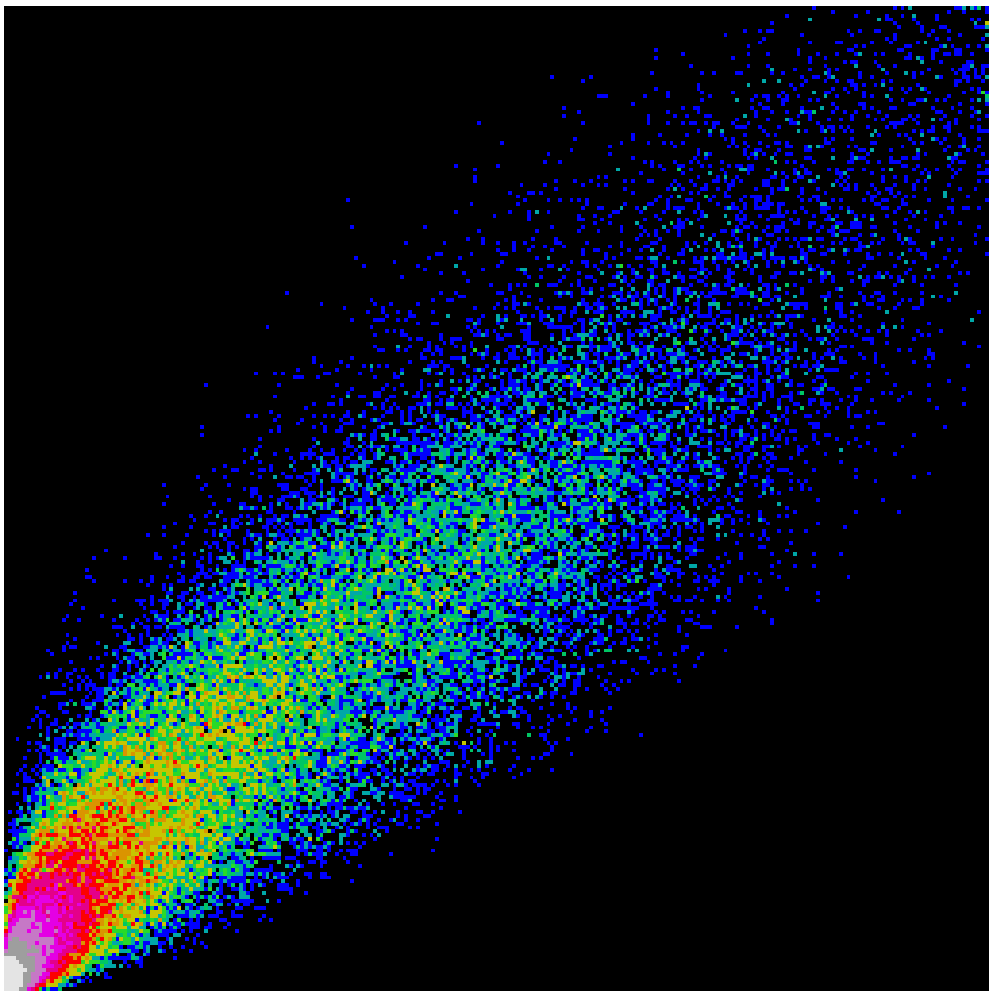
Colocalisation



McMaster Biophotonics Facility ▪ Dept. Biochemistry and Biomedical Sciences ▪ HSC 4H14, 1200 Main Street
McMaster University ▪ Hamilton, ON ▪ www.macbiophotonics.ca

Table of contents

Introduction

Colocalisation analysis involves comparison of the spatial localisation of two proteins. These two proteins have either been rendered intrinsically fluorescent by molecular-engineering or indirectly fluorescent using fluorescence immunocytochemistry.

We are ultimately interested in whether these two proteins occupy the same volume of interest. For some experiments, the volume of interest may be the same cell – are the two proteins expressed in the same cell? If this is the case then there is no better replacement for manual counting of "double-positive cells". For tissues, stereological approaches to determine numbers would be advised.

In other cases we may want to know if the two proteins occur in the same protein-complex or subcellular compartment. In this case we are limited by the resolution of the light microscope and, in a best case scenario, can only say whether the two proteins occupy the same 200×200×400 nm volume – or voxel. If we want higher resolution, we would have to use the technique of FRET which can resolve interactions of proteins 7 nm apart. As can be seen from this simple calculation, even if we can prove the proteins occupy the same voxel, this does not necessarily mean they are interacting; they may be up to 400 nm apart!

There are many techniques used to calculate image correlation. They can broadly be divided in to *Qualitative*; *Threshold-based* and *Intensity-based* approaches. Threshold-based approaches use only pixels above a certain threshold in their calculations while intensity based approaches use all the pixels regardless of their intensity.

Often the threshold-based analyses are more intuitive, not requiring expert knowledge of a particular coefficient and often reported as an easily comprehended "percentage colocalisation" value. However, since the user has to define the threshold the analyses may be subject to bias. There are algorithms which will automatically calculate the appropriate threshold for an image based on the image's intensity histogram. However, the image's histogram may vary test and control samples.

Intensity-based analysis removes user bias by analysing all the pixels based on of their intensity, however, it must be noted that some authors consider this a draw back rather than an advantage due to the intrinsic uncertainty of pixel intensity (Lachmanovich *et al.*, 2003). Intensity based approaches are also susceptible to the potential errors described in section *Confounds.*

Qualitative

This is often simply the presentation of an image where the red and green channels have been merged resulting in some yellow pixels which it is claimed to demonstrate colocalisation. One issue with this type of image is that it cannot be seen by 10% of the population with red-green colour blindness. Using magenta instead of red improves this and combining the images as cyan-magenta using a "difference" algorithm can also make the colocalisation more apparent (Demandolx *et al.*, 1997).

This approach has the advantage of being easily comprehended and providing, at a glance, some spatial information of the colocalisation. However, the amount of "yellow" is extremely susceptible to any image processing that modifies the intensity histogram of the images. This sort of merged image should always be considered an accompaniment to a qualitative analysis of the two channels.

Threshold-based analysis

Key to this technique id the user definition of the threshold. Pixels above this value are considered to be positive, pixels below this value are considered to be negative.

Simple segmentation (Fay et al., 1997) *threshold = meanBG +2x stdevBG*

Complex Tophat filter/watershed/ (Lachmanovich et al., 2003)

This sort of approach seems to be best suited to two images with discrete punctuate staining. Once thresholded the two channels can be analysed in a number of ways. Either simply the percentage of red channel above threshold that colocalise with a green pixel above threshold. More complex analysis can also be done. Lachmanocivh calculated the centre of mass for each puncta and considered it colocalised with another punctum if it fell within its area. Two values were therefore calculated: "% red colocalised with green" and "% green colocalised with red".

In each case thought eh authors took the important step of demonstrating what degree of overlap would be expected by random chance alone. Fay et al randomly displaced one channel with respect to the other while Lachmanovich et al took the neighbouring green area as a random field of puncta. Here we see where errors may arise when a threshold is set. Assuming the puncta are fainter at the edges, a high threshold will reduce their area and result in less colocalisation.

Confounds

While using intensity-based analysis can avoid potential user bias associated with setting a threshold, it does require that the intensities in the image are accurate. There are several ways in which the accuracy of the intensities in the image can be improved.

Photon shot-noise

Photon-detection is subject to inherent Poisson distributed shot-noise. This means that if 10 photons arrive at the detector (this is not an unusually small number for a pixel in a confocal image), the noise is equal to sqrt(10) ~3, i.e. the 10 photons may appear in our pixel between 7 to 13.

There is inherent uncertainty in how pixel intensity relates to emitted-photons, with the noise proportional to the square-root of the number of photons. Deconvolution of images can help maximise the signal to noise ratio (Landmann, 2002;Landmann *et al.*, 2004).

Background correction

A properly acquired image should have a certain amount of background signal to ensure all the "real signal" that is above background is detected.

This non-zero background needs to be removed. If not, some methods of quantification will assume it to represent red or green signal and assign it a positive correlation value. Due to intrinsic noise in the image, subtracting only the mean background intensity from the image often leaves a lot of the background above zero and skews the correlation calculation. We typically subtract the mean value plus 3× the standard deviation of the background.

Uneven background cannot be corrected using this simple technique. Ideally a background image should be acquired for each channel using the same hardware settings used to acquire the images. These background images should be subtracted from their respective image. This is often not possible so a background subtraction algorithm such as the rolling-ball algorithm is useful to flatten out the uneven background allowing the [mean+3×StdDev] correction.

Bleed-through a.k.a. Cross-talk

If your green dye is being detected in your red-channel you will get excellent colocalisation even if the red and green are in different cells! The hardware should be configured so that single labelled/expressing green

cells do not appear in the red channel or (less likely) single labelled/expressing red cells do not appear in the green channel.

Cross-talk can be minimised by using sequential acquisition (i.e. red then green images) and narrow bandwidth emission filters. Once the hardware is configured with these two control samples the hardware settings should not be changed.

Qualitative Colocalisation

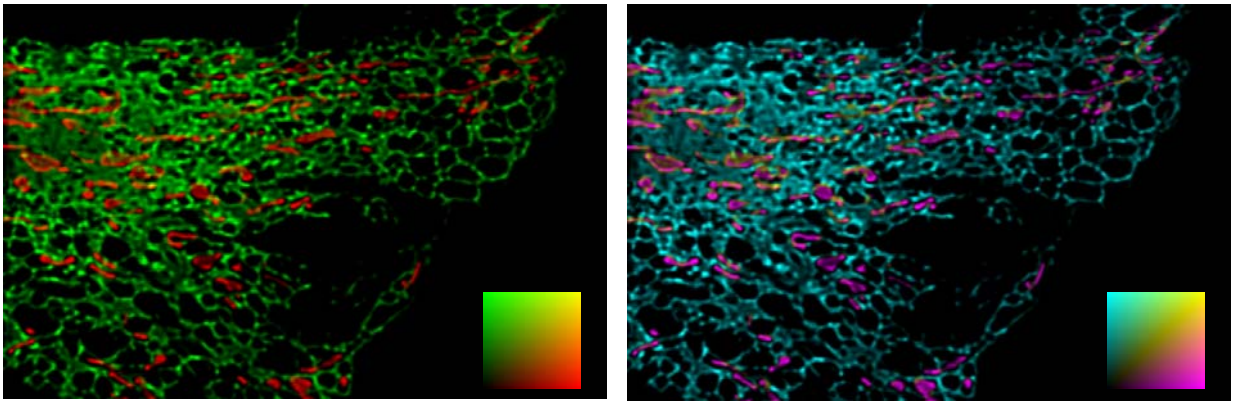
Qualitative colocalisation can be divided in to two techniques: *Dye-overlay* and *Threshold-overlap*. While both of these techniques can provide a useful visual aid to demonstrate colocalisation, they need to be accompanied by quantitative analysis to avoid bias.

Dye Overlay

This technique merges the red and green channels of an image. Where the red and green of the image overlap, the pixels appear yellow. The amount of yellow however is subject to how each channel has been processed. This can introduce bias when processing multiple images.

An alternative to the normal Red-Green merge is to merge the images based on Cyan and Magenta.

This can aid visualisation of colocalisation due to our better perception of red and green colours. The ImageJ function "*Plugins/Colour Functions /Colour Merge*" function will perform a 'difference' arithmetic processing on the image stacks you select. This is not strictly a merge (when cyan and magenta merge they produce white, not yellow) but facilitates visualisation of the separate channels (Demandolx *et al.*, 1997).

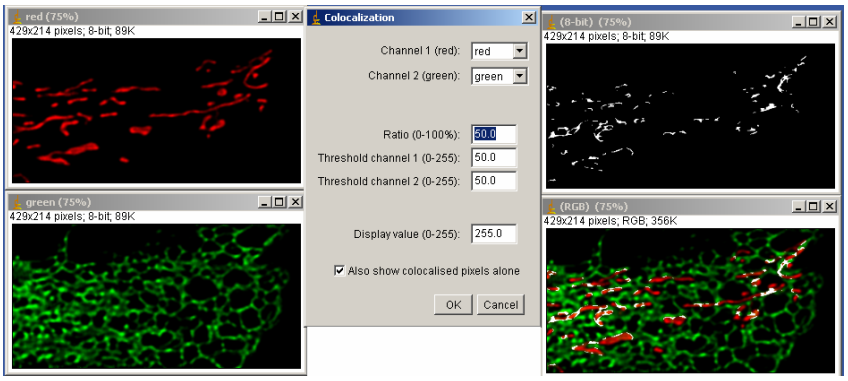


Often, showing yellow pixels in a red-green merged image is as far as colocalisation quantification goes. This is somewhat arbitrary, especially for the ~5% of the population that are colour-blind!

Threshold-overlap

One step further is to highlight those pixels that show certain red pixel intensity and green pixel intensity above a certain threshold value. However, this requires the user to decide a "threshold" value which can introduce bias and is sensitive to how each channel has been acquired and processed.

In the example below, pixels that have red and green values greater than 50 have been re-coloured white using ImageJ's *Plugins/Colour Functions/Colocalization* plugin.



Quantitative Colocalisation

More rigorous analysis of colocalisation requires its quantification. There are a number of colocalisation analyses that require thresholds to be set – below which, the pixels are ignored. The threshold is often set by the user and can lead to bias.

Noise can result in false-positive colocalising events so pre-processing of images is often desirable. Ideally the images should be deconvolved. If this is not possible and a median filter can improves the signal-to-noise without blurring the edges in the image (Landmann and Marbe 2004).

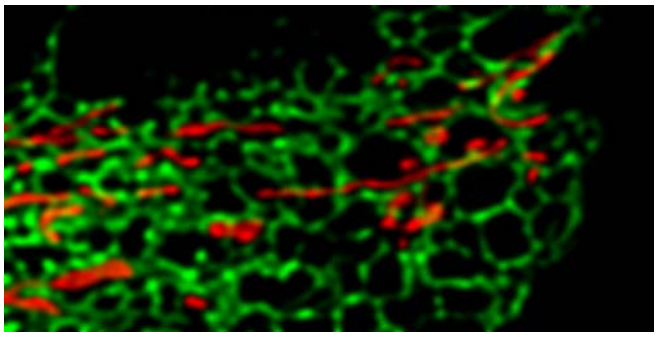
Scatter plots

Often, the relationship between the red and green pixels in an image is displayed as an intensity-scatter plot. For 8-bit images (zero to 255) a 256×256 scatter plot is generated. In each scatter plot, the first (typically red) image component is represented along the x-axis, the second image (typically green) along the y-axis. The intensity of a given pixel in the first image is used as the x-coordinate of the scatter-plot point and the intensity of the corresponding pixel in the second image as the y-coordinate.

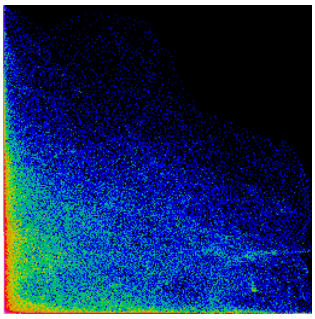
Scatter plots can be displayed in two ways: as "*Frequency plots*" or as "*Red-Green plots*".

In "*Frequency plots*" (e.g. Zeiss LSM, Bitplane ImarisColoc) the pixels are pseudocoloured so that their colour represents the frequency of the red-green pixel combination in the original image (hot colours representing high values by convention). This sort of plot contains the most information, but can be a little difficult to relate it back to the original image.
In "*Red-Green scatter plots*" (e.g. Biorad LasersharpNT, Media Cybernetics ImagePro plus), the colour of the scatter plot pixel represents the actual colour in the image. This does not tell you the frequency of the pixel, but is easier to relate to the original image.

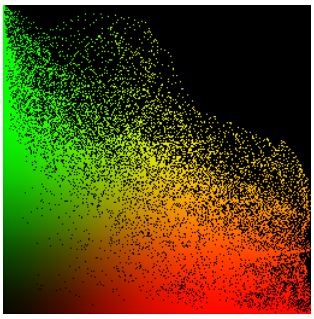
ImageJ will generate both types of scatter-plot. Examples of scatter plots for different types of colocalisation can be found in the ***Examples*** section.



Frequency scatter plot



Red-Green scatter plot



.

Pearson's Correlation coefficient - R_r

$$R_r = \frac{\sum (R_i - \overline{R}) \times (G_i - \overline{G})}{\sqrt{\sum (R_i - \overline{R})^2 \times \sum (G_i - \overline{G})^2}}$$

For pixel *i* in the images, *R* and *G* are intensities of the red and green channel respectively.

Popular method of quantifying *correlation* in many fields of research from psychology to economics. In many forms of correlation analysis the values for Pearson's will range from 1 to -1. A value of 1 represents perfect correlation; -1 represents perfect exclusion and zero represents random localisation. However, this is not the case for images. While perfect correlation gives a value of 1, perfect exclusion does not give a value of -1. Low (close to zero) and negative values for Pearson's correlation coefficient for fluorescent images can be difficult to interpret. However, a value close to 1 does indicate reliable colocalisation.

Overlap Coefficient - R

$$R = \frac{\sum_i (R_i \times G_i)}{\sqrt{\sum_i (R_i)^2 \times \sum_i (G_i)^2}}$$

This is easier than the Pearson's coefficient to comprehend. It ranges between 1 and zero with 1 being high-colocalisation, zero being low. However, *the number of objects in both channel of the image has to be more or less equal.*

The overlap coefficient is strongly influenced by the ratio of red to green pixels and should only be used if you have roughly equal numbers of red and green pixels (i.e. $N_{red} \div N_{green}$ pixels ~1). (Manders *et al.* 1993)

Overlap Coefficients for red and green – k_{red} and k_{green}

$$k_{red} = \frac{\sum_i (R_i \times G_i)}{(R_i)^2}$$

$$k_{green} = \frac{\sum_i (R_i \times G_i)}{(G_i)^2}$$

The problems of the overlap coefficient can be cancelled out by dividing it in to two separate coefficients – one for each channel. However, these values are very sensitive to the absolute fluorescence intensity. If a detector has been set too low in one channel, or one channel has bleached more than the other, this will affect the *k* value for that channel.(Manders *et al.* 1993)

Colocalisation Coefficients – M_{red}, M_{green}

$$M_{red} = \frac{\sum_i R_{i,coloc}}{\sum_i R_i} \qquad M_{green} = \frac{\sum_i G_{i,coloc}}{\sum_i G_i}$$

$R_{i,coloc} = Ri$ if $Gi > 0$; $G_{i,coloc} = Gi$ if $Ri > 0$.
i.e. M_{red} is the sum of the intensities of red pixels that have a green component divided by the total sum of red intensities.

{Manders, Verbeek, et al. 1993 2636 /id}. These split-coefficients avoid issues relating to absolute intensities of the signal, since they are normalised against total pixel intensity. We also get information as to how well each channel overlaps the other. There are cases where red may overlap significantly with green, but most of the may not overlap with the red (see Example 1).

If the assumption is made that greyscale number equates to dye molecules (***this is not necessarily correct***) then these coefficients represent the percentage of red dye molecules that share their location with a green dye molecule.

These coefficients are very sensitive to poor background correction and do not take in to account the intensity of the second channel, other than it is non-zero. For example, a bright red pixel colocalising with a faint green pixel is considered equivalent to a bright red pixel colocalising with a bright green pixel. Intuitively, a red-green pixel-pair of similar intensities should be considered "more colocalised" than a pixel pair of widely differing intensities.

Intensity Correlation Quotient – ICQ

If the intensities in two images vary in synchrony (i.e. they are *dependent*), they will vary around their respective mean image intensities together. So, if a pixel's intensity is below average in the red channel (i.e. R_i-R_{mean}< 0); it will be below average in the green channel (i.e. G_i-G_{mean}<0). Similarly, if a pixel is above average in one channel it will be above average in the other. Therefore, in an image where the intensities vary together, the *product of the differences from the mean* (PDM), will be positive. The converse is true. If the pixel intensities vary asynchronously, i.e. the channels are segregated so that when a red pixel is above average, the corresponding green pixel is below average; then most of the PDMs will be negative.

The ICQ is based on the non-parametric sign-test analysis of the PDM values and is equal to the ratio of the number of positive PDM values to the total number of pixel values. The ICQ values are distributed between -0.5 and +0.5 by subtracting 0.5 from this ratio.

$$PDM = (R_i - \overline{R}) \times (G_i - \overline{G})$$

PDM = Product of the Difference from the Mean for each channel.
For pixel *i* in the image, *R* and *G* are the respective intensities in the red and green channel.

$$ICQ = \left(\frac{N_{+ve}}{N_{total}} \right) - 0.5$$

N_{+ve} = number of positive values for PDM.
N_{total} = total number pixels that do not have a value of zero in each channel.

Random staining: ICQ~0; Segregated staining: 0> ICQ≥ -0.5; Dependent staining: 0<ICQ≤+0.5 (Li *et al.*, 2004).

Confounds for colocalisation analysis

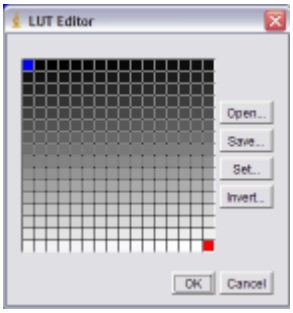
Bleed-through (a.k.a. cross-talk)

Colocalisation typically involves determining how much the 'green' and 'red' fluorophores spatially overlap. Therefore it is essential that the green emitting dye does not contribute to the red signal i.e. the emission from the "green" fluorophore *bleeds through* in to the red detection channel. Green emission dyes such as FITC emit a significant amount of yellow light (>565 nm) which may contribute to the image in the "red" channel. In this case red and green signals *will* colocalise because they are coming from the same source – the green-dye.

Typically, red dyes do not emit green fluorescence but this requires experimental verification. Each dual labelled specimen needs to be accompanied by singly-labelled and unlabelled controls to determine the amount of bleed-through and background fluorescence.

One possible way to avoid bleed-through is to acquire the red and green images ***sequentially***, rather than ***simultaneously*** (as with normal dual channel confocal imaging). Also, using narrower filters to exclude more of the green emission could help.

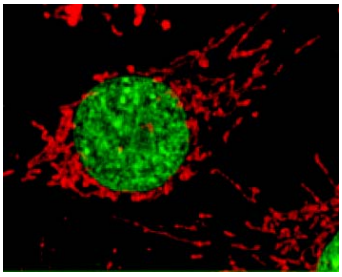
Background correction



When calculating correlation coefficients, the images must be background-corrected. If the background is not corrected and each channel has, say, a grey-value of 5 for the background, the equations that measure the colocalisation coefficients will consider this to represent colocalisation and overlap of the dyes even though, in reality, neither dye is present. A non-zero background is most easily seen with ImageJ's "*Plugins/LUT/Hi Lo Indicator*" LUT. Most image processing packages have a similar LUT/Palette/Colour table (e.g. Biorad systems have a "*SETCOL*" LUT; Zeiss a "*Range Indicator"* palette).

In this example coefficients were calculated for the same image, first with a background of 5 in each channel, and secondly after background correction. The image has a green-stained nucleus and red mitochondria – there should be little colocalisation (except for a couple of mitochondria under the nucleus – another possible confound for widefield images in particular).

	Uncorrected	Corrected
Pearson's correlation coeff. (r_p)	-0.039	-0.115
Overlap coeff. (r)	0.371	0.029
Ch1:Ch2 pixel ratio	1.000	1.461
Colocalisation coefficient for red-channel (Mred)	1.000	0.059
Colocalisation coefficient for green-channel (Mgreen)	1.000	0.173
ICQ	-0.019	-0.256

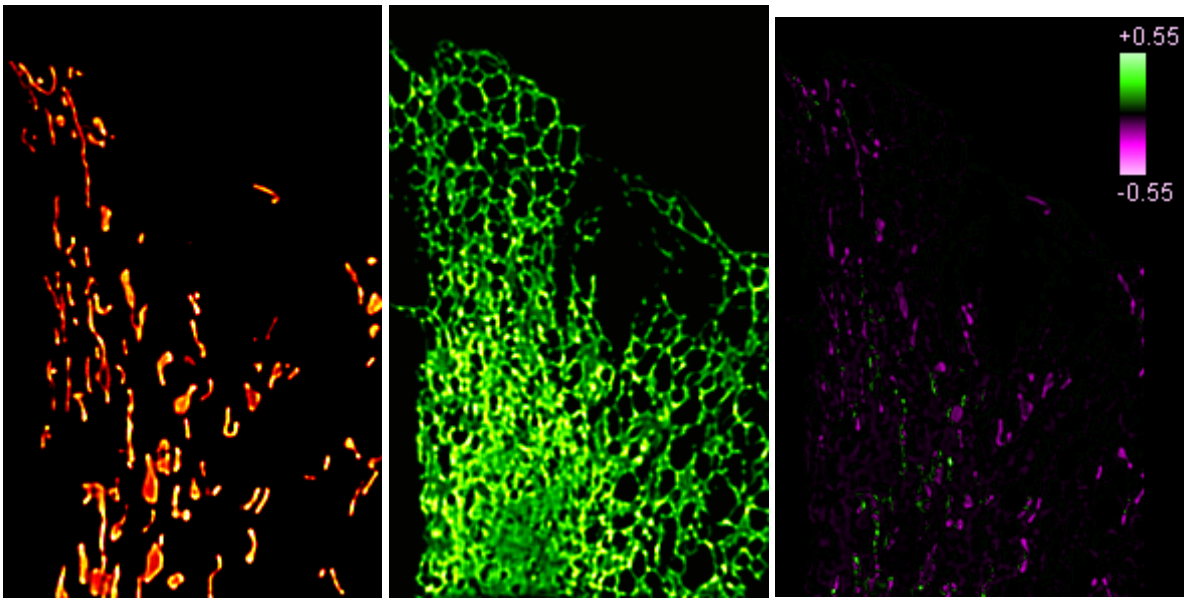


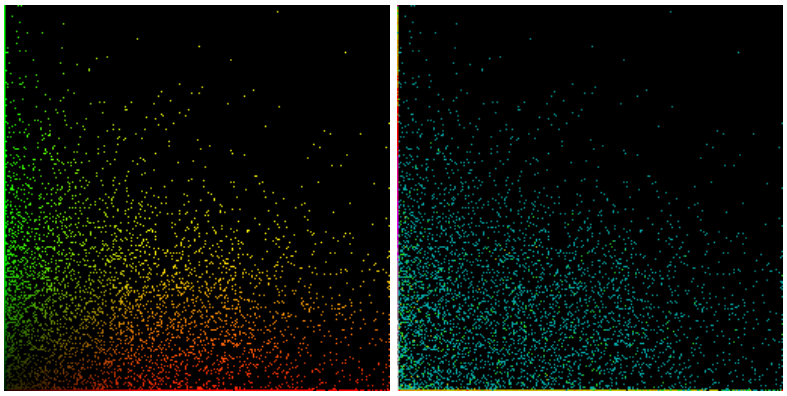
Poor choice of partners

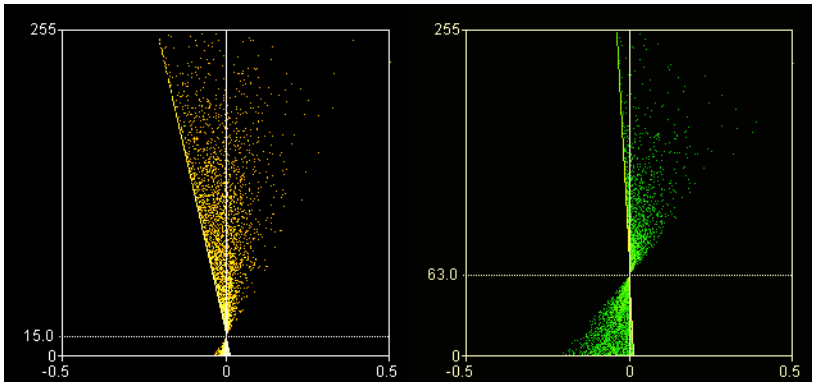
When we try to determine whether protein A is colocalised with protein B we need to ensure that this is sensible comparison. If protein A is ubiquitous in a cell, it will, simply by chance, colocalise with discretely localised protein B. No real information has been gained. Even if we get colocalisation, we must ask, would we have got this result by random chance alone? In some cases, randomly displacing the image from one of the channels can tell you whether the colocalisation you see is random or significant (Fay *et al.*, 1997).

Perhaps the most convincing colocalisation data is that where the control situation is compared to a test situation where the colocalisation is perturbed i.e. protein A vs protein B before and after perturbation. Alternatively, compare the colocalisation coefficients for protein A and B with protein A and unrelated protein C that shows no colocalisation with A.

Examples







Example 1: Partial colocalisation

Mito-DsRed; ER-EGFP

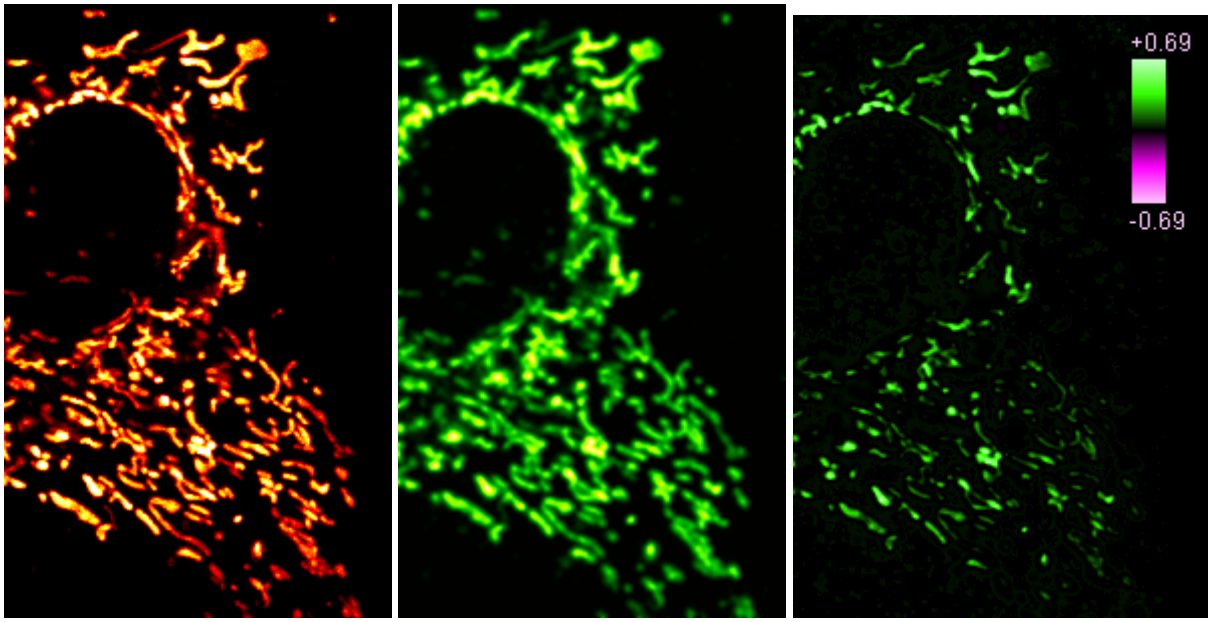
Pearson's correlation coeff. (Rr) = 0.051
Overlap coeff. (R) = 0.185
Ch1:Ch2 pixel ratio = 0.218
Colocalisation coefficient for channel 1 (M1) = 0.746
Colocalisation coefficient for channel 2 (M2) = 0.155
ICQ= -0.052

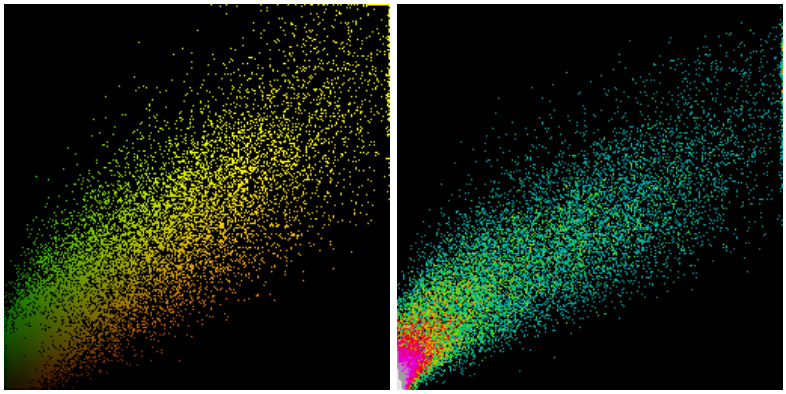
Here the *Pearson's coefficient* is correctly telling us that there is little correlation between the two channels. However, since this coefficient is unreliable in cases other than strong positive correlation, we cannot be sure whether the channels are random or segregated.

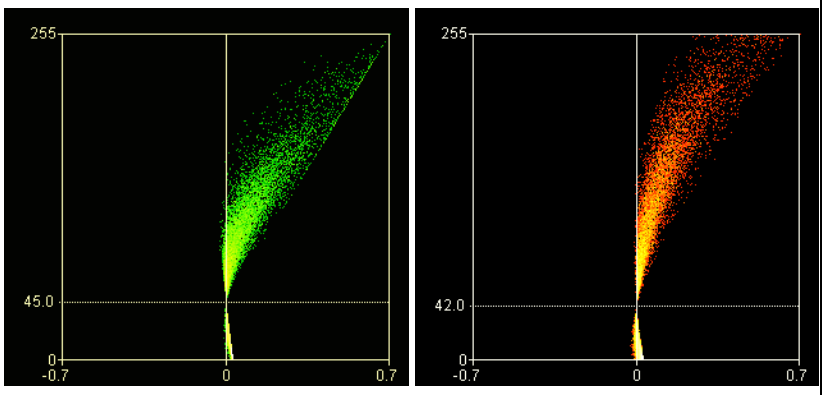
The *overlap coefficient* cannot be used here as the *Ch1:Ch2* ratio is not 1.

The *colocalisation coefficients* here tell us that there is significant overlap between red with green, but not much overlap of green with red.

The *ICQ* shows that despite there being overlap, the red and green intensities are not varying in synchrony (i.e. ICQ~0) and so are largely randomly associated.







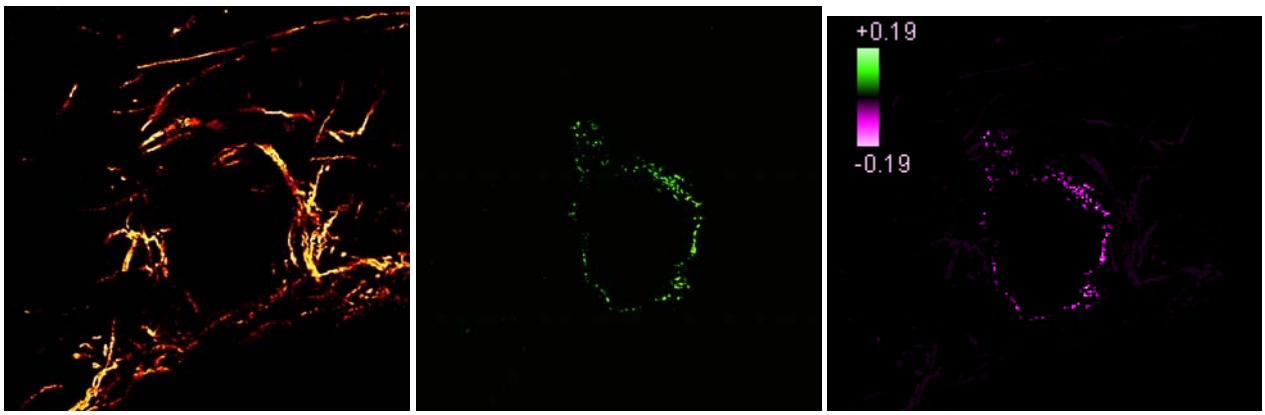
Example 2: Very high colocalisation

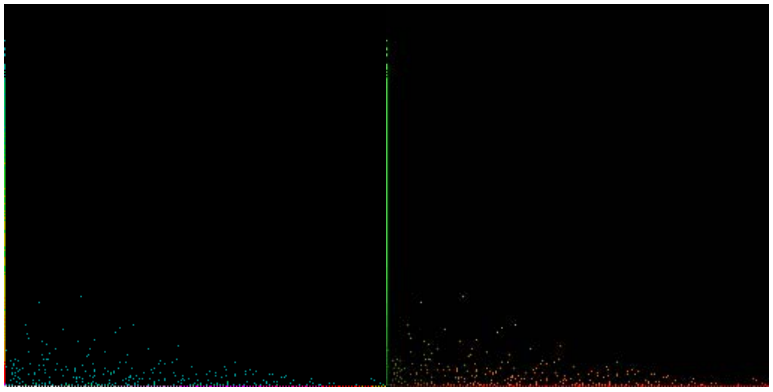
TMRE (red); Mito-pericam (Green)

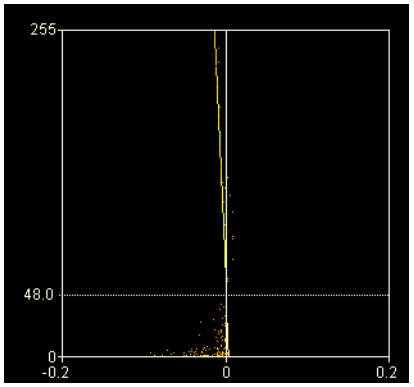
Pearson's correlation coeff. (Rr) = 0.920
Overlap coeff. (R) = 0.934
Ch1:Ch2 pixel ratio = 0.801
Colocalisation coefficient for channel 1 (M1) = 0.998
Colocalisation coefficient for channel 2 (M2) = 0.970
ICQ = 0.391

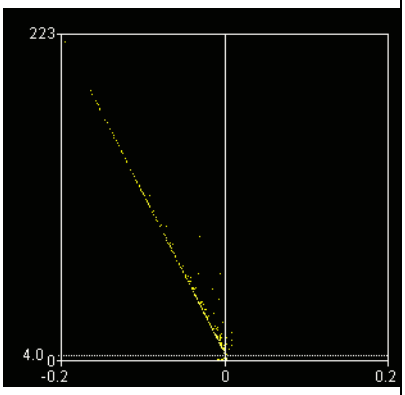
In this example, all coefficients correctly report the strong overlap between the two channels.

The *ICQ* is lower than we may expect given that both the red and green signals should be in the mitochondrial matrix. Careful examination of the merge image however does reveal heterogeneity in overlap of the colours. This could possibly reflect heterogeneity in TMRE staining due to heterogeneity in membrane potential, whereas the green channel will vary with protein import in to the mitochondrion. So although they overlap, their intensities do not vary together.









Example 3: Segregation

GFAP (red); Factor VIII (green)

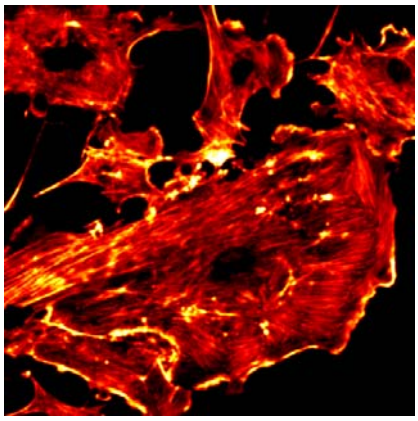
Pearson's correlation coeff. (Rr) = -0.015
Overlap coeff. (R) = 0.01
Ch1:Ch2 pixel ratio = 15.034
Colocalisation coefficient for channel 1 (M1) = 0.007
Colocalisation coefficient for channel 2 (M2) = 0.008
ICQ = 0.114

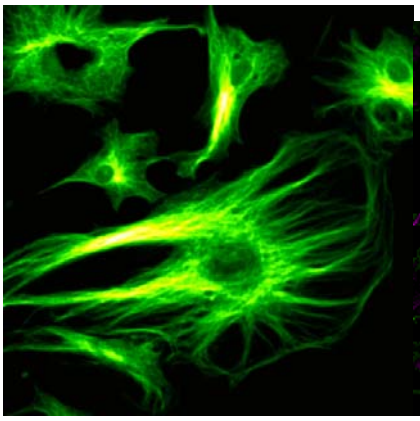
The *Pearson's coefficient* should equal -1 for a completely segregated image which illustrates the problems of using this coefficient on anything other than a strongly positive correlation.

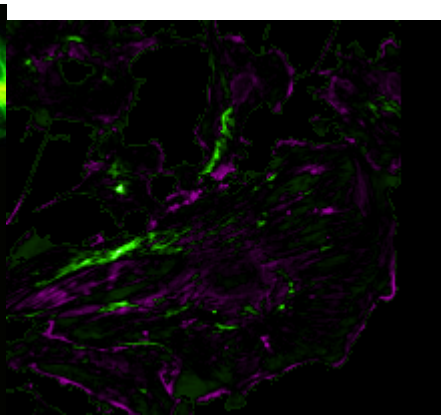
The *overlap coefficient* is not valid due to the CH1:Ch2 ratio.

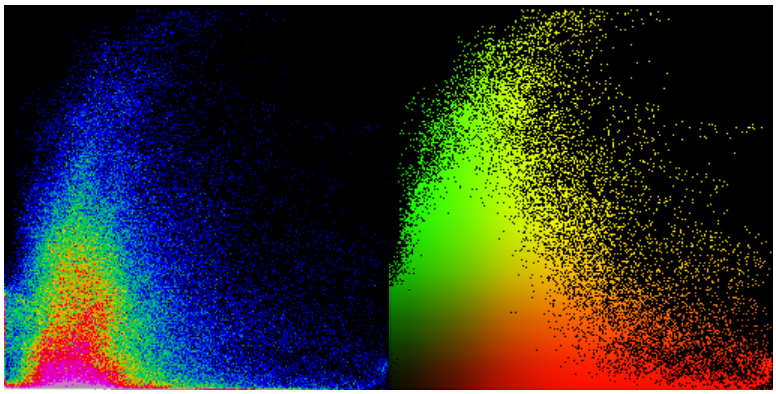
Given that we know, from the biology, that these two signals do not coincide in this example, the *colocalisation coefficients* are rather high. This may be due to overlap at the edges of each signal where blurring may cause red pixels to have a faint green component, and vice-versa.

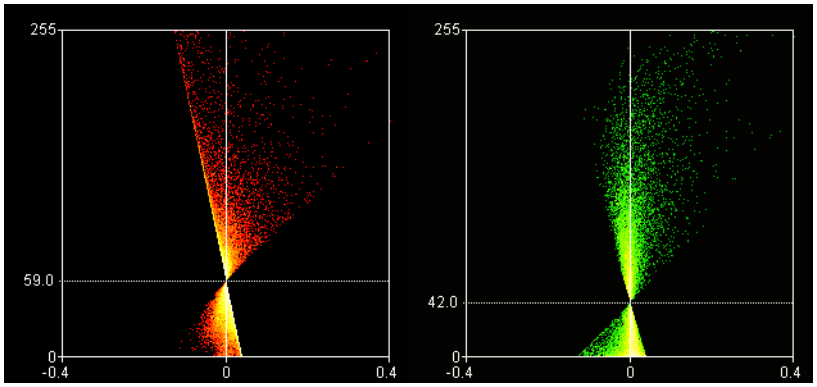
Here the *ICQ* is approximately zero indicating that the overlap is random.











Example 4: Random colocalisation

F-actin (red); microtubules (green)

Pearson's correlation coeff. (Rr) = 0.346

Overlap coeff. (R) = 0.605

Ch1:Ch2 pixel ratio = 1.163

Colocalisation coefficient for channel 1 (M1) = 0.879

Colocalisation coefficient for channel 2 (M2) = 0.993

ICQ = -0.028

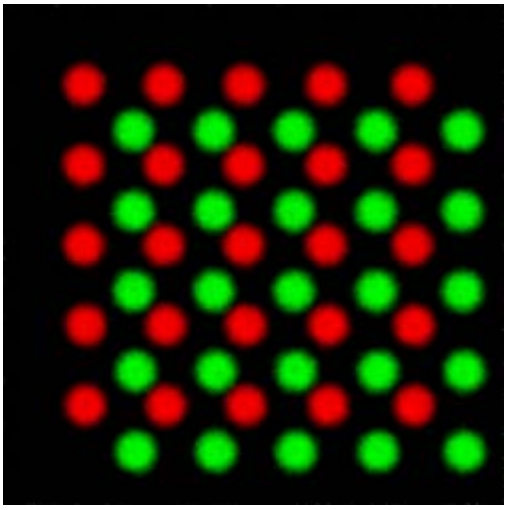
The *Pearson's coefficient* is reporting reasonable correlation, but not very strong.

The Ch1:Ch2 ration is close to 1 and the *Overlap coefficient* reports a random overlap (~0.5).

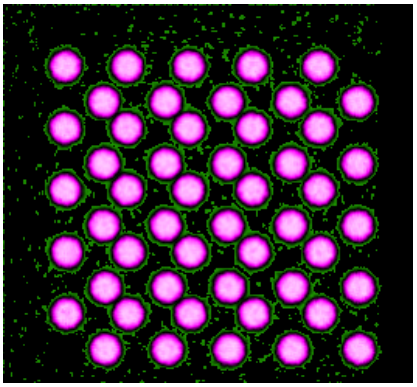
The *colocalisation coefficients* are high for each channel, i.e. most red pixels colocalise with a green pixel of some intensity.

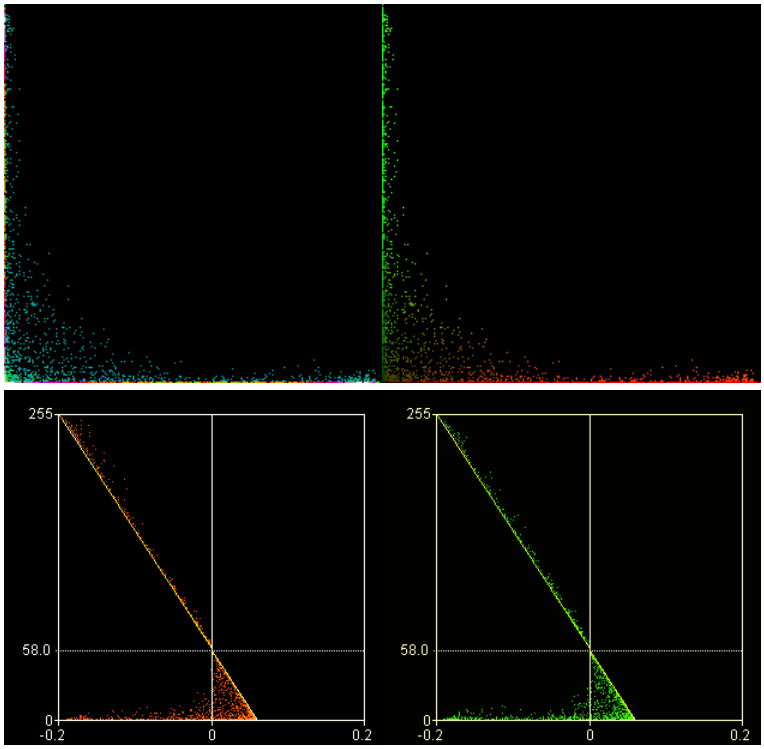
The *ICQ* shows us however, that the pixel

No-colocalisation

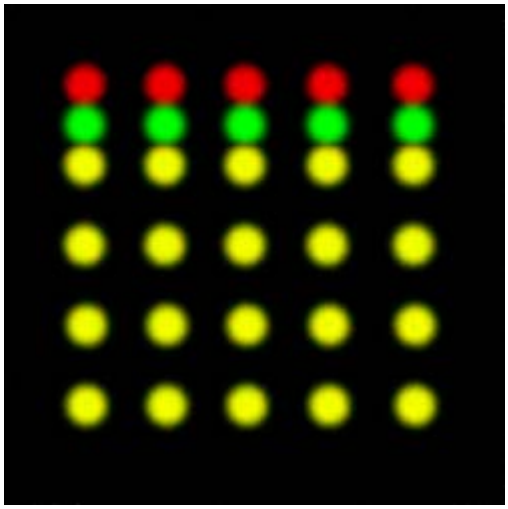


Pearson's correlation coeff. (Rr) = -0.176
Overlap coeff. (R) = 0.001
Ch1:Ch2 pixel ratio = 0.987
Colocalisation coefficient for red-channel (M1) = 0.031
Colocalisation coefficient for green-channel (M2) = 0.025
ICQ = -0.138

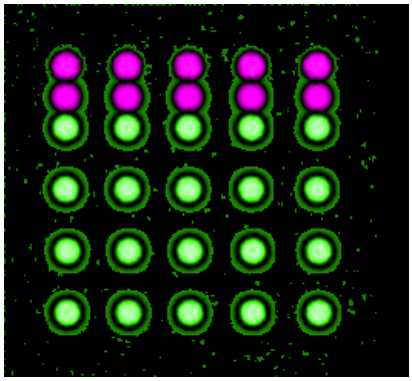


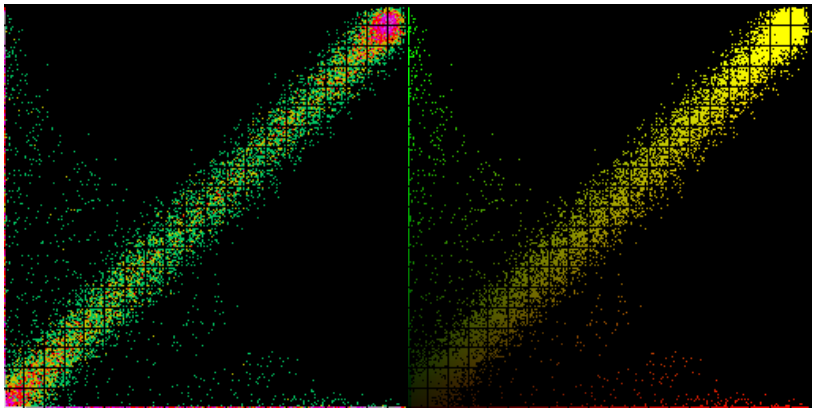


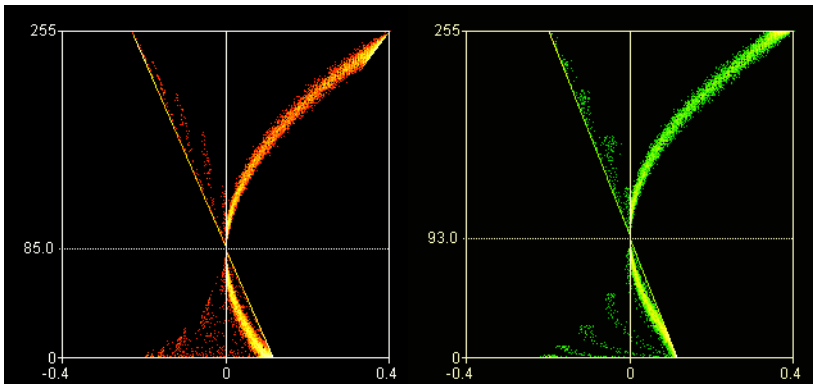
High red-green colocalisation



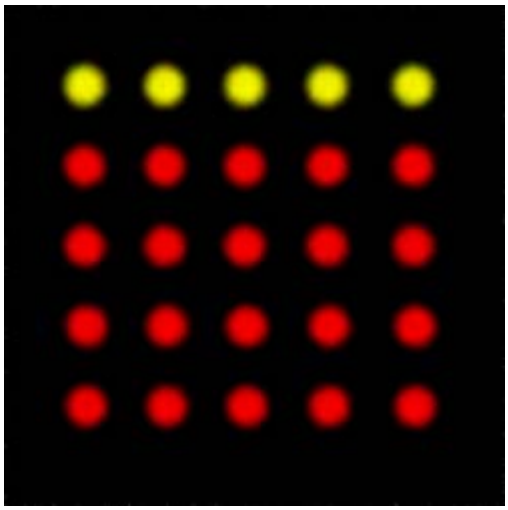
Pearson's correlation coeff. (Rr) = 0.770
Overlap coeff. (R) = 0.804
Ch1:Ch2 pixel ratio = 1.016
Colocalisation coefficient for red-channel (M1) = 0.821
Colocalisation coefficient for green-channel (M2) = 0.834
ICQ = 0.336



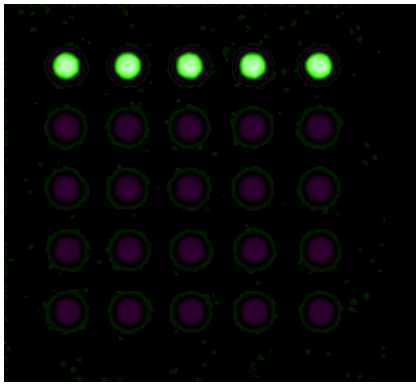


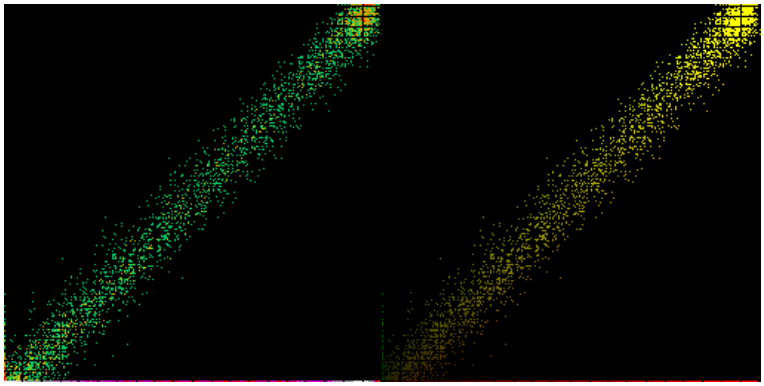


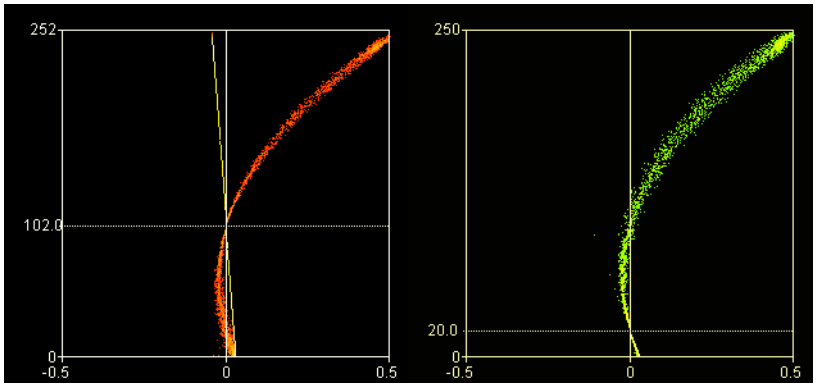
Little green staining, all colocalised with red



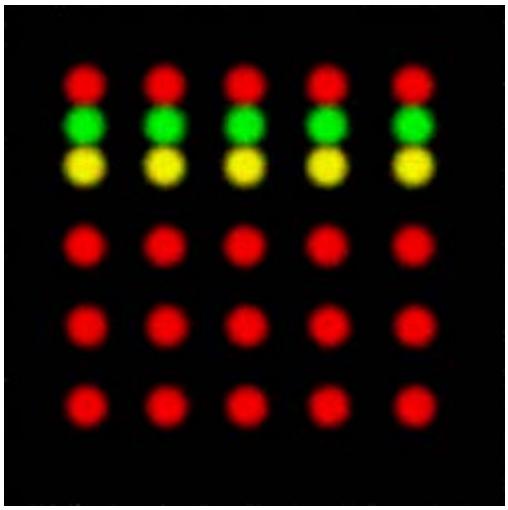
Pearson's correlation coeff. (Rr) = 0.414
Overlap coeff. (R) = 0.444
Ch1:Ch2 pixel ratio = 4.249
Colocalisation coefficient for red-channel (M1) = 0.199
Colocalisation coefficient for green-channel (M2) = 0.989
Sign test value (count +ve) = 7190
Sign test value (count -ve) = 55310
ICQ = 0.085



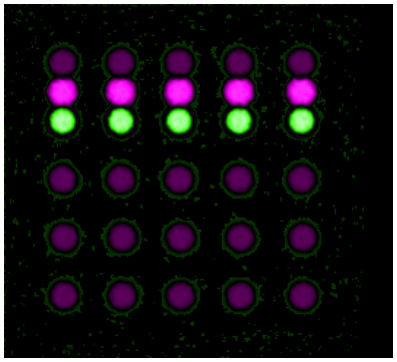


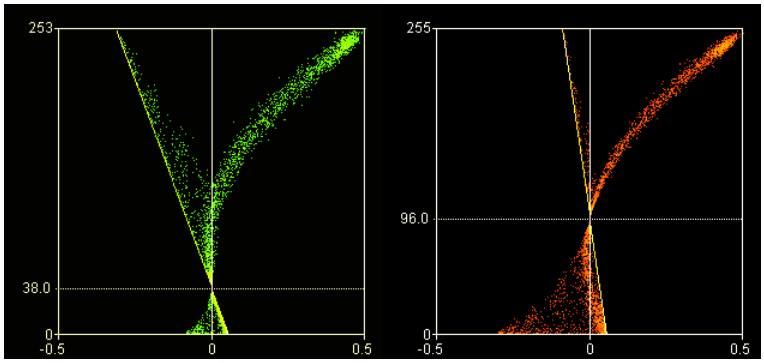


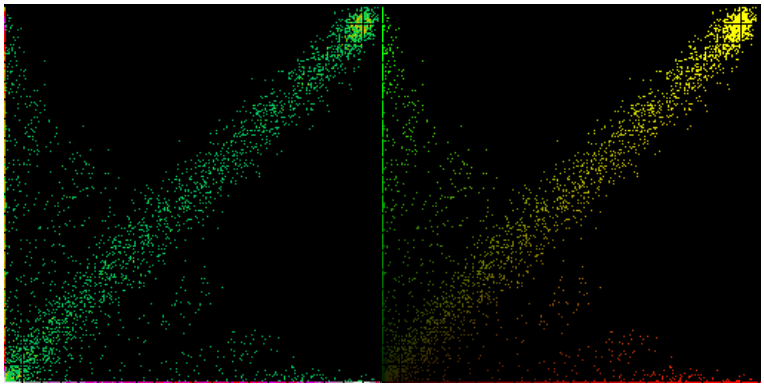
Green 50% colocalised with red; Red hardly colocalised with Green



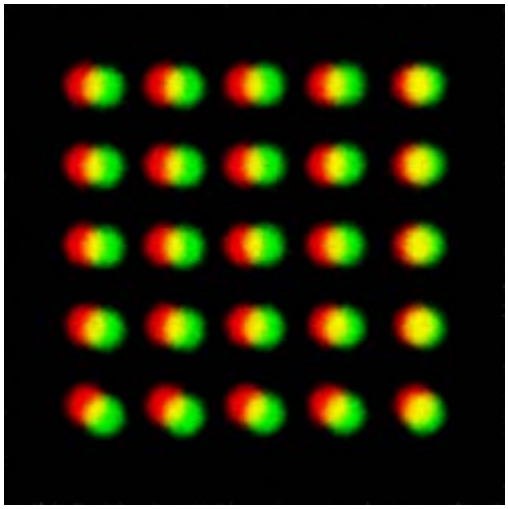
Pearson's correlation coeff. (Rr) = 0.258
Overlap coeff. (R) = 0.325
Ch1:Ch2 pixel ratio = 2.491
Colocalisation coefficient for red-channel (M1) = 0.223
Colocalisation coefficient for green-channel (M2) = 0.590
ICQ = 0.010



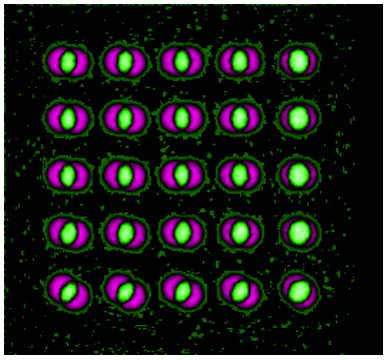


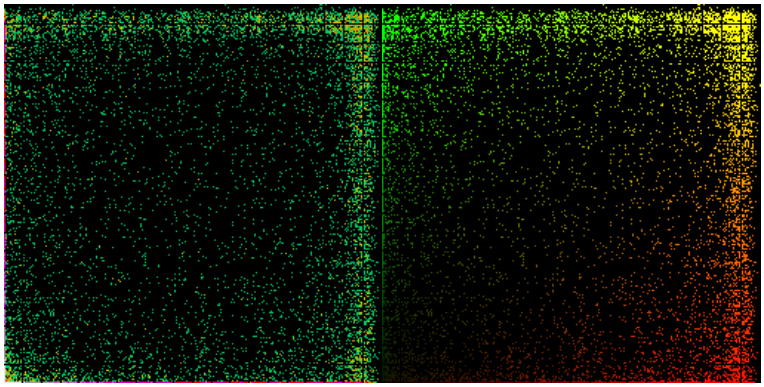


Random localisation (duplicate rotated 90°-right)

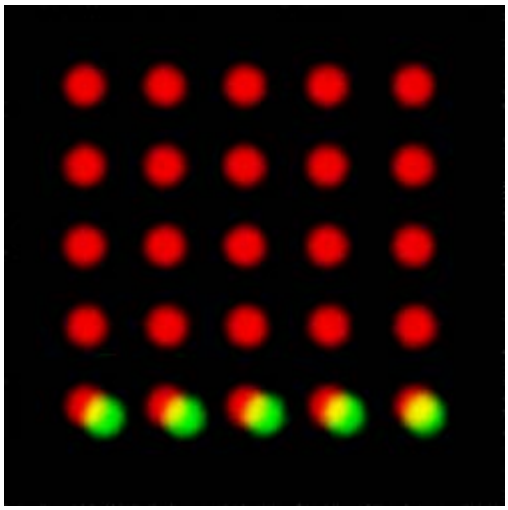


Pearson's correlation coeff. (Rr) = 0.506
Overlap coeff. (R) = 0.581
Ch1:Ch2 pixel ratio = 0.988
Colocalisation coefficient for red-channel (M1) = 0.737
Colocalisation coefficient for green-channel (M2) = 0.730
ICQ = 0.155

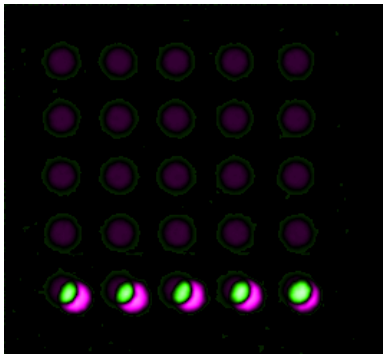


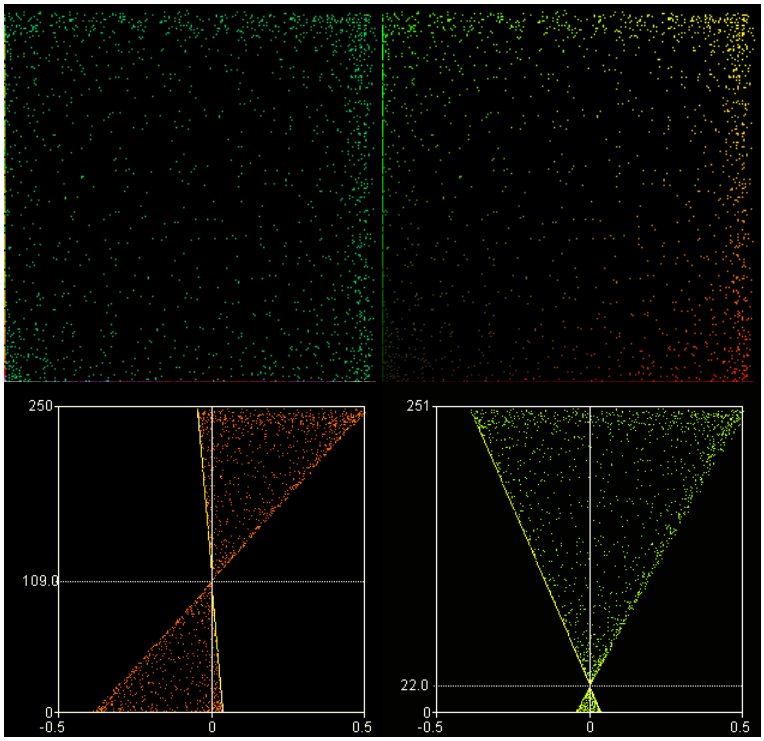


Random, partial localisation



Pearson's correlation coeff. (Rr) = 0.200
Overlap coeff. (R) = 0.250
Ch1:Ch2 pixel ratio = 4.488
Colocalisation coefficient for red-channel (M1) = 0.145
Colocalisation coefficient for green-channel (M2) = 0.707
ICQ=0.006





References

Media Cybernetics Application Note 1: Colocalization of fluorescent probes.
http://www.mediacy.com/apps/colocfluorprobes.pdf

Smallcombe A. (2001) Multicolor Imaging: The Important Question of Co-Localization. *BioTechniques* **30**, 1240-1246.REFERENCES

1. Demandolx D et al. (1997) Multicolour analysis and local image correlation in confocal microscopy. *Journal of Microscopy,* **185,** 21-36.

2. Fay FS et al. (1997) Quantitative Digital Analysis of Diffuse and Concentrated Nuclear Distributions of Nascent Transcripts, SC35 and Poly(A)*1. *Experimental Cell Research,* **231,** 27-37.

3. Lachmanovich E et al. (2003) Co-localization analysis of complex formation among membrane proteins by computerized fluorescence microscopy: application to immunofluorescence co-patching studies. *J Microsc,* **212,** 122-131.

4. Landmann L (2002) Deconvolution improves colocalization analysis of multiple fluorochromes in 3D confocal data sets more than filtering techniques. *Journal of Microscopy,* **208,** 134-147.

5. Landmann L et al. (2004) Colocalization analysis yields superior results after image restoration. *Microsc Res.Tech.,* **64,** 103-112.

6. Li Q et al. (2004) A Syntaxin 1, G{alpha}o, and N-Type Calcium Channel Complex at a Presynaptic Nerve Terminal: Analysis by Quantitative Immunocolocalization. *J.Neurosci.,* **24,** 4070-4081.