TECHNICAL NOTE 08

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Co-Localisation Analysis with the Lasersharp Software

Written by: Anna Smallcombe and Duncan McMillan, Bio-Rad Microscopy Division, Hemel Hempstead, UK

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 assesment 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.

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:

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- Any cross reactivity between any of the MoleculeA antibodies and the MoleculeB antibodies. A series of unlabelled and single labelled controls will allow any cross-reactivity to be asessed 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.
- Spectral overlap of the fluorescent emissions of the fluorochromes.

Fluorochrome selection

A pair of fluorochromes 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 one fluorochrome is compared with the other, the more problems relating to bleed-through will occur. The best result will be achieved if the green fluorochrome has a narrow emission spectrum and the red fluorochrome has an emission spectrum in the red or even far red. Care should be taken during the labeling protocol to ensure that the two fluorophores 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 fluorochromes 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 fluorochrome and a far red fluorochrome 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 fluorochrome then this combination should be used.

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

The best fluorochrome combinations for confocal microscopy co-localisation studies (with antibodies) are:

Argon ion laser: Alexa 488 or Cy2 + Cy3 (r-phycoerythrin is also good if it can penetrate the tissue to reach the target). You may also be able to use Cy2 with TRITC provided that TRITC staining is sufficiently strong.

Krypton/Argon laser: Alexa 488 + Lissamine rhodamine or XRITC or Texas red + Cy5

Argon + GreenHeNe: Alexa 488 or Oregon green or Bodipy-FL+ Lissamine rhodamine or XRITC

Argon + RedHeNe: Alexa 488 + Cy5

Argon + Green HeNe + RedDiode: Alexa 488 + Lissamine rhodamine + Cy5

If one of the probes is nuclear, then you have more options eg you could use 7-aminoactinomycin-D which is excited by either 543 or 568nm, but emits maximally at 655nm.

Image acquisition

Unless one has chosen two fluorophores with extremely well separated emission spectra then image acquisition should be made in sequential mode. If one is using a Krypton/Argon laser with an AOTF 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 point 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 intensisty but below saturation. Likewise, the black level should be adjusted to remove sub-signal noise.

In summary

- **Ensure antibody specificity and lack of cross-reactivity**
- **Fluorochromes should have well separated emissions e.g. Alexa 488 and Lissamine rhodamine or Texas Red**
- **Emission filters should be optimised to maximise emission collection whilst avoiding bleed-through from other fluorochromes**
- **Use sequential image collection where bleed-through is present**
- **Use AOTF control (where available) of excitation intensity.**

The theory behind co-localisation analysis

LaserSharp version 3.0 and later contain co-localisation analysis functionality. The program calculates two values which represent the proportion of colocalising objects in each component of a dual-colour image. These values are called co-localisation coefficients. The calculations are based on Pearson's correlation coefficient 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;

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

ΣR_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;

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

ΣG_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 an 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 blue channels and between the red and blue channels to obtain coefficients for each pair of colour combinations. The software caters for independent background (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.

How to use the software

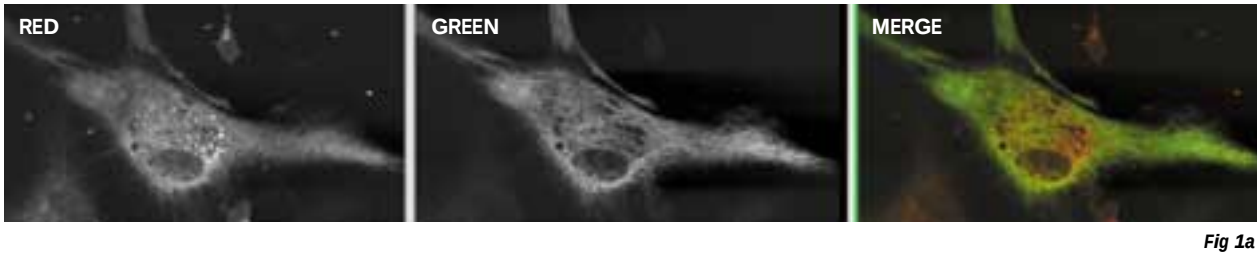

Fig 1a

Open the image of interest – (this needs to be a merged file containing both component images). If a particular region is required, SELECT a rectangle or polygon which includes the areas of interest. Then select Image|co-localisation.

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

Cells in culture stained with antibodies to two cytoskeletal proteins. The red and green images were collected sequentially using 568nm and 488nm excitation respectively (from a Krypton/Argon laser). See *Fig 1a*.

There is thus very little bleed-through to consider.

Fig1b shows the LaserSharp co-localisation dialog box and the fluorogram generated from the red and green intensities within the component images. Background and bleed-through is selected and removed from the data (not the image) and two coefficients are displayed (see arrow). These values are

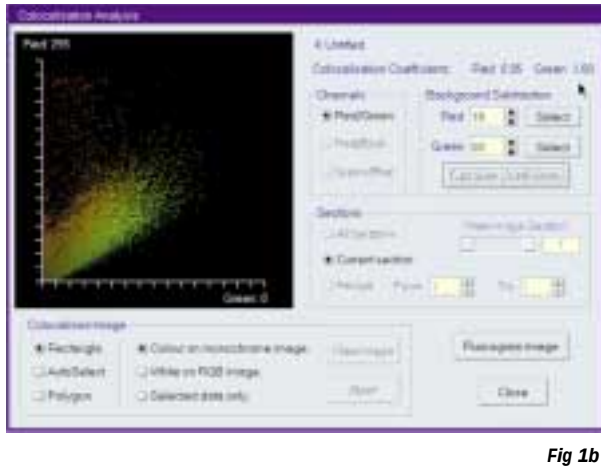

Fig 1b

Red = 0.95 and Green = 1.0. This means that there are some red pixels with no green component, but most of them show some co-localisation. All green pixels show co-localisation with the remaining red pixels.

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 ie 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. 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:

Fig 2a shows how part of the fluorogram can be selected to segment the image data and *Fig 2b* shows the image with the selected pixels highlighted in white.

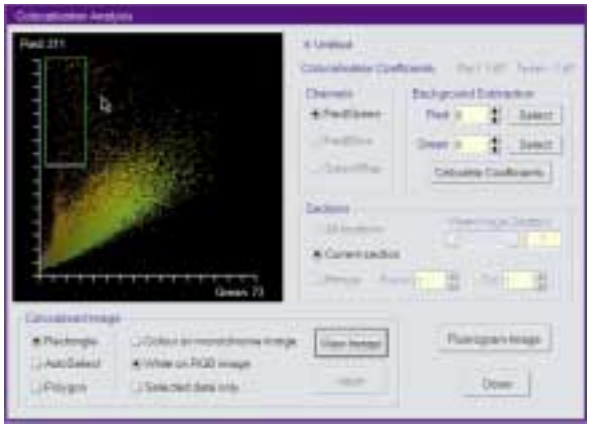

Fig 2a

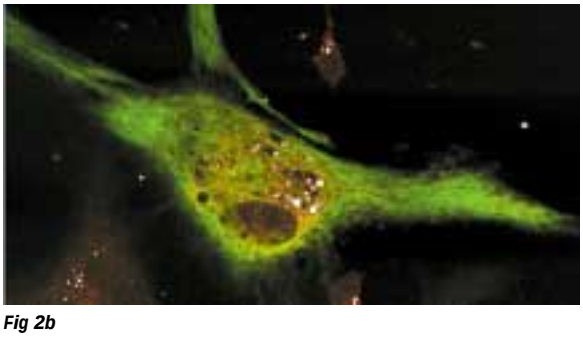

Fig 2b

Example of no co-localisation

Fig 3a shows some double labelled cells (sample from David Vaux and Mike Hollingshead, Oxford). The images were collected simultaneously with both 488nm and 568nm excitation from a Krypton/Argon laser. As can be seen, there is some 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. Thus in *Fig 3b* a measured value of 64 has been removed from the red image (which is the maximum intensity enclosed by the selected rectangle) and a background level of

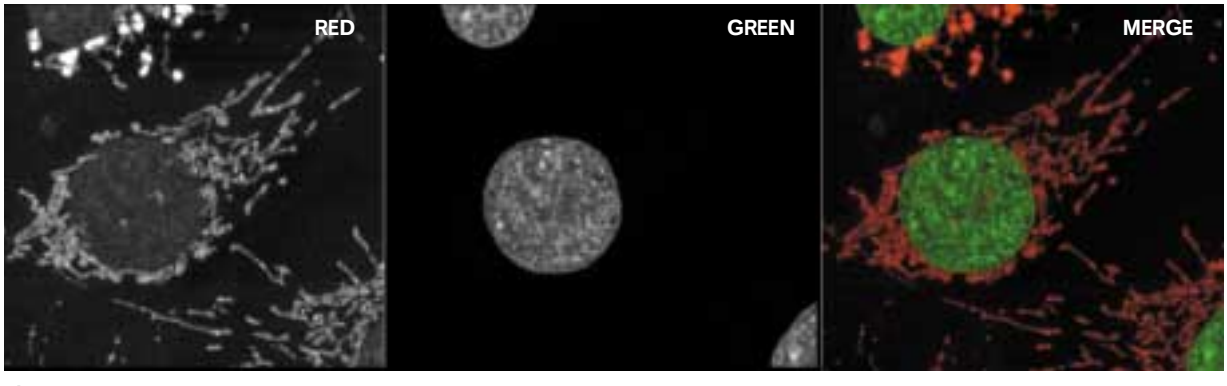

Fig 3a

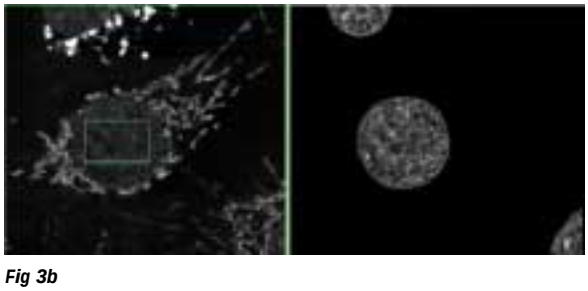

Fig 3b

4 removed from the green image. *Fig 3c* shows that the co-localisation coefficients are both extremely low as they should be. With AUTOSELECT (*Fig 3d*), when the background is subtracted, the fluorogram places a rectangle around the pixels which are above background levels in both the red image AND the green image. The VIEW IMAGE option then allows these pixels to be displayed in white *Fig 3e*.

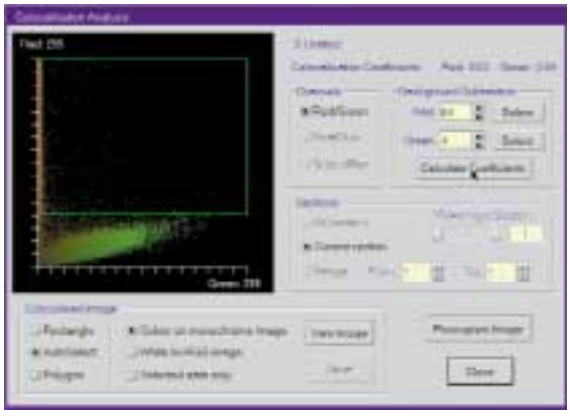

Fig 3c

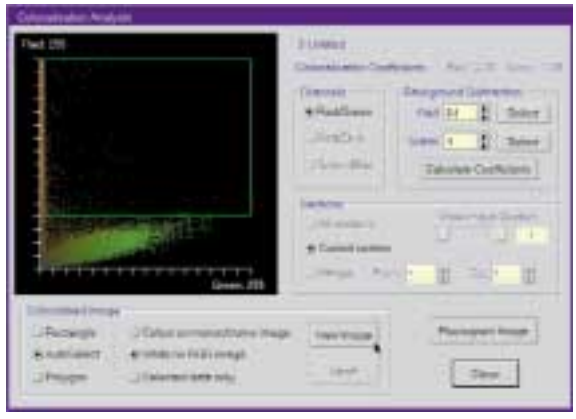

Fig 3d

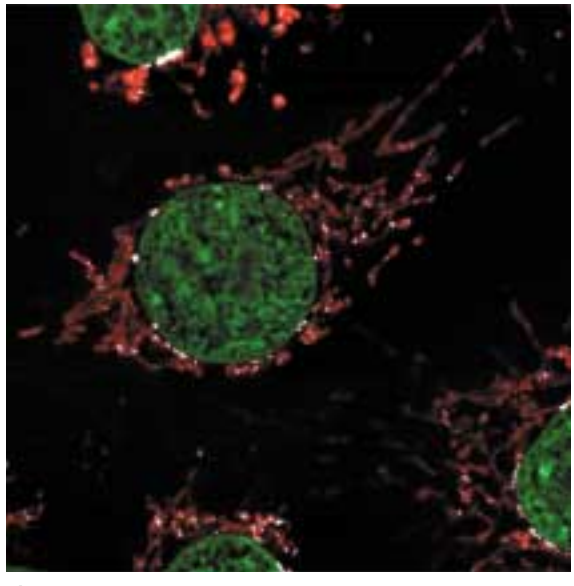

Fig 3e

Example of total co-localisation

Fig 4a 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.

Fig 4b shows the coefficients calculated without background subtraction – showing a falsely low red coefficient. *Fig 4c* shows the coefficients calculated following background subtraction. The values are both equal to 1.0 which indicates total co-localisation as expected.

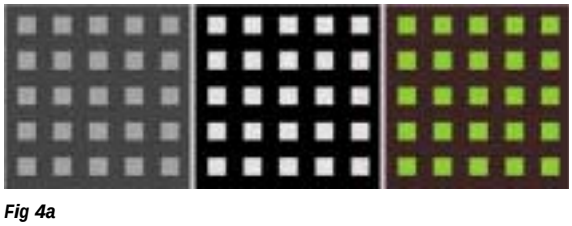

Fig 4a

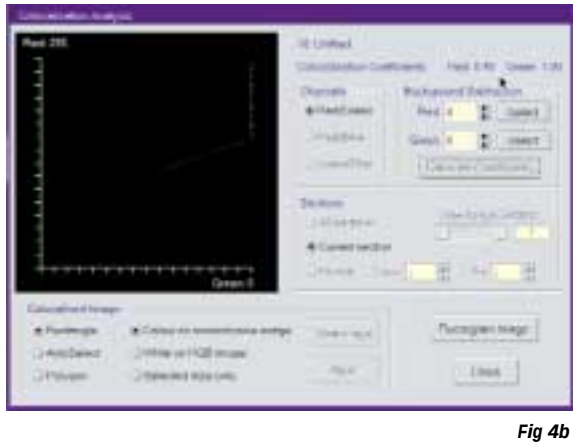

Fig 4b

Example of partial co-localisation

Fig 5a 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.

Fig 5b shows the co-localisation analysis giving 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.

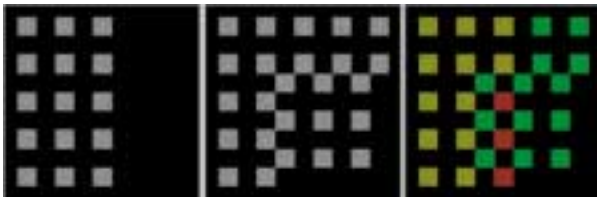

Fig 5a

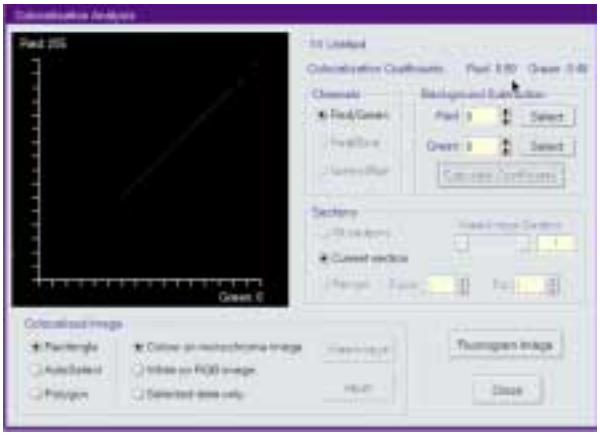

Fig 5b

Summary

In summary, the LaserSharp co-localisation package will provide valid coefficients provided that:

- Bleed-through has been avoided during acquisition
- Background staining has been subtracted from all images
- Analysis is done only on optical sections NOT projections.

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)

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Microscopy Division

U.S. (800) 4BIORAD **California** (510) 7411000 **Australia** +61 (2) 99142800 **Austria** +43 (1) 8778901
Belgium 0800 75489 **Canada** +1 (905) 7122771 **China** +86 (10) 62051860 **Denmark** +45 (39) 179947
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The Netherlands 0800 0226048 **New Zealand** +64 (9) 4433099 **Singapore** +65 (272) 9877 **Spain** +34 (1) 6617085
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