform slide. The uniform image requires a background subtraction, the intensity in the absence of illumination.
5. Autofluorescence differs between specimens, and may differ in intensity and spectra within a specimen. Moreover, it may also be altered by the fixation method. Autofluorescence is often lower at longer wavelengths, and thus choosing fluorophores in this part of the spectrum can reduce such problems. It is also possible to remove autofluorescence by photobleaching prior to the addition of fluorophores. If the spectrum of the autofluorescence is known, a technique called spectral unmixing can be used to separate the fluorophores from each other and from autofluorescence. The use of bright fluorophores is recommended since they will have relatively stronger emissions than the autofluorescence, to the extent that autofluorescence becomes insignificant.
6. Linearity declines as illumination intensity increases. After emitting a photon and returning to the ground state, a fluorophore in strongly illuminated areas (the point of focus) will be almost immediately re-excited. However, fluorophores in out of focus areas, that are more weakly illuminated, will fluoresce more as the illumination intensity is increased.
7. These criteria should be clearly stated in any publication.
8. The quality of a digital image can be assessed by visual inspection of the difference between consecutively acquired images by addressing the question

- whether pixels change as an image is reacquired, or simply by assessing whether individual pixels differ from their neighbours in a single image.
9. This differs from the more common combination of red and green to produce yellow. Red-green-yellow is unsatisfactory for people who have the most common forms of colour blindness [9].
 10. The display produced by overlaying images is sensitive to the acquisition settings and to changes in brightness and contrast of each channel. This means that the operator can produce white or grey colour in any part of the image where both fluorophores are present. For co-occurrence this is less important, but for correlation the display can be deceptive.
 11. There are large differences in the ease or even potential for selecting ROIs between different software. The MBF colocalization plugin allows the use of a ROI generated elsewhere.
 12. Correlation measurements should only be attempted between pixels that include both fluorophores, since one simply cannot measure the relationship between something that is not there [10,11]. Generally, very few of the pixels within a carefully defined ROI are devoid of fluorescence.
 13. The Manders Overlap coefficient is a confusing hybrid measurement with features of both correlation and co-occurrence [4].
 14. Correlation measurements need to be restricted to a ROI. It is useful to define regions of interest within cells or tissues since the correlation in different organelles may be completely different and the existence of multiple relationships make the readout meaningless. See ref. [4] for a discussion on how multiple relationships within a region of interest affects the correlation coefficient.
 15. The method described by Costes et al. sets a threshold using the reasonable assumption that pixels below the threshold will be uncorrelated and thus sets the threshold where the correlation is greater than zero. However, this method sometimes produces thresholds that exclude large numbers of pixels containing both fluorophores with intensities substantially above background. Moreover, this method will fail completely for fluorophores that show no correlation.
 16. The Pearson correlation coefficient for a replicate set of images also serves as a positive control. The measured correlation between two images of the same specimen should be one (unity) and any deviation from this number is caused by image noise.
 17. The method RBNCC [10] only applies to colocalization using the Pearson correlation and Spearman rank correlation coefficients. Measurements using the two Manders coefficients, M1 and M2, are affected both by noise and by the pixel size. Thus, when using these coefficients it is important to acquire images of high quality and only make comparisons between images with the same pixel size.
 18. RBNCC generates accurate correlation coefficients from noisy images [12]. This means that a time-course can be obtained from live cells without significantly bleaching the fluorophores and enhancing the phototoxicity, which can accompany prolonged exposure times. In other words, image acquisition can be

accomplished using lower levels of illumination, with a corresponding reduction in photobleaching and phototoxicity. The correction built into RBNCC will compensate for reductions in the signal caused by photobleaching.

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