

Deconstructing co-localisation workflows: *from co-expression assessment to super-resolved co-distribution analysis*

Fabrice P Cordelières, PhD

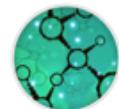
Bordeaux Imaging Center

Centre Broca Nouvelle-Aquitaine

146, rue Léo-Saignat

33077 Bordeaux

fabric.cordelier@u-bordeaux.fr

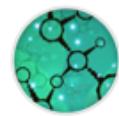


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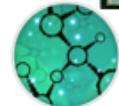
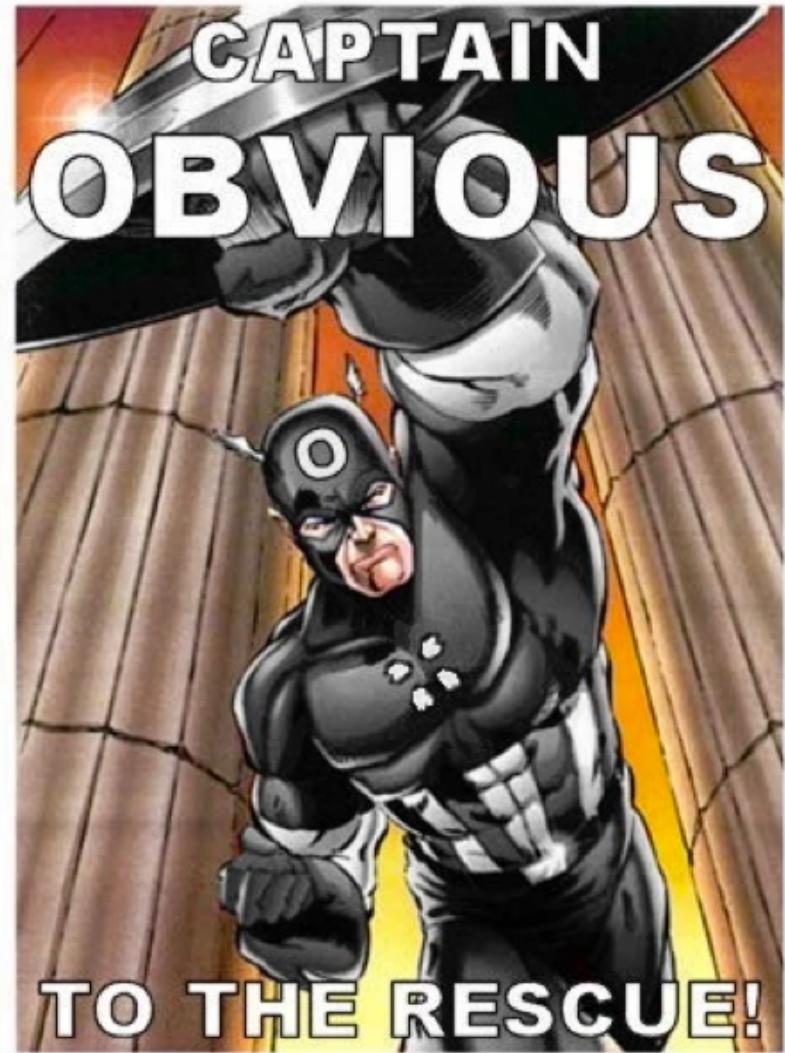
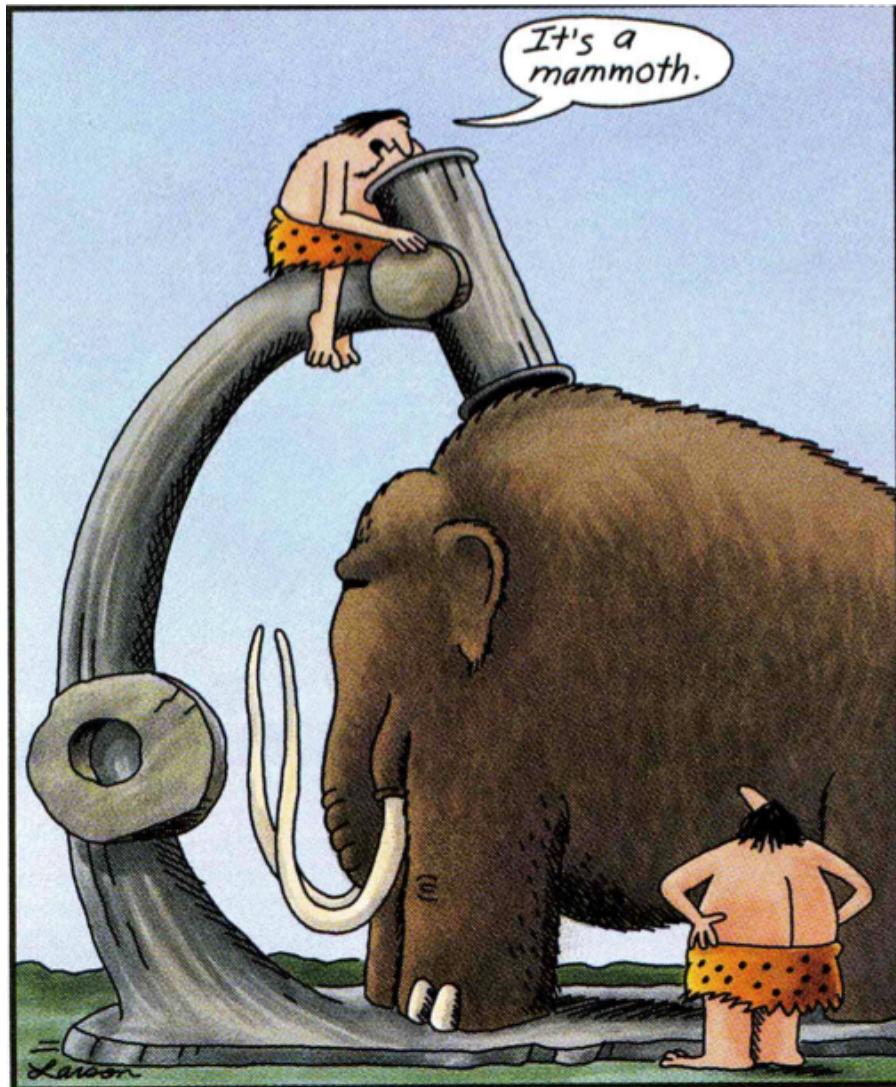
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Why should we quantify co-localisation ?

Don't trust your eyes !



Why should we quantify co-localisation ? Don't trust your eyes !

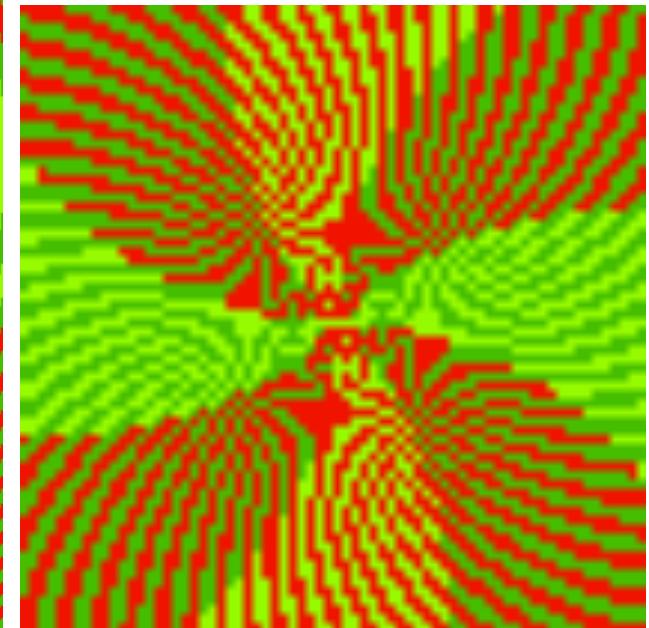


Why should we quantify co-localisation ?

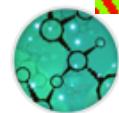
Don't trust your eyes !



Obvious, was it ?



File ▶ Open Samples ▶ Spirals
(Macro) in Fiji



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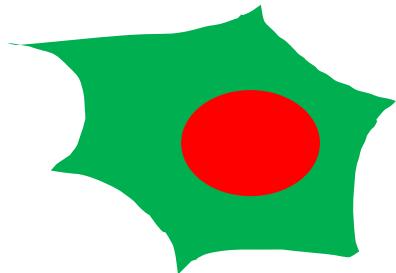
Co-localisation

One word, many meanings

Co-localisation

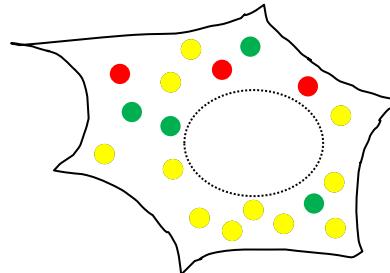
Co-expression

Two proteins are located within the same structure/cell



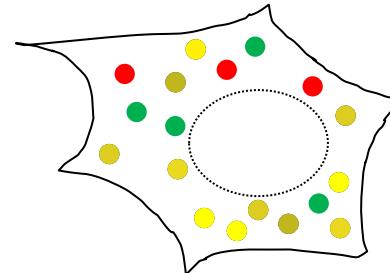
Co-occurrence

At the current resolution, the positions (of some) of the two labelling can't be distinguished



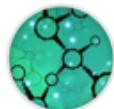
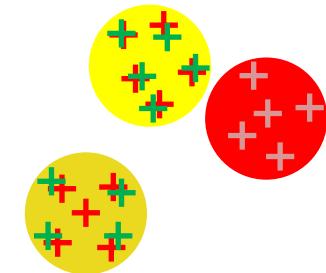
Correlation

At the current resolution, for (some) positions, the intensities of the two labelling are linked



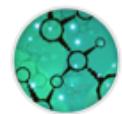
Co-distribution

At the current resolution, the spatial distributions of the two labelling are linked



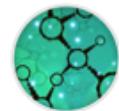
Co-localisation workflows

Overview



Checking data integrity

image
raster data
application 24-bit enc
used formats
smaller digital
JPEGe
file



Co-localisation workflows

Checking data integrity

Checking data integrity

Pre-processing

Choosing a reporter/ metric

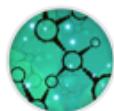
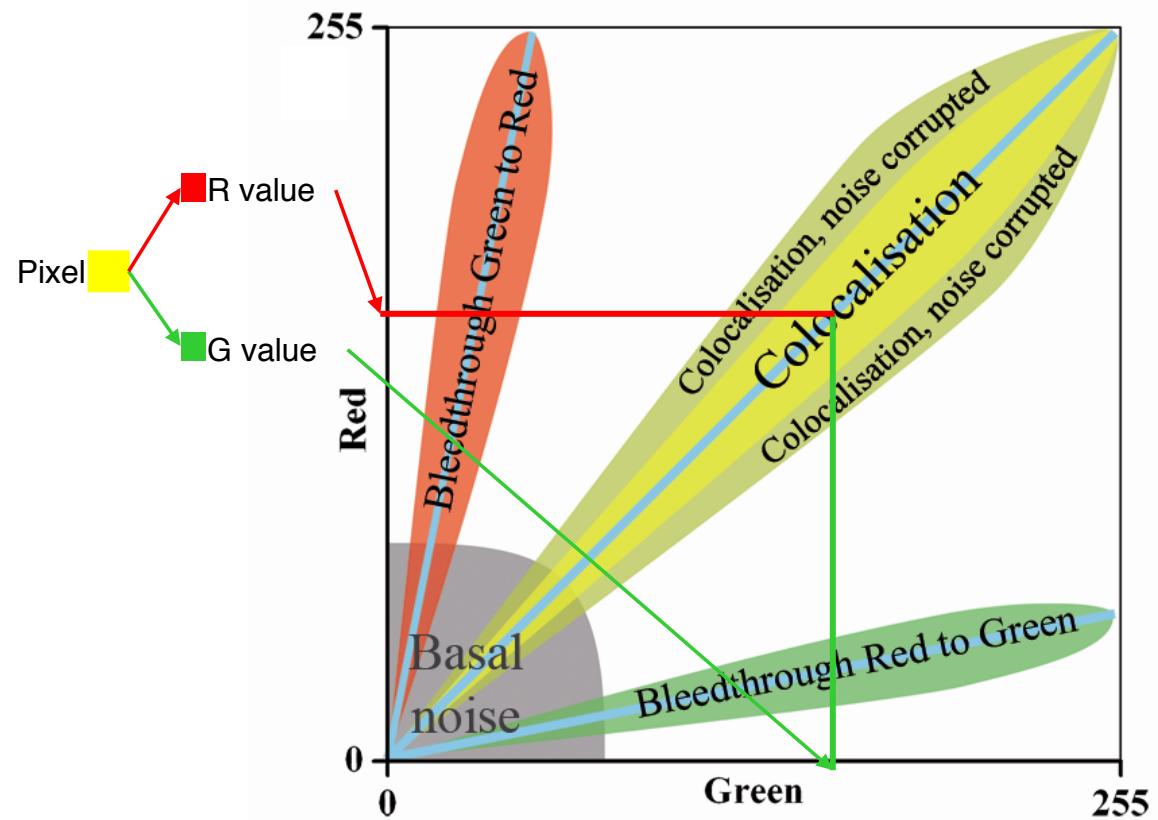
Comparing Interpreting

Assembling a workflow

Dyes

Check for bleethrough and/or cross-talk

One possible way is to use the cytofluorogram, looking for dots clouds close to the axis



Co-localisation workflows

Checking data integrity

Checking data integrity

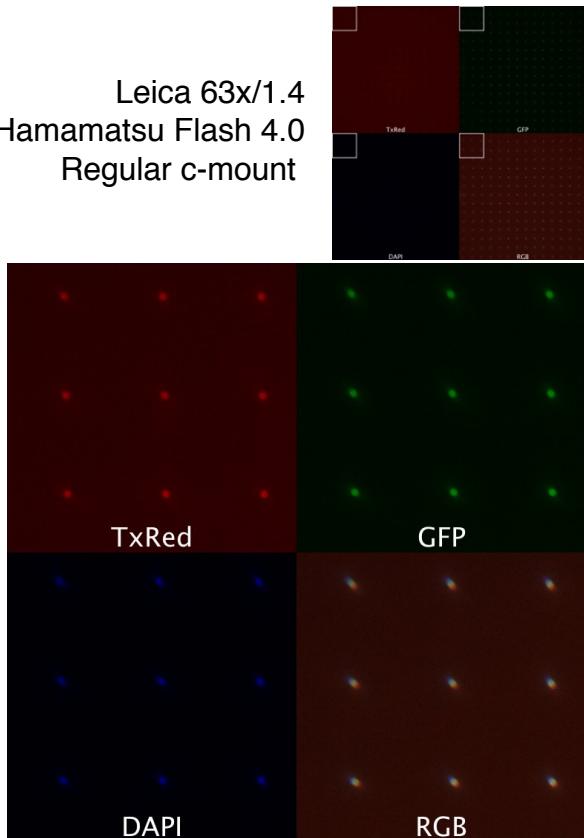
Pre-processing

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Comparing Interpreting

Assembling a workflow

Leica 63x/1.4
Hamamatsu Flash 4.0
Regular c-mount



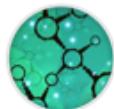
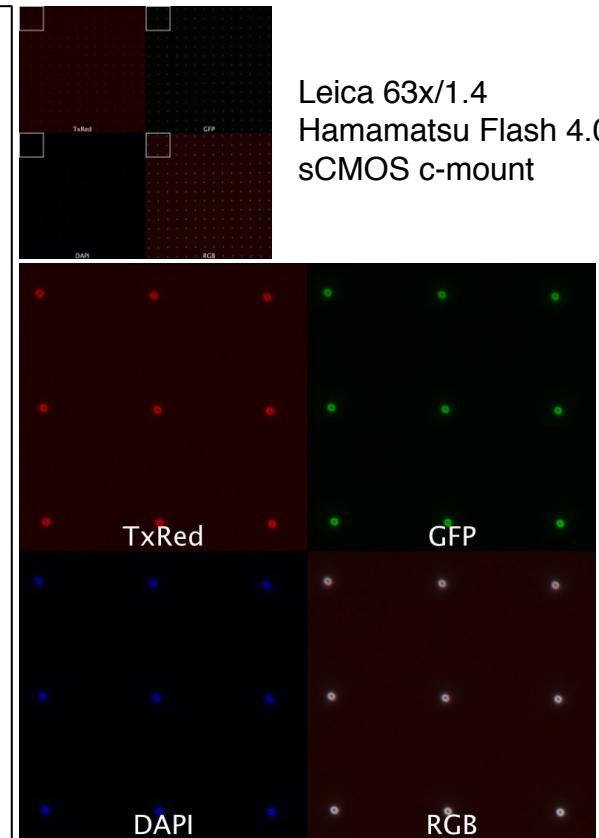
Co-registration

Make sure what should be co-localised is co-localised

Use reference slides (fluorescent beads, Argolight slide) to check for misregistration and aberrations

Have a look at the MetroloJ plugin !

Leica 63x/1.4
Hamamatsu Flash 4.0
sCMOS c-mount



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Co-localisation workflows

Checking data integrity

Checking data integrity

Pre-processing

Choosing a reporter/ metric

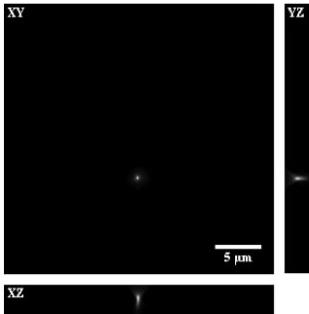
Comparing Interpreting

Assembling a workflow



16 avril 2009 21:24
PSF profiler report on My PSF.tif

Profile view:



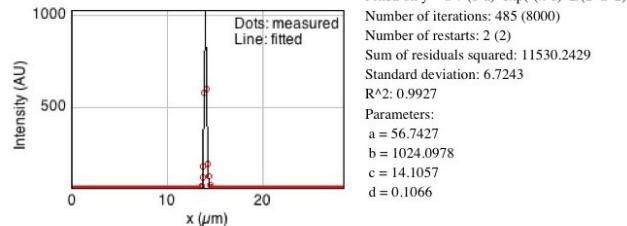
Microscope infos:

Microscope: Confocal
Wavelength: 580.0 nm
NA: 1.4
Sampling rate: 0.112x0.112x0.1 μm
Pinhole: 1.0 Airy Units

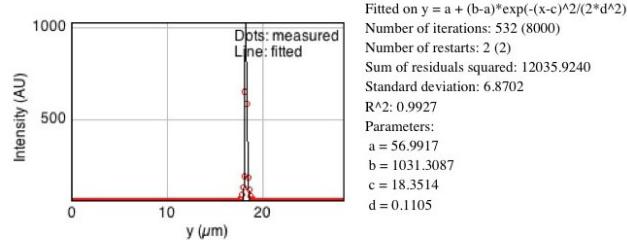
Resolution table:

	FWHM	Theoretical resolution
x	0.251 μm	0.166 μm
y	0.26 μm	0.166 μm
z	0.753 μm	0.414 μm

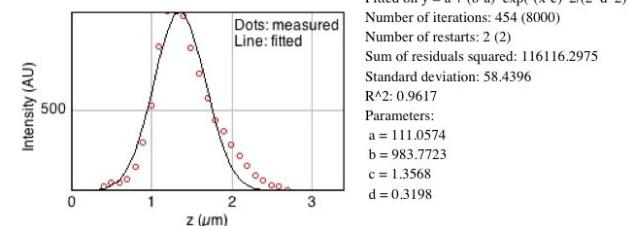
X profile & fitting parameters:



Y profile & fitting parameters:



Z profile & fitting parameters:

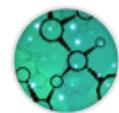


Resolution

Know your limits

Use reference slides
(fluorescent beads, Argolight slide) to measure resolution

Have a look at the MetroloJ plugin !



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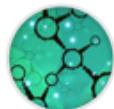
Have a look at the MetroloJ plugin !

Resolution

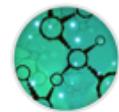
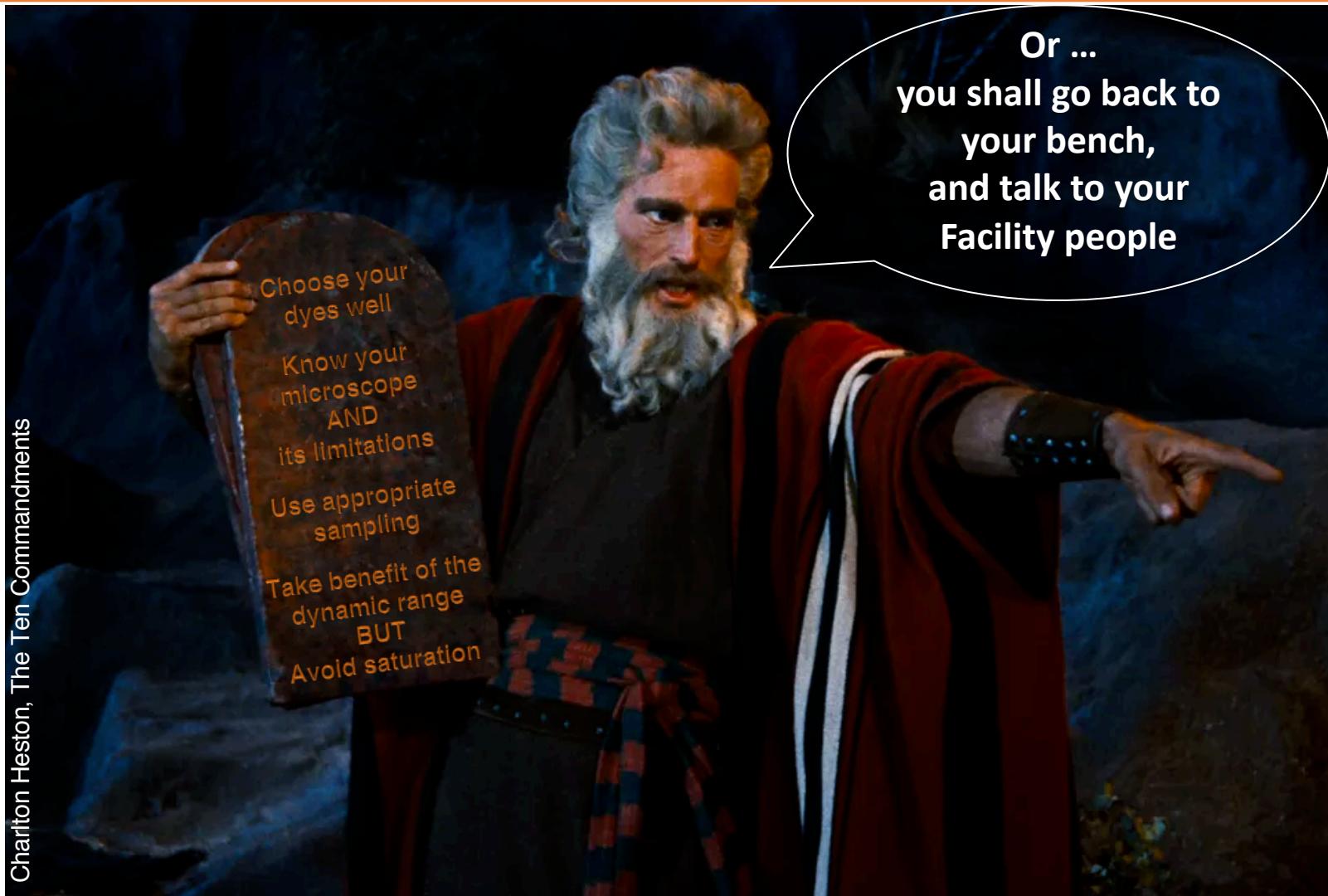
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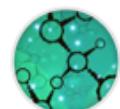


Without good images, there is no point going further !



Pre-processing

image
raster data used formats
application 24-bit erg
metafile
quality
representations
language
pixels
Portable
Format
Graphic
format
pixel
size
lossy
graphic
vector
information
number
original
algorithm
bit
GIF
page
Vector
supported
lossless
widely
wa
smaller
digital
SVG also
camera
losses
provide
based
color
Zvi
file
JPEG



Co-localisation workflows

Pre-processing

Checking data integrity

Pre-processing

Choosing a reporter/ metric

Comparing Interpreting

Assembling a workflow

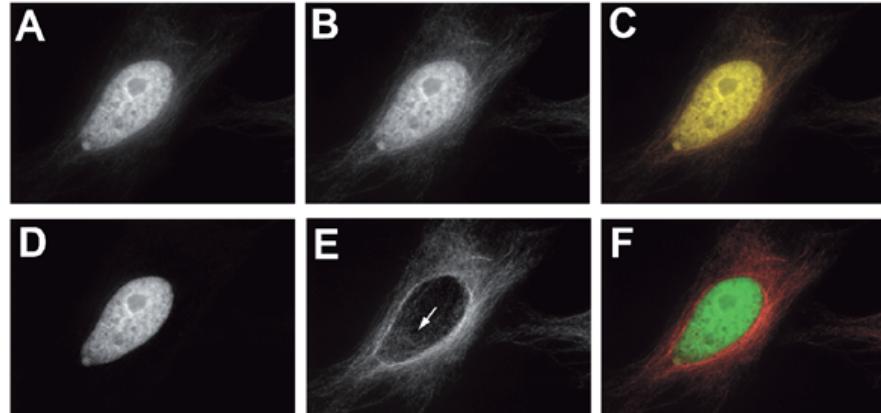
Corrections

Image acquisition-related

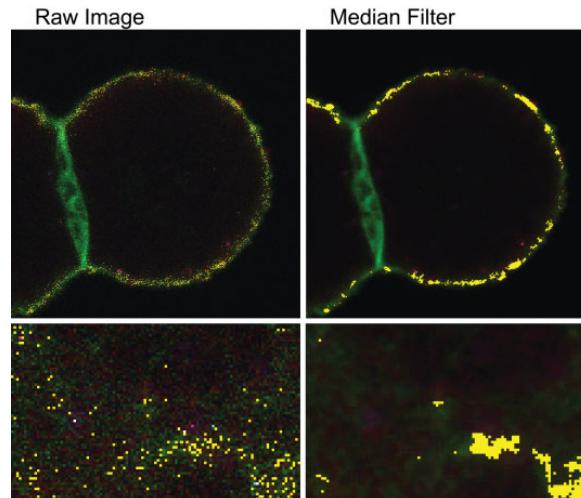
- Bleedthrough/crosstalk: better go back to the microscope before trying unmixing
- Chromatic shift: better be corrected on the microscope before trying to compensate by translation

Background and noise

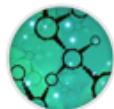
- Median filtering: Ok but impairs resolution
- Denoising: not to be used as a black box !



Zimmermann, Adv. Biochem. Eng. Biotechnol., vol. 95, pp. 245–265, 2005.



L. Landmann and P. Marbet, Microsc. Res. Tech., vol. 64, pp. 103–112, 2004.



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Co-localisation workflows

Pre-processing

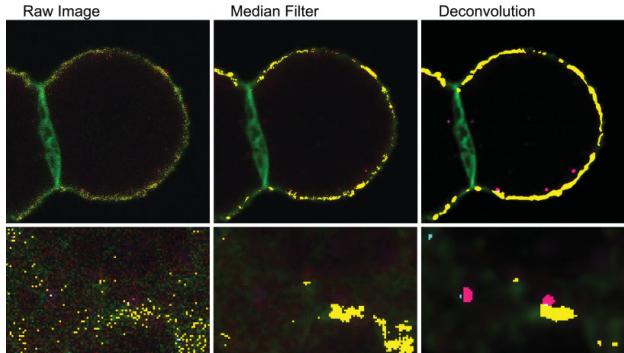
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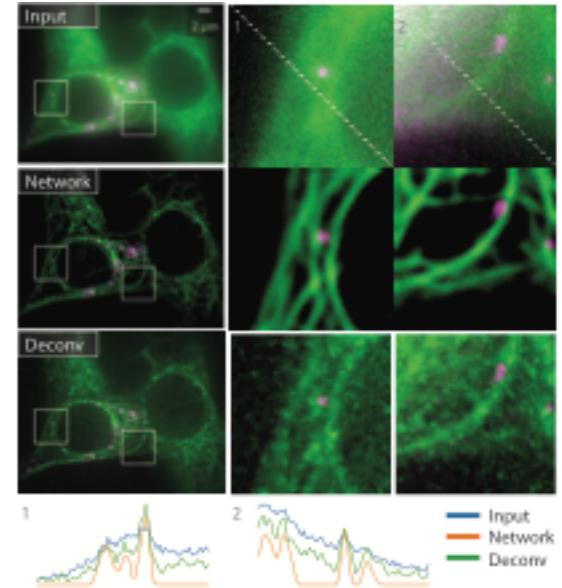
Restoration

Deconvolution:

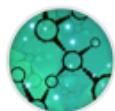
- Not to be used as a black box !
- Choose the algorithm well (conservative, nb iterations/stop criterion)
- Know your PSF
- Make sure the PSF is the same everywhere or use multi-PSF algo.
- Look for artefacts

Machine learning:

- Quite recently applied to microscopy images for restoration
- Definitely something that has to be tested



M. Weigert, et al., ‘Content-Aware Image Restoration: Pushing the Limits of Fluorescence Microscopy,’ *bioRxiv*, Jan. 2018.



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Co-localisation workflows

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Alternatively, you may ask a friend...



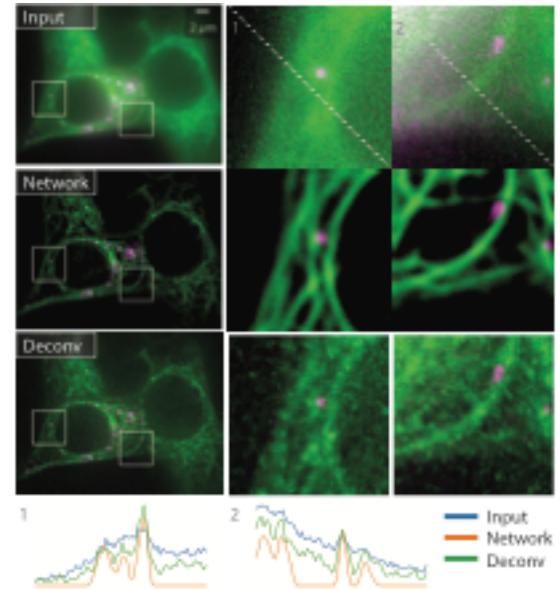
Restoration

Deconvolution:

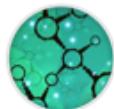
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Co-localisation workflows

Pre-processing

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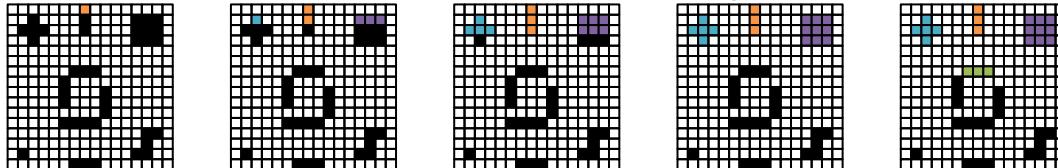
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Choosing a reporter/ metric

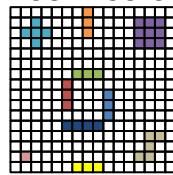
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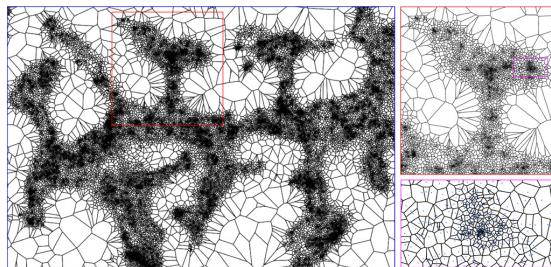
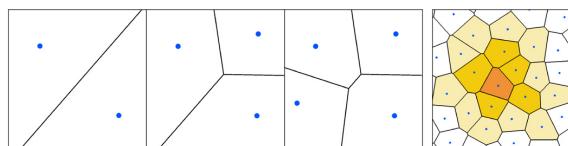
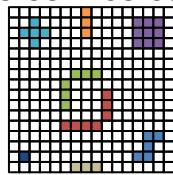
Pixel tagging, line per line → Objects' map



4-connected



8-connected



Segmentation

Differentiate objects' from background pixels:

- Simple threshold ?
- Adaptive/local threshold ?
- Other ?

Isolate/delineate objects

- Connexity analysis: tag each individual object
- Look for contours ? (snake etc)
- Extract points of interest (centre etc)

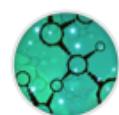
When working on detections:

- Group detections into objects ? (tessellation etc)

For a review on threshold algorithms: M. Sezgin and B. Sankur, *J. Electron. Imaging*, vol. 13, no. 1, pp. 146–165, 2004.

Illustration, bottom: F. Levet et al., *Nat. Methods*, 12(11), 1065–1071, 2015.

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Co-localisation workflows

Pre-processing

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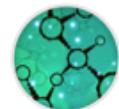
Unmixing: T. Zimmermann, Adv. Biochem. Eng. Biotechnol., vol. 95, pp. 245–265, 2005.

Deconv for coloc: L. Landmann and P. Marbet, *Microsc. Res. Tech.*, vol. 64, pp. 103–112, 2004.

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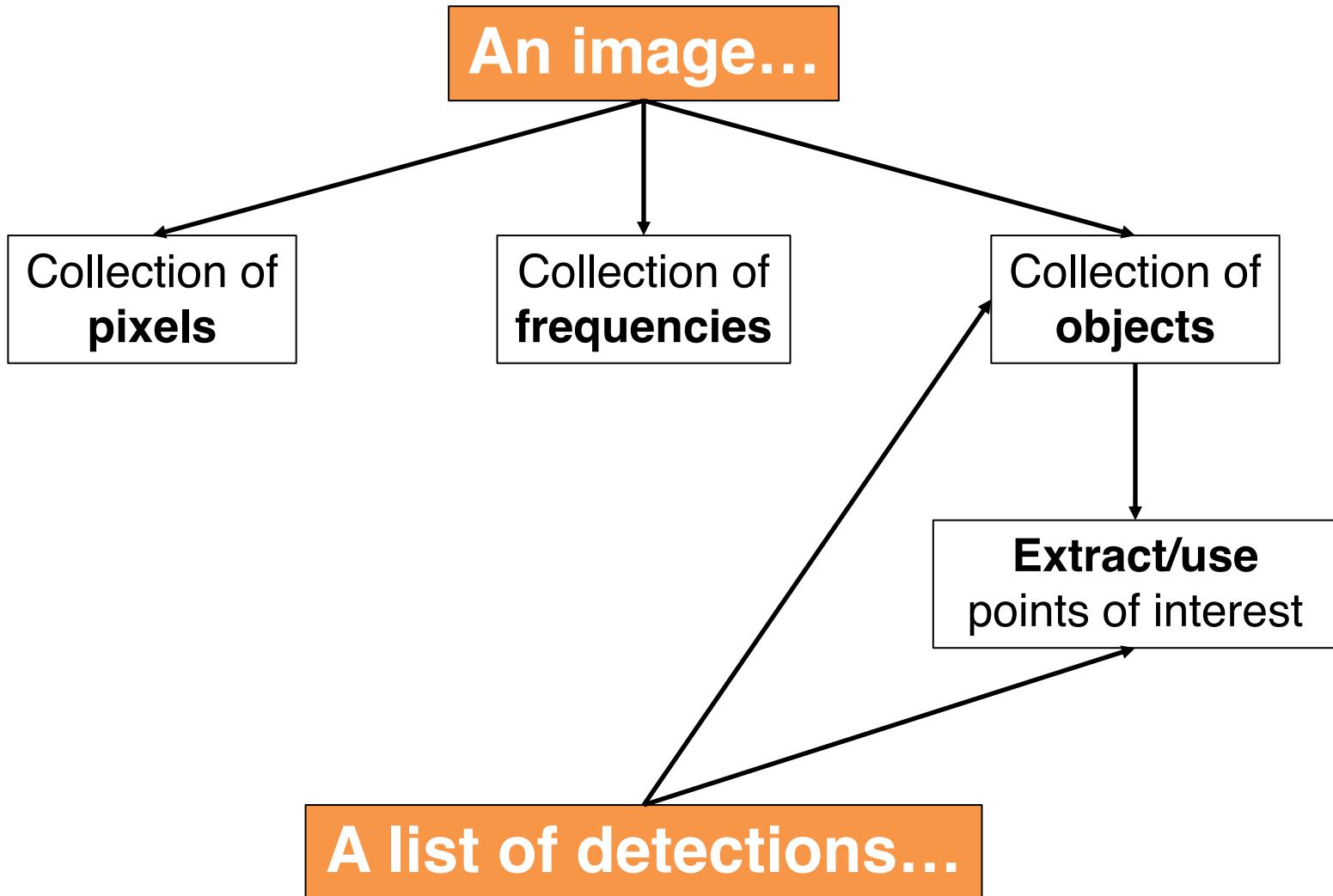
Choosing a reporter/metric

Image
format
file
JPEG



Co-localisation workflows

Data input



Co-localisation workflows

Choosing a reporter/metric

Checking data integrity

Pre-processing

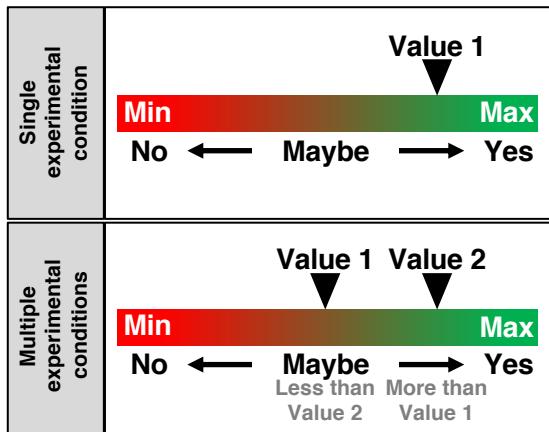
Choosing a reporter/ metric

Comparing
Interpreting

Assembling a workflow

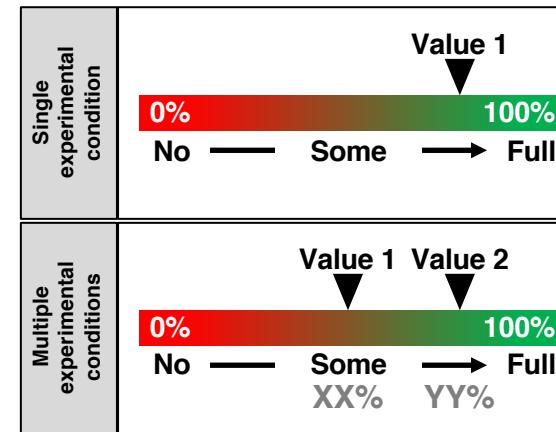
Indicators

« numerical values that evaluate a degree of signal coincidence over a predefined scale, but are not suitable to calculate a precise amount of overlap. They are suitable for relative comparisons, without being usable for direct quantitative studies »

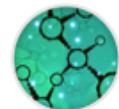


Quantifiers

« provide the experimenter with an absolute value, quantifying the overlap of signals, by using physical descriptors such as area or volume or by measuring distances between defined coordinates within the structures. »



Definitions from: F. P. Cordelières and S. Bolte, *Methods Cell Biol.*, vol. 123, pp. 395–408, Jan. 2014.



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Co-localisation workflows

Choosing a reporter/metric



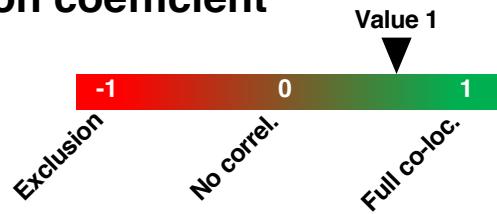
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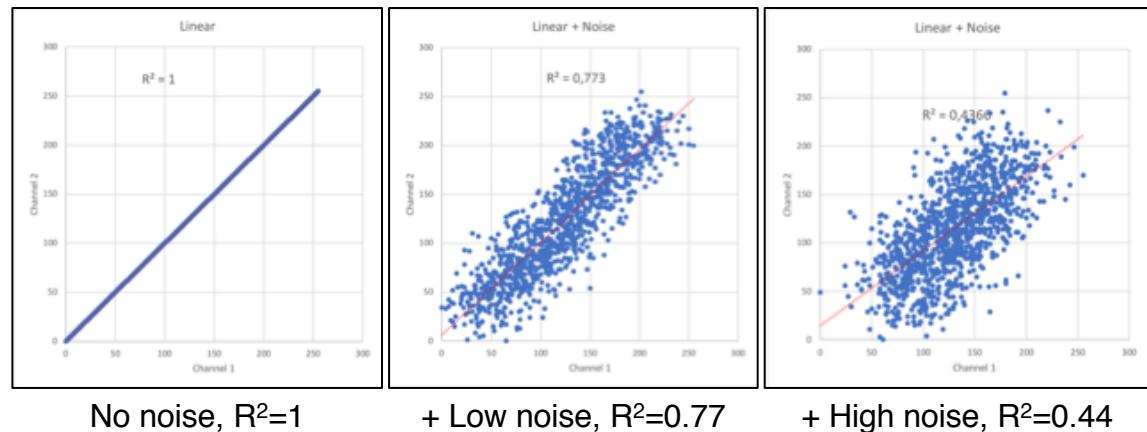
Single experimental condition	
Multiple experimental conditions	

Pearson's correlation coefficient

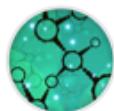
$$r_p = \frac{\sum_i (R_i - R_{aver}) \times (G_i - G_{aver})}{\sqrt{\sum_i (R_i - R_{aver})^2 \times \sum_i (G_i - G_{aver})^2}}$$



« The square PCC (generally denoted as R^2) is [...] a statistic that estimates the fraction of variability in G that can be explained by its linear regression with R »



Formula: E. M. Manders, et al., J. Cell Sci., vol. 103, pp. 857–62, Nov. 1992. / Link to R^2 : K. W. Dunn, et al., AJP Cell Physiol., vol. 300, pp. C723–C742, 2011.



Co-localisation workflows

Choosing a reporter/metric

Checking data integrity

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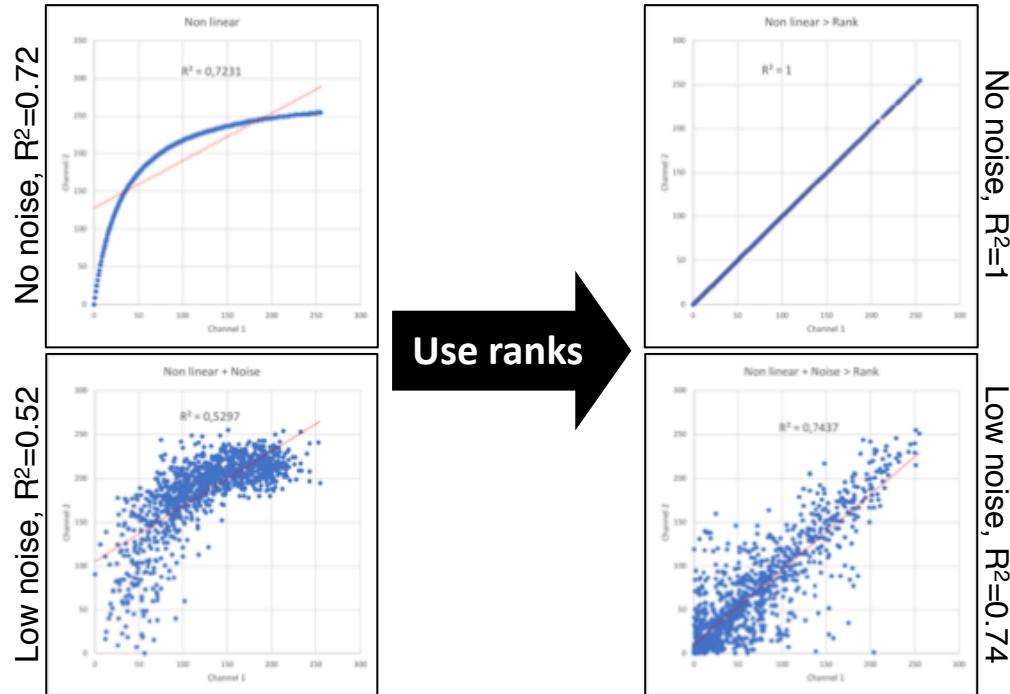
Single experimental condition	Value 1
	Min → Maybe → Yes
	No ← Maybe → Yes

Multiple experimental conditions	Value 1	Value 2
	Min → Maybe → Yes	
	No ← Maybe → Yes	
	Less than Value 2	More than Value 1

Spearman's correlation coefficient

- 1-Replace intensities by ranks to linearize data
- 2-Compute Pearson's coefficient

It's already a workflow !!!



C. Spearman, Am. J. Psychol., 1904 / J. Adler, et al., J. Microsc., 2008 / A. P. French, et al., Nat. Protoc., 2008.

Co-localisation workflows

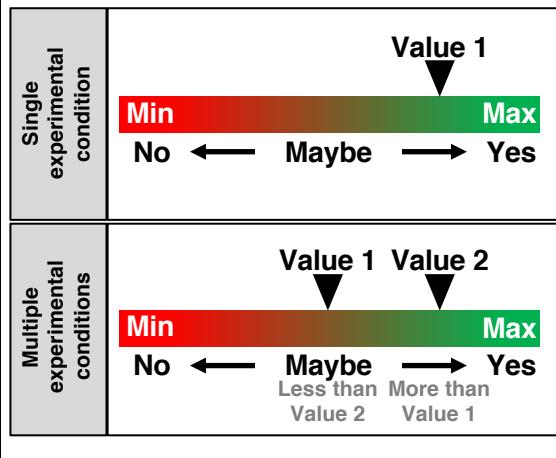
Choosing a reporter/metric



Many other indicators exist !

Indicators

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E. M. Manders, et al., J. Cell Sci., vol. 103, pp. 857–62, Nov. 1992.

Overlap coefficient

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



Numerator: becomes high when R_i and G_i belong to the same voxel (co-loc.)
Denominator: proportional to the overall number of non zero voxels

k_1 & k_2 coefficients

$$r^2 = k_1 \times k_2 \quad k_1 = \frac{\sum_i R_i \times G_i}{\sqrt{\sum_i R_i^2}} \quad k_2 = \frac{\sum_i R_i \times G_i}{\sqrt{\sum_i G_i^2}}$$



k_1 : sensitive to differences of intensities of green signal

k_2 : sensitive to differences of intensities of red signal

If $R_i = \alpha G_i$, $k_1 = 1/\alpha$ and $k_2 = \alpha$

→ Foundations of the Manders' coefficients

Co-localisation workflows

Choosing a reporter/metric

Checking data integrity

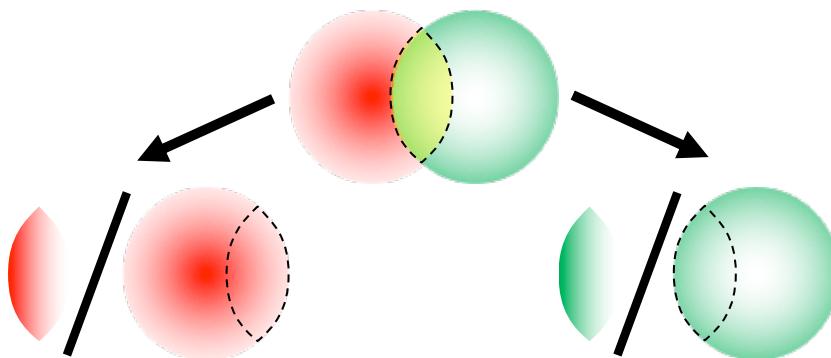
Pre-processing

Choosing a reporter / metric

Comparing
Interpreting

Assembling a workflow

Based on intensities' overlap



Manders' coefficients:

- 1-Select a threshold for each channel
- 2-Retrieve the total intensity in the overlapping zone, over the threshold
- 3-Retrieve the total intensity over the threshold
- 4-Compute the ratio

$$M_1 = \frac{\sum_i A_{i,coloc}}{\sum_i A_i}$$

Original paper: where $A_{i,coloc}=A_i$ if $B_i>0$, 0 otherwise
Modified: tM_1 , where $A_{i,coloc}=A_i$ if $B_i>\text{Thr}_B$, 0 otherwise

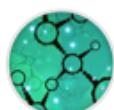
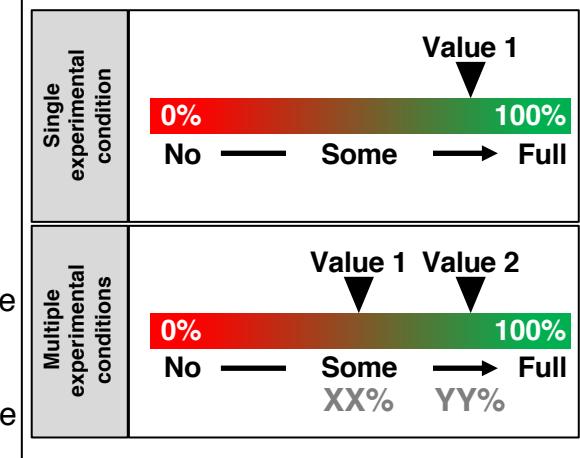
$$M_2 = \frac{\sum_i B_{i,coloc}}{\sum_i B_i}$$

Original paper: where $B_{i,coloc}=B_i$ if $A_i>0$, 0 otherwise
Modified: tM_2 , where $B_{i,coloc}=B_i$ if $A_i>\text{Thr}_A$, 0 otherwise

E. M. Manders, et al., *J. Cell Sci.*, vol. 103, pp. 857–62, Nov. 1992.

Quantifiers

« provide the experimenter with an absolute value, quantifying the overlap of signals, by using physical descriptors such as area or volume or by measuring distances between defined coordinates within the structures. »



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Co-localisation workflows

Choosing a reporter/metric

Checking data integrity

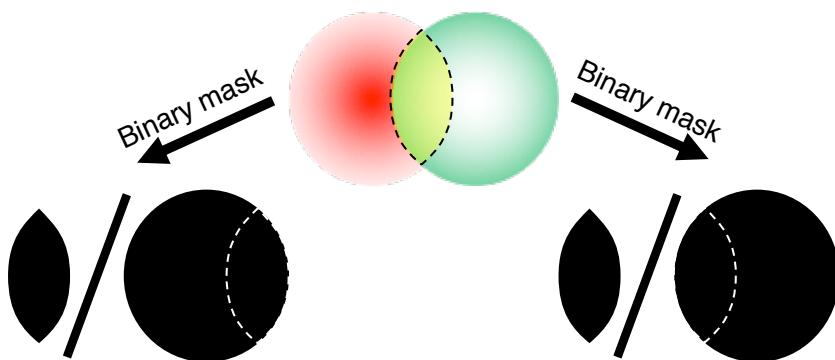
Pre-processing

Choosing a reporter / metric

Comparing
Interpreting

Assembling a workflow

Based on pixels' overlap



Modified Manders' coefficients / Intersection coefficients:

- 1-Select a threshold for each channel
- 2-Retrieve the total intensity in the overlapping zone, over the threshold
- 3-Retrieve the total intensity over the threshold
- 4-Compute the ratio

$$M_1 = \frac{\sum_i A_{i,coloc}}{\sum_i A_i}$$

Where $A_{i,coloc}=1$ if $B_i>Thr_B$, 0 otherwise
and $A_i=1$ if $A_i>Thr_A$, 0 otherwise

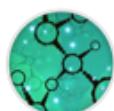
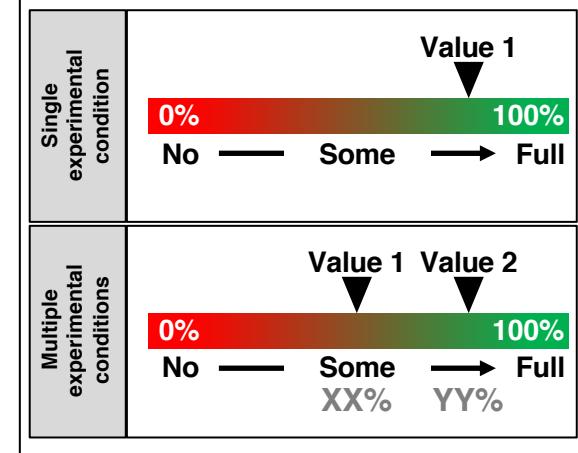
$$M_2 = \frac{\sum_i B_{i,coloc}}{\sum_i B_i}$$

Where $B_{i,coloc}=1$ if $A_i>Thr_A$, 0 otherwise
and $B_i=1$ if $B_i>Thr_B$, 0 otherwise

Modified from the original definitions found in: E. M. Manders, *et al.*, *J. Cell Sci.*, vol. 103, pp. 857–62, Nov. 1992.

Quantifiers

« provide the experimenter with an absolute value, quantifying the overlap of signals, by using physical descriptors such as area or volume or by measuring distances between defined coordinates within the structures. »



Co-localisation workflows

Choosing a reporter/metric

Checking data integrity

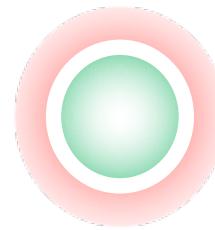
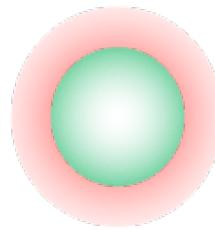
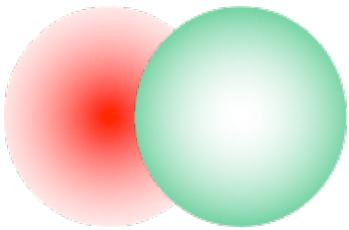
Pre-processing

Choosing a reporter/ metric

Comparing
Interpreting

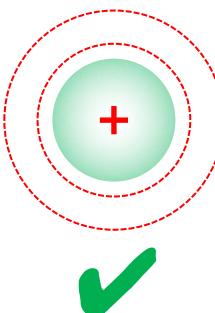
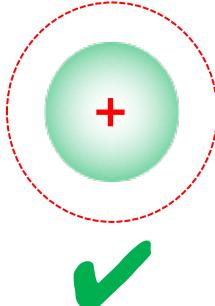
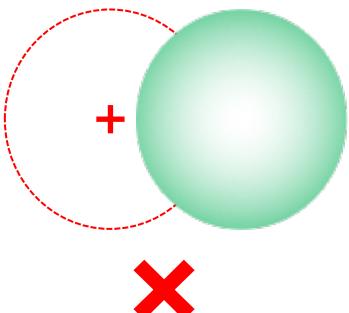
Assembling a workflow

Based on centre/object overlap



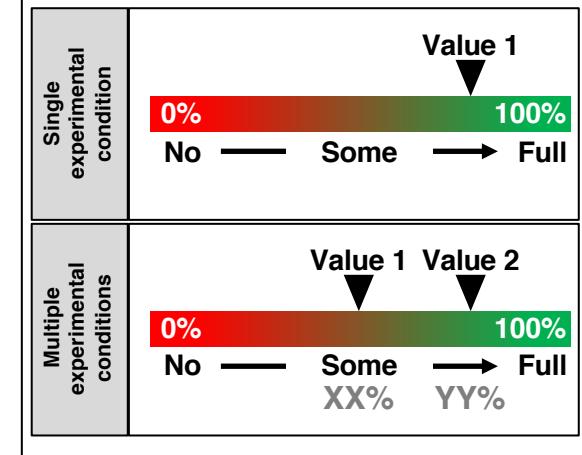
Method:

- 1-Compute the centres (mass or geometrical) of objects on channel 1
- 2-Compute the ratio centres from channel 1 falling on objects from channel 2
- 3-Repeat 1 & 2, using channel 1 for objects, channel 2 for centres



Quantifiers

« provide the experimenter with an absolute value, quantifying the overlap of signals, by using physical descriptors such as area or volume or by measuring distances between defined coordinates within the structures. »



E. Lachmanovich, et al., *J. Microsc.*, vol. 212, pp. 122–31, 2003.

Co-localisation workflows

Choosing a reporter/metric

Checking data integrity

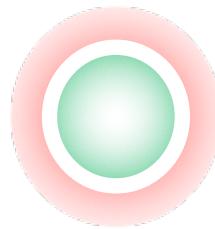
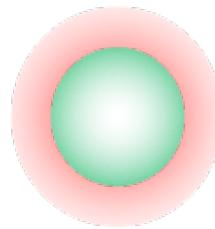
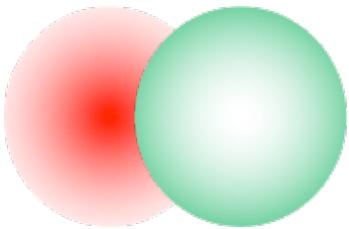
Pre-processing

Choosing a reporter/ metric

Comparing
Interpreting

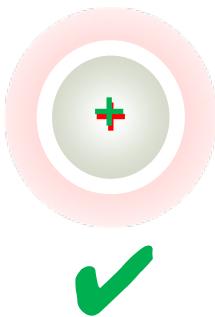
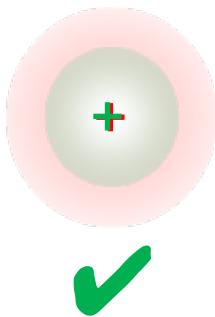
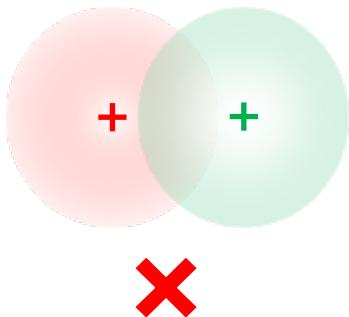
Assembling a workflow

Based on distances



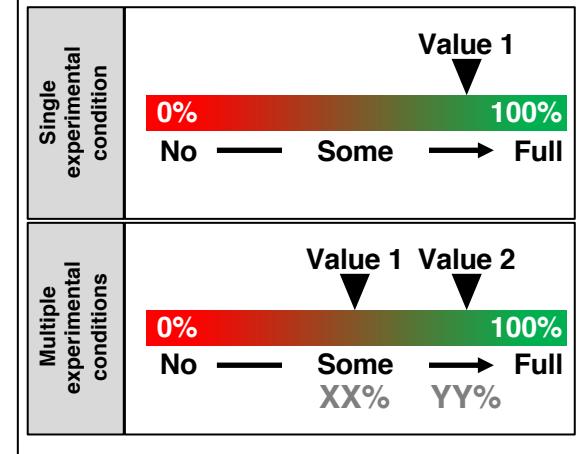
Method:

- 1-Compute the centres of objects on channel 1 & 2
- 2-Compute the distance between each centre from one channel and the closest from the other channel
- 3-Define a metric, ex: is the distance below the optical resolution ?

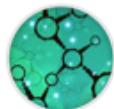


Quantifiers

« provide the experimenter with an absolute value, quantifying the overlap of signals, by using physical descriptors such as area or volume or by measuring distances between defined coordinates within the structures. »



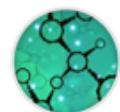
F.P. Cordelières and S. Bolte, JACoP v2.0: improving the user experience with co-localization studies, in *ImageJ User&Developer Conference*, 2008, 174–181.



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Co-localisation workflows

Comparing/interpreting

Checking data integrity

Pre-processing

Choosing a reporter/ metric

Comparing
Interpreting

Assembling a workflow

Getting significance out of a single dataset

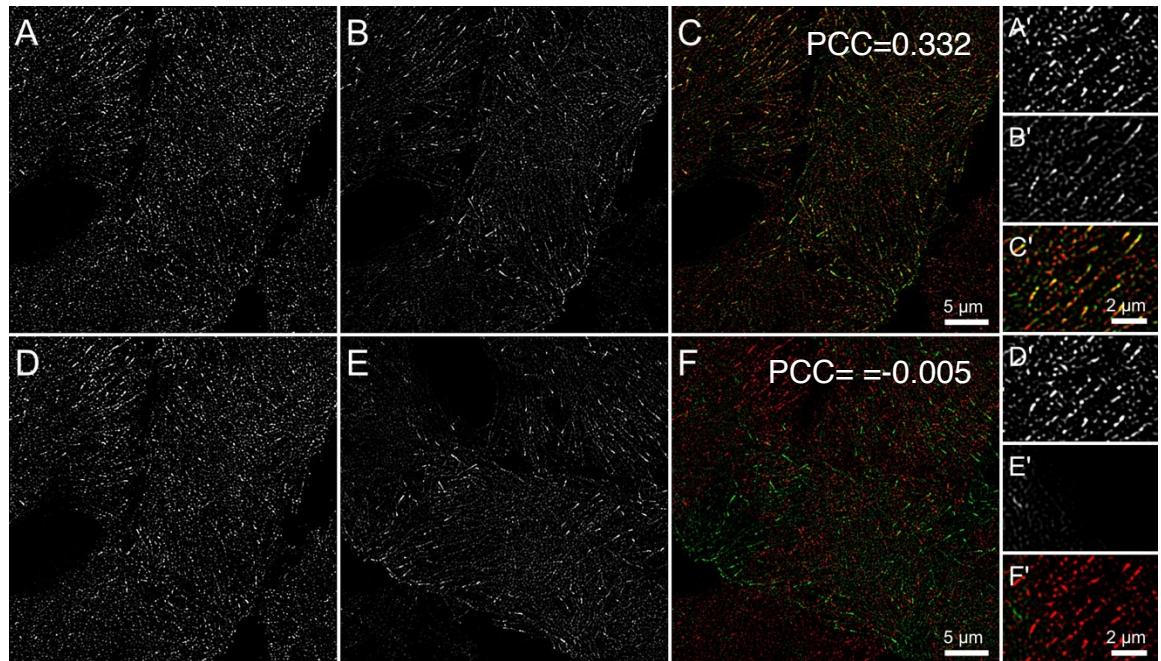
Methods:

- Generating a dataset where the content is the same, but localised differently
- Compare to original dataset

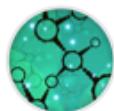
In practice:

- If image's width and height are the same: rotate one of the two images by 90°

Strategy 1: Rotate



You should read this: J.H. McDonald and K.W. Dunn, Statistical tests for measures of colocalization in biological microscopy., *J. Microsc.*, 252:295–302, 2013.



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Co-localisation workflows

Comparing/interpreting

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Pre-processing

Choosing a reporter/ metric

Comparing Interpreting

Assembling a workflow

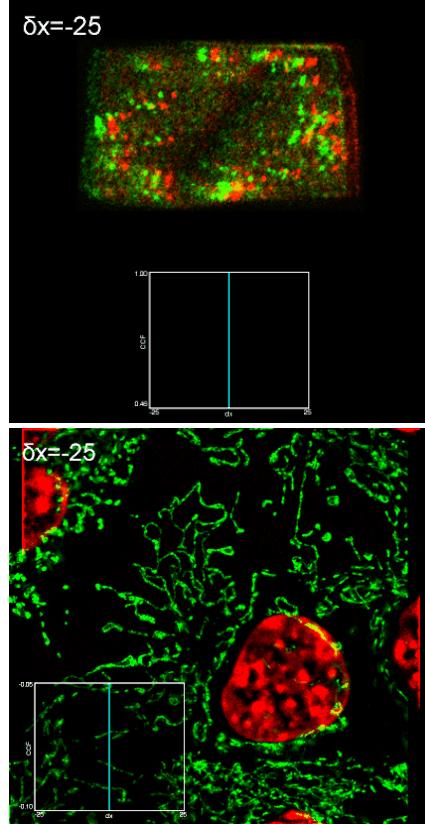
Getting significance out of a single dataset

Methods:

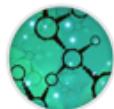
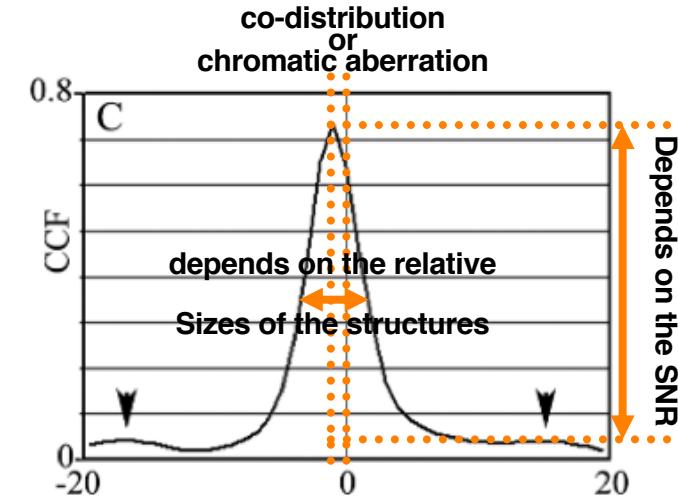
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

In practice:

- If image's width and height are the same: rotate one of the two images by 90°
- Translate one image relative to the other



B. van Steensel, et al., J. Cell Sci., vol. 792, pp. 787–792, 1996.



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Co-localisation workflows

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Comparing
Interpreting

Assembling a workflow

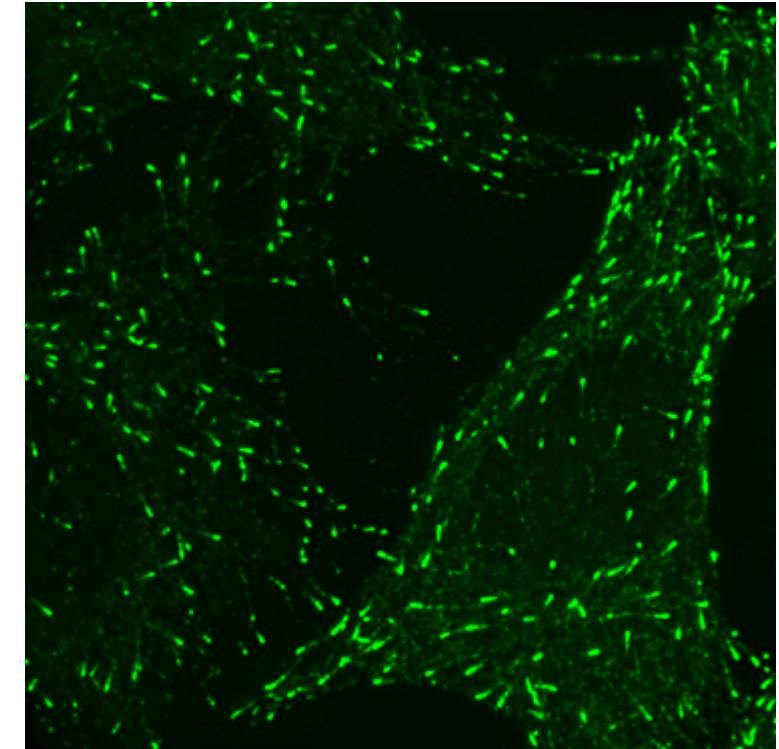
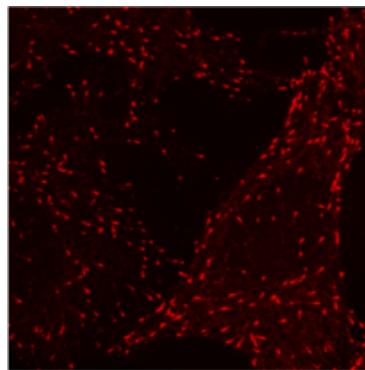
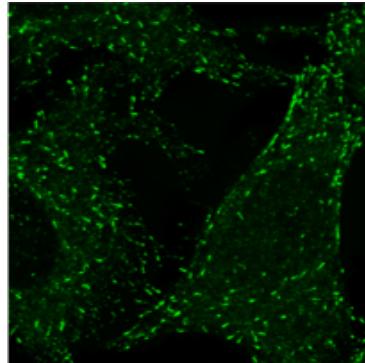
Getting significance out of a single dataset

Methods:

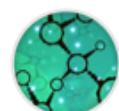
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

In practice:

- If image's width and height are the same: rotate one of the two images by 90°
- Translate one image relative to the other
- Randomise the image



S. V Costes, D. Daelemans, E. H. Cho, Z. Dobbin, G. Pavlakis, and S. Lockett, *Biophys. J.*, vol. 86, no. 6, pp. 3993–4003, Jun. 2004.



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Co-localisation workflows

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Choosing a reporter/ metric

Comparing
Interpreting

Assembling a workflow

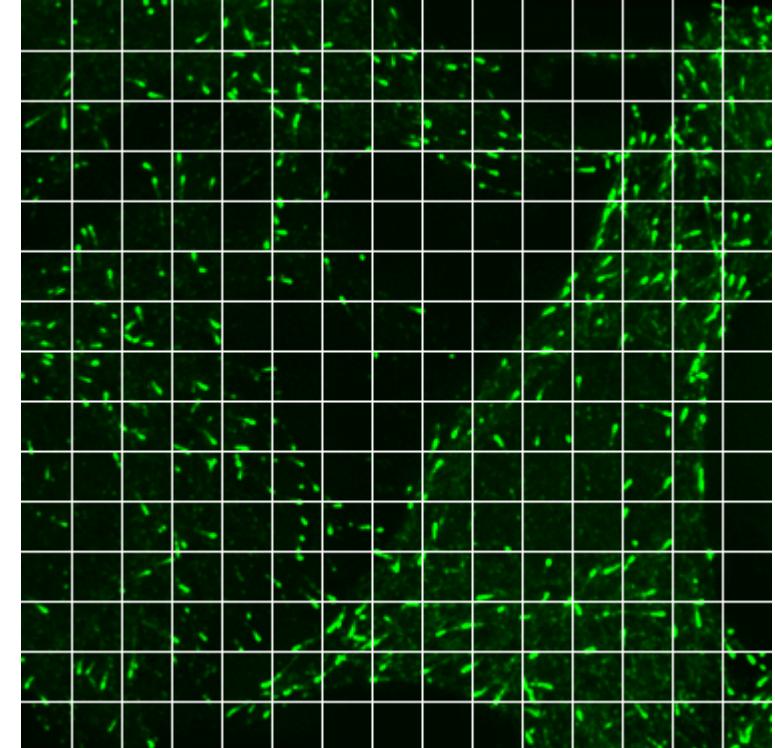
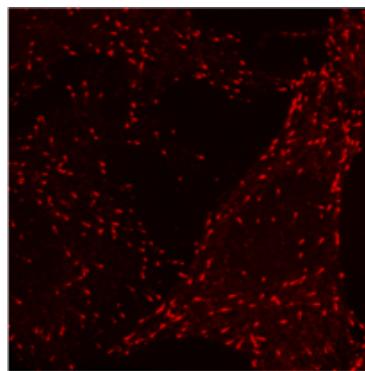
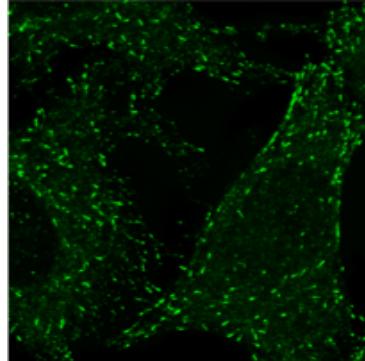
Getting significance out of a single dataset

Methods:

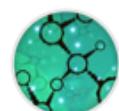
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

In practice:

- If image's width and height are the same: rotate one of the two images by 90°
- Translate one image relative to the other
- Randomise the image



S. V Costes, D. Daelemans, E. H. Cho, Z. Dobbin, G. Pavlakis, and S. Lockett, *Biophys. J.*, vol. 86, no. 6, pp. 3993–4003, Jun. 2004.



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Co-localisation workflows

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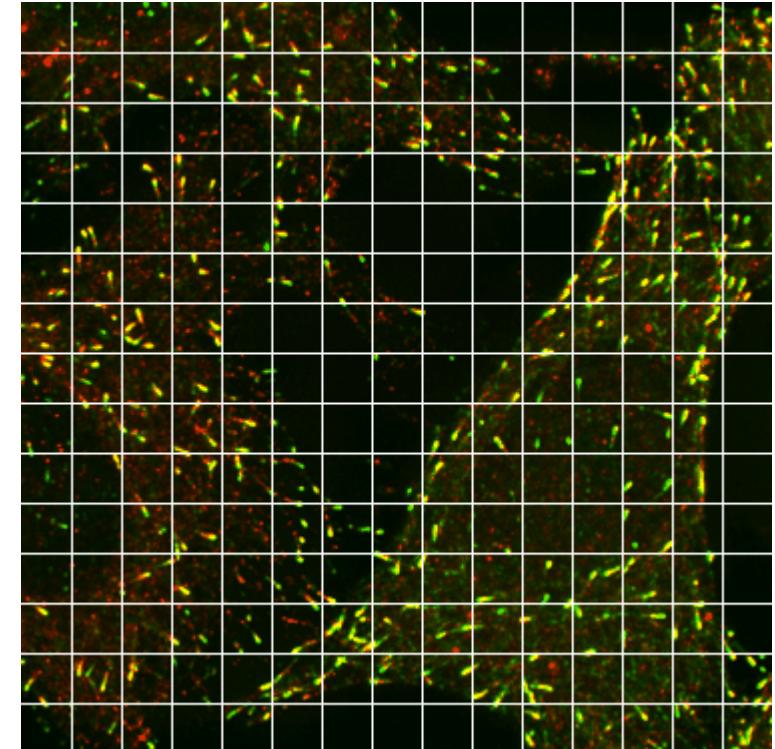
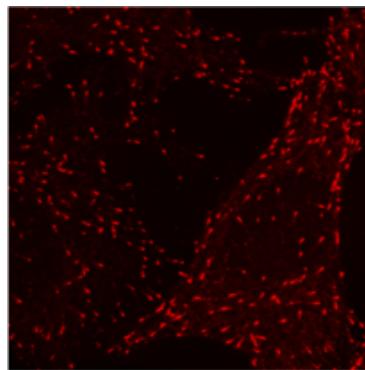
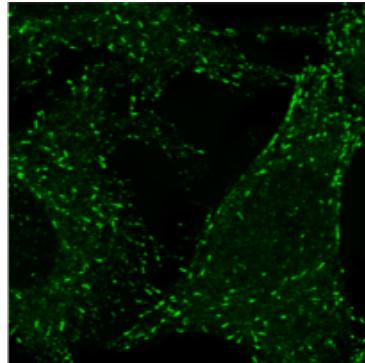
Getting significance out of a single dataset

Methods:

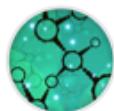
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

In practice:

- If image's width and height are the same: rotate one of the two images by 90°
- Translate one image relative to the other
- Randomise the image



S. V Costes, D. Daelemans, E. H. Cho, Z. Dobbin, G. Pavlakis, and S. Lockett, *Biophys. J.*, vol. 86, no. 6, pp. 3993–4003, Jun. 2004.



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Co-localisation workflows

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Choosing a reporter/ metric

Comparing Interpreting

Assembling a workflow

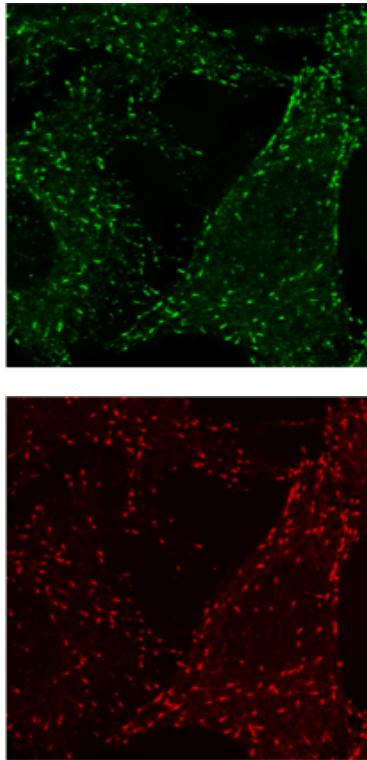
Getting significance out of a single dataset

Methods:

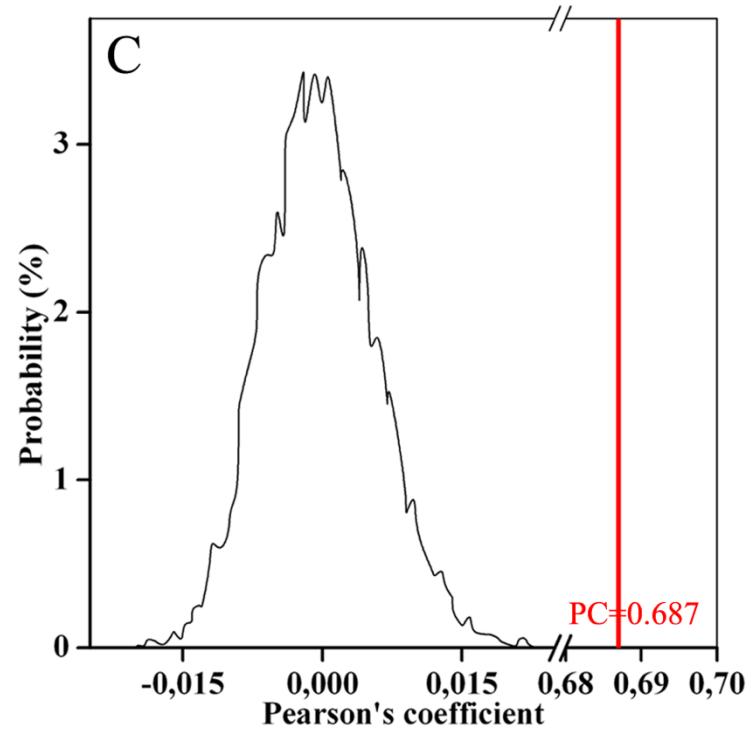
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

In practice:

- If image's width and height are the same: rotate one of the two images by 90°
- Translate one image relative to the other
- Randomise the image



Strategy 3: Randomise



S. V Costes, D. Daelemans, E. H. Cho, Z. Dobbin, G. Pavlakis, and S. Lockett, *Biophys. J.*, vol. 86, no. 6, pp. 3993–4003, Jun. 2004.

Co-localisation workflows

Comparing/interpreting



Getting significance out of a single dataset

Methods:

- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

In practice:

- If image's width and height are the same: rotate one of the two images by 90°
- Translate one image relative to the other
- Randomise the image

→ Pb: Local correlation due to

- ➔ 1-Point spread function
- ➔ 2-The size of structures

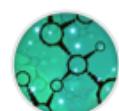
McDonald & Dunn 2013

One experimentation condition, using PCC:

*"When N data points are **statistically independent** of each other, the significance of a PCC value is tested by calculating $t = PCC\sqrt{[(N-2)/(1-PCC^2)]}$ which is t-distributed with **N-2 degrees of freedom** [...]. However, in images of cells, the pixels are not statistically independent data points. Instead, they are **autocorrelated**, meaning that each pixel is likely to have similar values to its neighbouring pixels."*

You should read this: J.H. McDonald and K.W. Dunn, Statistical tests for measures of colocalization in biological microscopy., *J. Microsc.*, 252:295–302, 2013.

See also: <https://www.r-bloggers.com/one-sample-students-t-test/> & <http://www.statisticssolutions.com/manova-analysis-one-sample-t-test>



Co-localisation workflows

Comparing/interpreting



Getting significance out of a single dataset

Methods:

- Ask an expert in statistics for help !
- Use the appropriate test

In practice:

According to MacDonald & Dunn, use:

- One-sample one tailed Student's t-test for testing mean PCC measurements.

McDonald & Dunn 2013

One experimentation condition, using PCC:

→ They propose:

- To repeat the experiment for the same condition
- Calculate the average PCC (m_0)
- Use the **one-sample one tailed Student's t-test**

→ The hypothesis for a Student's t test (infos not from their review):

- **Null hypothesis H_0 :** the difference between the true mean (μ) and the experimental observation (m_0) is equal to zero i.e. no co-localisation
- **Two-tailed alternative hypothesis H_1 :** assumes $\mu \neq m_0$ i.e. “not no co-localisation” (exclusion or co-localisation)
- **Upper-tailed alternative hypothesis H_1 :** assumes $\mu > m_0$ i.e exclusion
- **Lower-tailed alternative hypothesis H_1 :** assumes $\mu < m_0$ i.e co-localisation
- **Lower-tailed alternative hypothesis H_1 :** assumes $\mu < m_0$ i.e co-localisation

You should read this: J.H. McDonald and K.W. Dunn, Statistical tests for measures of colocalization in biological microscopy., *J. Microsc.*, 252:295–302, 2013.

See also: <https://www.r-bloggers.com/one-sample-students-t-test/> & <http://www.statisticssolutions.com/manova-analysis-one-sample-t-test>

Co-localisation workflows

Comparing/interpreting



McDonald & Dunn 2013

Compare several experimentation conditions, using PCC:

→ **They propose:**

- To calculate the average PCC for each experimental condition
- Use the **two-samples Student's t-test**

→ **The hypothesis for a Student's t test (infos not from their review):**

- **Null hypothesis H_0 :** the difference between two means is equal to zero i.e. same co-localisation properties
- **Alternative hypothesis H_1 :** assumes a difference between two means i.e. not the same degree of co-localisation.

Getting significance out of several datasets

Methods:

- Ask an expert in statistics for help !
- Use the appropriate test

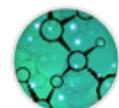
In practice:

According to MacDonald & Dunn, use:

- One sample one tailed Student's t-test for testing mean PCC measurements.
- Two-samples Student's t-test for comparing mean PCC measurements.

You should read this: J.H. McDonald and K.W. Dunn, Statistical tests for measures of colocalization in biological microscopy., *J. Microsc.*, 252:295–302, 2013.

See also: <https://www.r-bloggers.com/one-sample-students-t-test/> & <http://www.statisticssolutions.com/manova-analysis-one-sample-t-test>



Co-localisation workflows

Comparing/interpreting



Getting significance out of dataset(s)

Methods:

- Ask an expert in statistics for help !
- Use the appropriate test

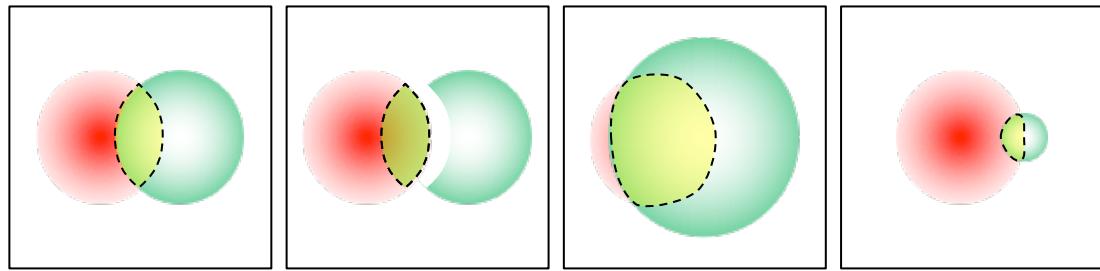
In practice:

According to MacDonald & Dunn, use:

- One-sample one tailed Student's t-test for testing mean PCC measurements.
- Two-samples Student's t-test for comparing mean PCC measurements.
- Two-samples Student's t-test for comparing mean MCC measurements.

McDonald & Dunn 2013

Using Mander's coefficients:

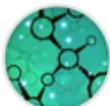


"This makes MCC values difficult to interpret in isolation: an MCC₁ of 0.60 would mean **strong colocalization** if only 5% of the image was green, **no association** if 60% of the image was green and **strong anticolocalization** if 95% of the image was green. Evidence for colocalization or anticolocalization comes from the difference between observed and expected MCC, not from MCC itself. **Thus, any statistical test must analyse the difference between the observed and expected MCC**".

→ Two-sample Student's t-test performs well to compare both expected and observed sets of values or 2 observed sets of values.

You should read this: J.H. McDonald and K.W. Dunn, Statistical tests for measures of colocalization in biological microscopy., *J. Microsc.*, 252:295–302, 2013.

See also: <https://www.r-bloggers.com/one-sample-students-t-test/> & <http://www.statisticssolutions.com/manova-analysis-one-sample-t-test>



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Co-localisation workflows

Comparing/interpreting

Checking data integrity

Pre-processing

Choosing a reporter/ metric

Comparing
Interpreting

Assembling a workflow

Getting significance out of a single dataset v1

Methods:

- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

In practice:

- If image's width and height are the same: rotate one of the two images by 90°
- Translate one image relative to the other
- Randomise the image

→ **Pb: Local correlation due to**
→ **1-Point spread function**
→ **2-The size of structures**

Getting significance out of a single dataset v2

Methods:

- Ask an expert in statistics for help !
- Use the appropriate test

In practice:

According to MacDonald & Dunn, use:

- *One-sample one tailed Student's t-test* for testing mean PCC measurements.
- *Two-samples Student's t-test* for comparing mean MCC measurements (observed vs expected).

Getting significance out of several datasets

Methods:

- Ask an expert in statistics for help !
- Use the appropriate test

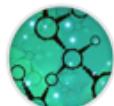
In practice:

According to MacDonald & Dunn, use:

- *Two-samples Student's t-test* for comparing mean PCC measurements.
- *Two-samples Student's t-test* for comparing mean MCC measurements (observed 1 vs observed 2).

You should read this: J.H. McDonald and K.W. Dunn, Statistical tests for measures of colocalization in biological microscopy., *J. Microsc.*, 252:295–302, 2013.

See also: <https://www.r-bloggers.com/one-sample-students-t-test/> & <http://www.statisticssolutions.com/manova-analysis-one-sample-t-test>



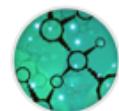
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Assembling a workflow

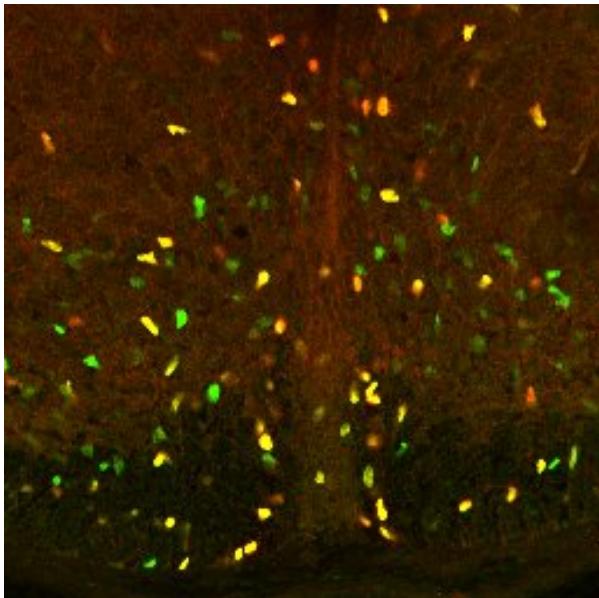
image
raster data used formats
application 24-bit etc
uncompressed
smaller digital
JPEG file



Co-localisation workflows

Co-expression analysis

Original image



Synopsis:

- The input dataset is composed of 2 images, showing a population of cells expressing either:
 - Marker A only
 - Marker B only
 - Both Marker A and marker B
- A user comes to the facility asking:
 - How to isolate each type of cell ?
 - How to count each type of cell ?
 - How to estimate the percentage of co-expressing cells ?

How would you do ???

Co-localisation workflows

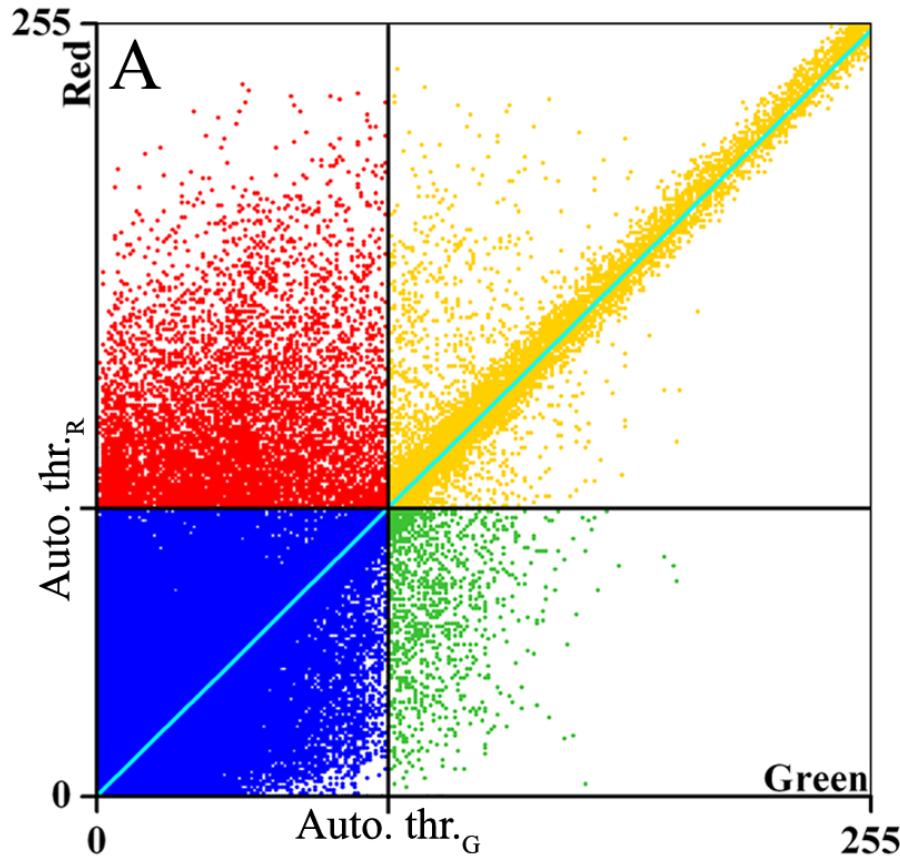
Co-expression analysis

How to set the threshold ? Costes' automatic threshold

Principle:

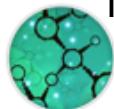
- Set threshold at max of the range
- Compute PCC below thresholds
- If $PCC > 0$, lower thresholds
- Stop and get thresholds just before $PCC \leq 0$

→ Sets thresholds by maximising the number of pixels with correlated intensities / minimising the number of pixels with uncorrelated pixels



Original paper: S. V Costes *et al.*, "Automatic and quantitative measurement of protein-protein colocalization in live cells.", *Biophys. J.*, vol. 86, 3993–4003, 2004.

Illustration from: S. Bolte and F. P. Cordelières, "A guided tour into subcellular colocalization analysis in light microscopy.", *J. Microsc.*, vol. 224, 213–32, 2006.



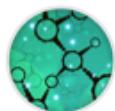
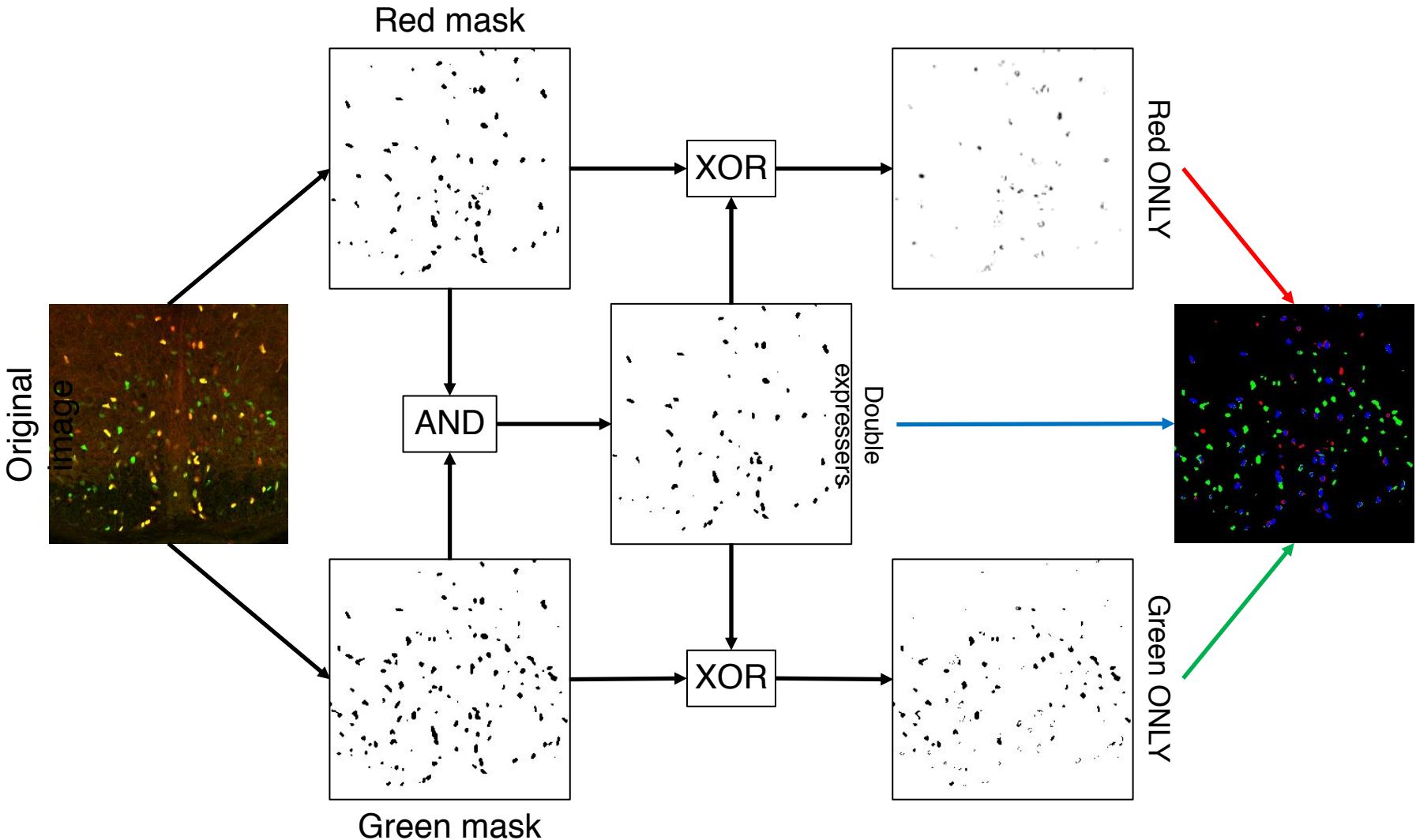
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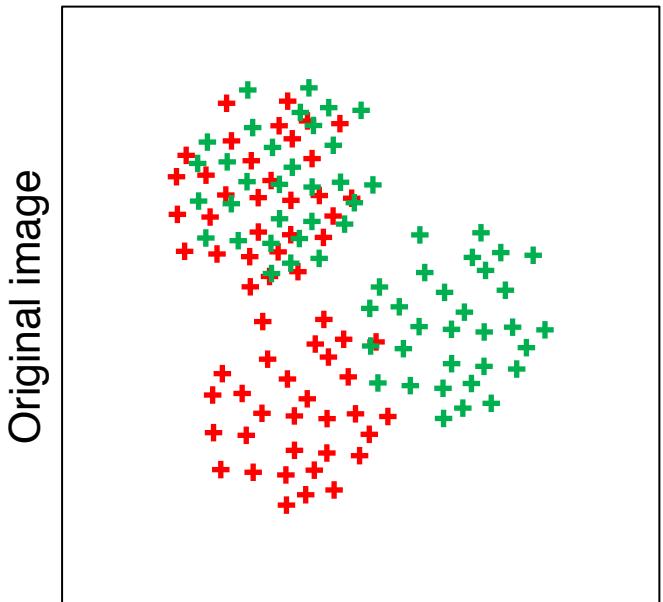
Co-localisation workflows

Co-expression analysis



Co-localisation workflows

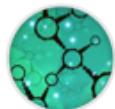
Working with detections



Synopsis:

- The image presents population of proteins, acquired using a pointillist method
- The input dataset is composed of two lists of coordinates for positions of
 - Marker A
 - Marker B
- A user comes to your facility asking “how to do co-localization on that ???”

How would you do ???



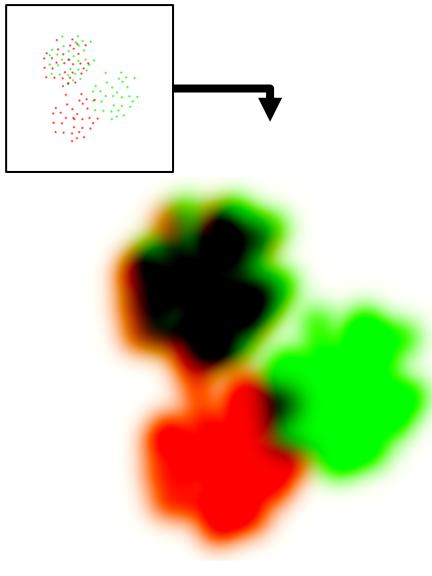
Co-localisation workflows

Working with detections

Getting back to something we “know” v1

Method:

- Assign to each point the precision of localisation as intensity
- Convolve with Gaussian blur or the acquisition PSF
- Use regular indicators/quantifiers

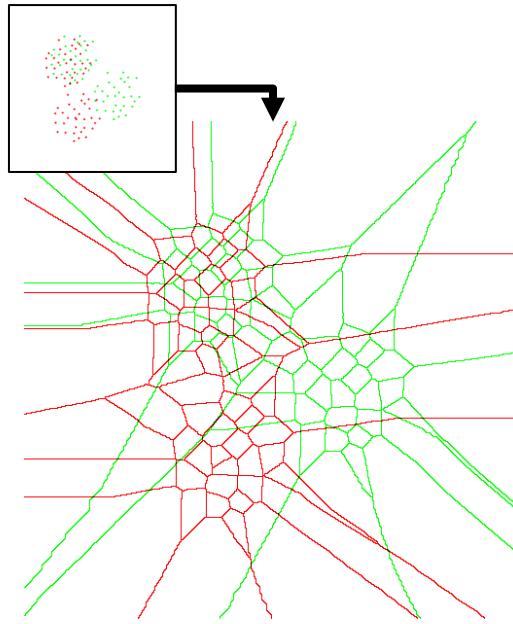


E. Betzig *et al.*, *Science*, 313, 1642–5, 2006.

Getting back to something we “know” v2

Methods:

- Partition space, based on the detections: tessellation. (Ex: Voronoï)
- Only retain pertinent tiles
- Use overlap measurement between the tiles

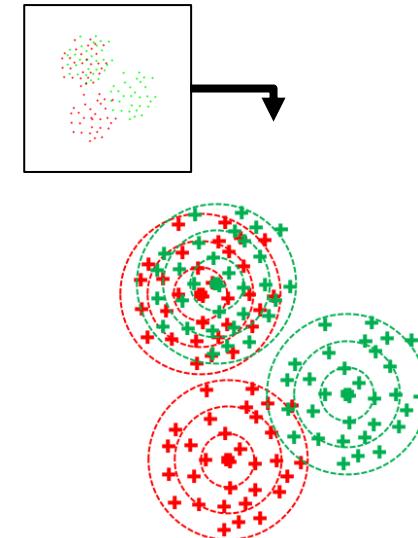


F. Levet *et al.*, *Nat. Methods*, 12, 1065–1071, 2015.
L. Andronov, *et al.* *Sci. Rep.*, 6, 1–9, 2016.

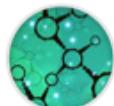
Working on distances

Methods:

- For each detection:
 - Evaluate its surrounding (same channel)
 - Evaluate how it relates to detections in the second channel



S. Malkusch *et al.*, *Histochem. Cell Biol.*, 137, 1–10, 2012.



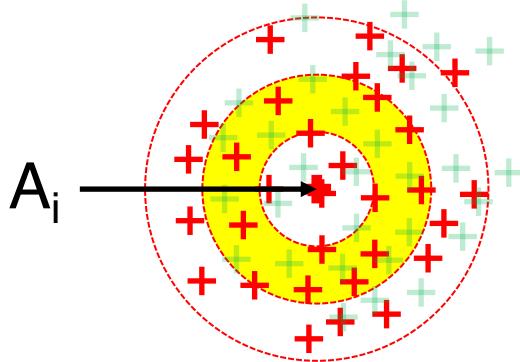
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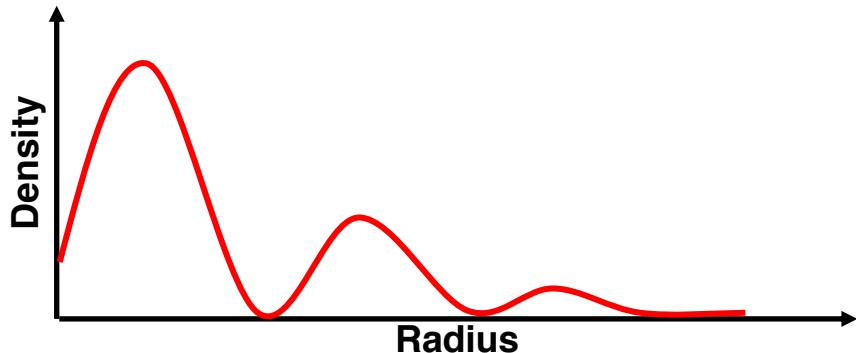
Co-localisation workflows

Working with detections



Define the density of detections from **A**, around A_i :

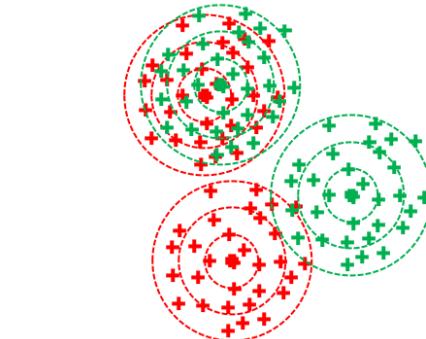
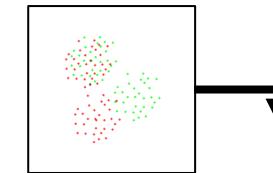
$$D_{A_i,A}(r) = \frac{N_{A_i,A}(r)}{\pi r^2} \times \frac{\pi R_{max}^2}{N_{A_i,A}(R_{max})} = \frac{N_{A_i,A}(r)}{N_{A_i,A}(R_{max})} \times \frac{R_{max}^2}{r^2}$$



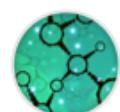
Working on distances

Methods:

- For each detection:
 - Evaluate its surrounding (same channel)
 - Evaluate how it relates to detections in the second channel

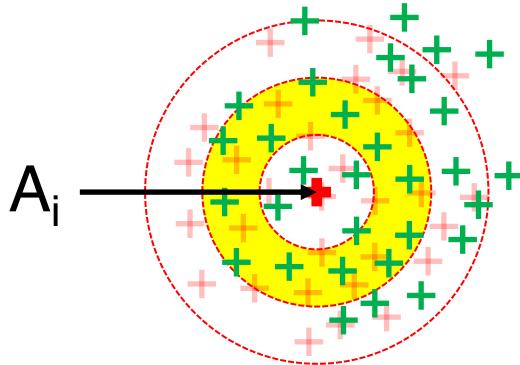


S. Malkusch *et al.*, *Histochem. Cell Biol.*, 137, 1–10, 2012.



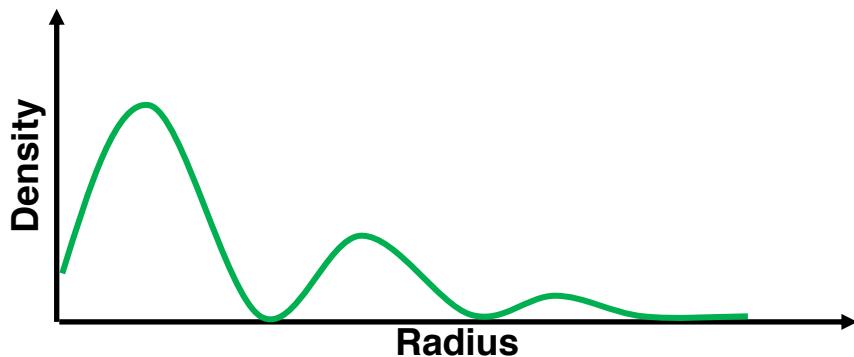
Co-localisation workflows

Working with detections



Define the density of detections from **B**, around A_i :

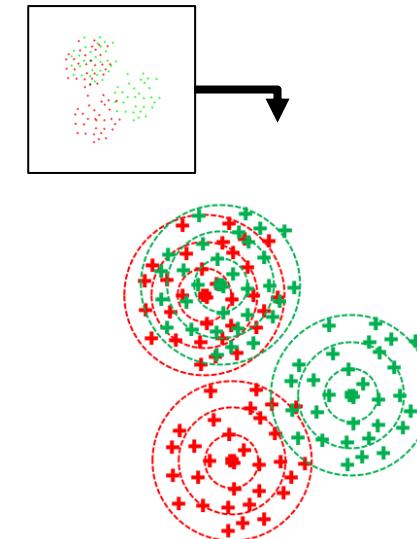
$$D_{A_i,B}(r) = \frac{N_{A_iB}(r)}{N_{A_iB}(R_{max})} \times \frac{R_{max}^2}{r^2}$$



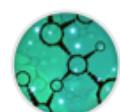
Working on distances

Methods:

- For each detection:
 - Evaluate its surrounding (same channel)
 - Evaluate how it relates to detections in the second channel



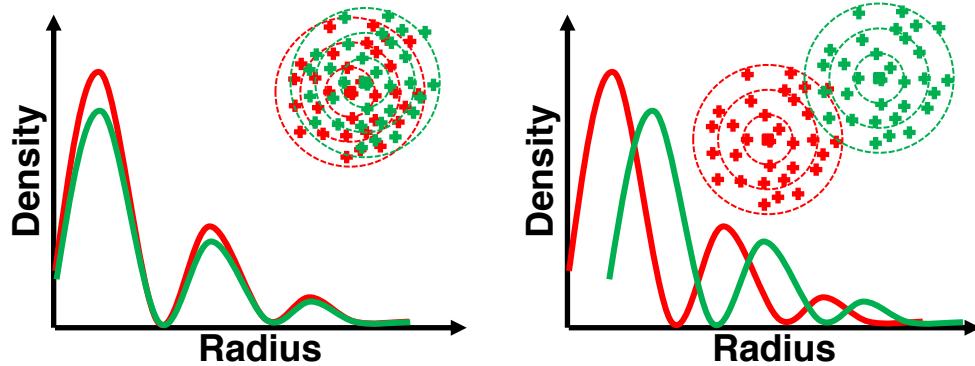
S. Malkusch *et al.*, *Histochem. Cell Biol.*, 137, 1–10, 2012.



Co-localisation workflows

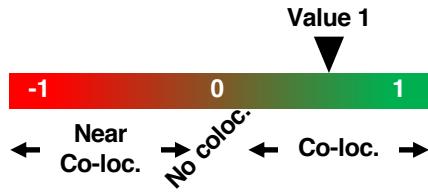
Working with detections

For each radius, correlate $D_{Ai,A}$ and $D_{Ai,B}$ using Spearman's coefficient



To penalise for the distance from A_i to the closest B, a correction coefficient is introduced

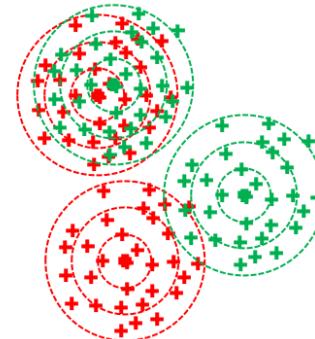
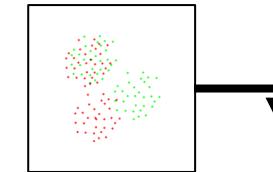
$$C_{Ai} = S_{Ai} \cdot e^{\left(-\frac{E_{Ai,B}}{R_{max}} \right)}$$



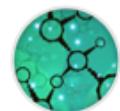
Working on distances

Methods:

- For each detection:
 - Evaluate its surrounding (same channel)
 - Evaluate how it relates to detections in the second channel

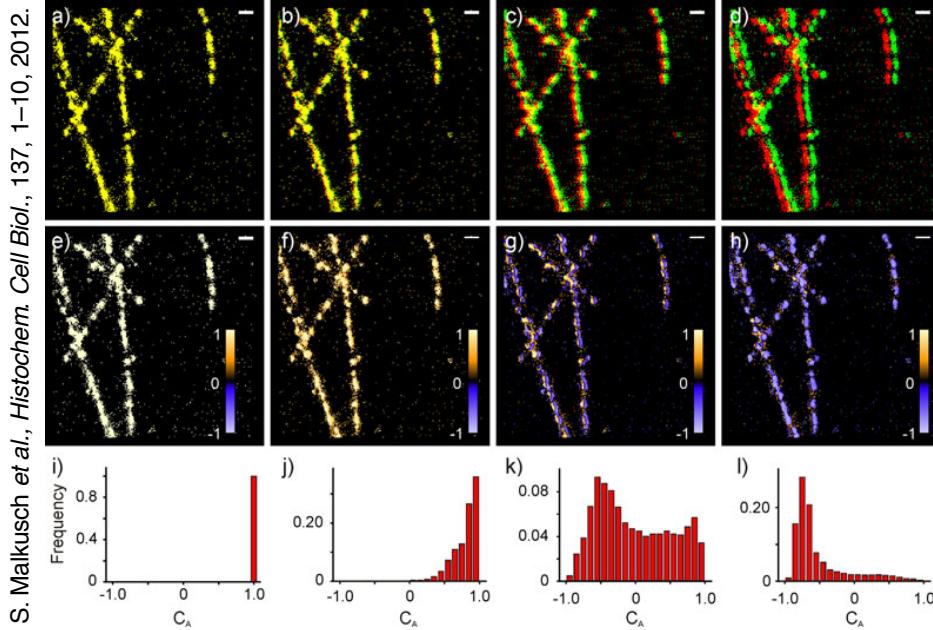


S. Malkusch *et al.*, *Histochem. Cell Biol.*, 137, 1–10, 2012.



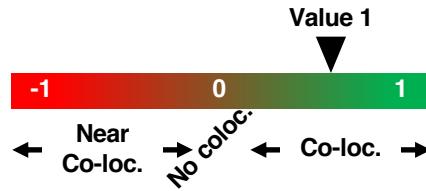
Co-localisation workflows

Working with detections



To penalise for the distance from A_i to the closest B , a correction coefficient is introduced

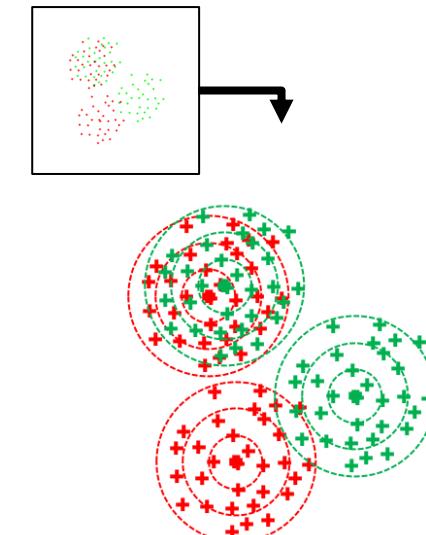
$$C_{A_i} = S_{A_i} \cdot e^{\left(\frac{-E_{A_i, B}}{R_{max}} \right)}$$



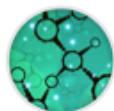
Working on distances

Methods:

- For each detection:
 - Evaluate its surrounding (same channel)
 - Evaluate how it relates to detections in the second channel



S. Malkusch et al., *Histochem. Cell Biol.*, 137, 1–10, 2012.



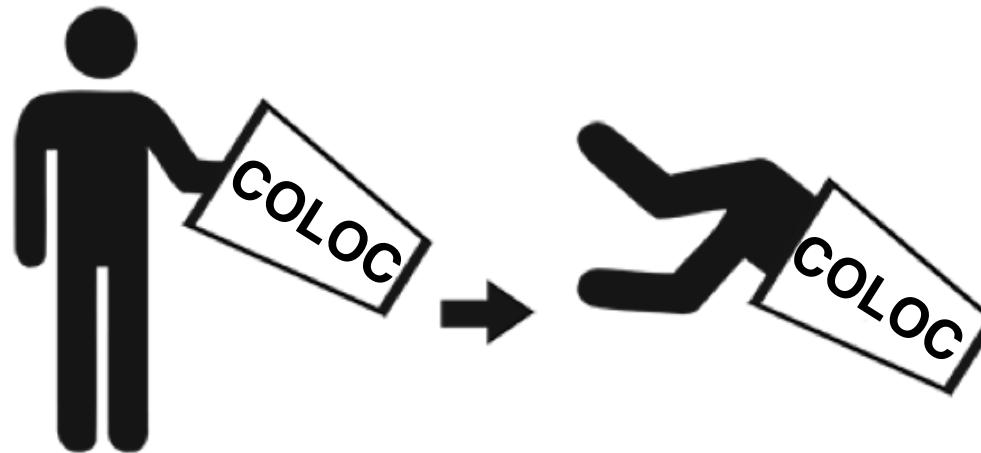
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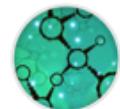
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Last advice
Think, be creative, test, get help... repeat

DANGER



**THIS MACHINE
HATES IDIOTS**



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