TECHNICAL NOTE 11

TECHNICAL

Co-localisation: how is it determined, and how is it analysed with the Bio-Rad *LaserPix* image analysis software?

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NB Bleed-through correction (if required) should always be performed prior to co-localisation analysis. For details of Bleed-through correction, see Technical Note 10.

What is co-localisation?

In a biological sense, co-localisation describes the existence of two or more molecule types in precisely the same spatial position. These molecules are often proteins and are frequently visualised using fluorescent antibodies or probes.

Typically, molecule A would be labelled with a green fluorescent antibody and molecule B with a red fluorescent antibody. Any co-localised points within the image would therefore appear yellow/orange to the eye or to an imaging system. If the sample is extremely thin, then a conventional epifluorescence microscope can be used to make an unambiguous assessment of the degree of co-localisation. However, if the sample is any thicker than a few microns, an optical sectioning technique is required to determine that the yellow/orange points are a consequence of loci in the same focal plane and not one locus vertically superimposed upon another. For this reason, confocal and multi-photon microscopes are well suited to the analysis of co-localisation.

The output from most confocal microscopes is a digital image made up of a multi-dimensional array of many voxels. Co-localisation of molecule A and molecule B at some point in the sample will be represented in the image by a voxel or voxels with a green intensity value above one particular level AND a red intensity value above another particular level.

How do we determine what is bleed-through and what is co-localisation?

During image acquisition:

It is possible to get bleed-through both from green fluors into the red channel and from red fluors into the green channel. The same is true for autofluorescence. In a dual labelled green/red sample, these two situations will both make the pixels appear yellow/orange, but **not** due to co-localisation. Please refer to *Technical Note 10* for details of how to deal with bleed-through.

True co-localisation can only be determined when bleed-through and all non-specific fluorescence has been removed from the images.

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Sample preparation and image acquisition

In order to reliably determine the degree of co-localisation, very careful attention must be paid to sample preparation and image acquisition. Additionally, knowledge of the sample preparation protocol must be applied during the subsequent image analysis.

It must be understood that if sufficient care is not taken during sample preparation and acquisition the software may generate values and images which are misleading. The following points must be carefully considered.

Antibody controls

All antibody controls should be stringently observed.

In the case where two molecules are being labelled immunofluorescently then it is strongly suggested that the indirect technique is used which helps improve specificity as well as allowing signal amplification.

Yellow/orange pixels will appear if there is co-localisation **or** under the following circumstances:

- Any cross reactivity between any of the molecule A antibodies and the molecule B antibodies. A series of unlabelled and single labelled controls will allow any cross-reactivity to be assessed prior to any assumptions about co-localisation are made.
- Misinformation about protein localisation can also occur if the primary antibody interacts with any molecule other than the target protein. This can also lead to very high background staining which will make analysis more difficult.

Fluor selection

A pair of fluors should be selected which have the least emission overlap possible. This is because the green emission and red emission may mutually bleed-through leading to many points within the sample appearing orange in the absence of co-localisation. The brighter that one fluor is compared with the other, the more problems relating to bleed-through will occur. The best result will be achieved if the green fluor has a narrow emission spectrum and the red fluor has an emission spectrum in the red or even far red. Care should be taken during the labelling protocol to ensure that the two fluors are of similar brightness.

With conventional epifluoresence, there is some limitation on using far red emitters due to the general insensitivity of the human eye to far red light. This makes rapid ocular assessment very difficult. Therefore, most users tend to use a conventional pair of fluors such as FITC and TRITC (which overlap very badly) or FITC and Texas red (which overlap a great deal less). The use of lasers with confocal microscopy permits the combination of a green fluor and a far red fluor since the detectors are not human eyes, but photomultiplier tubes which are more sensitive to far red light. If one can cope with the difficulties of trying to view a far red emitting fluor then this combination should be used.

There are not many far red emitting fluors available for immunological use – Cy5 is one and allophycocyanine another. Moreover, a red laser is required (a 3 line Krypton/Argon or a Red HeNe or Red diode).

Suggested fluor combinations for confocal microscopy co-localisation studies (with antibodies) are:

2 line Argon ion laser (488/514 nm)

Cy2, Alexa 488 or Bodipy-FL + Cy3, Alexa 532 or YFP (r-phycoerythrin is also good if it can penetrate the tissue to reach the target).

You may also be able to do Cy2 with TRITC provided that TRITC staining is sufficiently strong. For nucleic acid staining in combination with a nuclear probe, Alexa 488 + Propidium iodide will work provided that you use a high cut-off filter for the PI and a narrow band pass filter for the Alexa. Note that the 488 nm laser line will excite PI as well as Alexa 488, but the emissions can be adequately separated.

4 line Argon ion laser (457/477/488/514 nm)

CFP + Alexa 488 + Alexa 532

CFP + YFP

GFP + Alexa 532

Krypton/Argon laser (488/568/647 nm)

Alexa 488 + Alexa 568, Lissamine rhodamine, XRITC or Texas red + Cy5

GFP+RFP (DsRed)

Argon + Green HeNe (488/514/543 nm)

Alexa 488, Oregon green or Bodipy-FL + Alexa 546, Lissamine rhodamine or XRITC

GFP + RFP (DsRed)

Argon + Red HeNe

Alexa 488 + Cy5

Argon + Green HeNe + Red Diode

Alexa 488 + Alexa 546, Lissamine rhodamine + Cy5

If one of the probes is nuclear, then you have more options e.g. you could use 7-aminoactinomycin-D which is excited by either 543 nm or 568 nm, but emits maximally right up at 655 nm, or TOTO-3 which is excited by 647 nm (or 638 nm) and emits at 660 nm.

Image acquisition

Unless one has chosen two fluors with extremely well separated emission spectra then image acquisition should be made in sequential mode. If one is using a Krypton/Argon laser or 3 separate lasers with an AOTF control, then it is possible that careful balancing of the excitation intensities will produce images with sufficient spectral separation even in simultaneous mode.

One can normally make a quick assessment of co-localisation in an image – if all points which are red are also green, then there is probably some bleed-through. However, if there are distinctly green points, distinctly red points and also orange points, the chances are that there is some co-localisation. With sequential collection, it is important to set the gains such that the red and green signals are about the same intensity but below saturation. Likewise, the black level should be adjusted to remove sub-signal noise (but not background fluorescence). If bleed-through persists, you may either need to change your fluors (preferred method) or use the bleed-through correction module available in the *LaserPix* software prior to co-localisation analysis.

In summary

- Ensure antibody specificity and lack of cross-reactivity.
- Fluors should have well separated emissions e.g. Alexa 488 and Alexa 568, Lissamine rhodamine, Texas Red or Cy5.
- Emission filters should be optimised to maximise emission collection whilst avoiding bleed-through from other fluors.
- Use sequential image collection where bleed-through is present.
- Use AOTF control (where available) of excitation intensity. For interactive fluor spectra, laser lines and emission filters, please visit our fluorescence web page on **fluorescence.bio-rad.com**

The theory behind co-localisation analysis

LaserPix (optional software for NT systems) contains co-localisation analysis functionality. The program calculates two values, which represent the proportion of co-localising objects in each component of a dual-colour image. These values are called co-localisation coefficients. The calculations are based on Pearson's correlation co-efficient, which is a well trusted means of describing the degree of overlap between patterns or images. The co-localisation coefficients are calculated according to the following equations:

$$C_{red} = \frac{\sum_i R_{i,\,coloc}}{\sum_i R_i}$$

Where;

$\sum R_{i,coloc}$ = The sum of intensities of all red pixels which also have a green component

$\sum R_i$ = The sum of intensities of all the red pixels in the image.

So, $R_{i,coloc} = R_i$ if $G_i > 0$

and $R_{i,coloc} = 0$ if $G_i = 0$

$$C_{green} = \frac{\sum_i G_{i,\,coloc}}{\sum_i G_i}$$

Where;

$\sum G_{i,coloc}$ = The sum of intensities of all green pixels which also have a red component

$\sum G_i$ = The sum of intensities of all the green pixels in the image.

So, $G_{i,coloc} = G_i$ if $R_i > 0$

and $G_{i,coloc} = 0$ if $R_i = 0$

These two coefficients C_{red} and C_{green} are proportional to the amount of fluorescence of the co-localising objects in each component of the image, relative to the total amount of fluorescence in that component. These coefficients can be determined even when the signal intensities between the two components differ strongly. However, as seen from the equations, there is intensity weighting applied to the co-localised pixels to reflect the relationship between intensity and the quantity of labelled sites.

For 3 channel images, analysis can be done between the green and red channels, the green and blue channels and between the red and blue channels to obtain coefficients for each pair of colour combinations. The software caters for independent threshold (below desired signal) selection and subtraction from each image before reading out the coefficients. A fluorogram can be generated from either the entire image or a selected part of the image. This simply displays the intensity and distribution of different coloured pixels within the merged image as a scattergram and allows

the user to select a subset of pixels from the scattergram which meet certain intensity criteria in both colours and for these pixels then to be highlighted on the image. A unique 3D fluorogram can also be generated in which the z axis of the plot represents pixel frequencies. This enables visual assessment (with data) of which combinations of green/red intensities are typified by the sample. Co-localised pixels can be highlighted throughout a Z series and a co-localisation map can be generated.

Example of partial co-localisation – The double labelled cells shown in *Fig 1a* illustrate a situation where there are some distinctly red pixels, but almost no distinctly green pixels. All greenish pixels appear to have some red component i.e. co-localisation.

Figure 1a

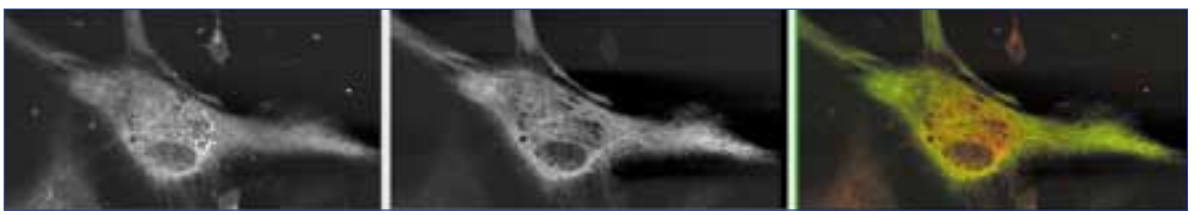


Figure 1b

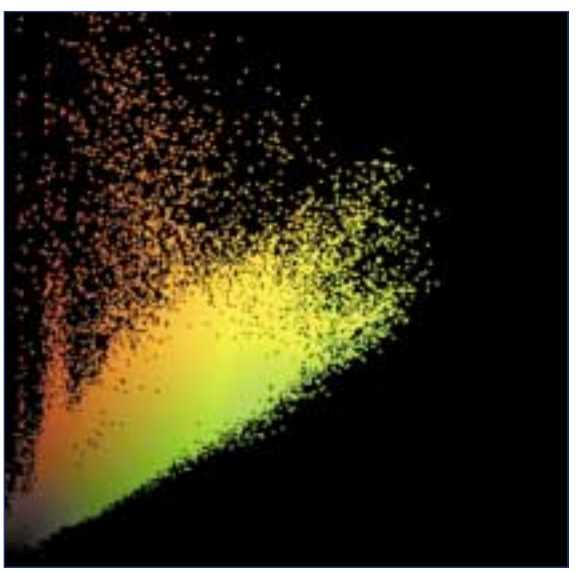


Figure 1c

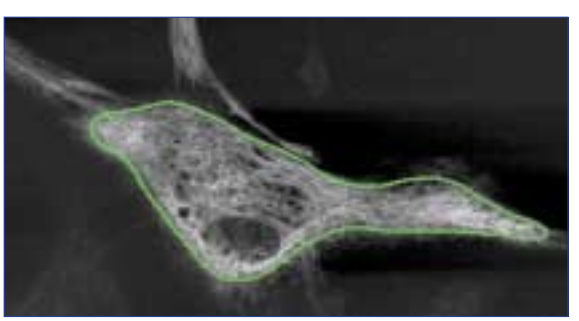


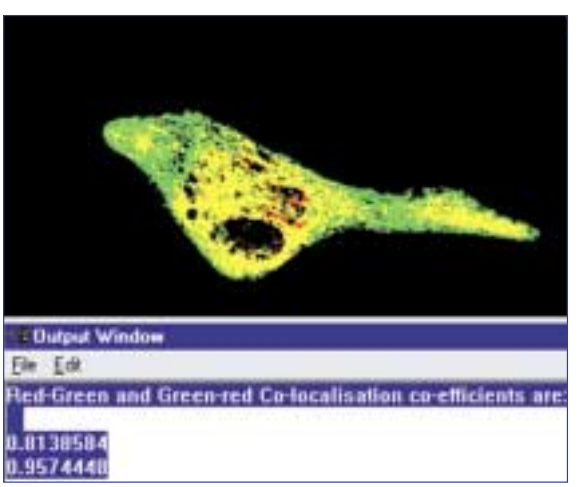
Figure 1a Cells in culture stained with antibodies to two cytoskeletal proteins. The red and gren images were collected sequentially using 568 nm and 488 nm excitation respectively (from a Krypton/Argon laser). There is thus very little bleed-through to consider.

Figure 1b Shows the *LaserPix* localisation 2D fluorogram generated from the red and green intensities within the component images.

Figure 1c Shows an area of interest (AOI) drawn around the cell.

Figure 1d Shows a co-localisation MAP where the yellow pixels wihtin the Area of Interest are co-localised and the small number of red pixels and green pixels are not. The coefficients generated reflect this situation by their high values. Note that proportionally, fewer red pixels are co-localised with green (0.81) than green pixels co-localised with red (0.95).

Figure 1d



What do the numbers mean?

The coefficients generated will always be between zero and one. A value of zero means that there is no co-localisation and a value of 1.0 means there is complete co-localisation.

A coefficient is generated for each colour of a two colour merge, e.g. Red 0.9 Green 0.45 would mean that the ratio of all the red intensities which showed a green component divided by the sum of all the red intensities in the selected area is 0.9 i.e. a very high degree of co-localisation and that the ratio of all the green intensities which showed a red component divided by the sum of all the green intensities is 0.45 which, is half the co-localisation value. So there is twice the degree of co-localisation of red pixels with green as there is of green pixels with red.

See the following examples:

Example of extremely low co-localisation

Fig 2a shows some double labelled cells (sample from David Vaux and Mike Hollingsworth, Oxford). The images were collected simultaneously with both 488 nm and 568 nm excitation from a Krypton/Argon laser. Prior to thresholding, there was a tiny amount of bleed-through from the green fluorescence into the red image (nucleus). If co-localisation analysis is performed without subtracting this bleed-though, the calculation will show false co-localisation. *Fig 2b* shows the 2D plot of red and green intensities from the entire image and how one can use a rectangle to select out certain thresholds.

Figure 2a

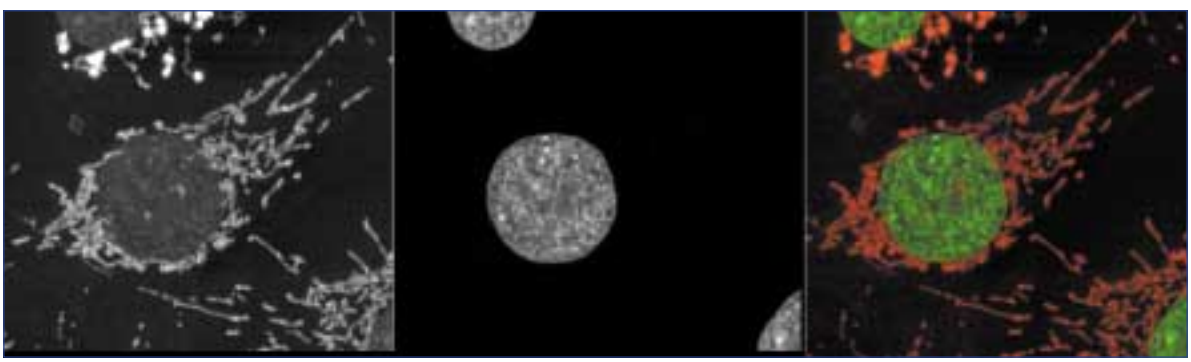
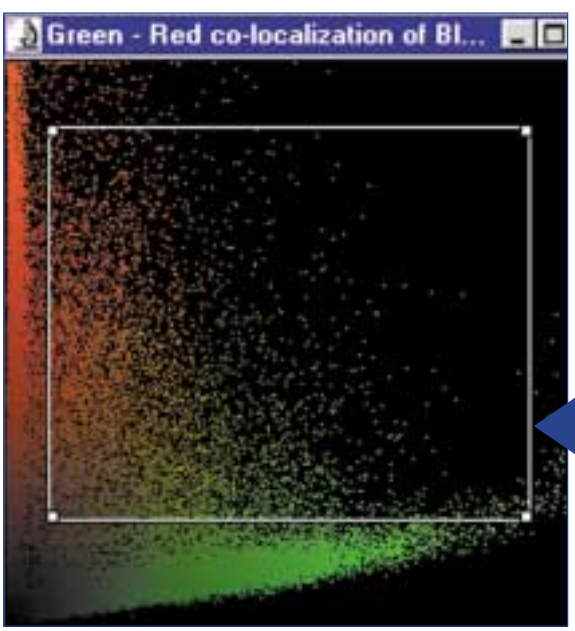


Figure 2a Double labelled culture cells showing a little bleed-through and almost no col-localisation.

Figure 2b



2D colour intensity plot

Rectangular AOI for thresholding pixels above background staining.

Figure 2b Shows the red/green *LaserPix* co-localisation plot using a rectangle to threshold out lower intensities in both colours.

Fig 2c shows the 3D plot where the z axis displays pixel frequency. *Fig 2d* shows a co-localisation MAP (upper section) where co-localised pixels are shown in yellow, and non-co-localised in bright red or bright green. The middle section shows how a white mask of co-localised pixels can be superimposed onto the original RGB image. The lower section shows that the co-localisation coefficients are both extremely low, as they should be.

Figure 2c

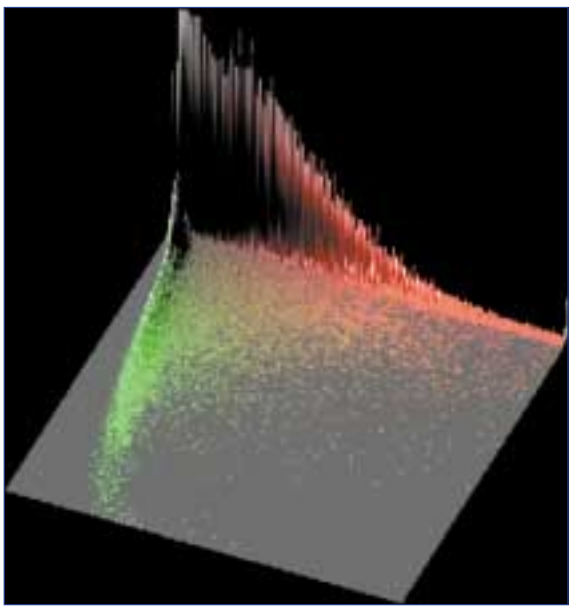


Figure 2d

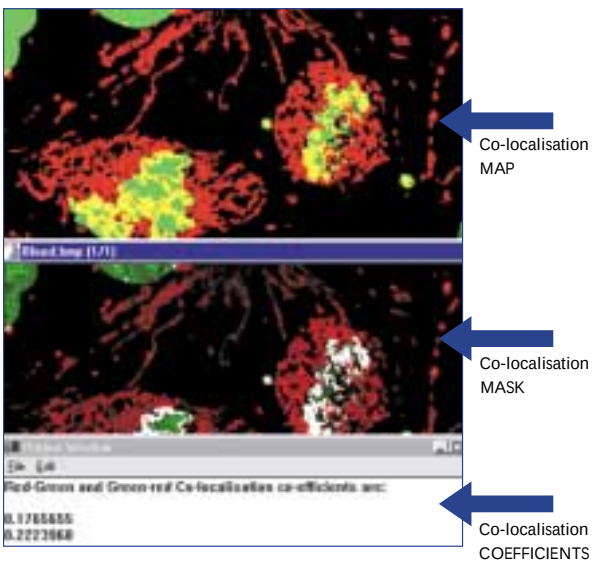


Fig 3a below shows a triple labelled sample and *Fig 3b* shows the red/blue co-localisation MAP using zoom to analyse pixel by pixel.

Figure 3a

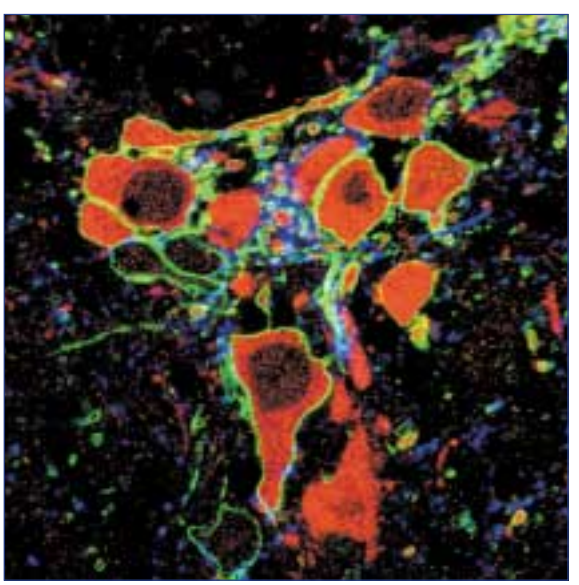
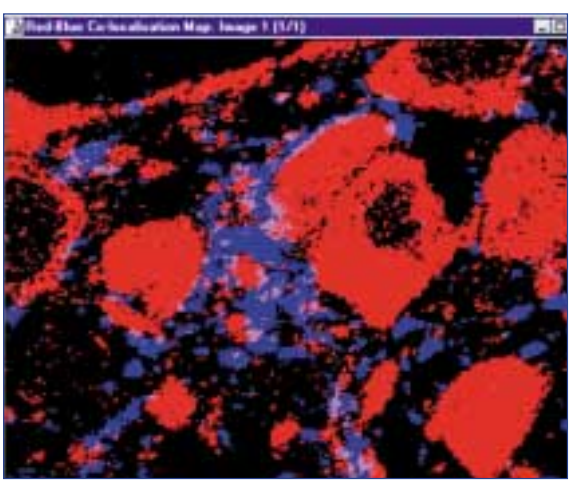


Figure 3b



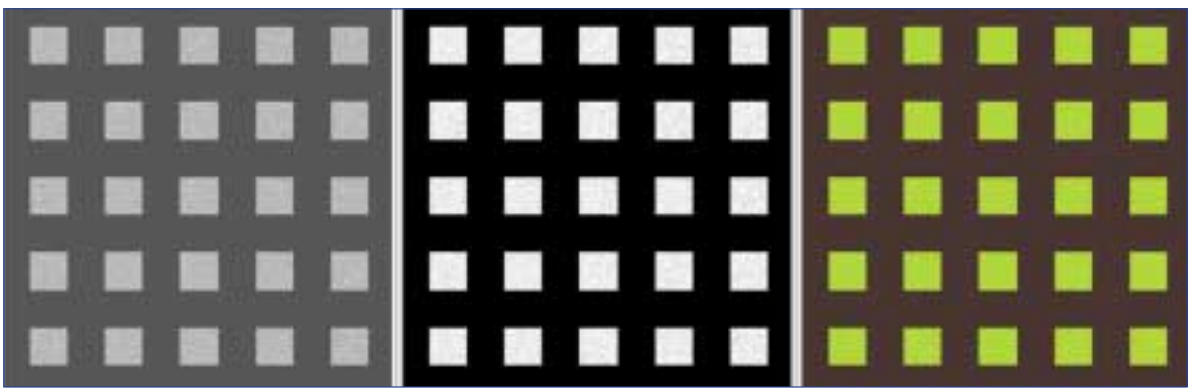
Co-localisation using test images

Total co-localisation

Fig 4 shows a red image with a high background and a green image with no background. There should be almost total co-localisation when this background is removed – even though the intensity of the red and green signals is very different.

The coefficients calculated without background subtraction show a falsely low red coefficient of 0.49. When the coefficients are calculated following background subtraction, the values are both equal to 1.0 which indicates total co-localisation as expected.

Figure 4

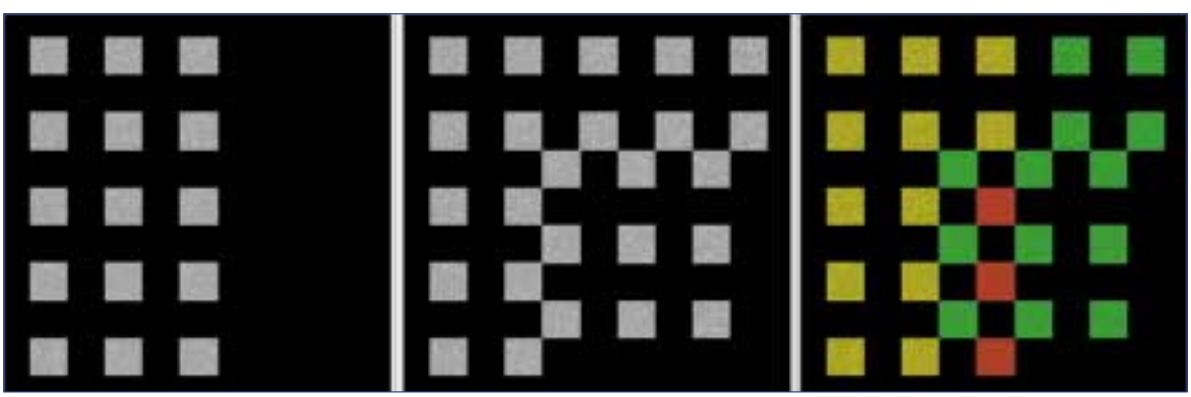


Partial co-localisation

Fig 5 shows images which have a purely red component, a purely green component, some co-localisation and no background. The red and green intensities are very similar.

The co-localisation analysis gives coefficients of RED = 0.8 and green = 0.48. This would be expected, since a greater proportion of the red pixels are co-localised than the proportion of green pixels which are co-localised. Since the intensities are fairly uniform (there is a little noise in the images), the intensity-weighted values almost exactly correspond to the overlapping areas in the image.

Figure 5



References

Manders, EEM., Verbeek, FJ., Aten, JA. *Measurement of co-localisation of objects in dual-colour confocal images*. Journal of Microscopy 169, Pt 3, pp **375-382** (1993)

Bio-Rad Technical Note 08. *Co-localisation Analysis with the LaserSharp Software*.

Bio-Rad Technical Note 10. *Fluorescence Bleed-through: How to reduce or avoid it*.

Further information

For more information about *LaserPix*, please contact your local Bio-Rad Representative.

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