Co-localisation

Formation microscopie 25-28 Novembre

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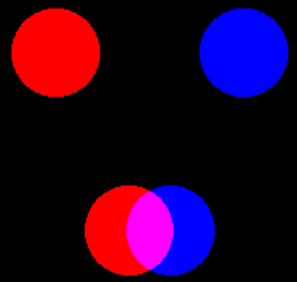
Co-localisation

Relation entre deux biomolcules ?



First of all

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- Looking for interaction ? (e.g. protein biding to each other)
  $\Rightarrow$ Concentrations (intensities) are correlated
  $\Rightarrow$ Also need biochemistry validation
- Looking for exclusion ?
  $\Rightarrow$ Avoid each other: signals non overlapping

Be aware !

Your conclusion is limited by the resolution of the detection system used:

"Distance between the two objects is under the limit of detection"

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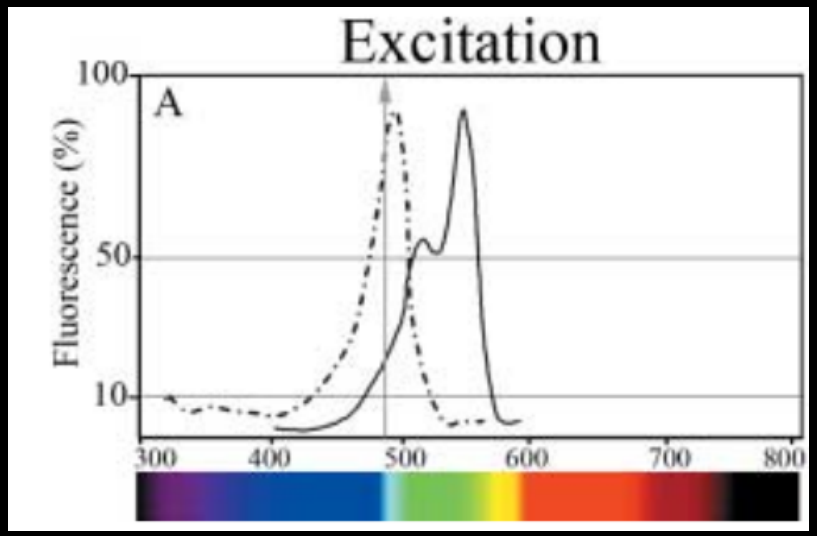
Colocalisation: Preparation

Choice of fluorochrome

Choose good combination of fluorochrome: selective excitation

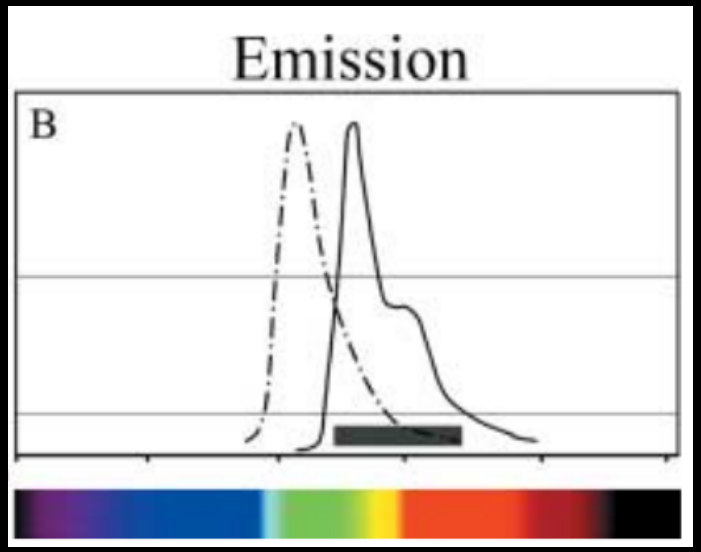


From Bolte et Cordelieres 2006

Crosstalk: 2 dyes can be excited by the same laser line

Choice of fluorochrome

Choose good combination of fluorochrome: selective emission



From Bolte et Cordelieres 2006

Bleed-through: 2 dyes can emit signals in the same spectral region

Preparation

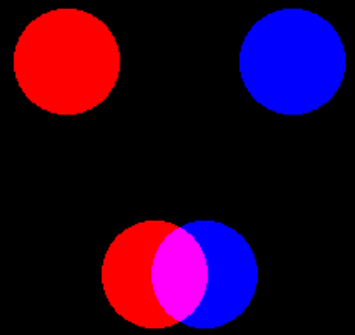
⚠ Use single labelled control for each fluorochrome before to check !

- As for most image analysis process, pre-process for image quality: Correct uneven illumination (with empty-field image), background removal, reduce noise with filtering, deconvolution.

Colocalisation: Visually

Straightforward method: overlay

Sur-impose the two channels, look at common area

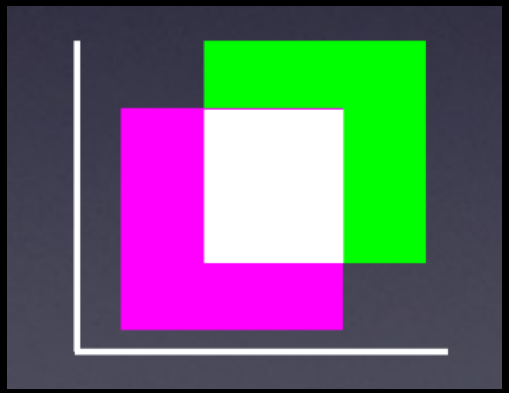


Standard way to **illustrate** co-localisation

But not a measure/proof !

Straightforward method: issues

Projection of 3D-stack, overlay



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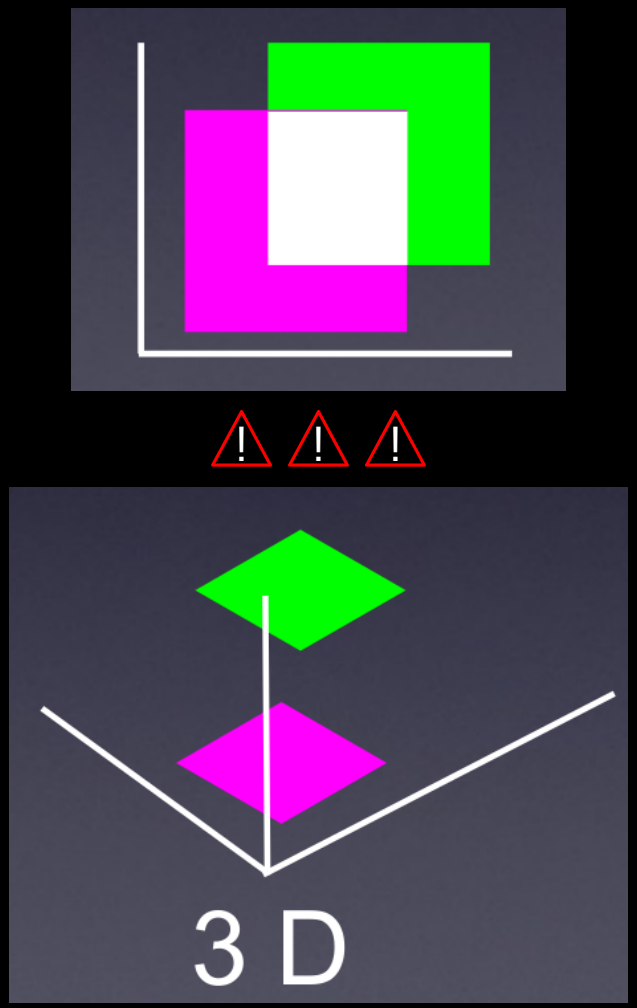
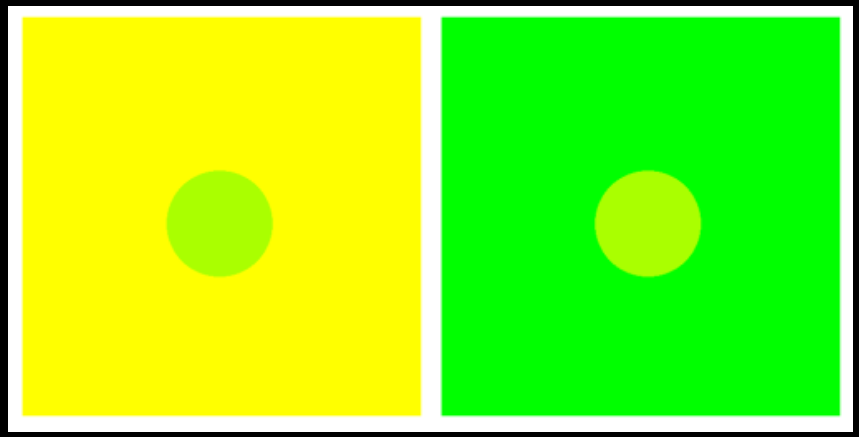


Adapted from course of D.J. White

Straightforward method: issues

Optical effects:



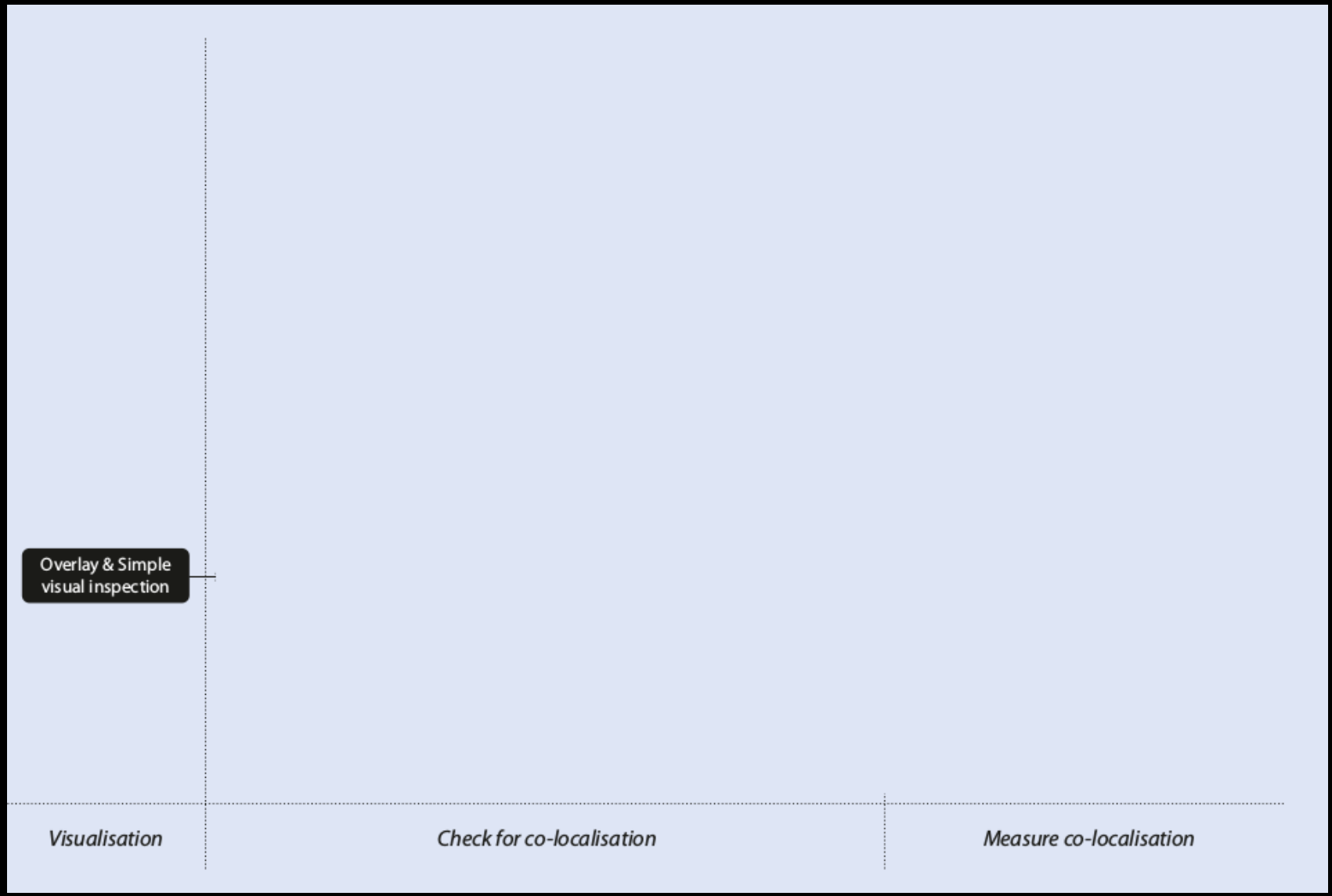
from imagej.net/Colocalization_Analysis

The two circles have the same color (if measured same intensity)

Straightforward method: issues

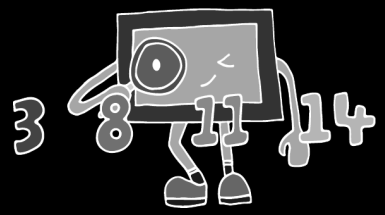
- Visual errors: 3D projection, optic illusions, difference intensities
- Main issues, lack of quantification:
  - Could observed overlay be due to chance only ?
  - Does low (null) amount of overlap come from proteins exclusion or occured just by chance ?
  - How much signals overlap ? E.g. usefull to compare to other conditions

Colocalization workflow



Adapted from Cordelieres and Zhang 2020

Colocalisation: Quantitatively



Quantitative measures of colocalisation

2 types of colocalisation (Aaron et al 2018):

- **Co-occurence**:

- **Correlation**:

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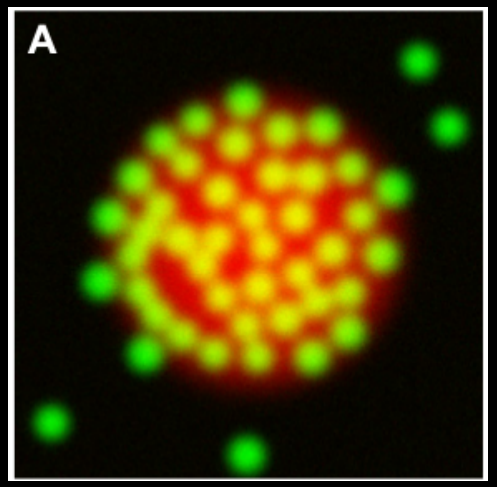

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  e.g. assessing functionnal relationship between two overlapping molecules

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$\Rightarrow$ Choice of the measure depends on the biological question. Both can be complementary.

Co-occurence

Threshold overlap

Idea: threshold each signals and count overlap

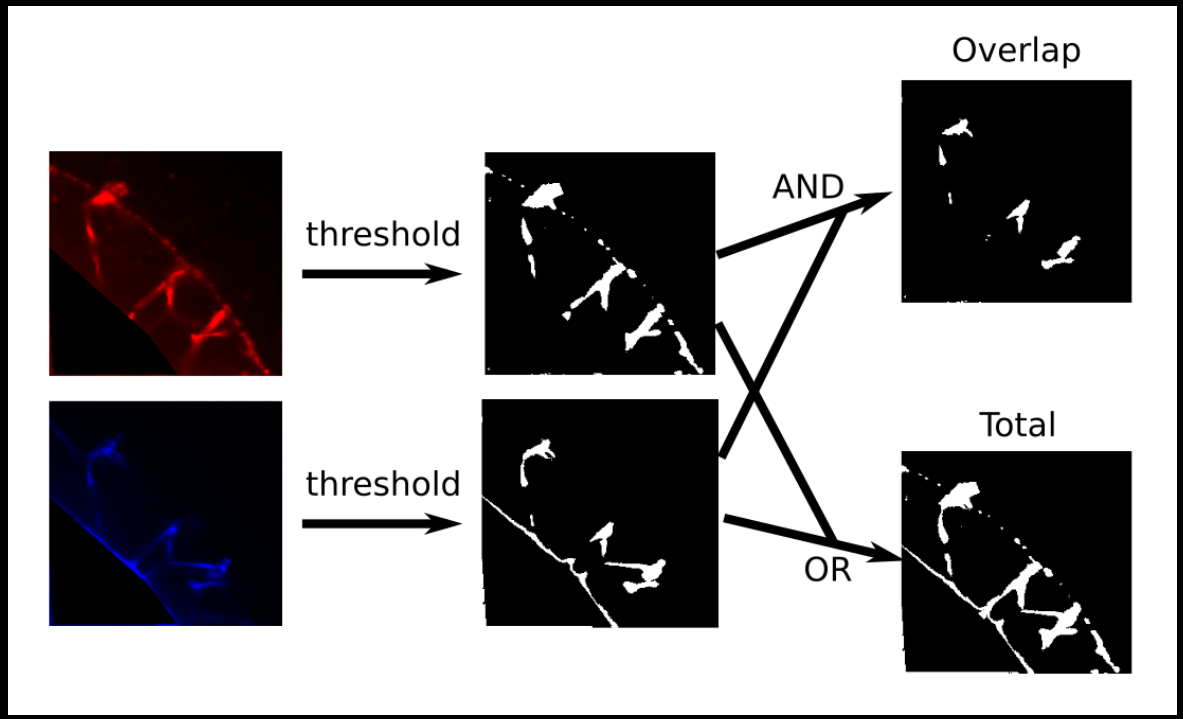

Image from Flora Crozet

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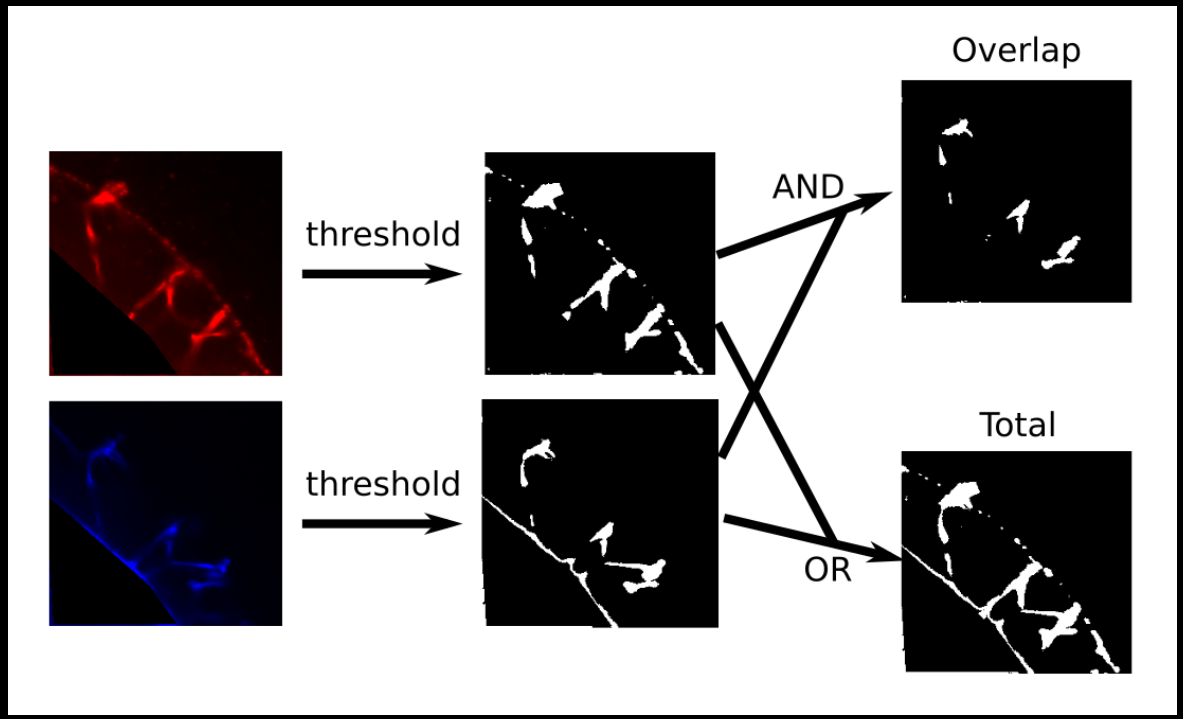
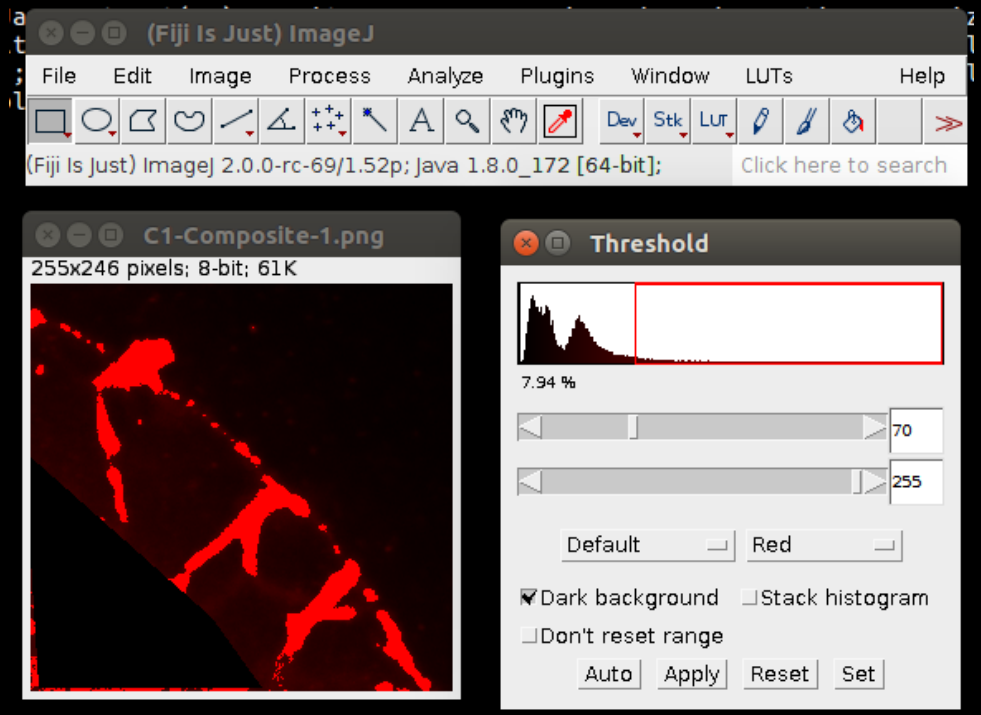


Image from Flora Crozet

Measure: $\frac{A_{overlap}}{A_{total}} = 0.26$ $\frac{A_{overlap}}{A_{red}} = 0.38$ $\frac{A_{overlap}}{A_{blue}} = 0.46$

How to do with ImageJ

- Threshold the 2 channels (`Image->Adjust->Threshold..`)
- Choose threshold manually or use an automatic method (preferred in general)



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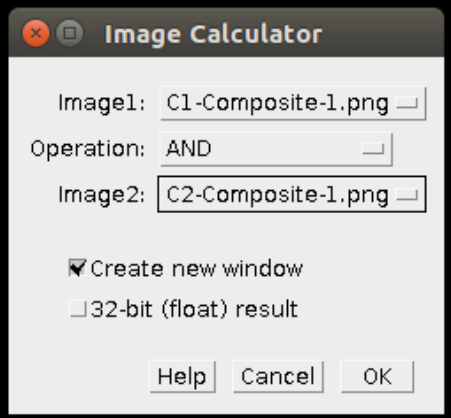

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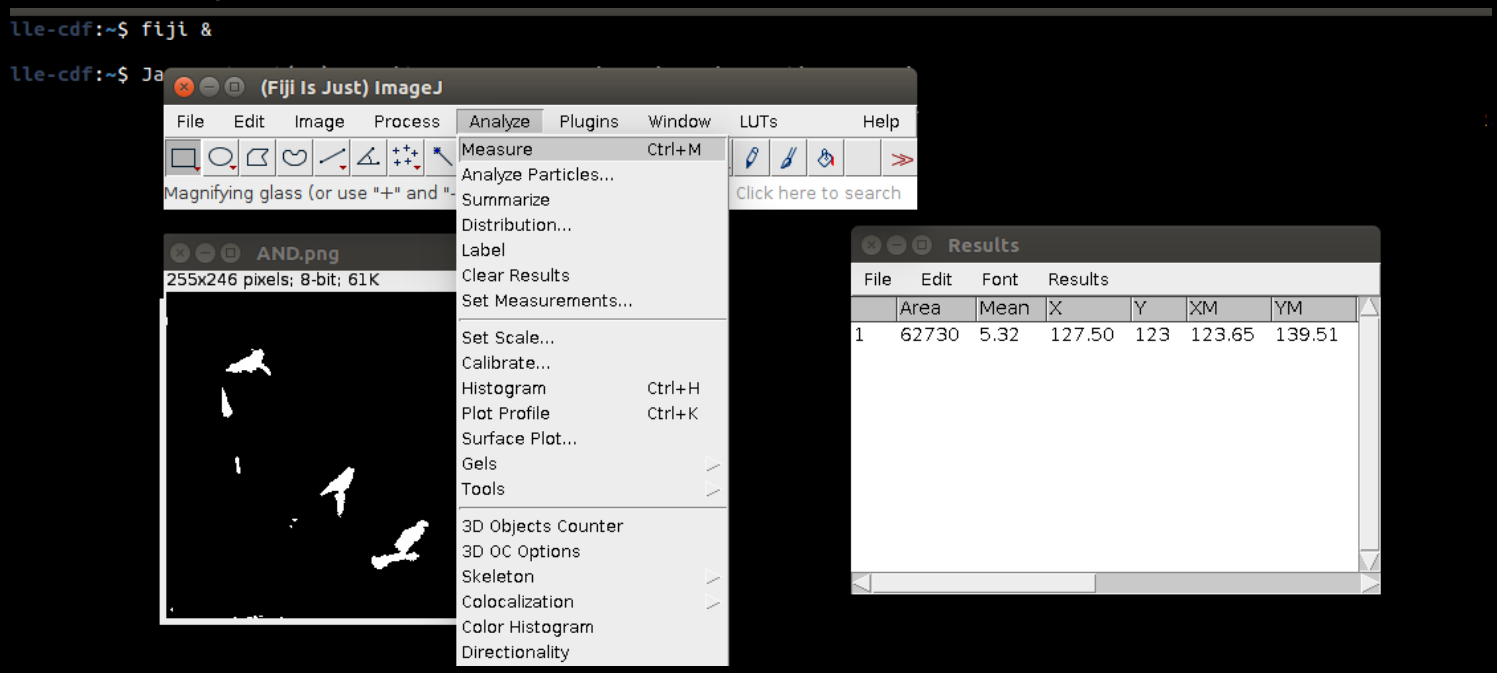
- Measure the area of positive pixels in each image and compare them (e.g. mean) `Analyze->Measure`



Threshold overlap

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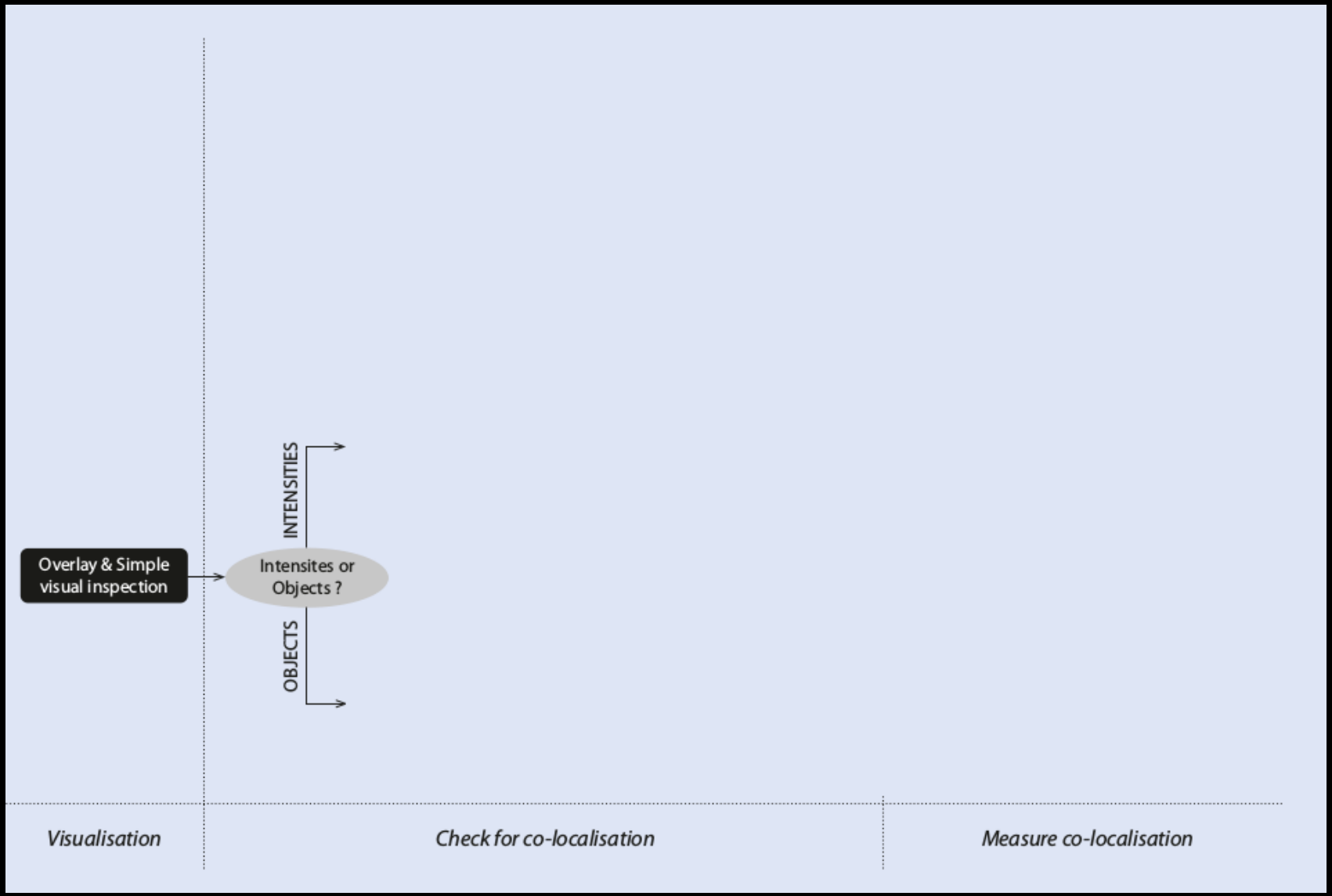
BUT

- ⚠ Limit of diffraction: preferable for large objects (cell, nuclei, large organelles)
- ⚠ Depends on threshold (objects not easy to segment, thresholding method biais)
- ⚠ No information on pixels intensities in the overlap areas

Colocalization workflow



Adapted from Cordelieres and Zhang 2020

Intensities: pixel-based methods

Manders coefficient (Pixel based)

Manders coefficient (Manders et al. 1993)

Proportion of each signal colocalized with the other

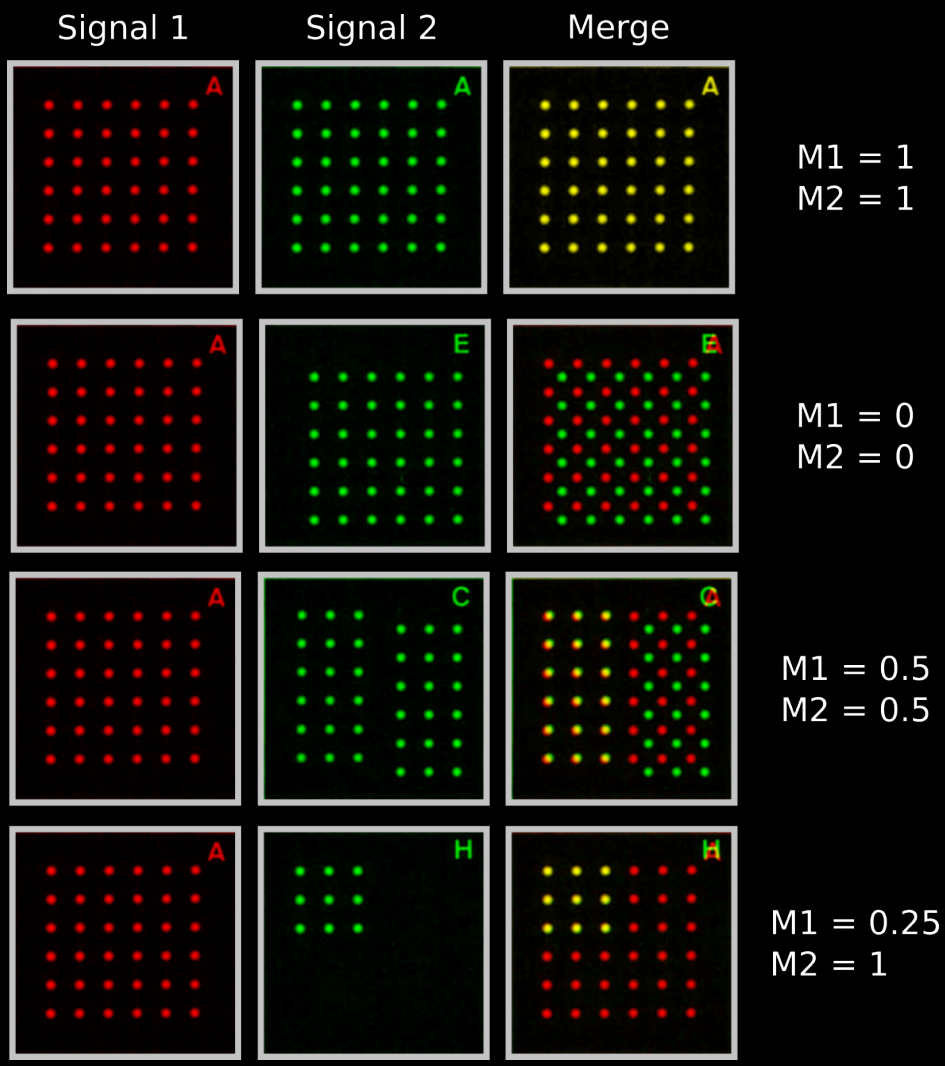
$$M_{Red} = \frac{\sum_{i=1}^{n} I_{i,coloc}^{Red}}{\sum_{i=1}^{n} I_{i}^{Red}}$$

I_i^{Red} intensity of pixel i in channel Red, $I_{i,coloc}^{Red} = \begin{cases} I_i^{Red} & if\ I_i^{Blue} > 0 \\ 0 & else \end{cases}$

- ⚠ Both chanels have to be thresholded to define colocalised pixels
- 🔧 Automatic methods to define the thresholds exist (see later)
- ✚ Manders coefficients give greater importance to bright pixels and less to small intensities values close to threshold

Manders coefficient (Pixel based): example

M_1 is the co-occurence fraction of color 1 with color 2:
"Of all the signal Red, how much of it is colocalized with signal green"



Adapted from Manders et al. 1993

Manders coefficient (Pixel based)

- Gives the co-occurence fraction for each signal (M_1, M_2)
- Overall overlap coefficient:

$$MOC = \frac{\sum_{i=1}^{n} x_i y_i}{\sum_{i=1}^{n} x_i^2 \sum_{i=1}^{n} y_i^2}$$

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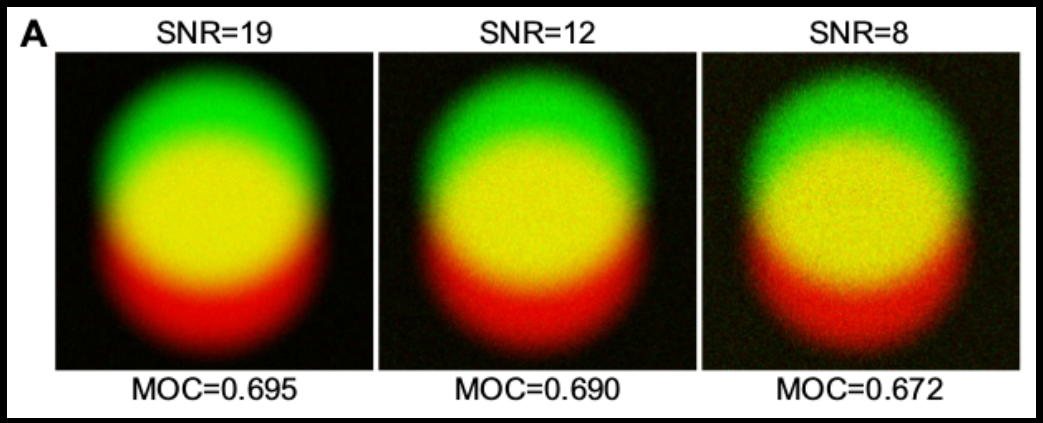

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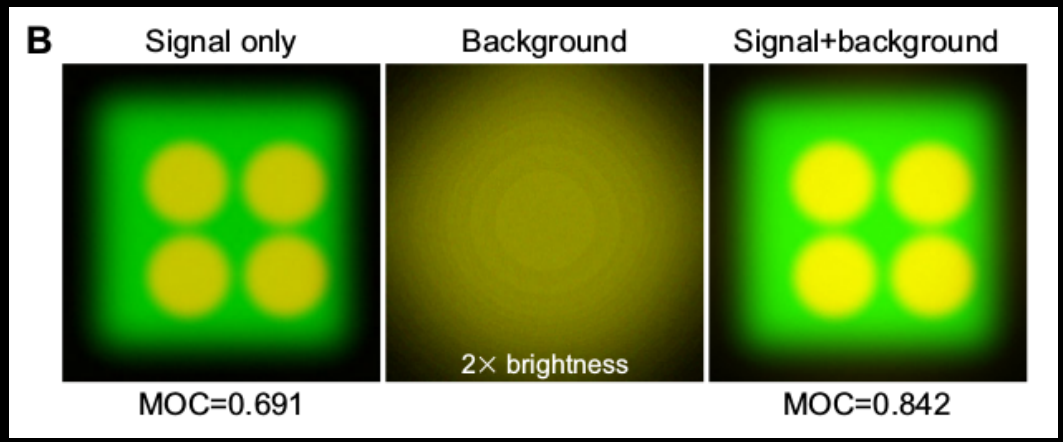
⚠ Sensitive to unwanted signals (e.g. out of focus light, saturation...):



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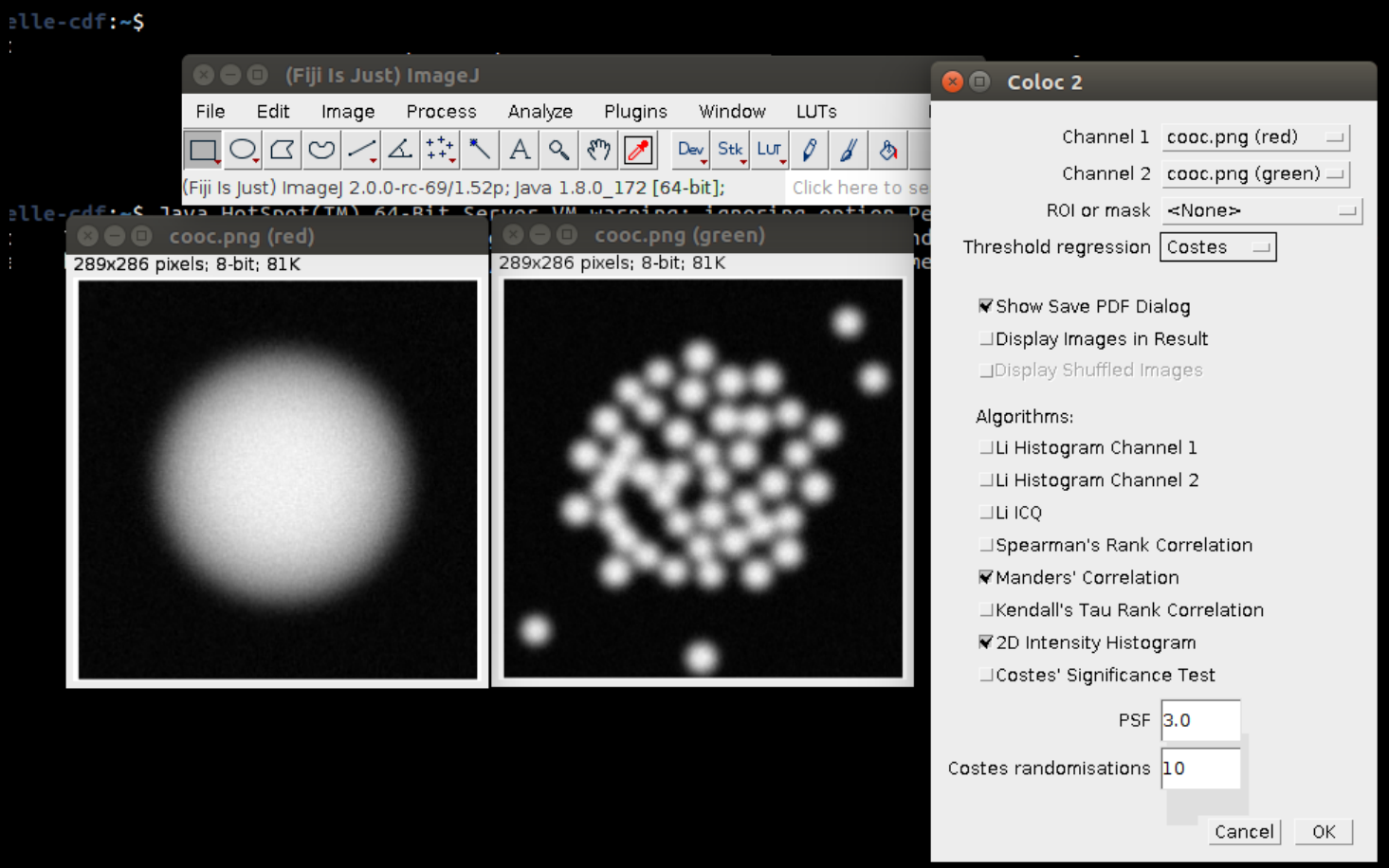
🔧 Pre-process the image to remove such signals (e.g. unsharp masking)

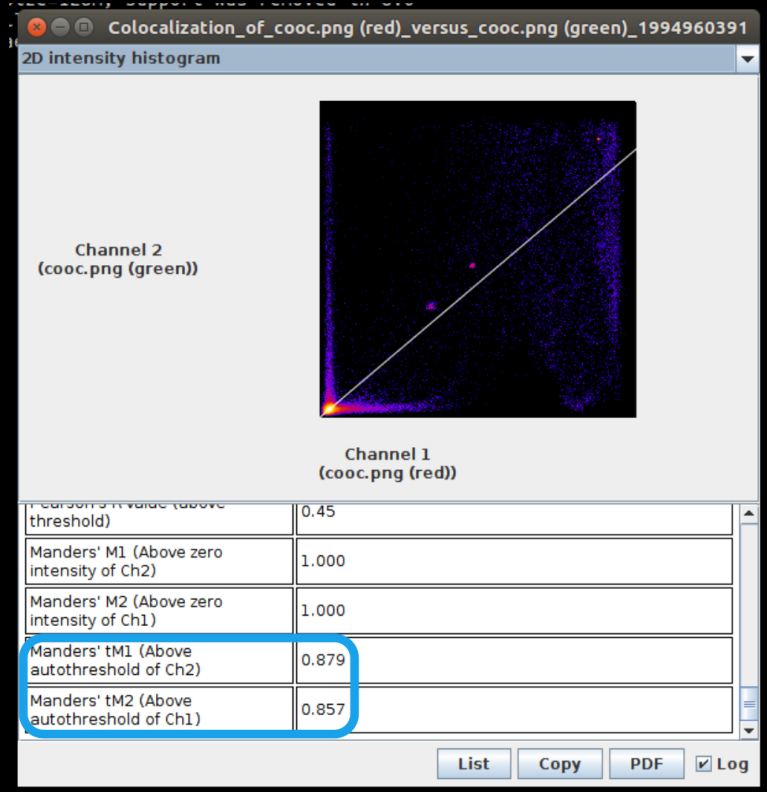
How to do with ImageJ

- With the `Coloc2` plugin `Analyze->Colocalization->Coloc 2`
- Possibility to use a Mask or ROI to concentrate only on relevant area (advised)



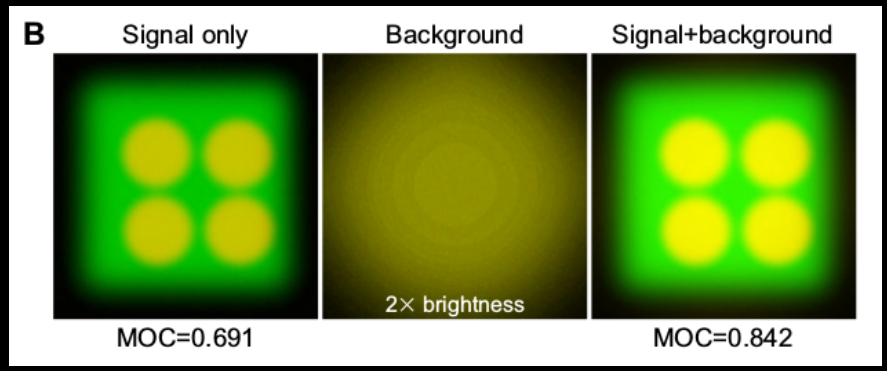
How to do with ImageJ

- With the `Coloc2` plugin `Analyze->Colocalization->Coloc 2`
- Possibility to use a Mask or ROI to concentrate only on relevant area (advised)
- Read the thresholded Manders coefficient (unthresholded: over-estimation as count all black pixels)



How to do with ImageJ

- Coloc2 gives you warnings ⚠ when it detects possible images artefacts



- ⚠ Always read carefully those warnings !

Warnings

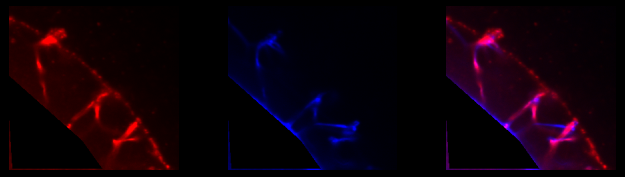
Type	Message
y-intercept far from zero	The ratio of the y-intercept of the auto threshold regression line to the mean value of Channel 2 is high. This means the y-intercept is far from zero, implying a significant positive or negative zero offset in the image data intensities. Maybe you should use a ROI. Maybe do a background subtraction in both channels. Make sure you didn't clip off the low intensities to zero. This might not affect Pearson's correlation values very much, but might harm other results.
Threshold of ch. 1 too high	Too few pixels are taken into account for above-threshold calculations. The threshold is above the channel's mean.
Threshold of ch. 2 too high	Too few pixels are taken into account for above-threshold calculations. The threshold is above the channel's mean.

Results

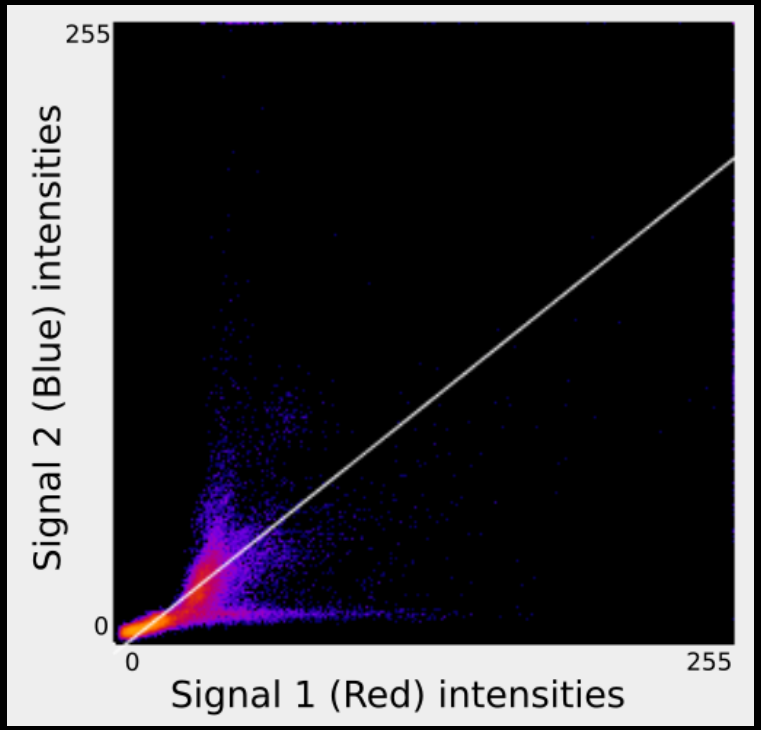
Correlation

Correlation visualisation

Goal: look for a relation between pixels intensities of both signal.



Plot the intensity of signal 1 (red) against the intensity of signal 2 (blue) for all pixels (called Scatterplot or Cytofluorogram).



Pearson's coefficient (Pixel based)

Pearson's coefficient

How much both signals are correlated:

$$r = \frac{\sum_i (R_i - \bar{R}).(B_i - \bar{B})}{\sqrt{\sum_i (R_i - \bar{R})^2.(B_i - \bar{B})^2}}$$

R_i is the intensity in Red channel of pixel i, B_i its intensity in Blue channel, $\bar{R}$ is the mean pixels intensity in the red channel.

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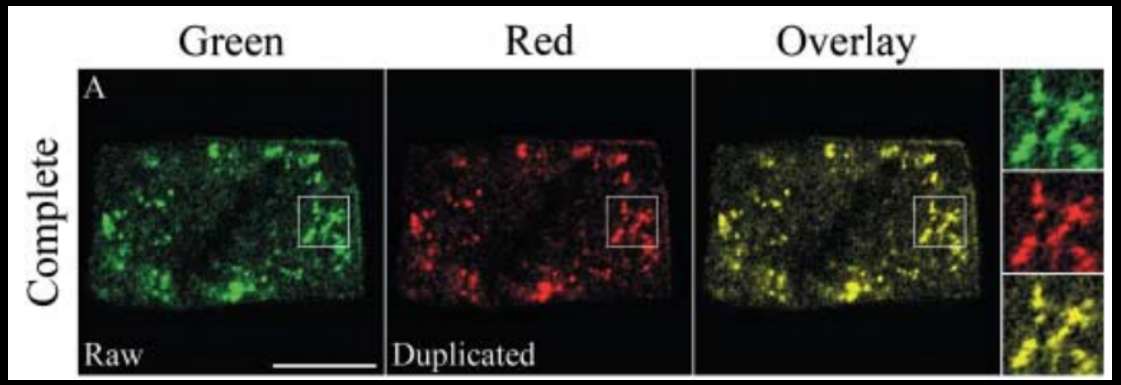
R_i is the intensity in Red channel of pixel i, B_i its intensity in Blue channel, $\bar{R}$ is the mean pixels intensity in the red channel.

r value can go from -1 to 1:

- $+1$: full correlation (images are the same)
- 0: no correlation (two signals are independant from each other
- -1: anti-correlation: when a pixel is red, there is no blue. Could be exclusion, but not necessarly.

Pearson's coefficient: examples

Strong interaction, nearly indentical signals (Golgi staining): duplicated image



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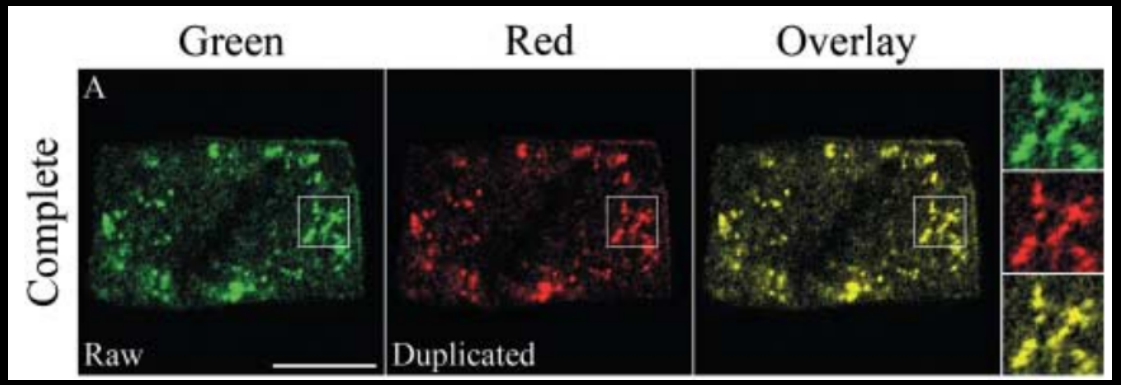
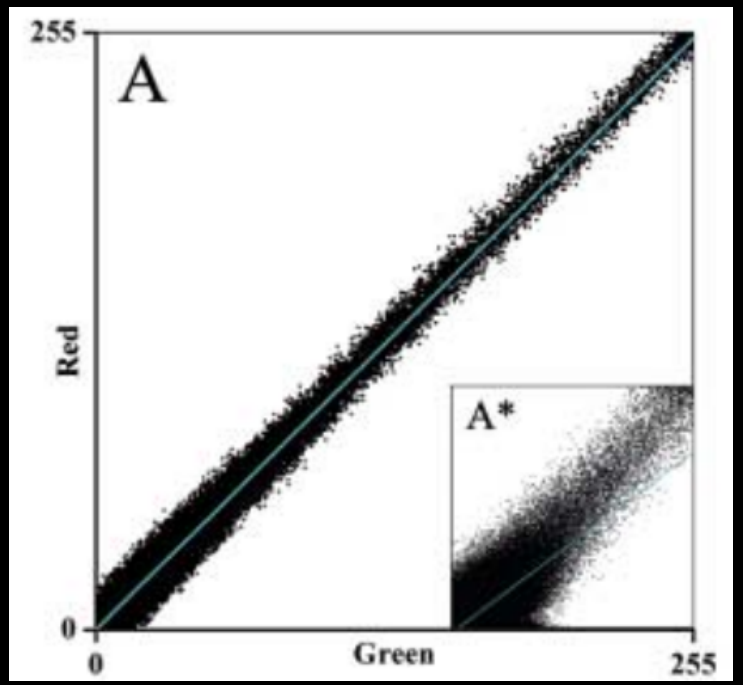




Full correlation:

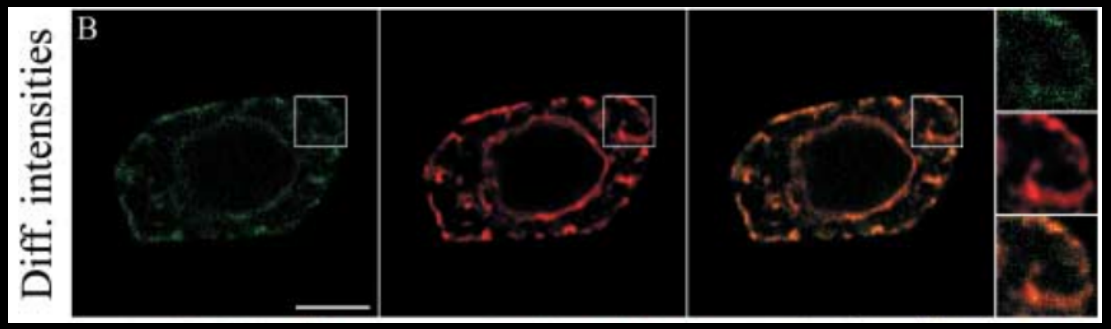
$$r \approx 1$$

Variation around the correlation line due to noise

From Bolte et Cordelieres, 2006

Pearson's coefficient: examples

Complete colocalization wtih different intensities: lower intensity in green channel



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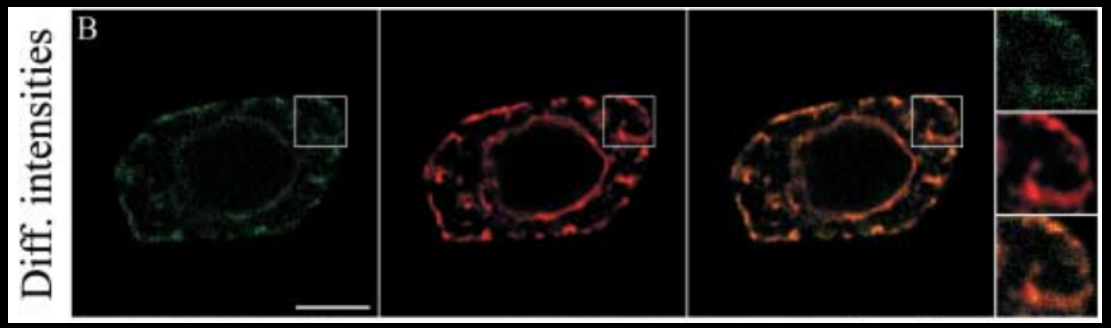
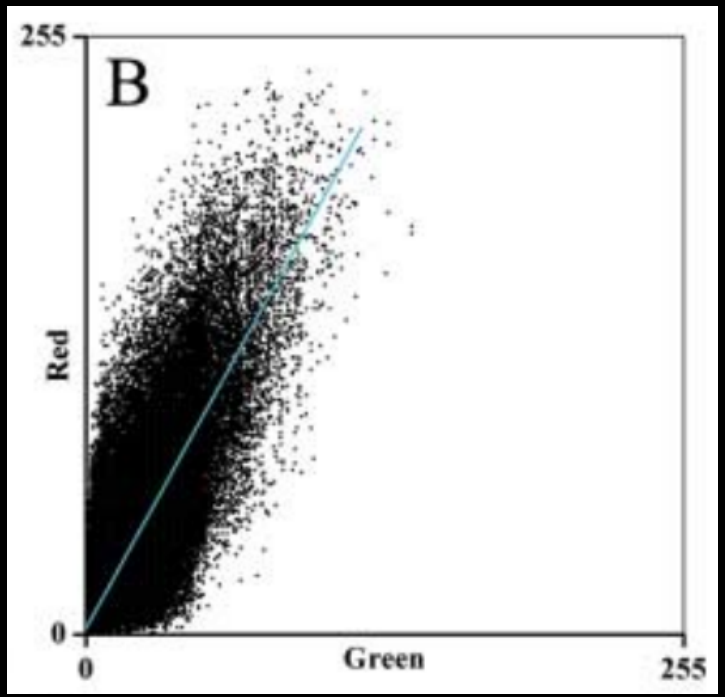


Strong correlation:

$$r \approx 0.85$$

Correlation still detected (rotated line, tilted toward stronger signal)



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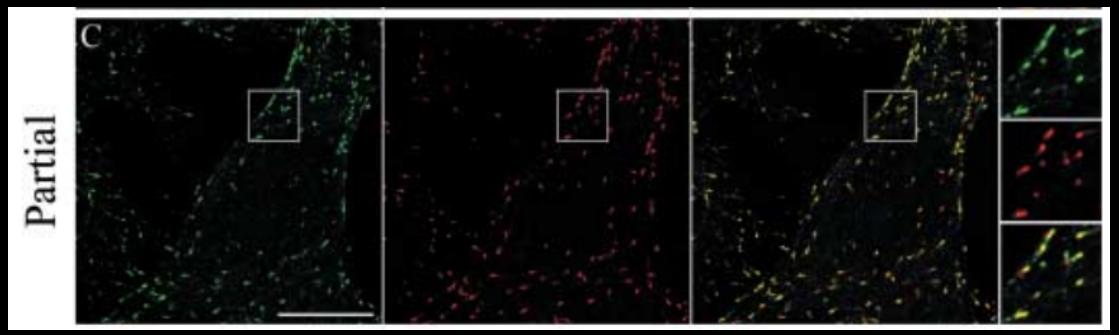
Pearson's coefficient: examples

Partial colocalization (two different MT plus-end tracking)



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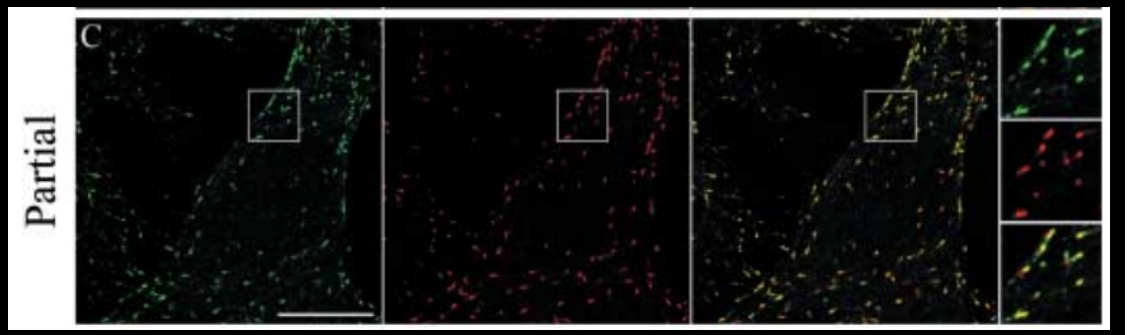
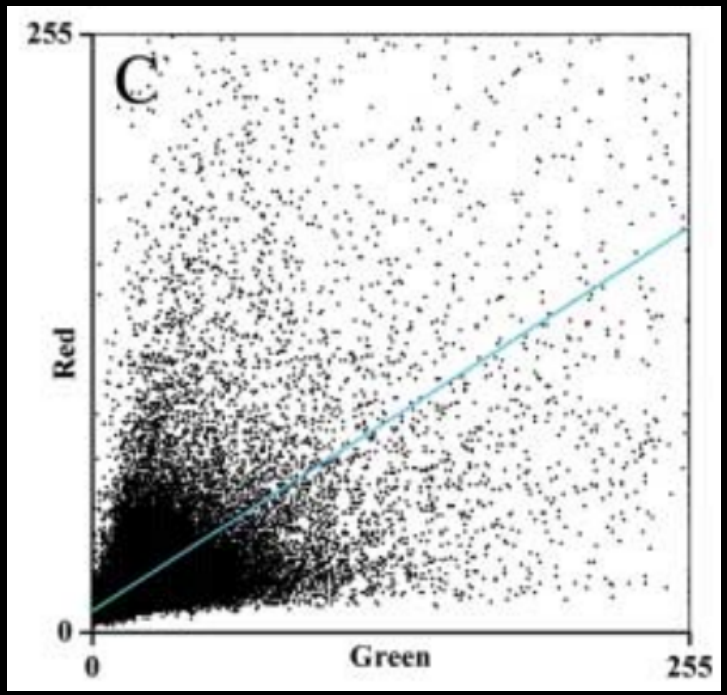




Good correlation:

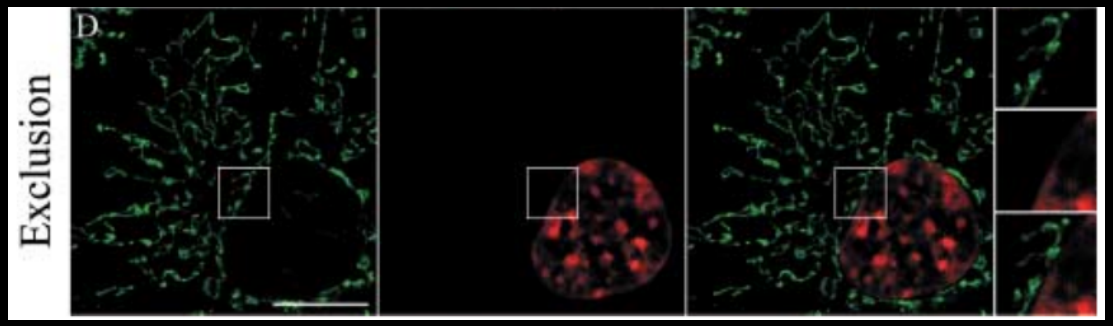
$$r \approx 0.69$$

Detect well partial colocalization

From Bolte et Cordelieres, 2006

Pearson's coefficient: examples

Exclusion: nuclear and mitochondrial staining.



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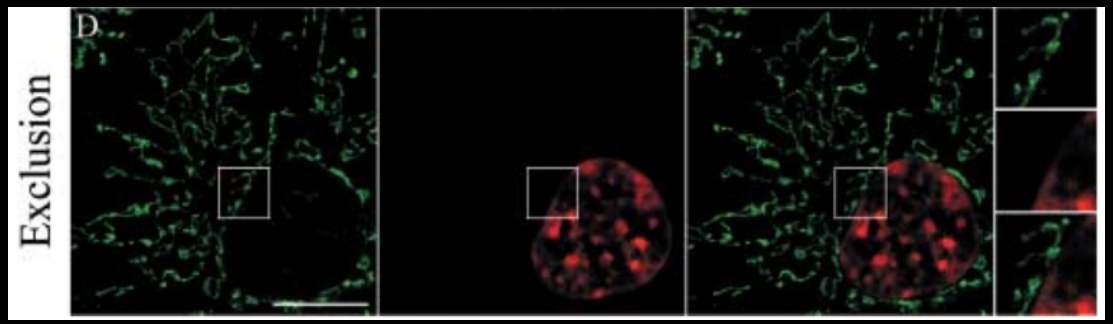
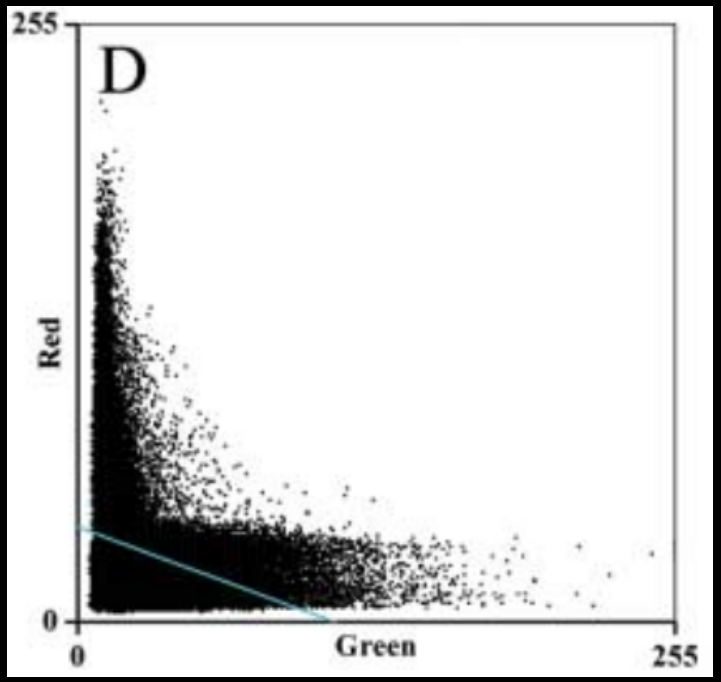
Exclusion: nuclear and mitochondrial staining.





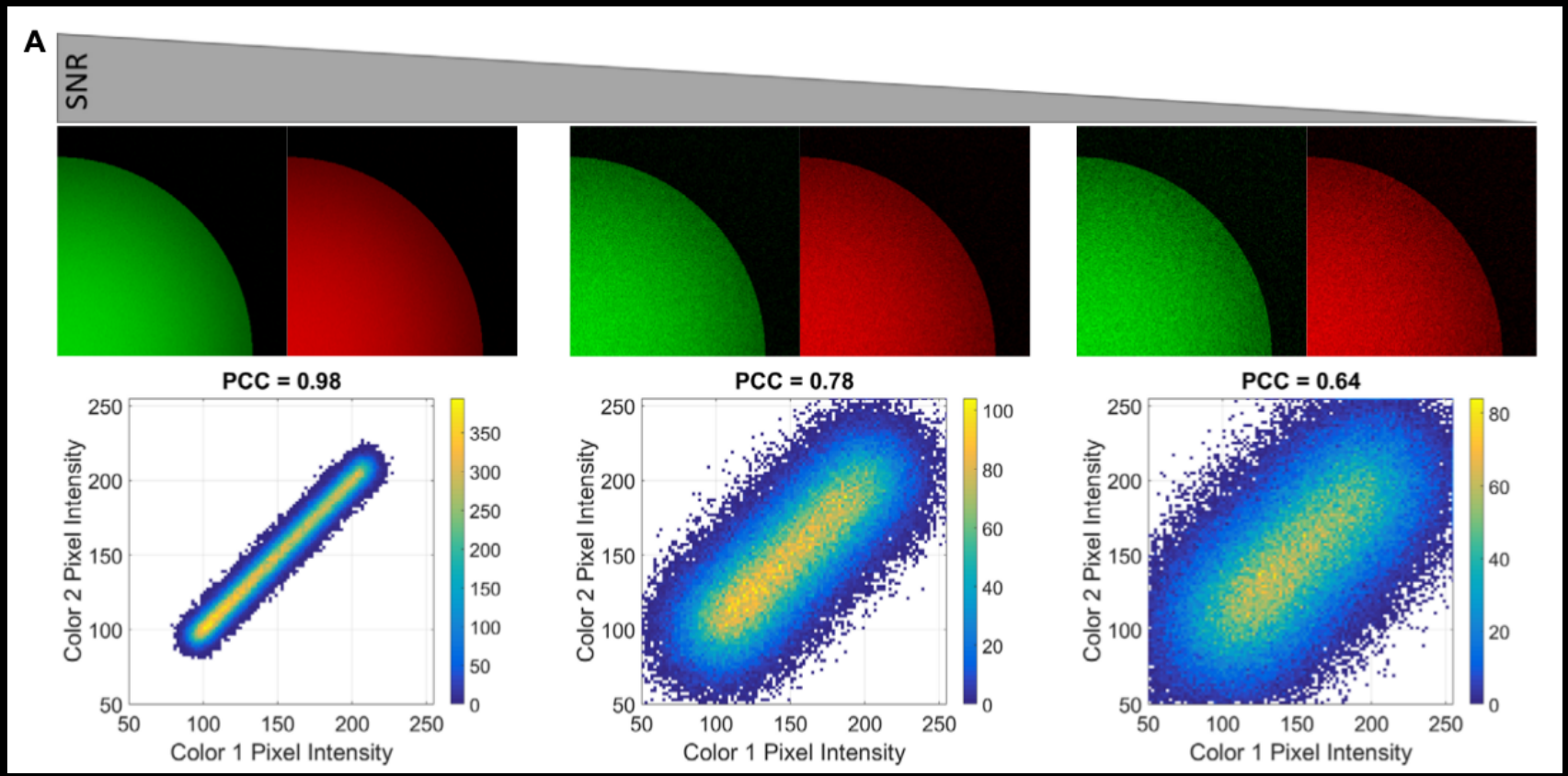
Anti-correlation:

$$r < 0$$

Can be exclusion, separate localization
(here inside/outside nucleus)

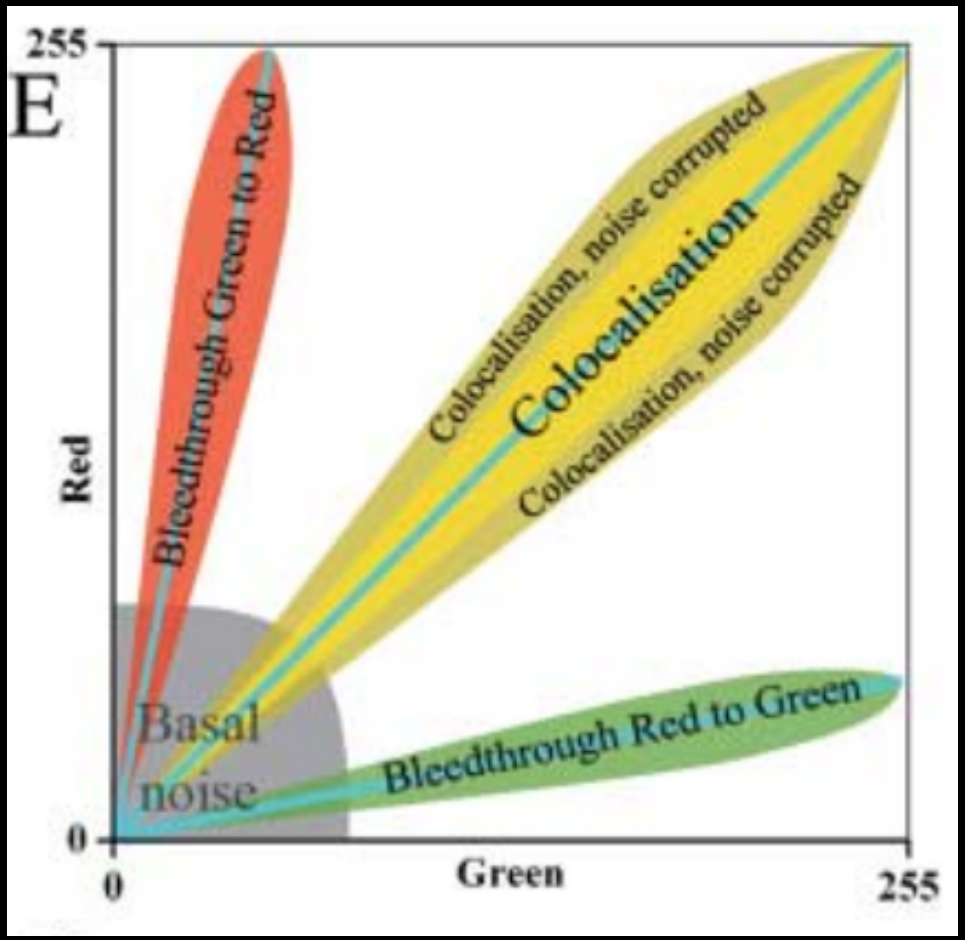
From Bolte et Cordelieres, 2006

Pearson's coefficient: sensitivity to noise



From Aaron et al., 2018

Pearson's coefficient: bleedthrough



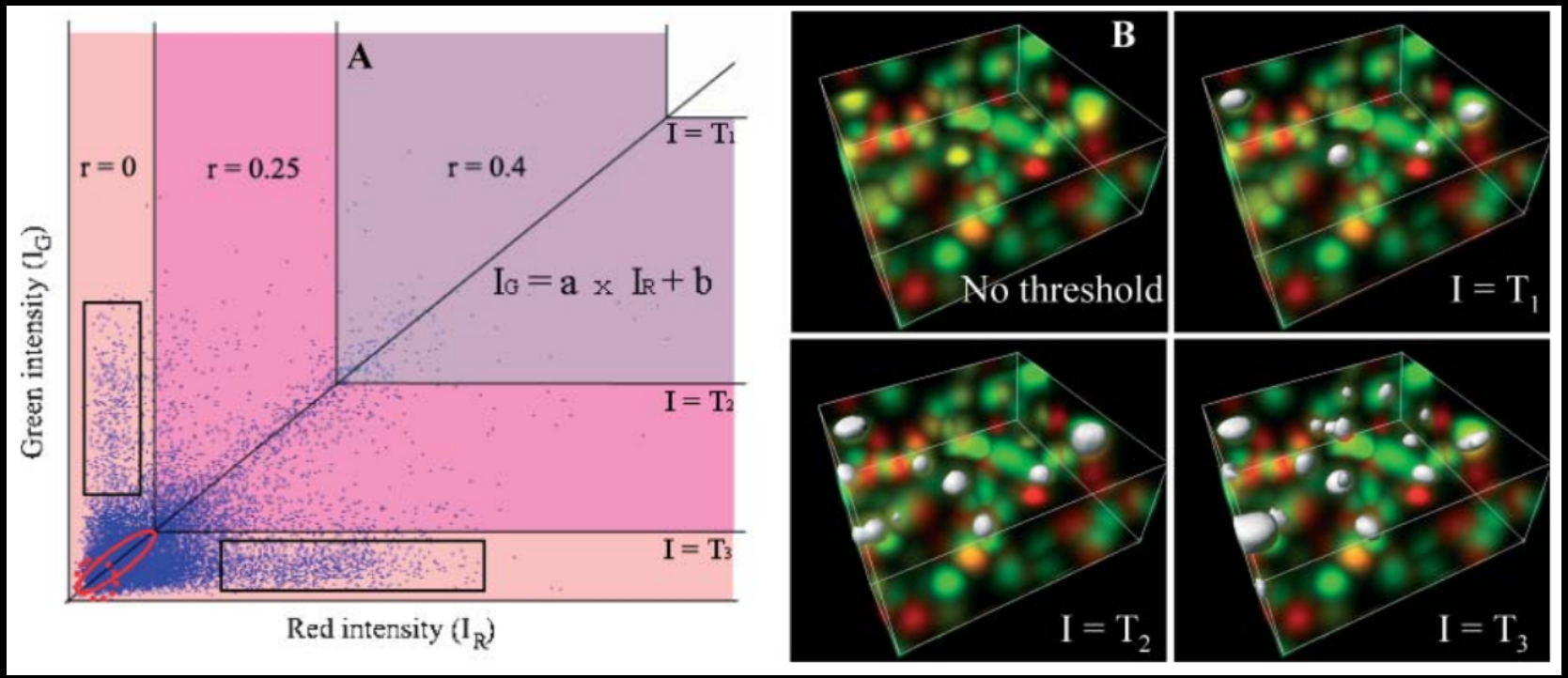
From Bolte et Cordelieres, 2006

Pearson's coefficient: remarks

- ✚ Easy to use, fast
- ✚ Not sensitive to difference of intensities
- ✚✚ Allows to define an automatic threshold for both channels (next slide)
- ⚠ Sensitive to noise
- ⚠ Not conclusive for exclusion
- ⚠ Sensitive to **non-linearity** of correlation
- 🔧 Use Spearman coefficient (see later)

Pearson's coefficient: automatic threshold

Idea: colocalized pixels are bright in both channels. Noise pixels are mainly low intensities values and without correlation.
Fix a threshold and calculate Pearson coef r with only pixels **under** the threshold. Decrease the threshold value until coefficient ≤ 0.



From Costes et al. 2004

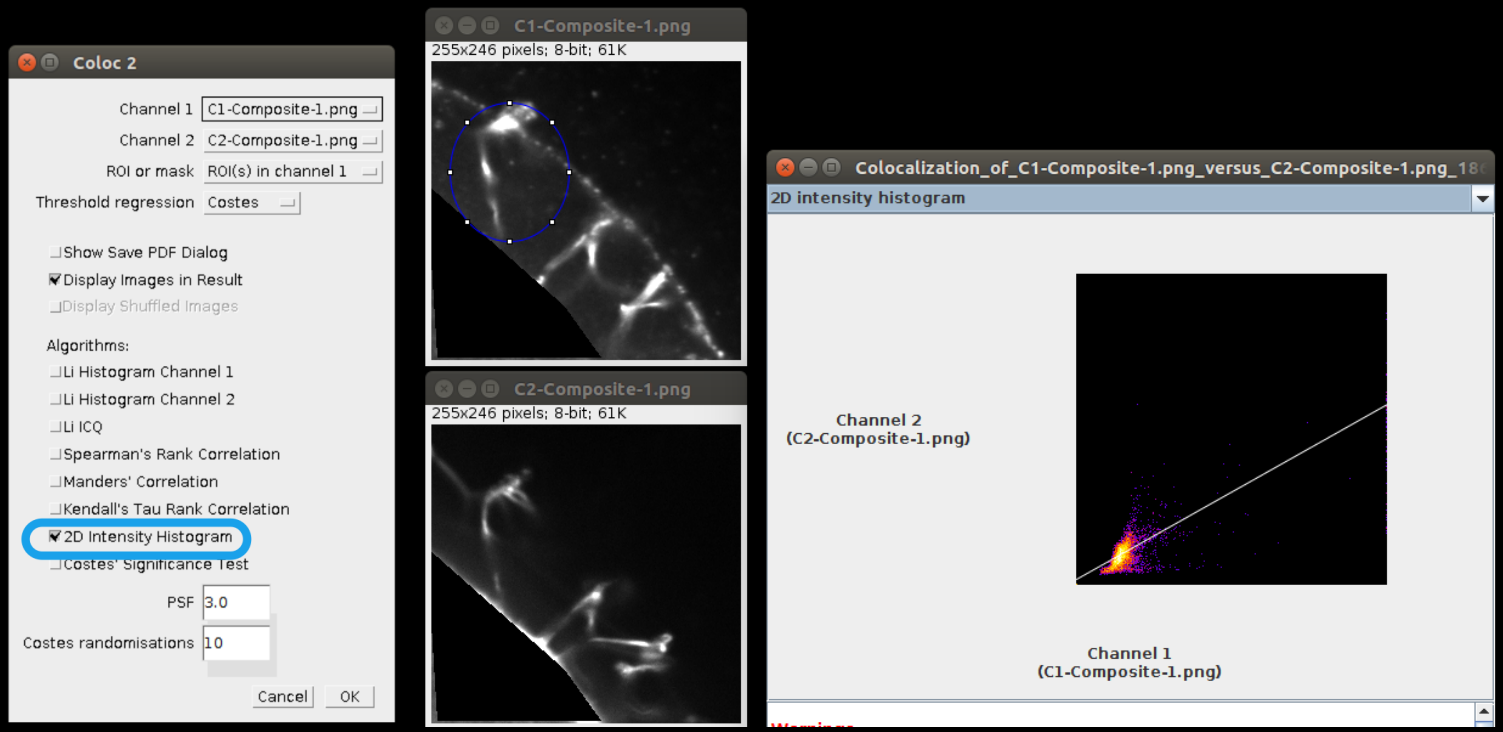
Pearson's coefficient: automatic threshold

After obtaining automatic thresholds:

- Keep only pixels above the resulting threshold
- Can calculate Pearson coefficient (without noise pixels)
- Can calculate Manders coefficient with these thresholds (seen previously)

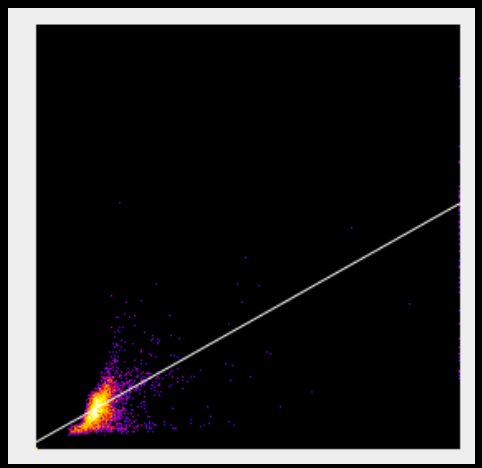
How to do with ImageJ

- Again with the `Coloc2` plugin
  `Analyze->Colocalization->Coloc 2`
- Generate the Scatterplot



How to do with ImageJ

- Again with the `Coloc2` plugin
  `Analyze->Colocalization->Coloc 2`
- Generate the Scatterplot
- Pearson informations in the `Coloc2` output



m (slope)	0.57
b (y-intercept)	3.01
b to y-mean ratio	0.09
Ch1 Max Threshold	43.00
Ch2 Max Threshold	27.00
Threshold regression	Costes
Pearson's R value (no threshold)	0.65
Pearson's R value (below threshold)	-0.01
Pearson's R value (above threshold)	0.56

Spearman's coefficient (Pixel based)

Spearman's coeffiecient usefull for **non-linear** correlations.
Similar to Pearson coefficient, but based on pixel intensities **rank** and not values.

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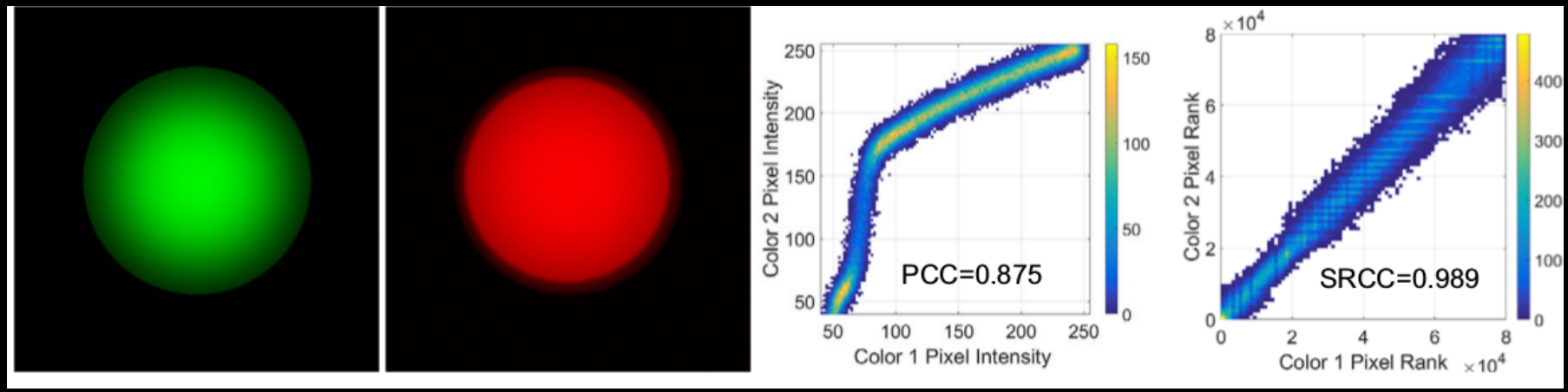

From Aaron et al. 2018

- ✚ Not too sensitive on the shape of the correlation
- ✚ Reduces impact of saturated pixels

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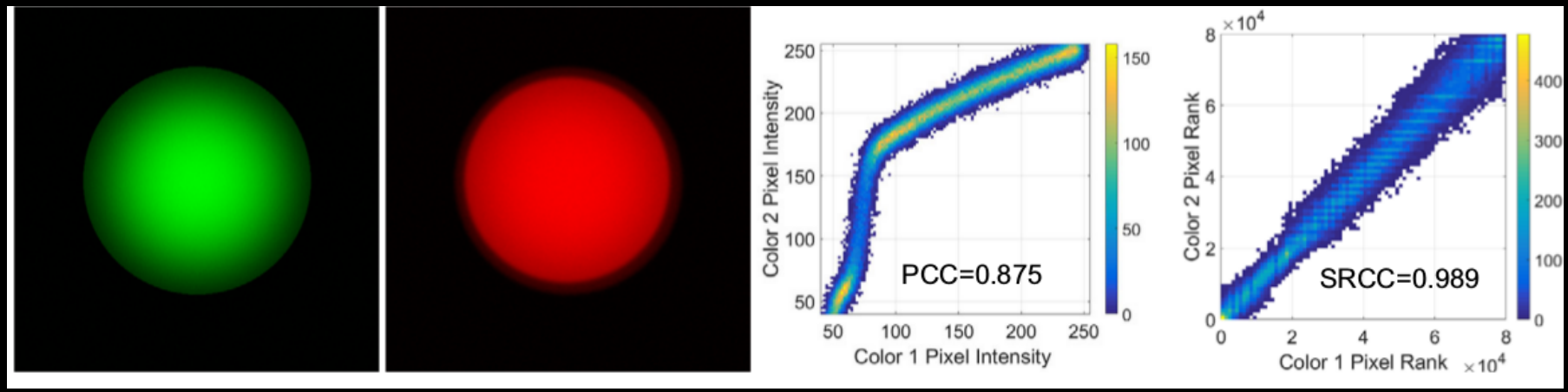
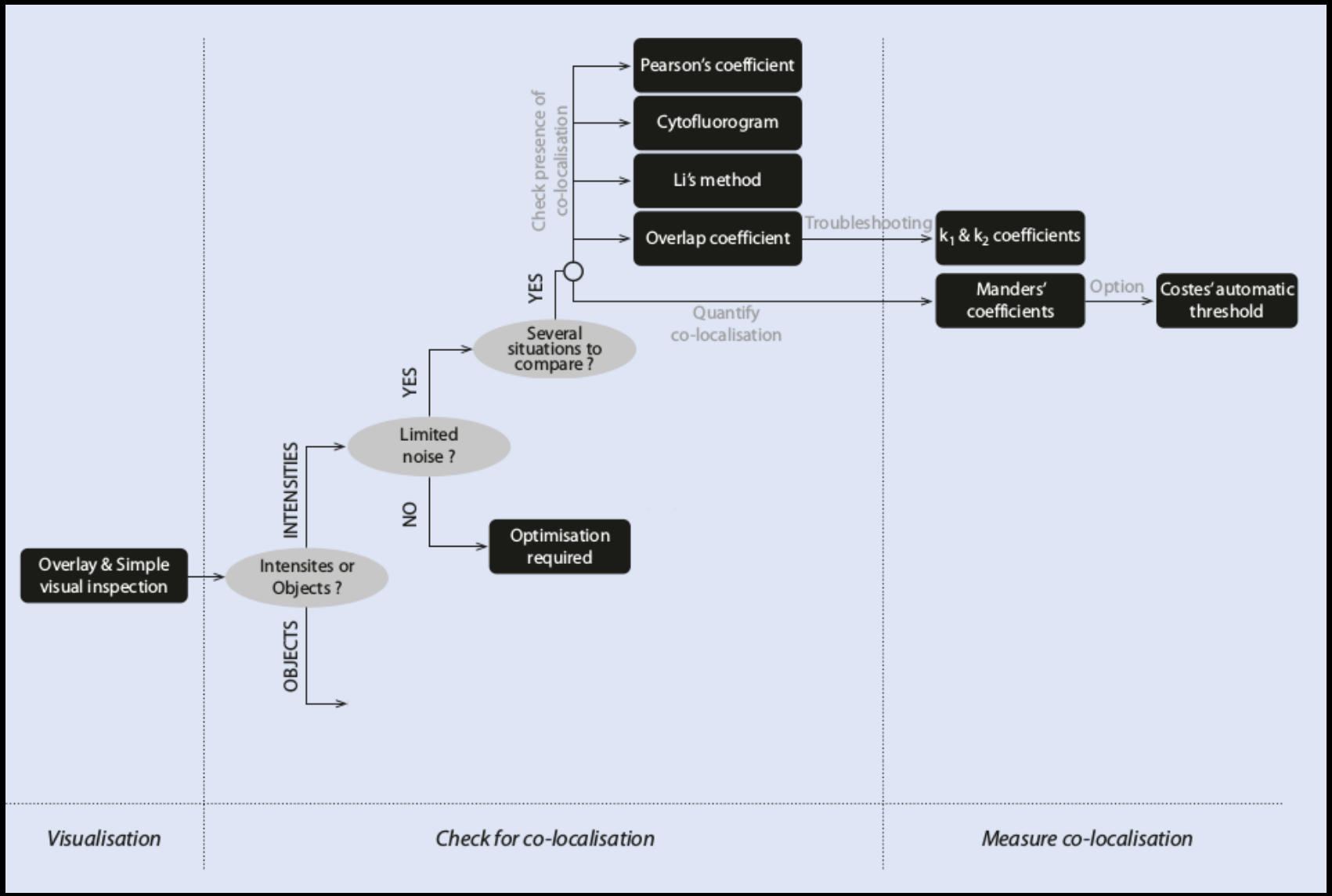


From Aaron et al. 2018

- ✚ Not too sensitive on the shape of the correlation
- ✚ Reduces impact of saturated pixels
- 🖼 Also possible to have it directly from colocalization plugins (e.g. `Coloc2`)

Colocalization workflow

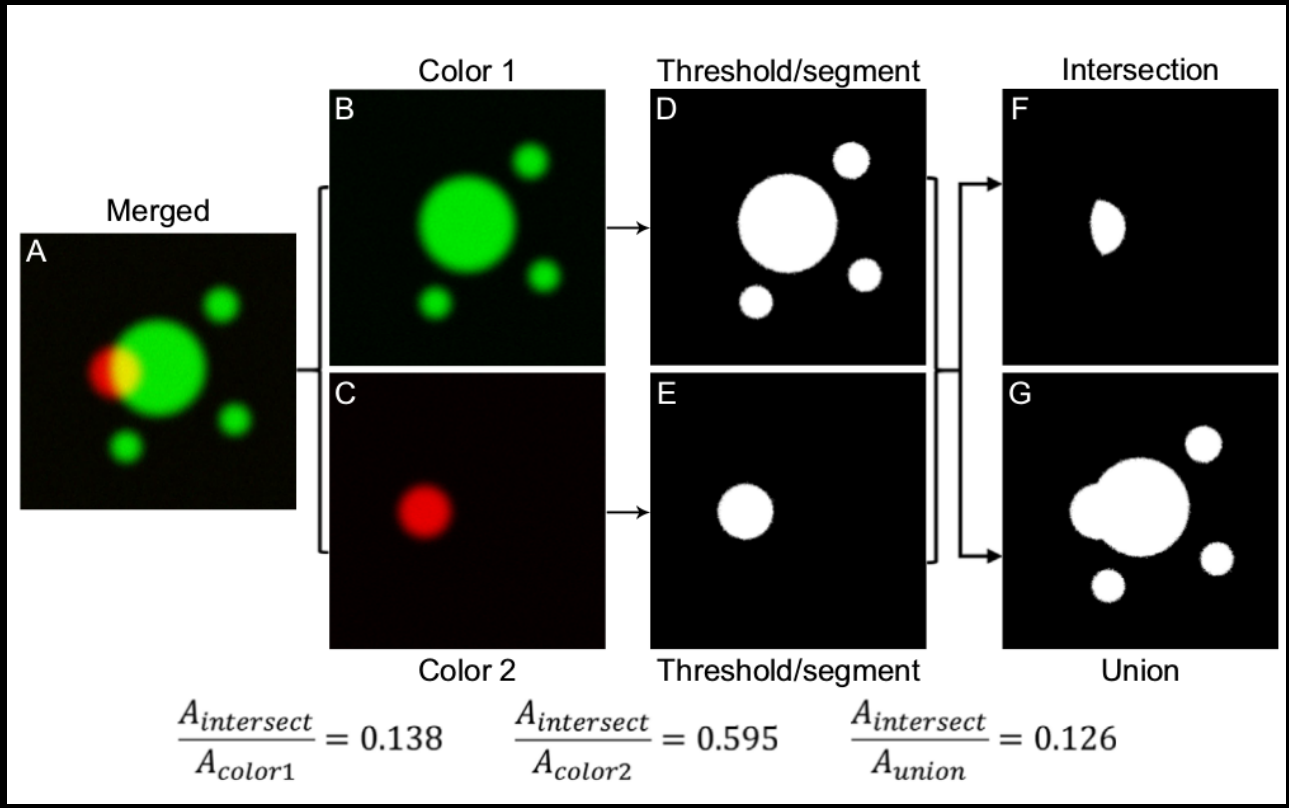


Adapted from Cordelieres and Zhang 2020

Object-based methods

Threshold overlap

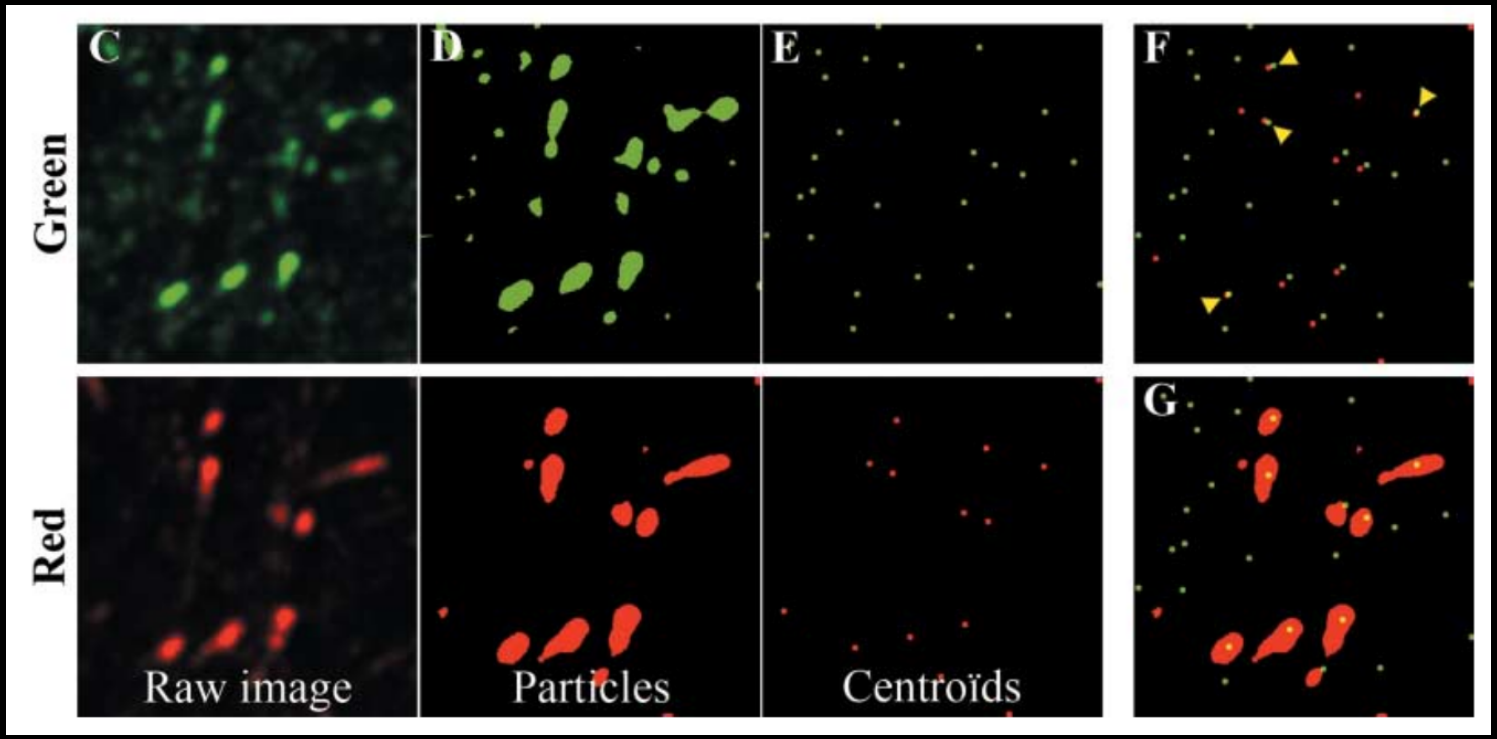
As seen before, segment objects and count overlap (area/volume)



⚠ Strongly depend on the object segmentation: size should be above resolution

Distance-based

Segment object, and measure the distance between the object centers. Can also be distance between center-to-edge, or edge-edge..., depending on objects properties (spheres, not same size, not same density...)



From Bolte et Cordelieres, 2006

How to do with ImageJ

- With the `DiAna` plugin (Gilles et al. 2017)
- First segment the images (possible with DiAnA `Plugins->DiAna->DiAna_Segment`, or manually, other plugins..)

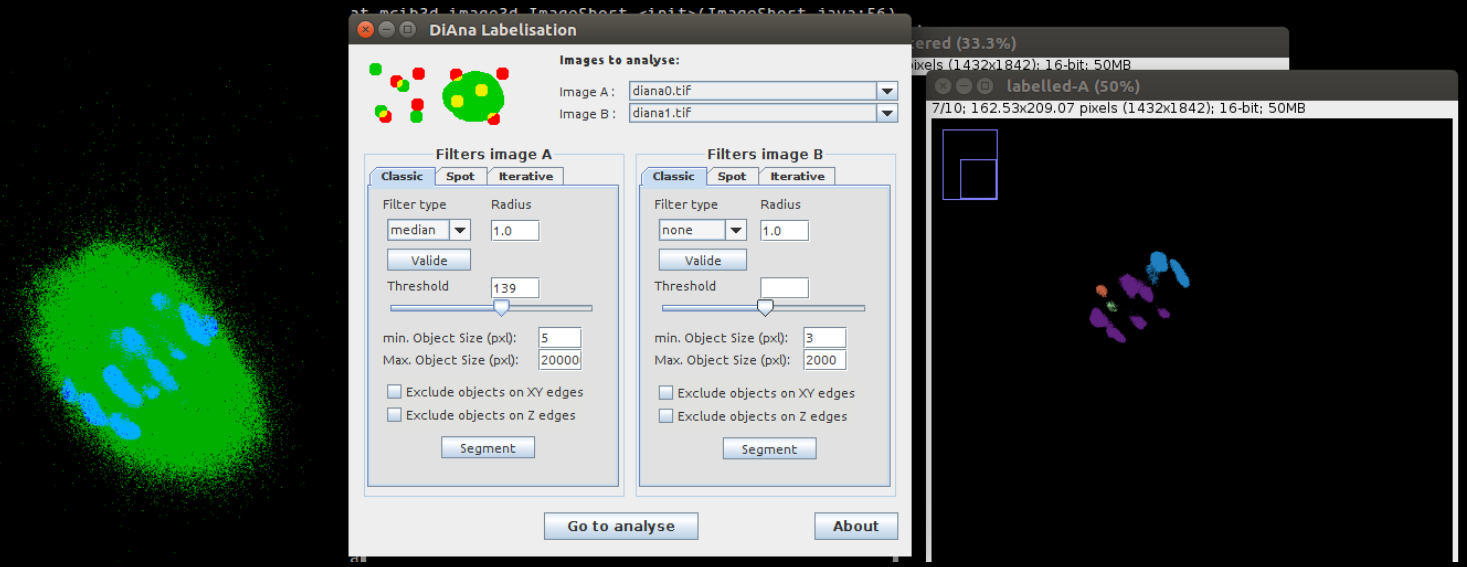
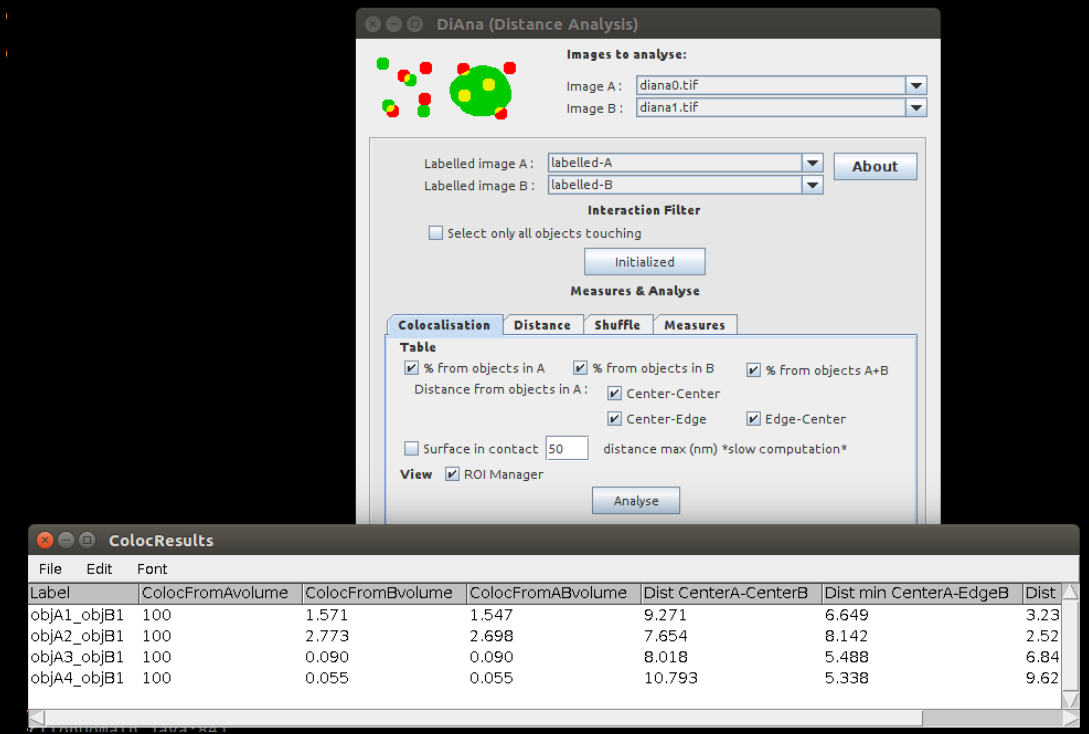


Image from Isma Bennabi

How to do with ImageJ

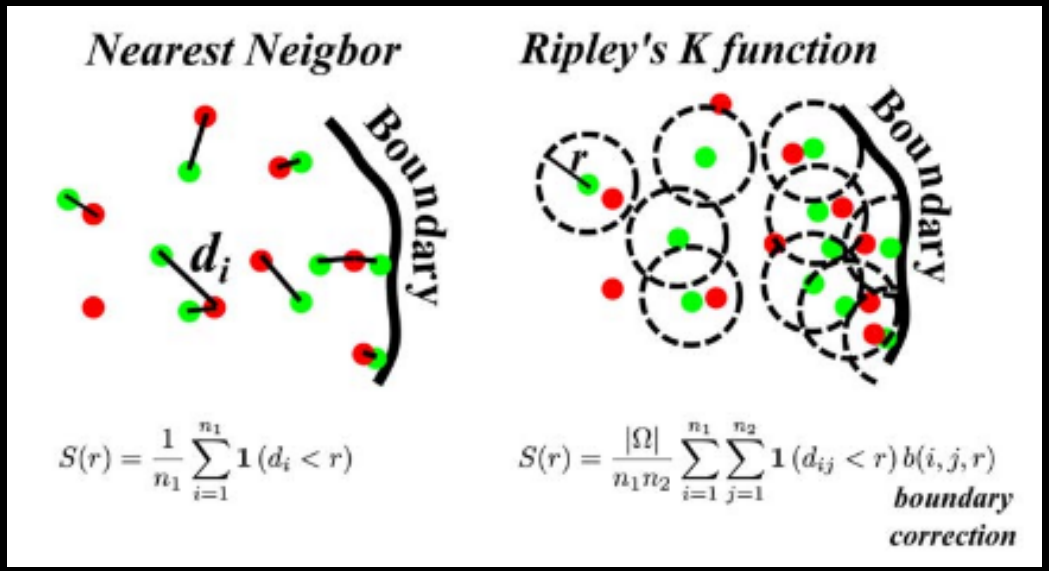
- With the `DiAna` plugin (Gilles et al. 2017)
- First segment the images (possible with DiAnA `Plugins->DiAna->DiAna_Segment`, or manually, other plugins..)
- Analysis of object distances with the plugin `Plugins->DiAna->DiAna_Analyze`



Label	ColocFromAvolume	ColocFromBvolume	ColocFromABvolume	Dist CenterA-CenterB	Dist min CenterA-EdgeB	Dist
objA1_objB1	100	1.571	1.547	9.271	6.649	3.23
objA2_objB1	100	2.773	2.698	7.654	8.142	2.52
objA3_objB1	100	0.090	0.090	8.018	5.488	6.84
objA4_objB1	100	0.055	0.055	10.793	5.338	9.62

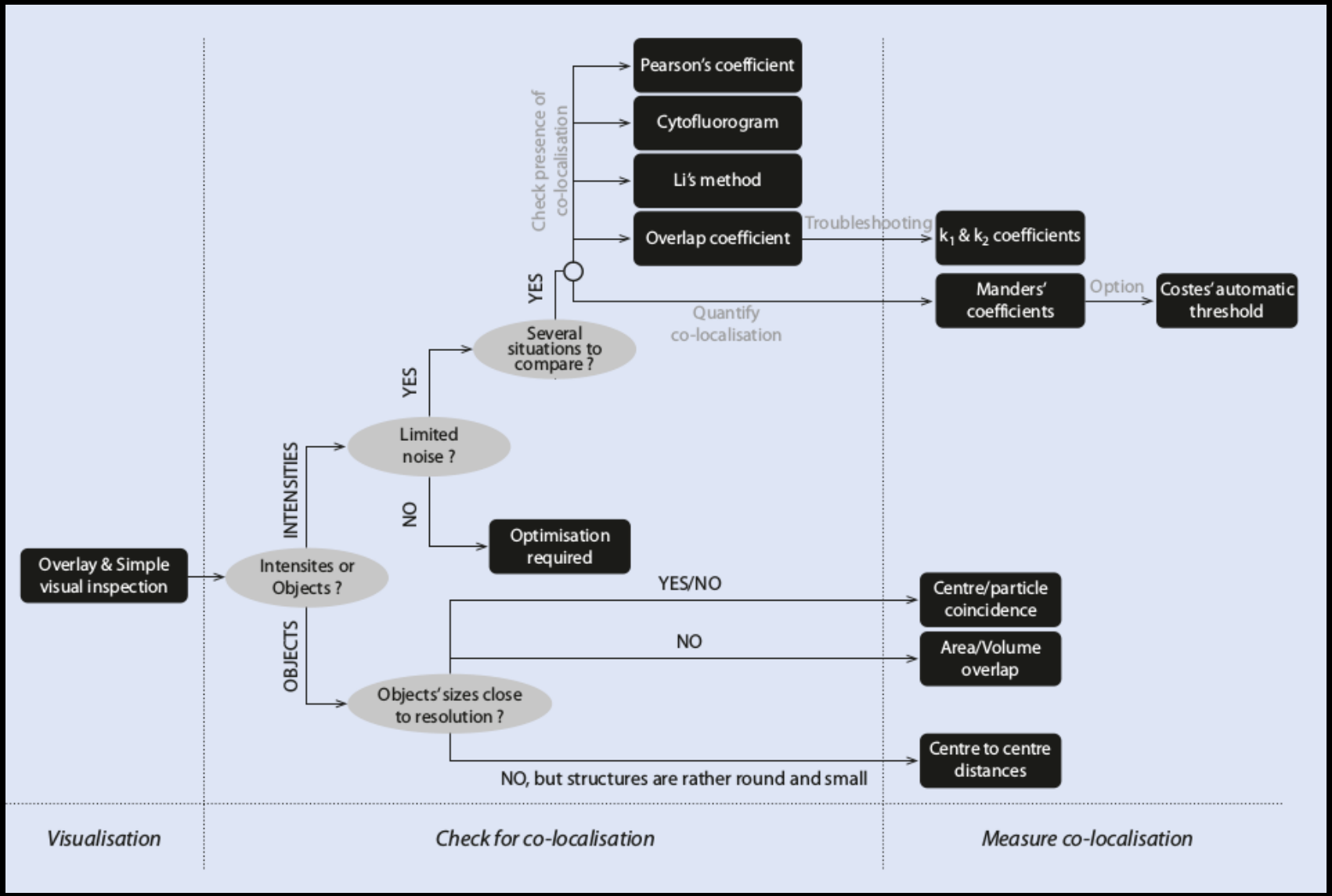
Neighboring measure

Segment object, and measure the number of neighbors of signal 2 in given area around each object of signal 1.
Can also be corrected to take into account boundaries (e.g. cell shape).



From Lagache et al., 2015

Colocalization workflow



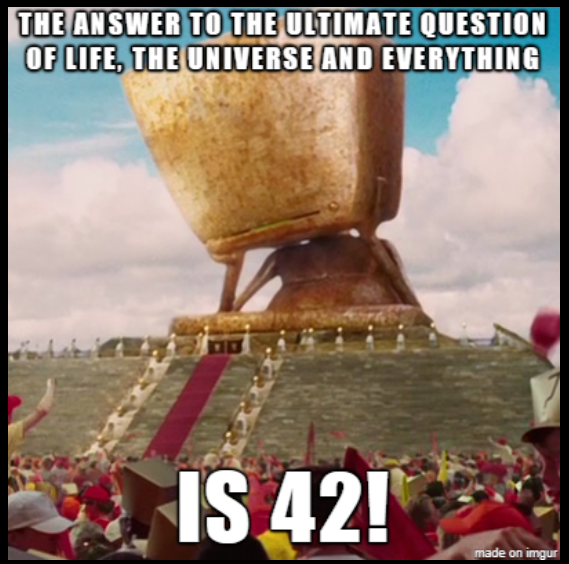
Adapted from Cordelieres and Zhang 2020

Interpretation

Is it significative ?

The colocalization methods give a score of colocalization. What should we make of it ?

- If goal is to compare different conditions
  $\Rightarrow$ Compare the distribution of the scores in each conditions



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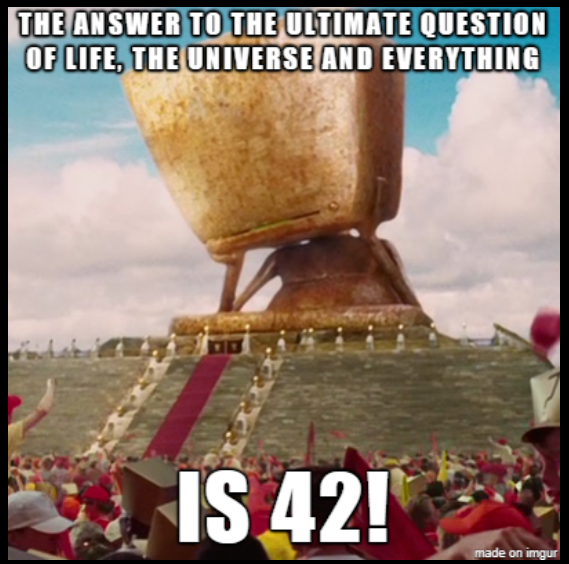
  - e.g. Obtained a Pearson coefficient of 0.6: is there correlation ?
  - Obtained a negative Pearson coefficient: could it be just random (few signals compared to area) or is there exclusion ?
  - Could it happen by chance given the two signals properties ?



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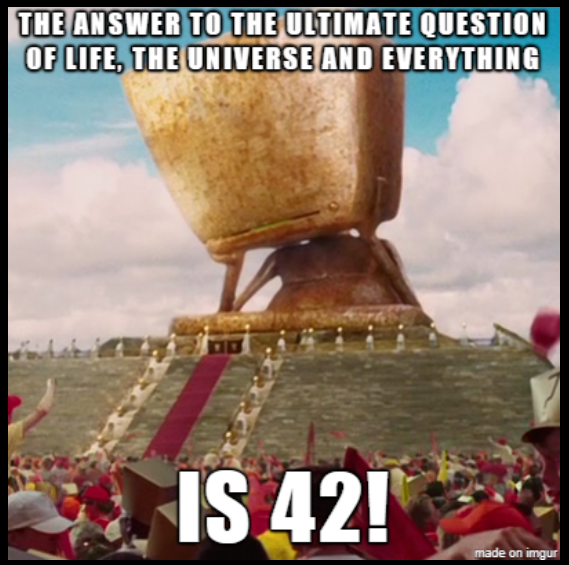
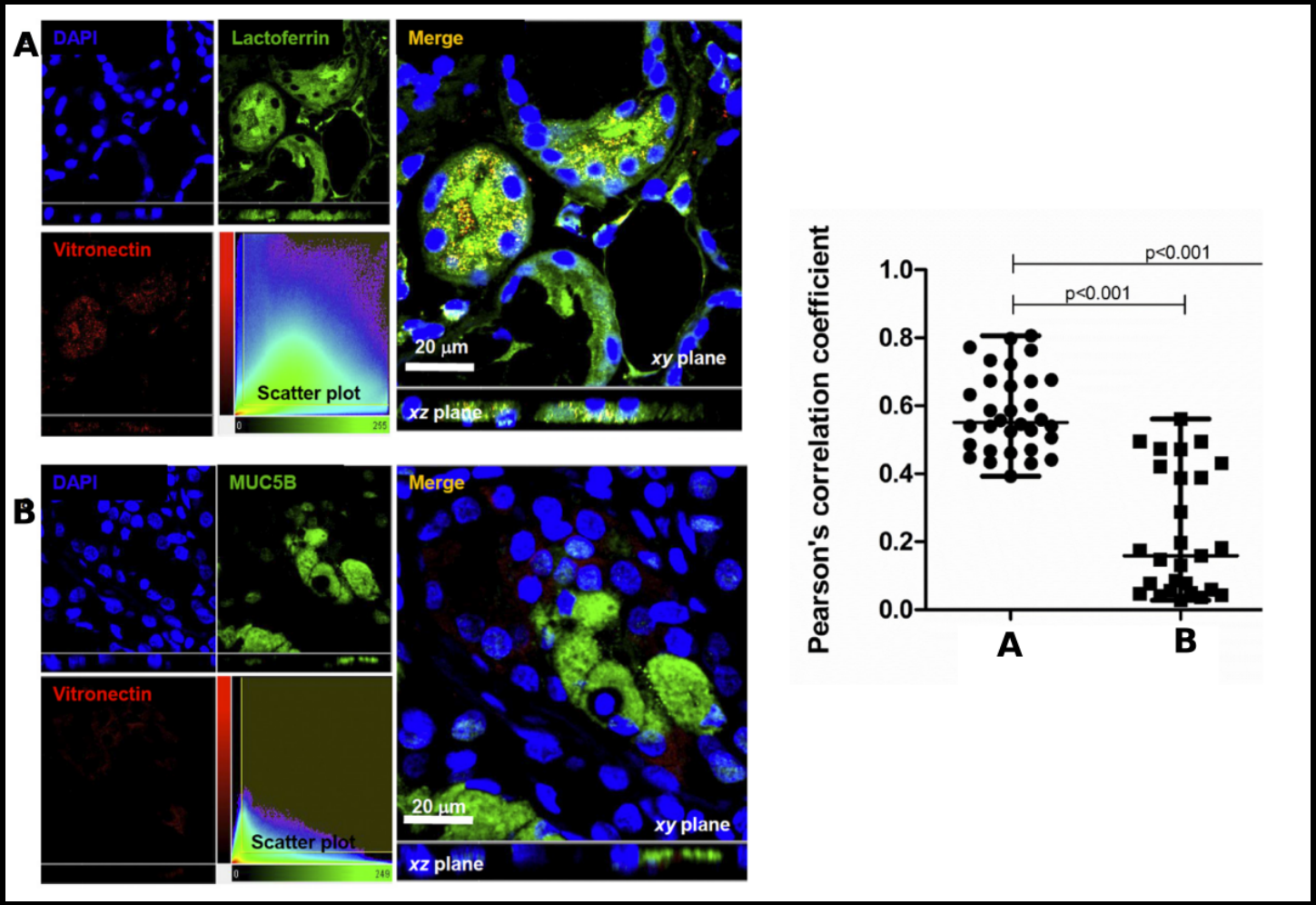
  $\Rightarrow$ Compare obtained score to scores that would be obtained randomly (e.g. Costes randomization method, cross-correlation).



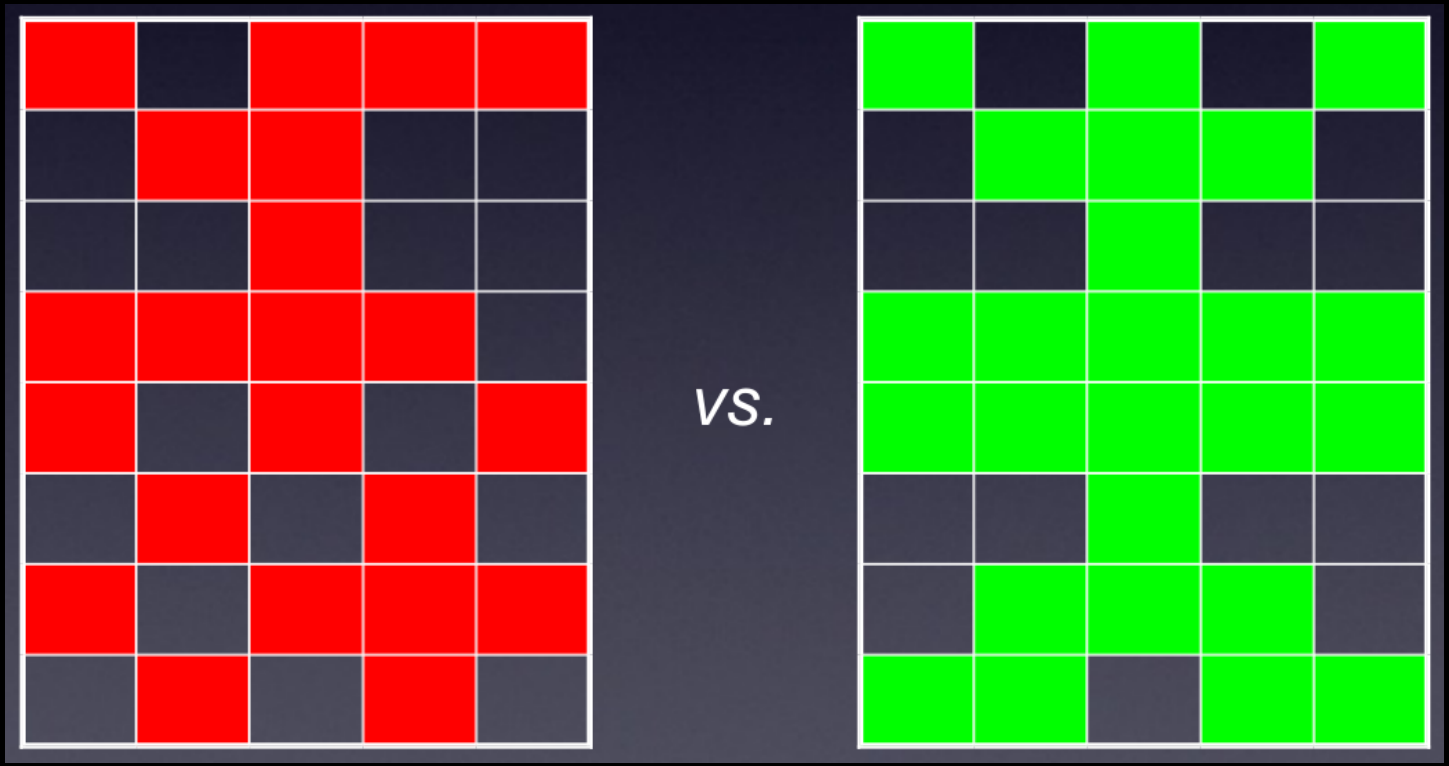
Comparing different conditions

Comparison between multiple conditions: exemple



From Salazar-Pelaez et al. 2015

Could it happen by chance ?

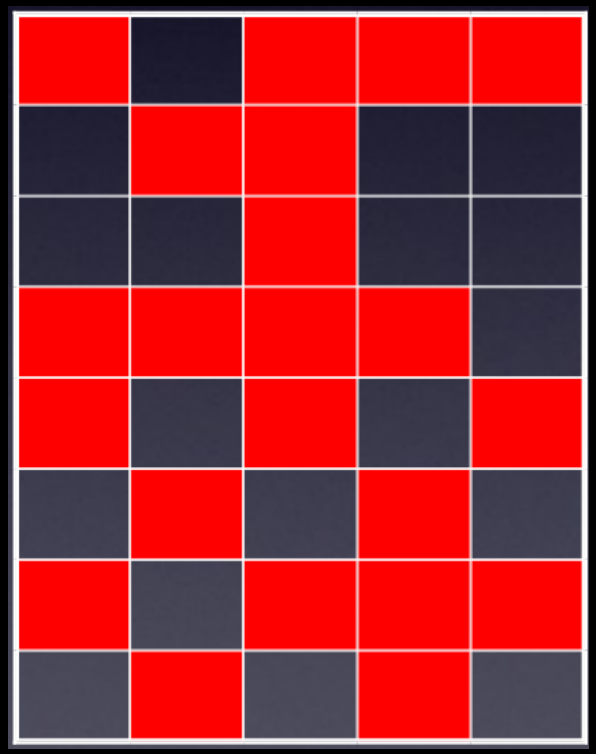


From colocalization course of D.J. White 2011

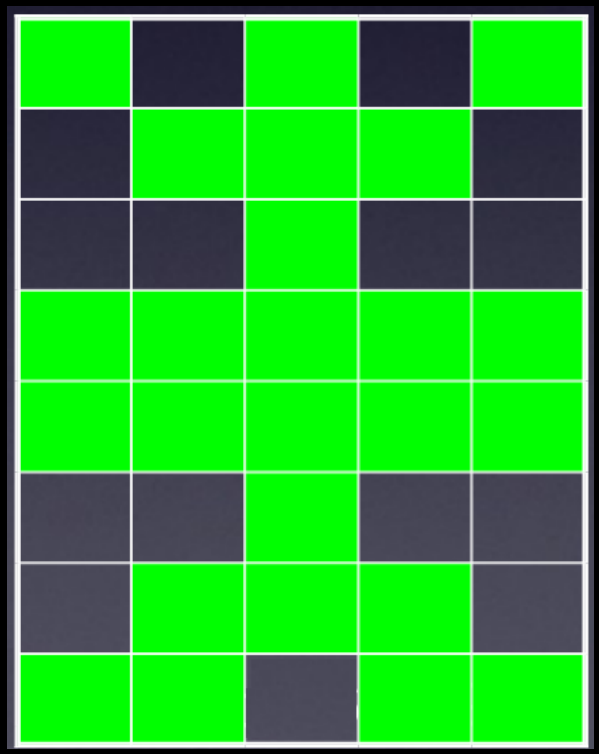
- 17/40 pixels overlap
- Lot of signal, could it happen just by chance ?

Could it happen by chance ?

- Costes method: shuffle the image pixels of one channel (e.g. Red)



vs



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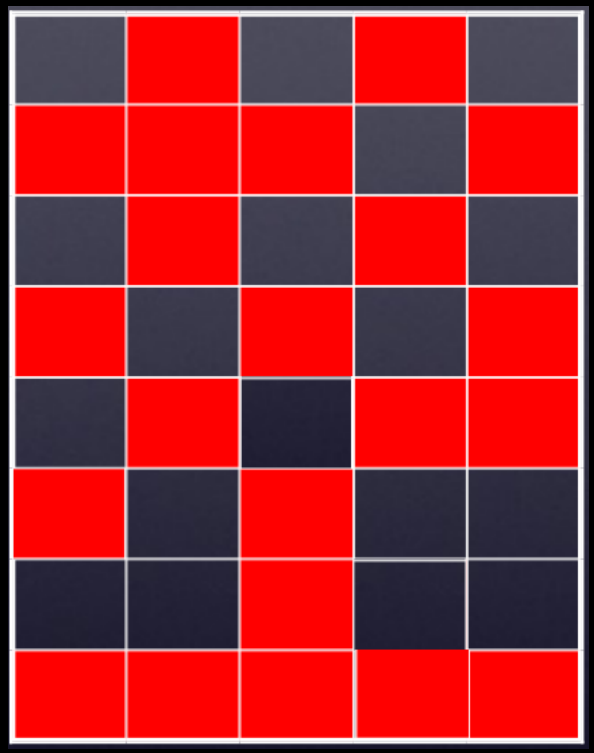
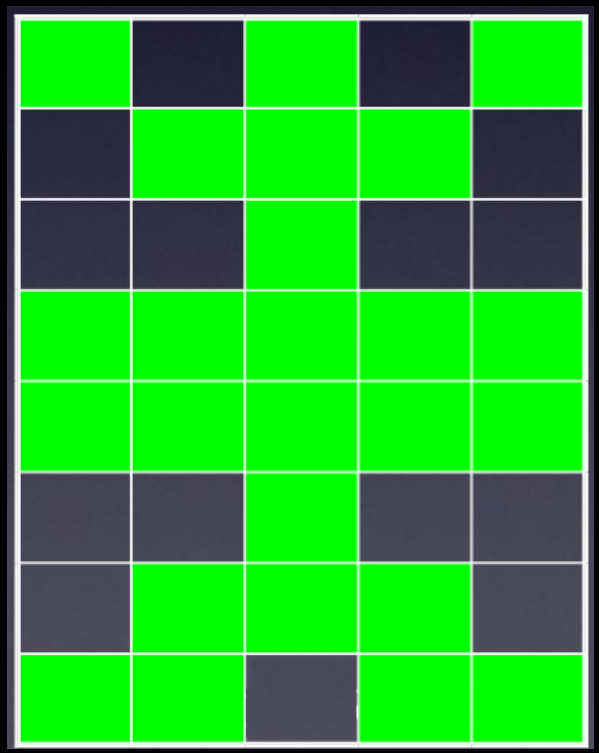


vs



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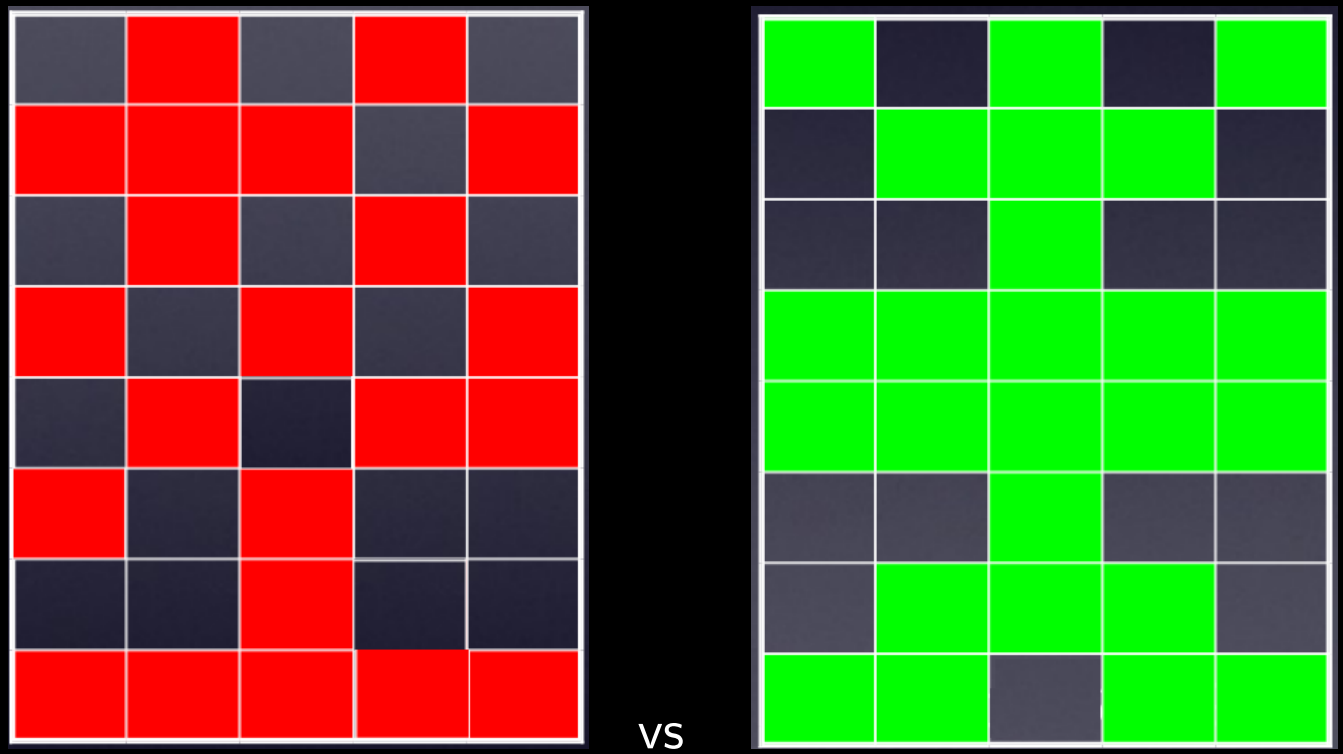

vs

- Calculate the correlation score with the new image and Green signal: 15/40

Could it happen by chance ?

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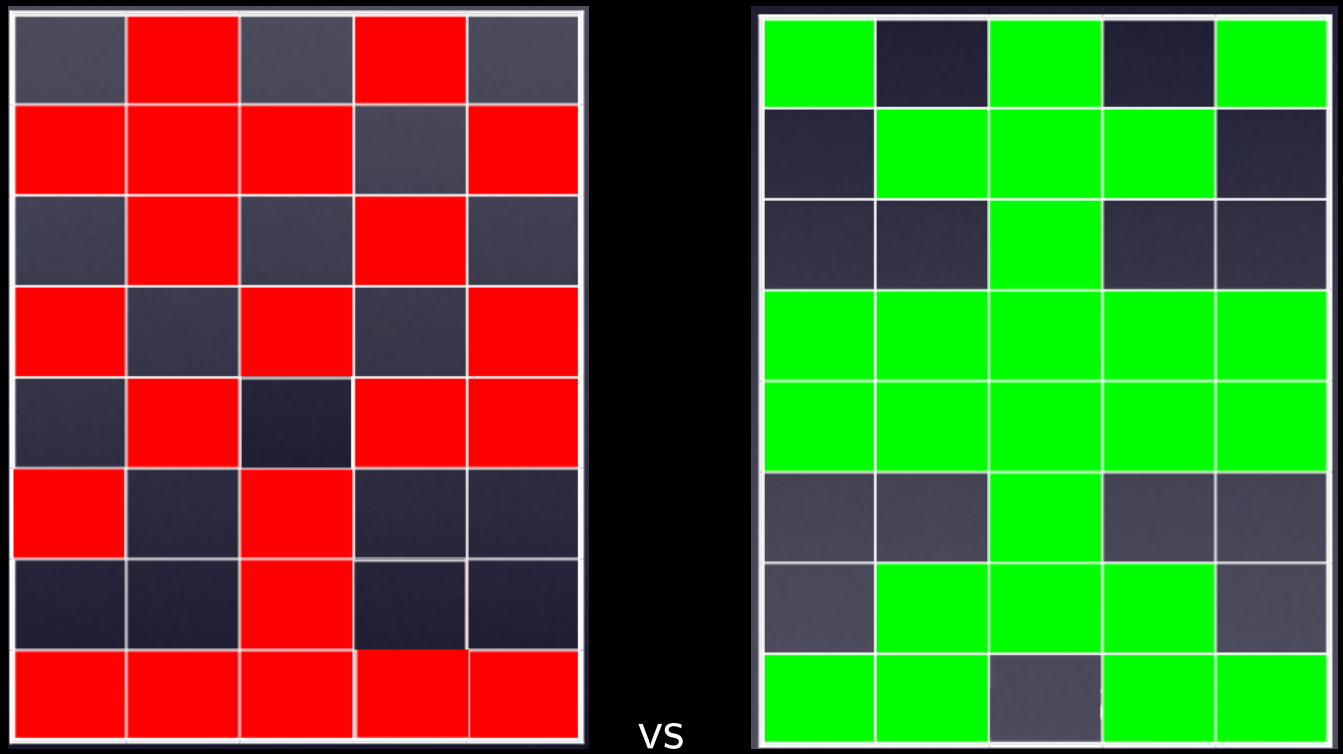
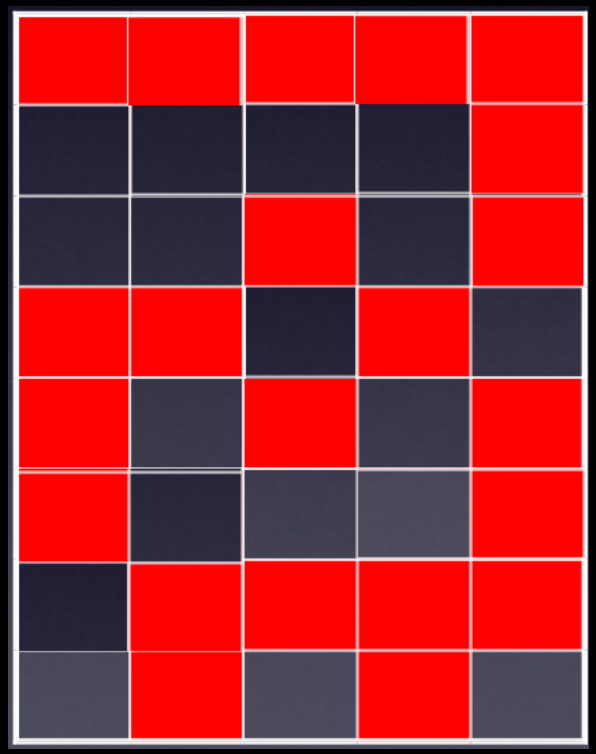
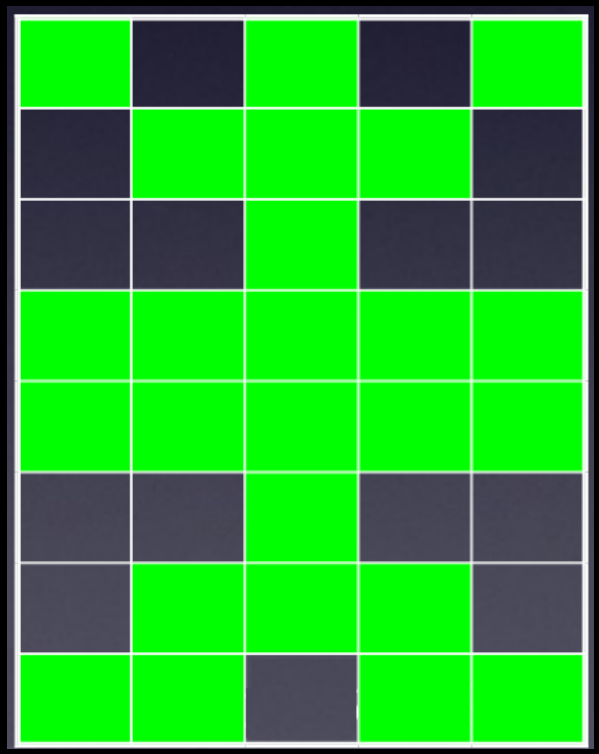


vs

- Calculate the correlation score with the new image and Green signal: 15/40
- Repeat

Could it happen by chance ?

- Costes method: shuffle the image pixels of one channel (e.g. Red)


vs


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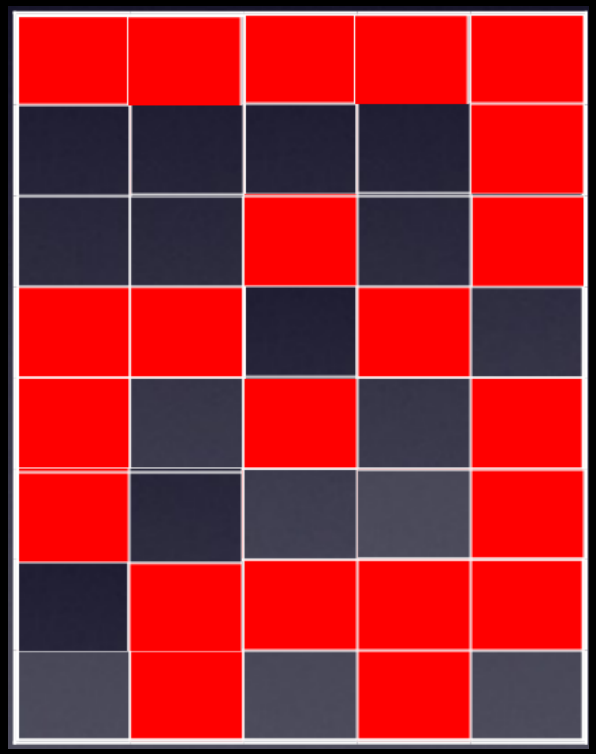
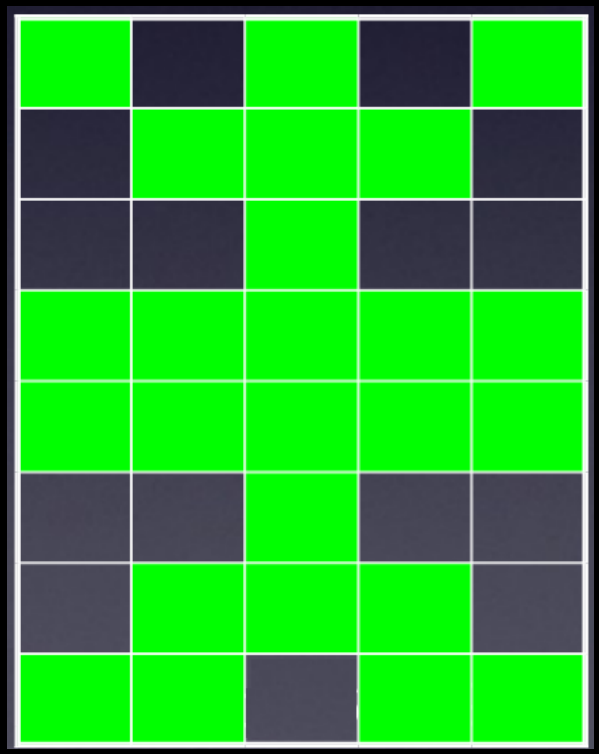
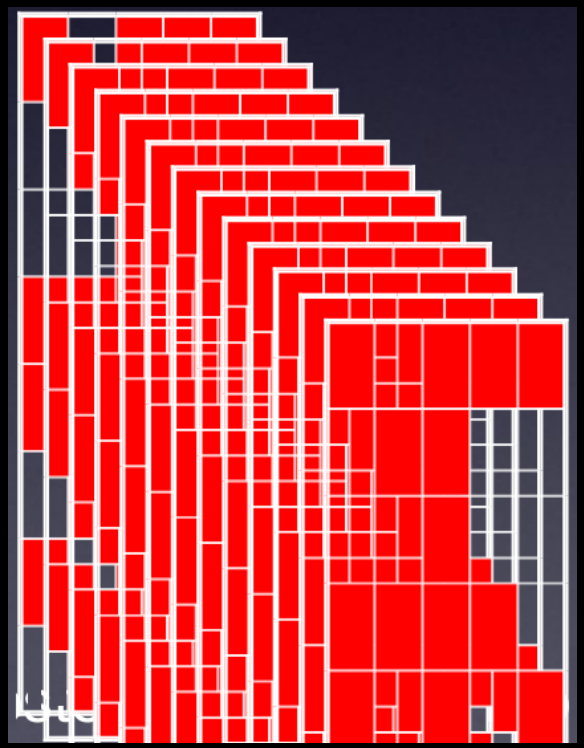

vs


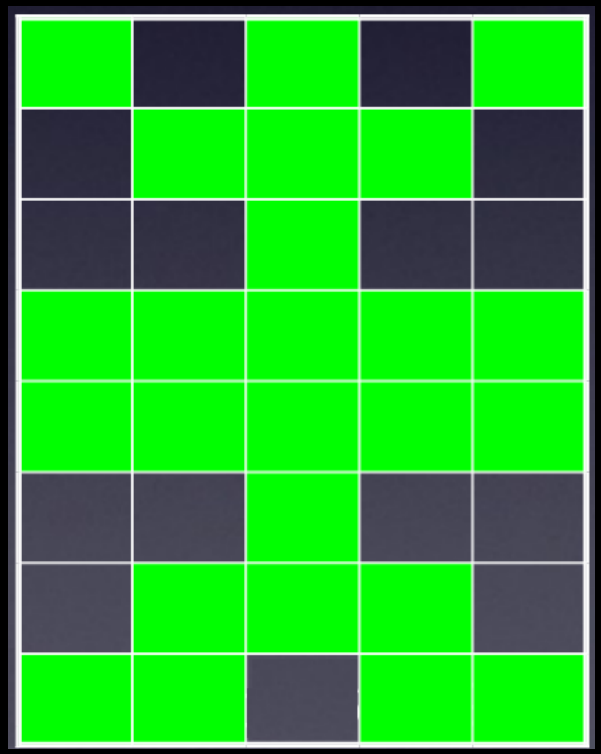
- Calculate the correlation score with the new image and Green signal: 15/40 16/40
- Repeat

Could it happen by chance ?

- Costes method: shuffle the image pixels of one channel (e.g. Red)



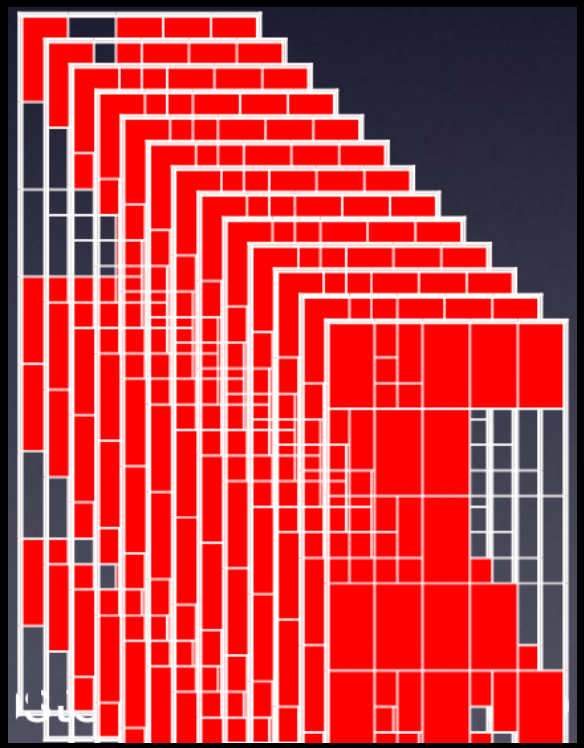
vs



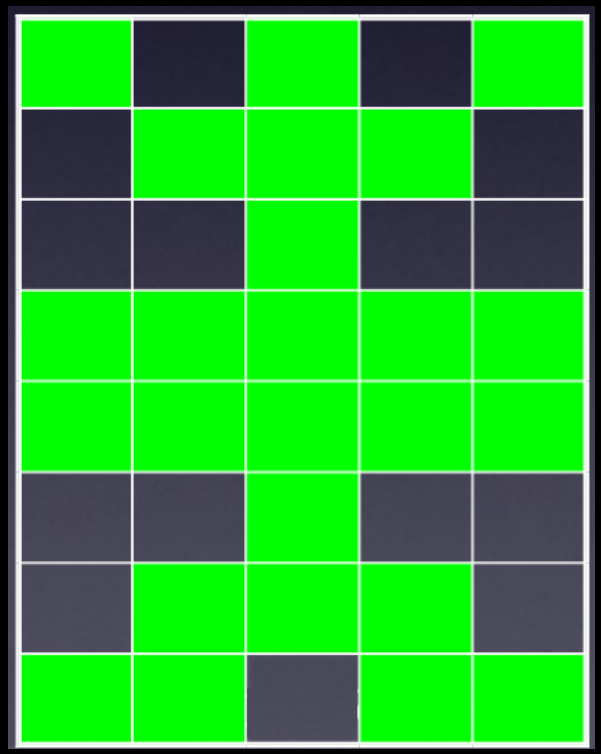
- Calculate the correlation score with the new image and Green signal: 15/40 16/40 18/40 20/40 13/40 25/40 11/40...
- Repeat a lot of times, N (N>100)

Could it happen by chance ?

- Costes method: shuffle the image pixels of one channel (e.g. Red)



vs



- Calculate the correlation score with the new image and Green signal: 15/40 16/40 18/40 20/40 13/40 25/40 11/40...
- Repeat a lot of times, N (N>100)
- Compare the original score 17/40 to the N generated ones
- If correlation $>$ 95% of the calculated scores, then there is correlation with 95% chance

Costes method

Remark:

- Here objects were simply squares so we shuffled the image by squares
- In practice, objects are larger than one pixel (size of their PSF)
- Neighbor pixels are thus correlated

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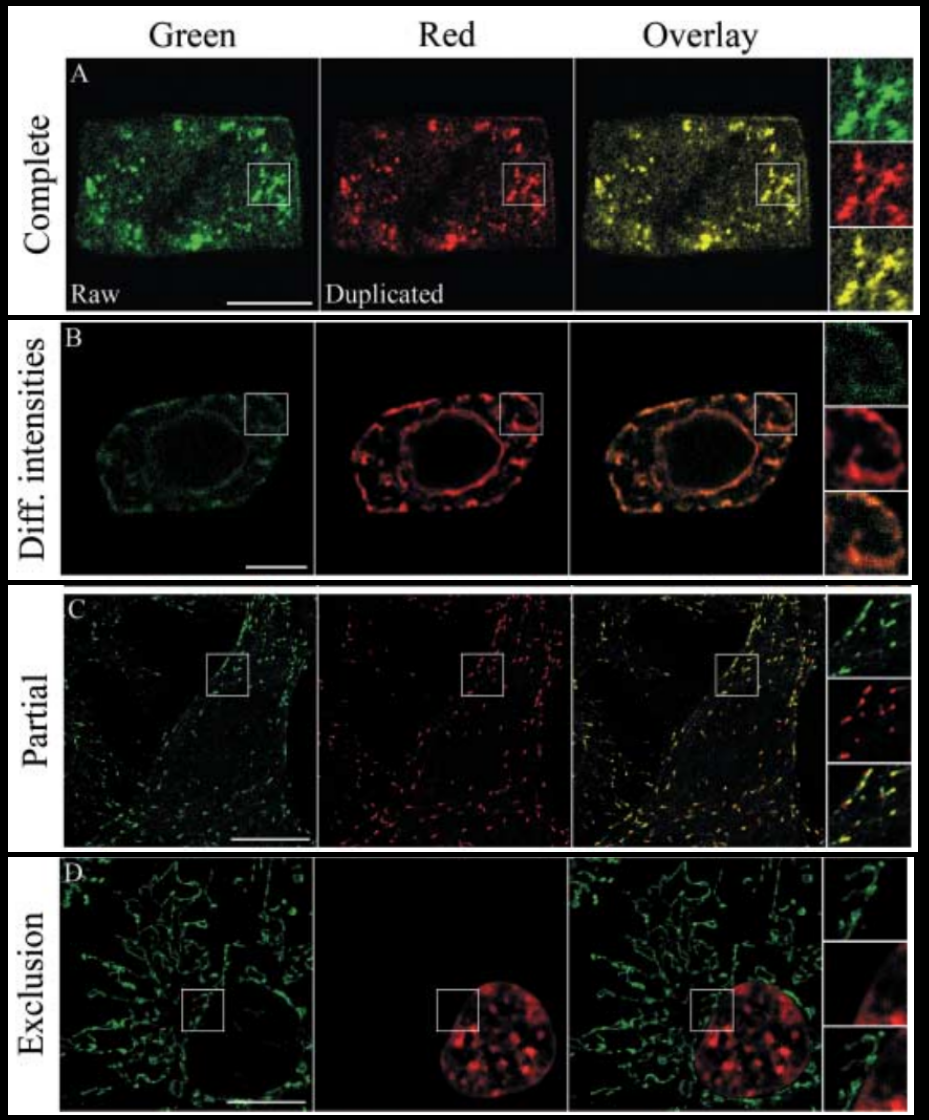
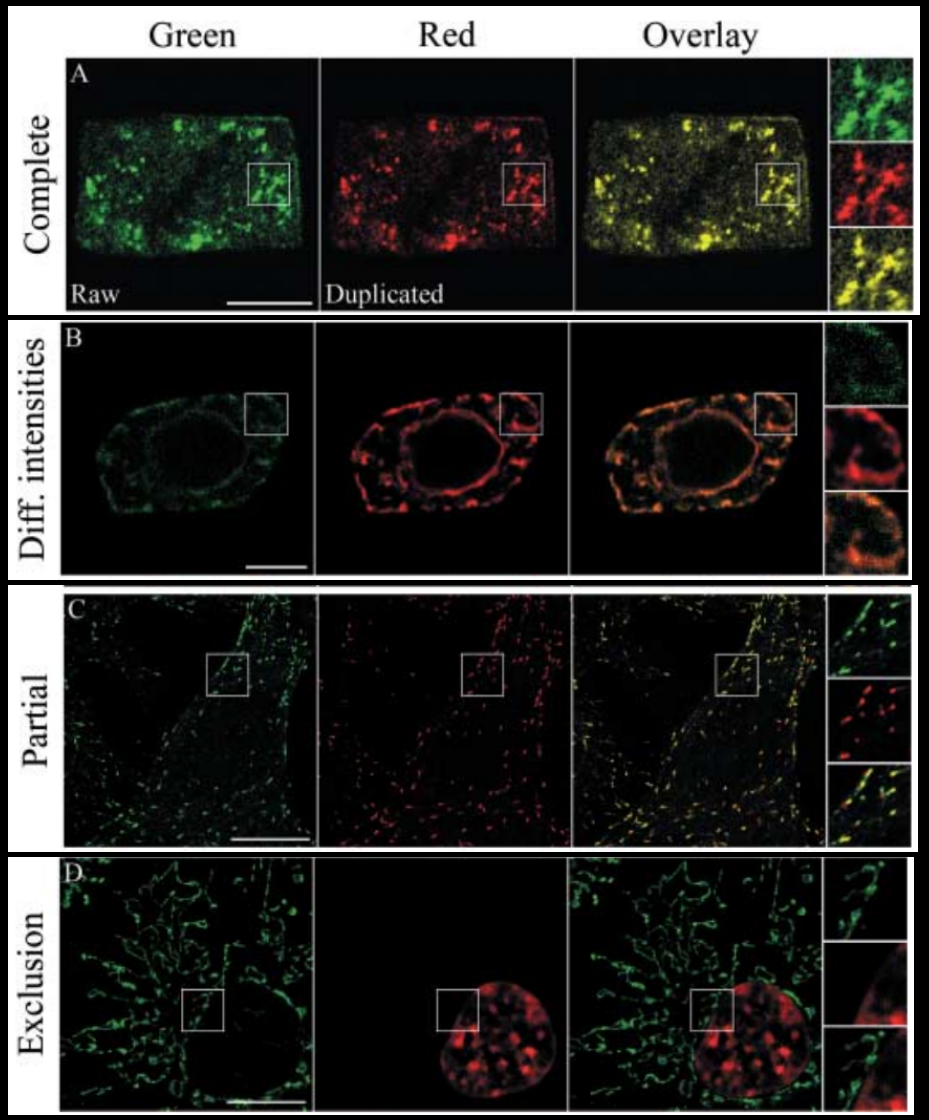
- Here objects were simply squares so we shuffled the image by squares
- In practice, objects are larger than one pixel (size of their PSF)
- Neighbor pixels are thus correlated
- Shuffle the images by PSF-sized chunks, not by pixels

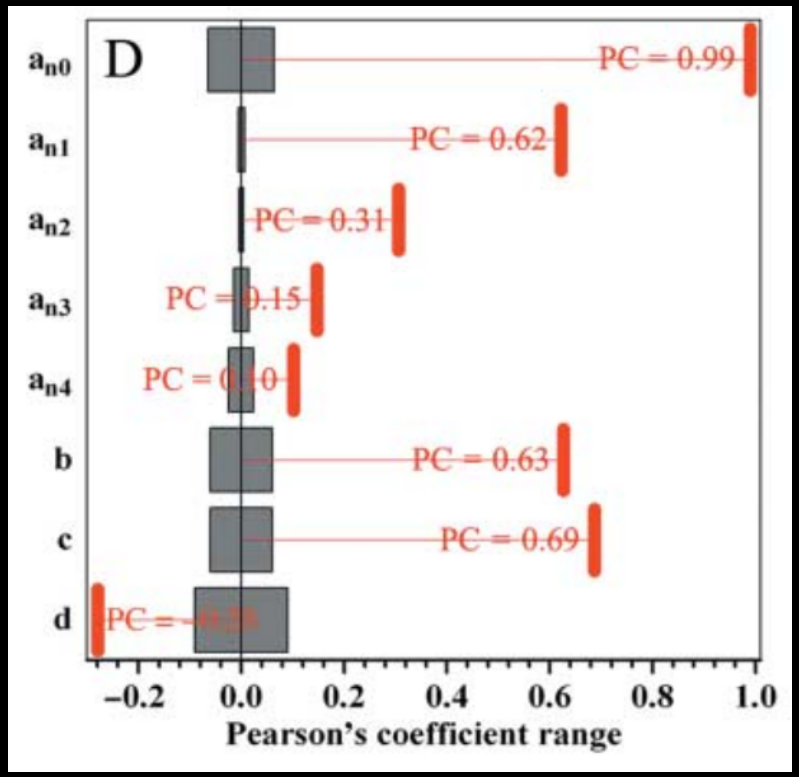
Costes method: example



Costes method: example



From Costes et al. 2004



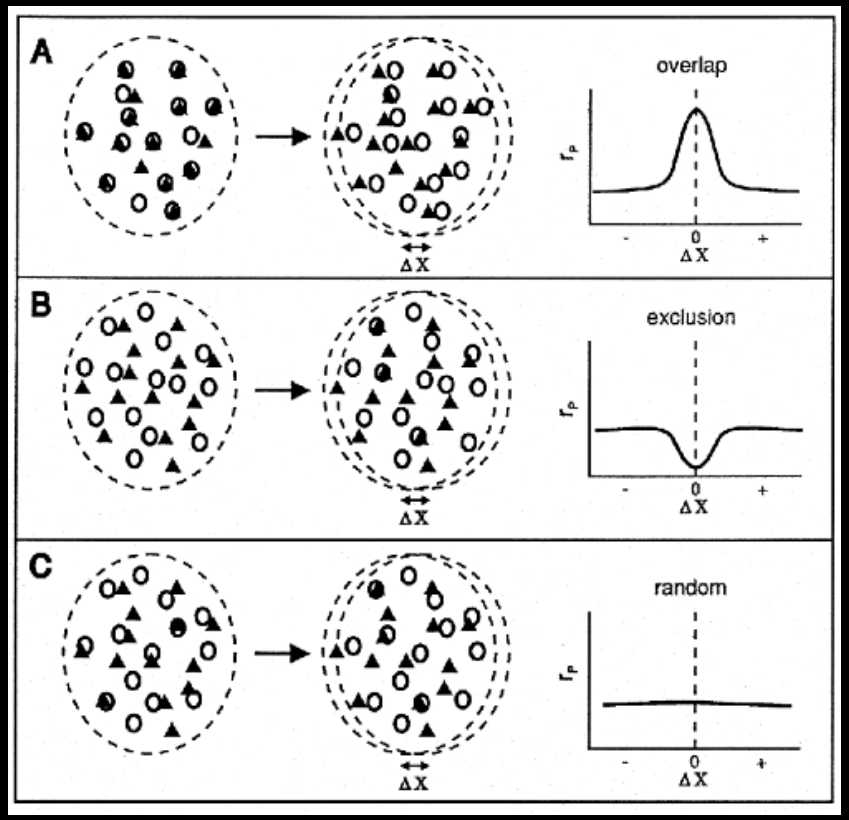
A, B, C: 100 % chance of correlation

D: 0 % chance, and anti-correlation (below random)

Van Steensel's method: cross-correlation

- Shift spatially all the image by a displacement horizontal vector ΔX
- Calculate new correlation coefficient
- Shift by a higher ΔX, repeat

⚠ Only on small and isotropic particles (orientation of the shift)

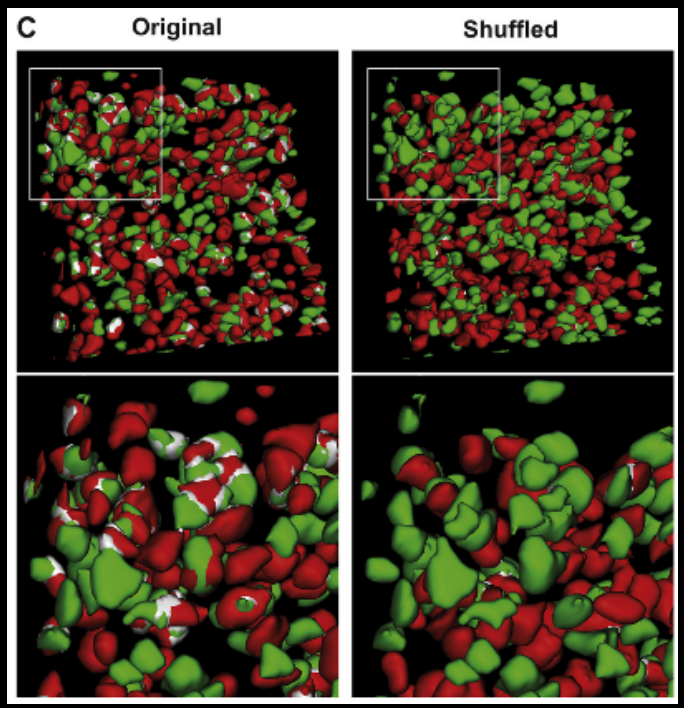


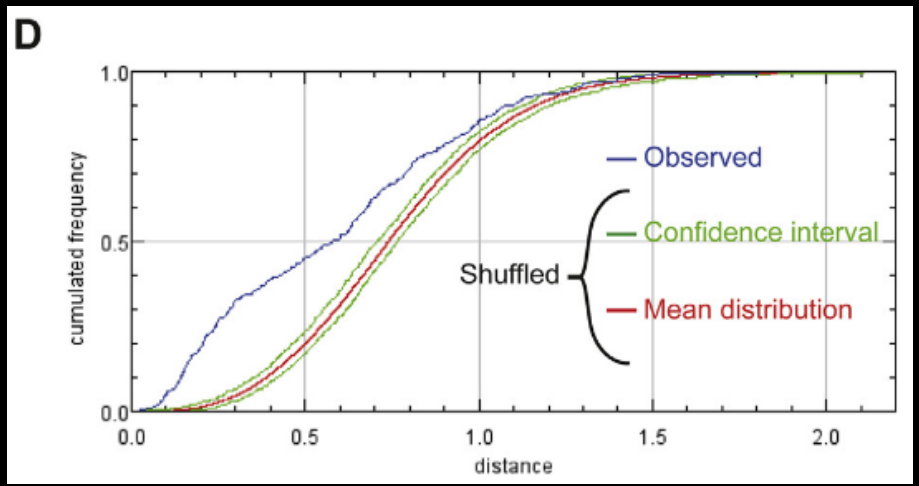
From Van Steesel et al. 1996

Shuffling method for object-based distance

Same principle for object-based method (Andrey et al. 2010):

- Shuffle the objects position
- Calculate center-center (or other) distance in shuffled image
- Compare distribution of distances between original image and shuffled ones

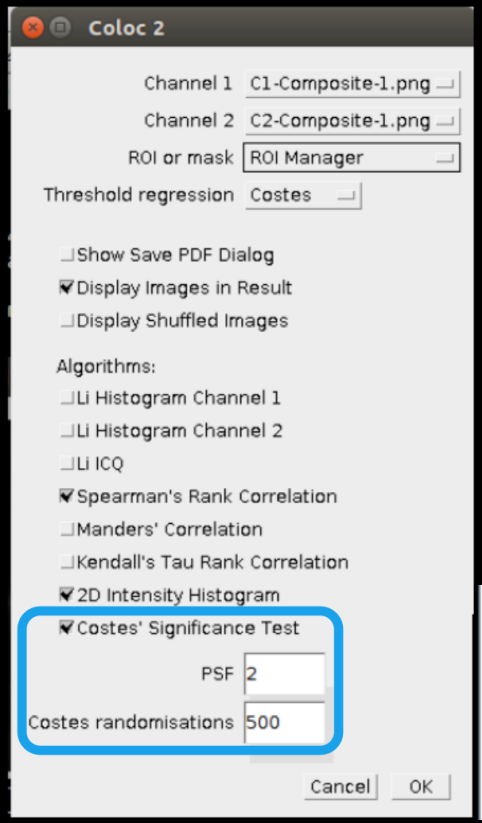




From Gilles et al. 2016

How to do with ImageJ

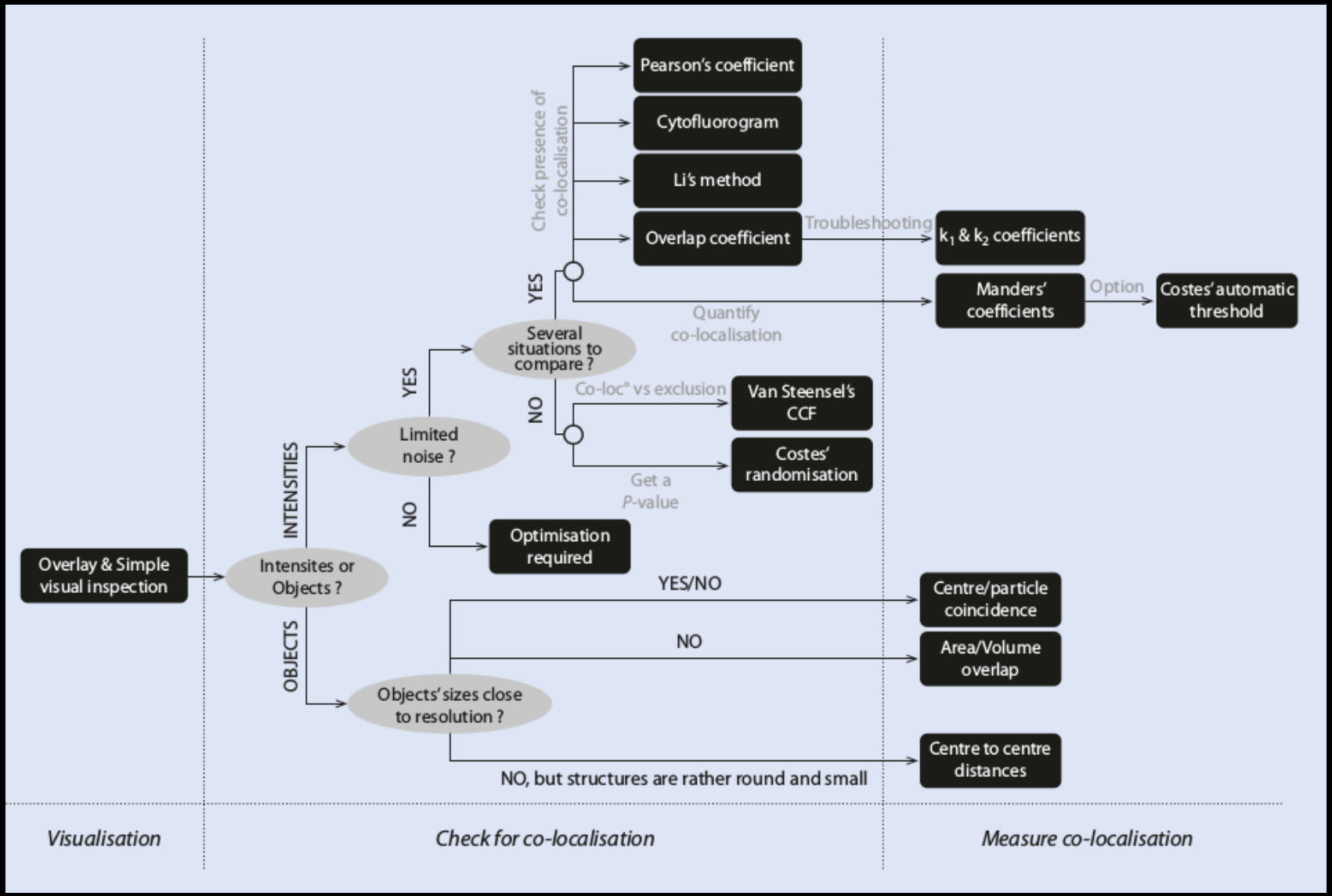
- Costes's randomization method, Van Steensel method, object based shuffling are proposed in ImageJ plugins
- Number of generated random images, ideally > 100



Costes P-Value	0.91
Costes Shuffled Mean	-0.02
Costes Shuffled Std.D.	0.05
Ratio of rand. Pearsons >= actual Pearsons value	10.00

Conclusion

Colocalization workflow



Adapted from Cordelieres and Zhang 2020

Some tools-courses

- Use your favorite image analysis framework (`ImageJ/Fiji`, `Icy`, `Python`, `Matlab`, `Imaris...`)
- Common plugins in ImageJ: `Coloc2`, `Colocalization_Threshold`, `JaCoP`, `DiAnA`, `ComDet`, `RG2B Colocalization`

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- Course: Quantitative imaging for colocalization analysis:
- ImageJ Colocalization plugin description:
- Icy colocalization studio plugin:
- Documentation for a suite of Colocalization plugins in ImageJ:
- *A guided tour into subcellular colocalization analysis in light microscopy*, Bolte et Corderlieres, 2006
- These slides: github.com/gletort/CoursColocalisation/