Colocalisation/Correlation

The past:
“I see yellow - therefore there is colocalisation”
but published images “look” over exposed.
No colocalisation definition + No stats = No Science.

Complementary methods: BioChemical, Optical (FRET, FLIM)
Colour Merge Images? Only for Art!

Channel Merge Images? What are they good for?
- Apart from looking pretty... not much.
- Scientific conclusions from the image below?
- Colour blind people - see green and red the same!

![Image of merged channel images](image-url)
Colour Merge + Projection = Danger!

Never make colour merge / overlay images from projections of 3D / z stacks... why not?

Lose 3D info - are the objects overlapping in 3D, or is one in front of the other one, in the z-stack.

False overlaps!!! Easy to make false interpretation
What does “Colocalisation” mean anyway…?

That depends who you ask…

... and what BIOLOGY you are thinking about
Colocalisation/Correlation?  
Think about the biology!

What is the biological/biochemical question?

- Are you looking for Co-Compartmentalisation?
- Are you looking for exclusion / anti correlation?
- Are you looking for interacting molecules?

  - Then you must also do biochemistry (Immuno Co-precip, Fluo Correlation Spectroscopy)
  - FRAP / FLIM might be very informative
"Colocalisation" covers two qualitatively different conditions:

1) that objects have both fluorophores present (Object Based Coloc) Segmentation needed. Biology?

2) there is some relationship between the intensities of the fluorophores in a pixel. (Pixel Intensity Based Coloc) Interaction - BioChemistry?
Colocalisation / Correlation / Concurrence?

2 fluorophores are there in a pixel

Binary information

Is it Random?

Is it Real?

Little or no biological meaning?

…unless you are confident about how to segment objects out from the background.
Definition of Terms

“Concurrence” = “co-presence” “there is red and green”

“Colocalisation” = Relationship between channel intensities

Eg. “Red is only found with Green”

Special case - “Correlation”

Intensity Correlation over Space
Define what is Colocalisation/Correlation?

Colocalisation is #1

2 objects overlap
Binary information
No intensity information

Concurrence?
Image Segmentation!

Biological Meaning?
Colocalisation is #2

Some objects appear to overlap with another object
Binary information
No intensity information

Colocalisation?

Biological Meaning?
Colocalisation is: #3

Intensity profiles overlap

Image “Correlation”

Biological Meaning?
Co-compartmentalisation?
Physical interaction?
Colocalisation/Correlation - Think about:

- Are your “objects” smaller than optical resolution?
  - Vesicles? Small Organelles?
    - Check channel overlap with sub resolution beads!

- Are your objects large?
  - Large single homogenous blobs?
  - Large reticular networks / membranes
  - Resolution required?

- Complementary “correlation” methods
  - Fluorescence correlation spectroscopy (FCS in live cells)
Colour Merge Images = Bad … so what should I do instead?

“Colocalisation Analysis”

Statistical Significance of Colocalisation

- Single image - random / insignificant.
- Statistical P value (significance), Manders coefficients, and Scatter Plot. (ImageJ, BioImageXD, Imaris and others)

But remember…

- Don’t merge projections of stacks (you lose 3D info, false coloc)
- Don’t believe your eyes, they lie. Machines don’t make mistakes…
How can I measure the amount of colocalisation or rather “correlation” between these two images?

BiolmageXD, ImageJ and others have methods to do that!
Colocalisation Analysis

Scatter plot
2D histogram
Publish it?

Coloc stats:
Pearsons $r$
$M_1, M_2,$
Costes P-val,

Automatic thresholding

Coloc Stats - Costes et al. 2004  Biophysical J.  vol 86 p3993
Pearson’s Image Correlation Coefficient (Manders et al., 1993)

\[
r = \frac{\sum_{i} (R_i - R_{av})(G_i - G_{av})}{\sqrt{\sum_{i} (R_i - R_{av})^2 \cdot (G_i - G_{av})^2}}
\]

Don’t panic - it’s not that complicated!

Correlation between images, \( r \) ranges from -1 to +1
+1 means full correlation (images are the same)
0 means no correlation (random)
-1 means full anti correlation (no red where there is green)
Pearson’s Image Correlation Coefficient

In English…per pixel and summed for the whole image:

\[ r = \frac{\text{sum of (red intensity - average red) x (green intensity - average green)}}{\text{sqrt of squares of above}} \]
The Problem with Pearson’s Image Correlation Coefficient is…

- Sensitive to diff intensity of the 2 images. Why?
- If red is 1/2 as bright as green…
  - Impossible to get \( r = 1 \), even if 100% correlated really.
  - … so Pearsons \( r \) is not robust for biological imaging…
- Need a method that handles this problem…
- Manders!!!
Manders Coefficients

Biologically meaningful coloc coefficients:

\[ M_R = \frac{\sum_i R_{i,\text{coloc}}}{\sum_i R_{i,\text{total}}} \]

\[ M_G = \frac{\sum_i G_{i,\text{coloc}}}{\sum_i G_{i,\text{total}}} \]

Proportion of each dye colocalised with the other (Manders et al., 1993)

\( R_{i,\text{coloc}} = \) colocalised red signal
\( R_{i,\text{total}} = \) total red signal

Great! … but how do I know which pixels are colocalised and which are not…?
“Thresholding” and “% colocalisation”

The calculated “% colocalisation” depends on what thresholds you set.

... so how should one set them?

.. until you get the result you want?

No science here!
Automatic Thresholding?

How should I set the thresholds of the 2 channels?

- Manually? No! Subjective user bias, not reproducible...
- Need a robust reproducible method!
- Find thresholds where Pearson correlation below thresholds $\leq 0$

Auto Threshold - Costes et al. 2004  Biophysical J.  vol 86 p3993
2D Histograms / Scatterplots

Display 2 colour channel image data in 2D:
- colour merge / overlay or 2D histogram?
- 2D histogram: Ch1 - y axis (left), Ch2 - x axis (bottom)
- Colour mapped to number of pixels with that R and G value (right)
Fig. 4. Reference images for colocalisation analysis. Images for colocalisation analysis were acquired from fixed maize root cells with Golgi staining (A) (Boutilé et al., 2006) or endoplasmic reticulum staining (B) (Kluge et al., 2004) and on fixed mammalian HeLa cells with microtubule plus-end tracking proteins EB1 and CLIP-170 staining (C) (Cordelieres, 2003), and nuclear and mitochondrial staining (D). Scale bars, 10 μm. These images illustrate the four commonly encountered situations in colocalisation analysis. (A) Complete colocalisation. (B) Complete colocalisation with different intensities. (C) Partial colocalisation. (D) Exclusion. Grey level images of the green and red image pairs (A–D) were used for subsequent treatments with ImageJ. A zoomed view of the insets is shown on each side of the colour panels.
Fig. 5. Colocalisation analysis with |ACol|: Pearson and Manders’ scatter plots and correlation coefficients. Scatter plots (A–D) correspond to the colocalisation events as shown in Fig. 4. (E) Model scatter plot explaining the effects of noise and bleed-through. (F) Pearson’s and Manders’ coefficients in the different colocalisation situations. A complete colocalisation results in a pixel distribution along a straight line whose slope will depend on the fluorescence ratio between the two channels and whose spread is quantified by the Pearson’s coefficient (PC), which is close to 1 as red and green channel intensity distributions are linked (E, a, black bar). (B) A difference in fluorescence intensities leads to the deflection of the pixel distribution towards the red axis. Note that the PC diminishes even if complete colocalisation of subcellular structures is still given (E, b, black bar). (C) In a partial colocalisation event the pixel distribution is off the axes and the PC is less than 1 (E, c, black bar). (D) In exclusive staining, the pixel intensities are distributed along the axes of the scatter plot and the PC becomes negative (E, d, black bar). This is a good indicator for a real exclusion of the signals. (E) The effect of noise and bleed-through on the scatter plot is shown in the general scheme. (F) The influence of noise on the PC was studied by adding different levels of random noise (a1–a6) to the complete colocalisation event (A = 0, no noise). A Note that the PC (black bar) tends to 0 when random noise is added to complete colocalising structures. The inset (A*) in (A) shows the scatter plot for the n2 noise level. Note that all of the mentioned colocalisation events (A–D) may only be detected faithfully once images are devoid of noise. (F) Manders’ coefficients were calculated for (A–D). The thresholded Manders’ tM1 (cross-hatched bars) and tM2 (diagonal hatched bars) are shown. Compare complete colocalisation (a0), complete colocalisation with random noise added (a1–a6), and complete colocalisation with different intensities (b), partial colocalisation (c) and exclusion (d). Note that the original Manders’ coefficients are not adapted to distinguish between these events, as they stay close to 1 for all situations (not shown). *Signal-to-noise ratios are: n1 = 12.03 dB, n2 = 6.26 dB, n3 = 4.35 dB and n4 = 3.52 dB.
2D Histograms / Scatterplots

- See correlation qualitatively - better than colour merge
- See problems from imaging:
  - Wrong offset
  - Bleed through
  - Saturated
  - Noisy
  - No correlation?
Does it work in a biological experiment? Yes!

Time course of Rev-CRM1 dissociation, nucleolus to nucleus

The dissociation rate constant $k_d = 1.25 \pm 0.31 \times 10^{-3}$ s$^{-1}$
One more thing...

**Statistical significance!**
- Are coloc results better than random chance?
- A busy image might give high correlation and Manders
  - Lots of signal = larger chance of random signal overlap.

![Image 1](red_grid.png) vs. ![Image 2](green_grid.png)

17 / 40 pixels overlap !!!

Is that significant or just random?

Statistical confidence P - Costes et al. 2004  Biophysical J.  vol 86 p3993
Costes Method - Randomisation...

Measure Pearson’s correlation for:
- Randomised 1st channel image data (PSF sized chunks)
- Repeat 100 times
- How many randomised have <= correlation than real image.

If > 95% of randomised are worse, then we believe Manders.

P = 0.5 = 50% (no)
P = 0.95 = 95% (yes)
P = 1 = 100% (YES!) confidence

Statistical confidence P - Costes et al. 2004  Biophysical J.  vol 86 p3993
Colocalisation example: *virus entry to caveolae*

Without significance test, we wrongly assume virus is colocalised with caveolae at 10 min P.I.

It is not! Only at 20 min is there significant correlation.
Examples:
No Correlation?

Pearson r 0.024
M1 0.0354
M2 0.0471

Why high Thresholds?
Noisy Saturated Images
Good Correlation?

Pearson $r$ 0.747
M1 0.7291
M2 0.7420

Thresholds
Include noise?

Badly Saturated!
Bad detector settings
Good Correlation?

Pearson r 0.68
M1 0.77
M2 0.63

Offset wrong
+ Saturated

Thresholds
Handle it?
No?
Bleed Through!
DAPI into GFP
Bad detector settings
Good Correlation? Bleed through?
Bad detector settings... gives wrong results!!!
Software for Colocalisation

ImageJ - Colocalisation plugins

BiolmageXD (Coloc Task - Pixel Intensity and Object based methods)

Imaris (Coloc module)

Matlab (J-Y. Tinevez MPI-CBG)
Thanks to: MPI-CBG LMF and IPF
Heino, Pahajoki,
Kankaanpää, Marjomäki
Uuksalainen, Paavolainen,
TEKES,

Thanks for listening