

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

Fabrice P Cordelières, PhD

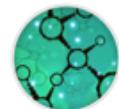
Bordeaux Imaging Center

Centre Broca Nouvelle-Aquitaine

146, rue Léo-Saignat

33077 Bordeaux

[fabric.cordelier@u-bordeaux.fr](mailto:fabric.cordelier@u-bordeaux.fr)

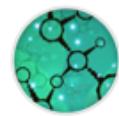
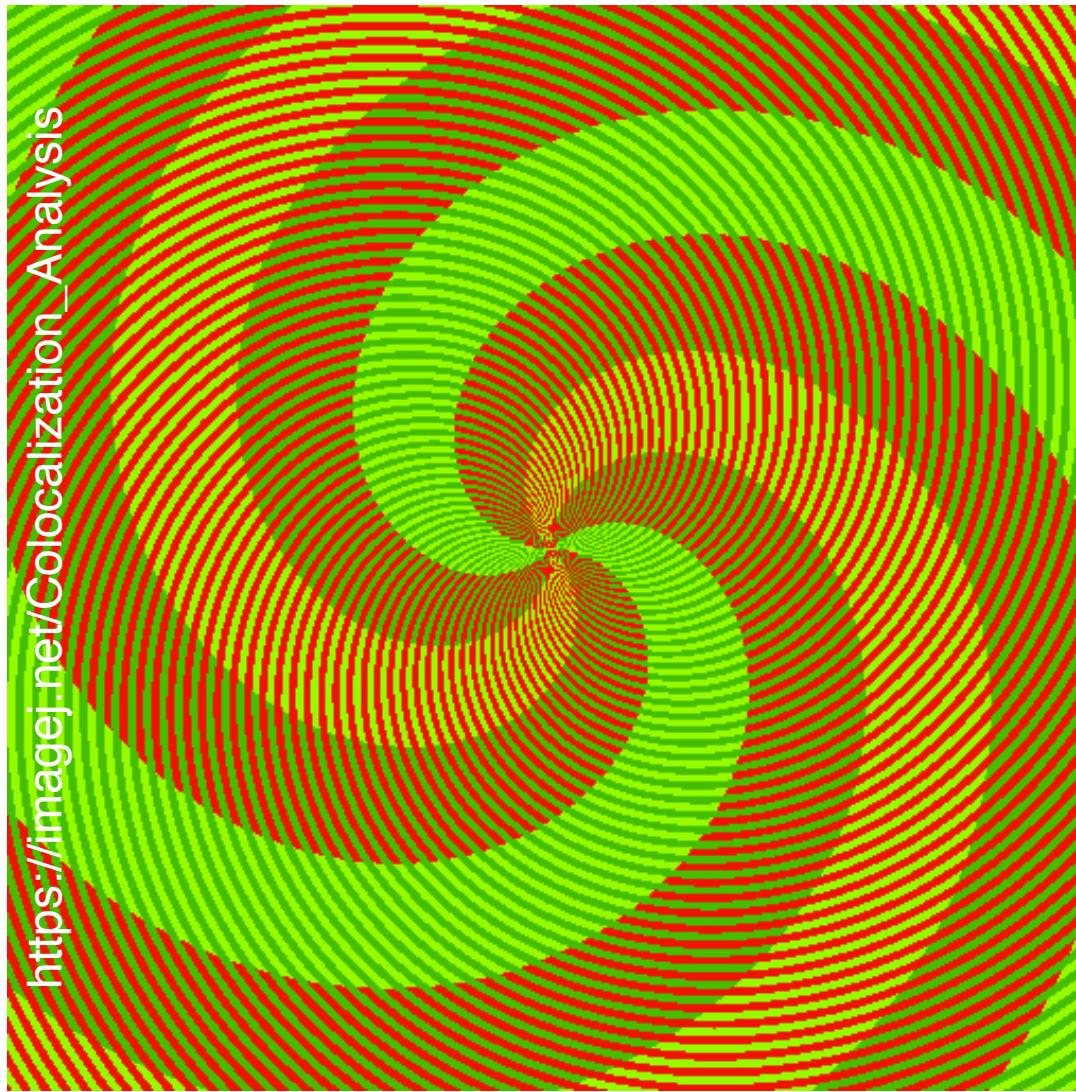


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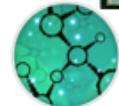
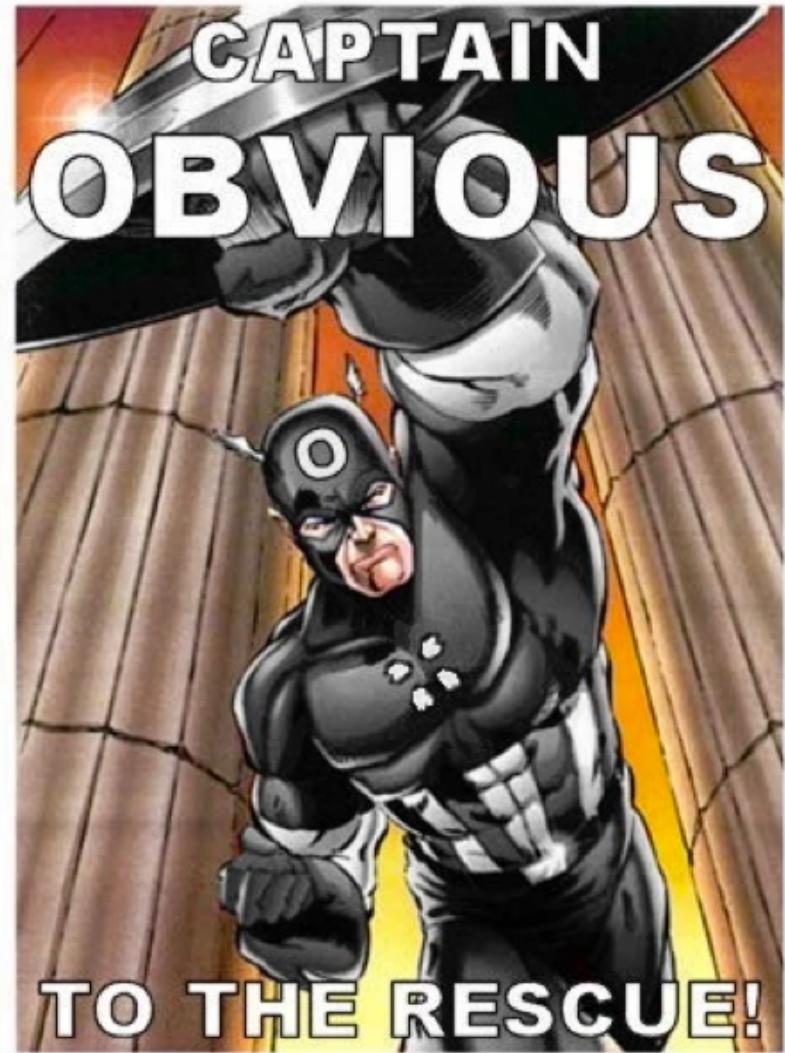
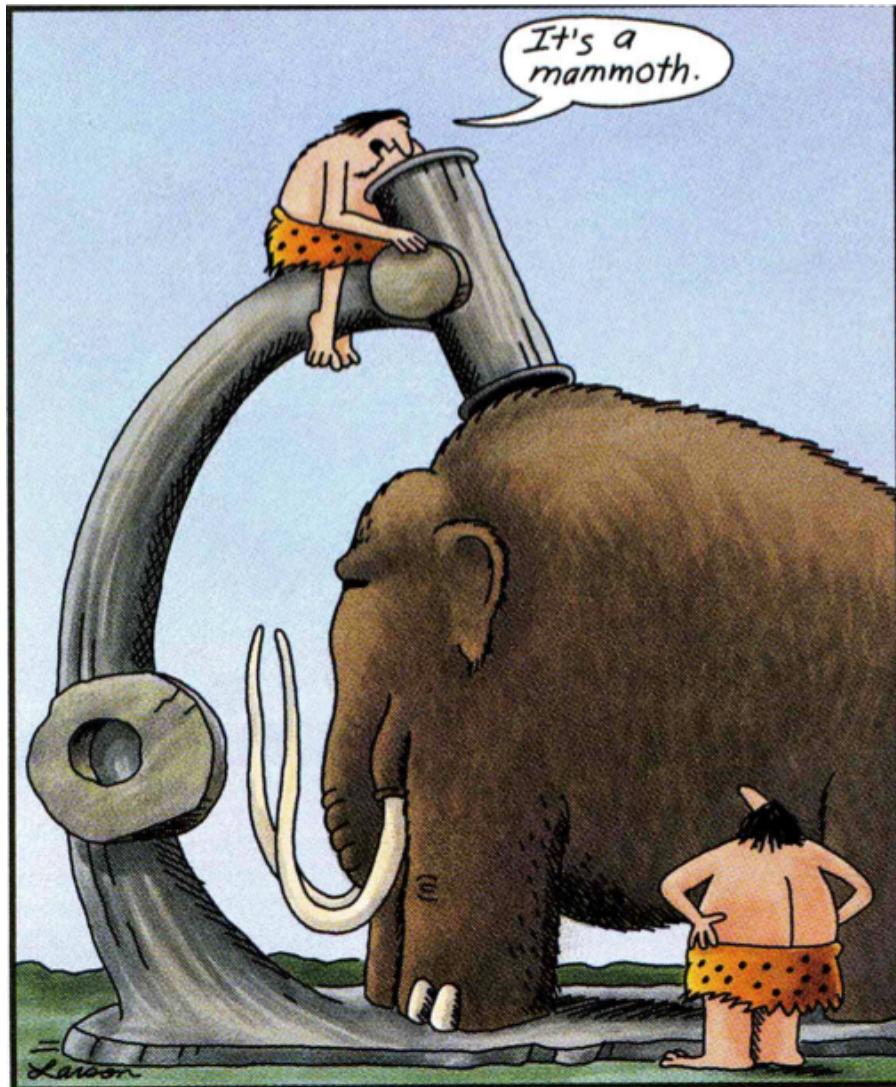
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# Why should we quantify co-localisation ?

## Don't trust your eyes !



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

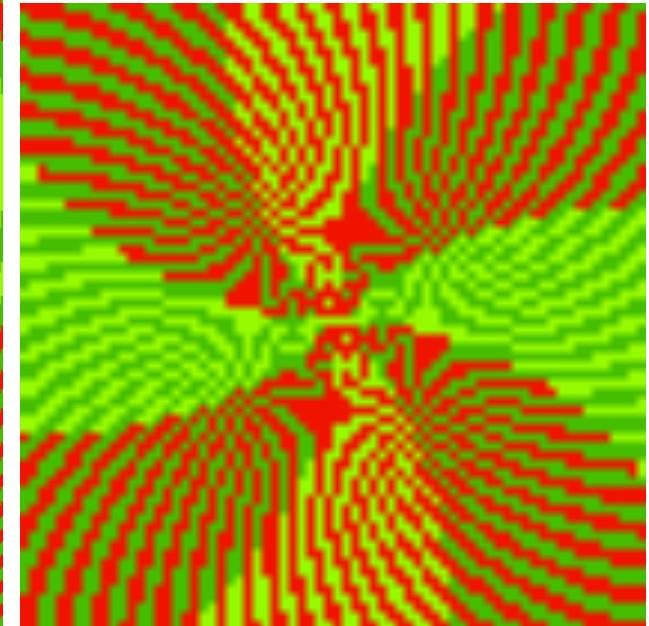


# Why should we quantify co-localisation ?

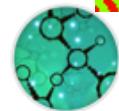
## Don't trust your eyes !



**Obvious, was it ?**



File ▶ Open Samples ▶ Spirals  
(Macro) in Fiji



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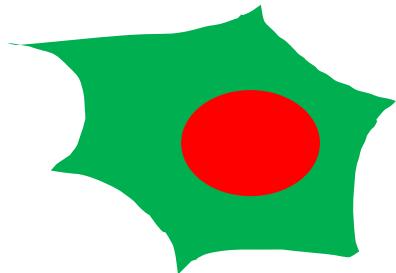
# Co-localisation

*One word, many meanings*

## Co-localisation

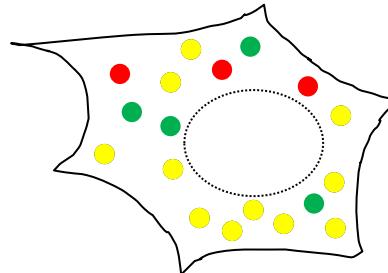
### Co-expression

*Two proteins are located within the same structure/cell*



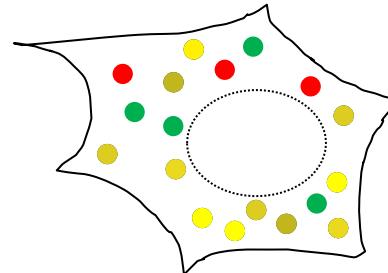
### Co-occurrence

*At the current resolution, the positions of the two labelling can't be distinguished*



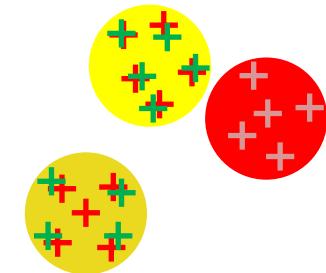
### Correlation

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



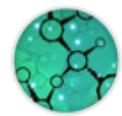
### Co-distribution

*At the current resolution, the spatial distributions of the two labellings are linked*



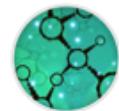
# Co-localisation workflows

## 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

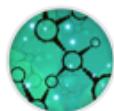
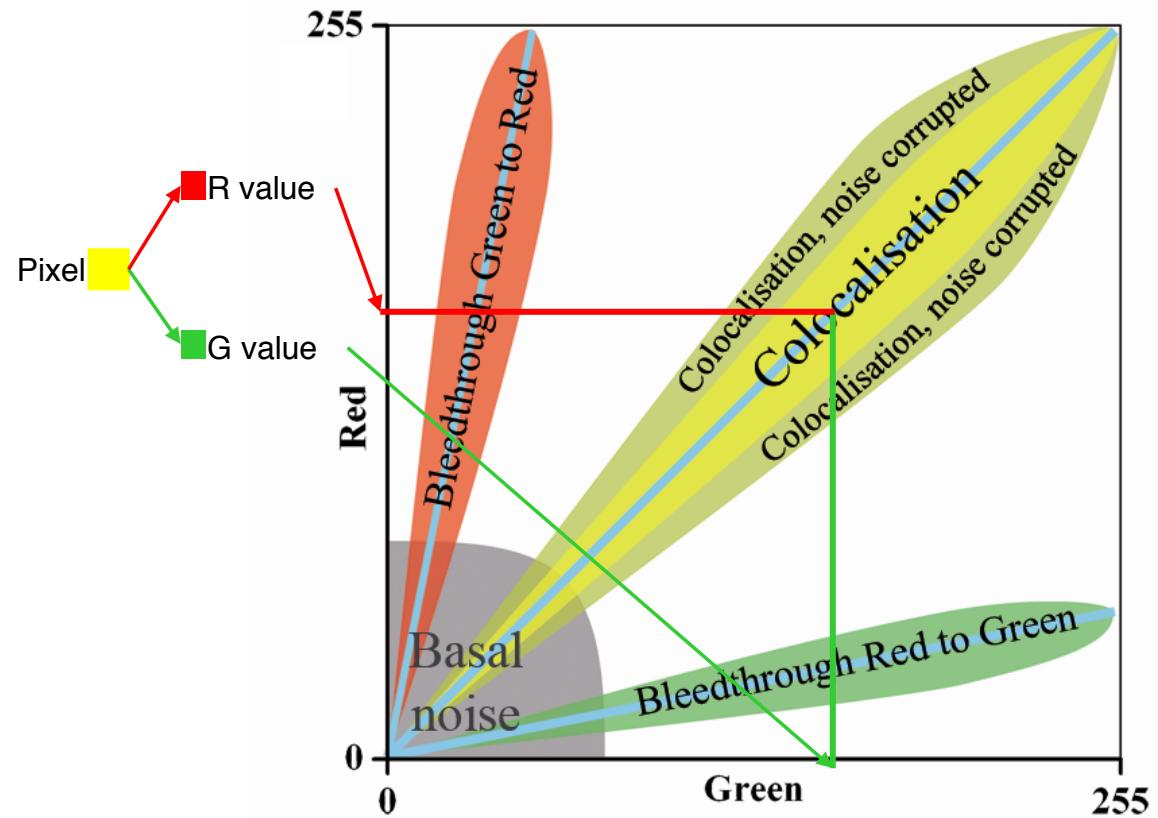
Comparing Interpreting

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

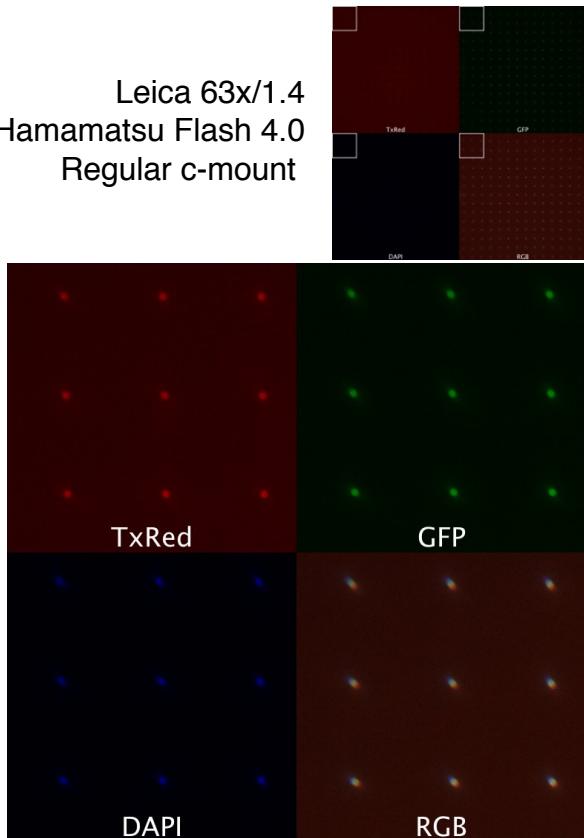
Pre-processing

Choosing a reporter/ metric

Comparing Interpreting

Assembling a workflow

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



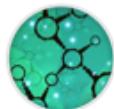
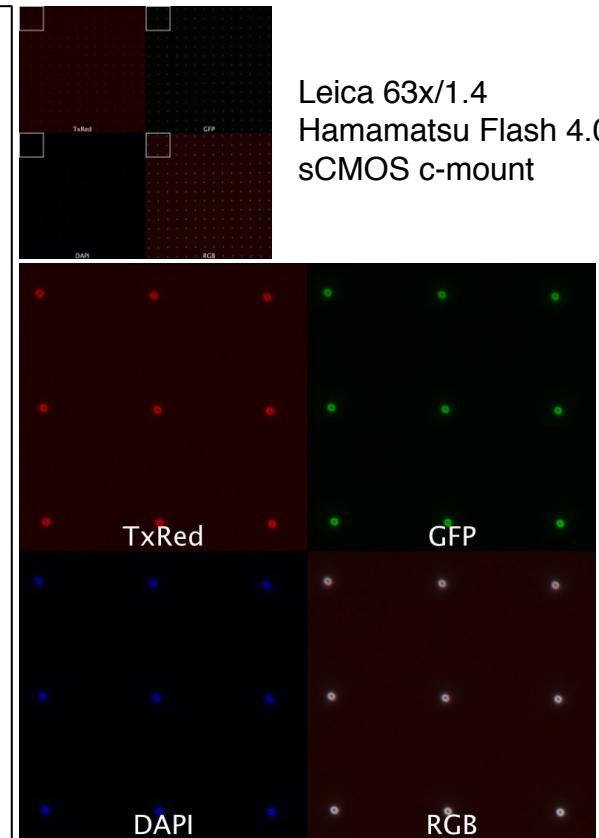
## Co-registration

*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

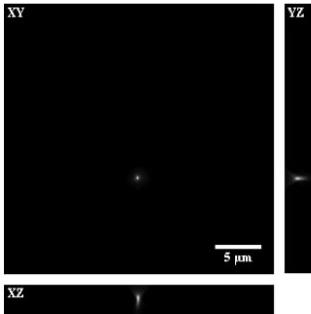
Comparing Interpreting

Assembling a workflow



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

### Profile view:



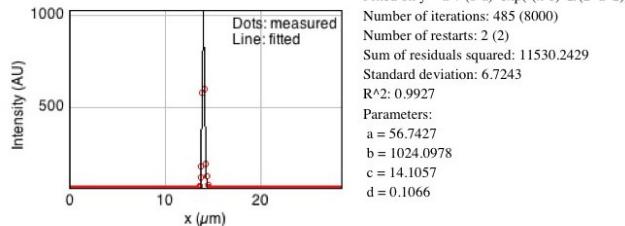
### Microscope infos:

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

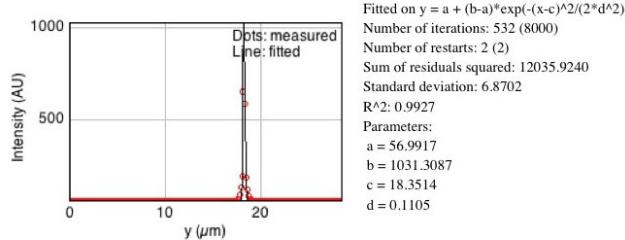
### Resolution table:

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

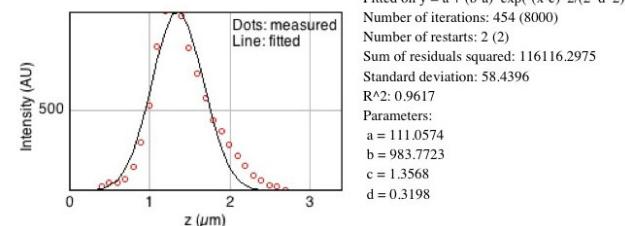
### X profile & fitting parameters:



### Y profile & fitting parameters:



### Z profile & fitting parameters:

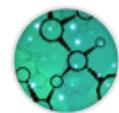


## Resolution

Know your limits

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

Have a look at the MetroloJ plugin !



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# Co-localisation workflows

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Checking data integrity

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### Dyes

*Check for bleedthrough and/or cross-talk*

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

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

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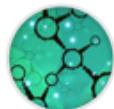
Have a look at the MetroloJ plugin !

### Resolution

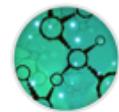
*Know your limits*

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

Have a look at the MetroloJ plugin !



# Without good images, there is no point going further !



# Pre-processing

# Co-localisation workflows

## Pre-processing

Checking data integrity

Pre-processing

Choosing a reporter/ metric

Comparing Interpreting

Assembling a workflow

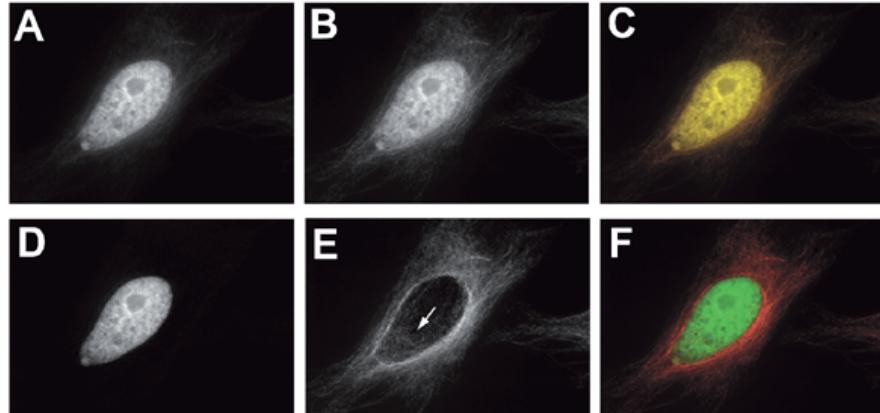
## Corrections

### *Image acquisition-related*

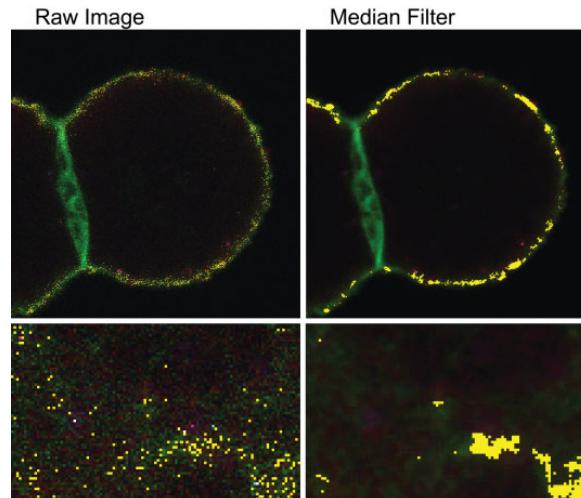
- Bleedthrough/crosstalk: better go back to the microscope before trying unmixing
- Chromatic shift: better be corrected on the microscope before trying to compensate by translation

### *Background and noise*

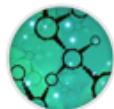
- Median filtering: Ok but impairs resolution
- Denoising: not to be used as a black box !



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

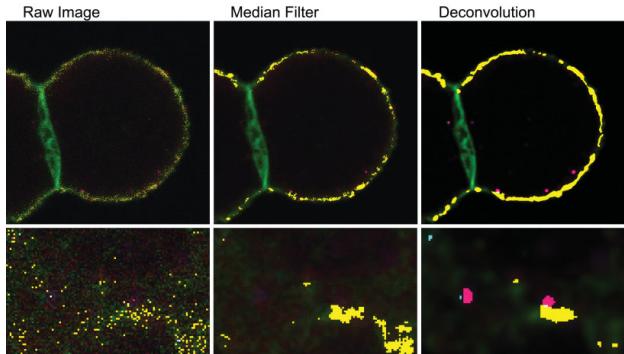
Checking data integrity

Pre-processing

Choosing a reporter/ metric

Comparing  
Interpreting

Assembling a workflow



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

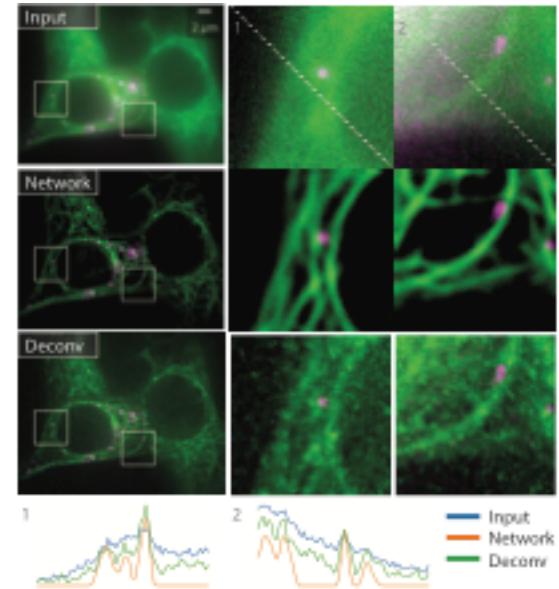
## Restoration

### *Deconvolution:*

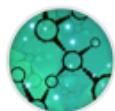
- Not to be used as a black box !
- Choose the algorithm well (conservative, nb iterations/stop criterion)
- Know your PSF
- Make sure the PSF is the same everywhere or use multi-PSF algo.
- Look for artefacts

### *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

## Pre-processing

Checking data integrity

Pre-processing

Choosing a reporter/ metric

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**Alternatively, you may ask a friend...**



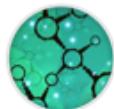
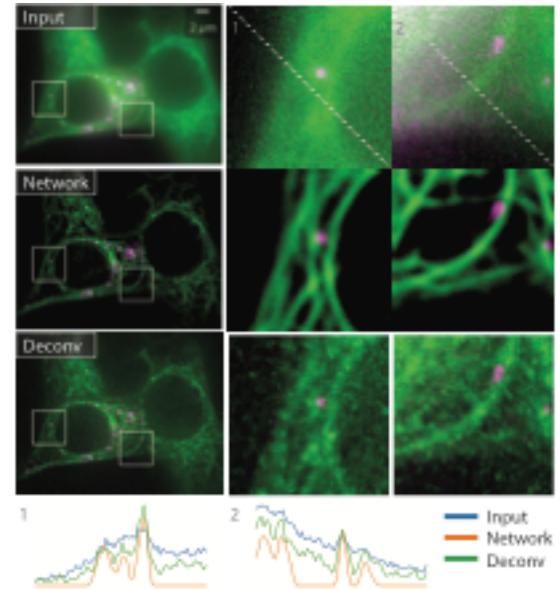
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# Co-localisation workflows

## Pre-processing

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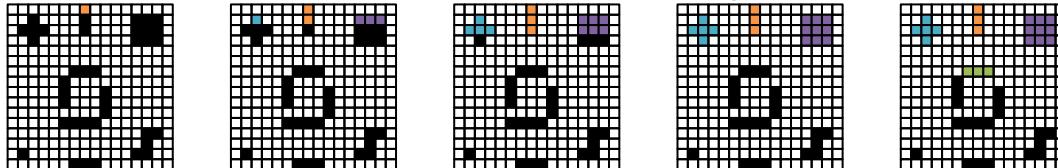
Pre-processing

Choosing a reporter/ metric

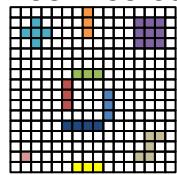
Comparing  
Interpreting

Assembling a workflow

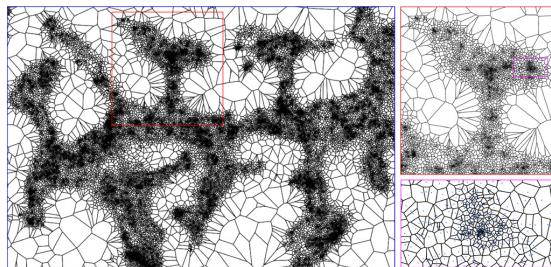
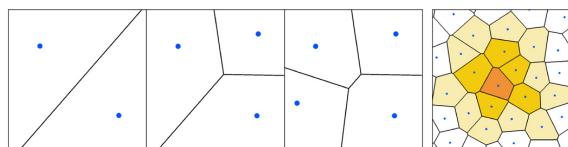
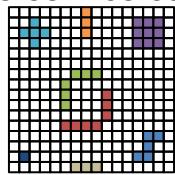
**Pixel tagging, line per line → Objects' map**



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)

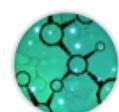
*When working on detections:*

- 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

Checking data integrity

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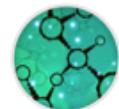
Unmixing: T. Zimmermann, Adv. Biochem. Eng. Biotechnol., vol. 95, pp. 245–265, 2005.

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

Thr. algos.: M. Sezgin and B. Sankur, *J. Electron. Imaging*, vol. 13, pp. 146–165, 2004.

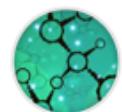
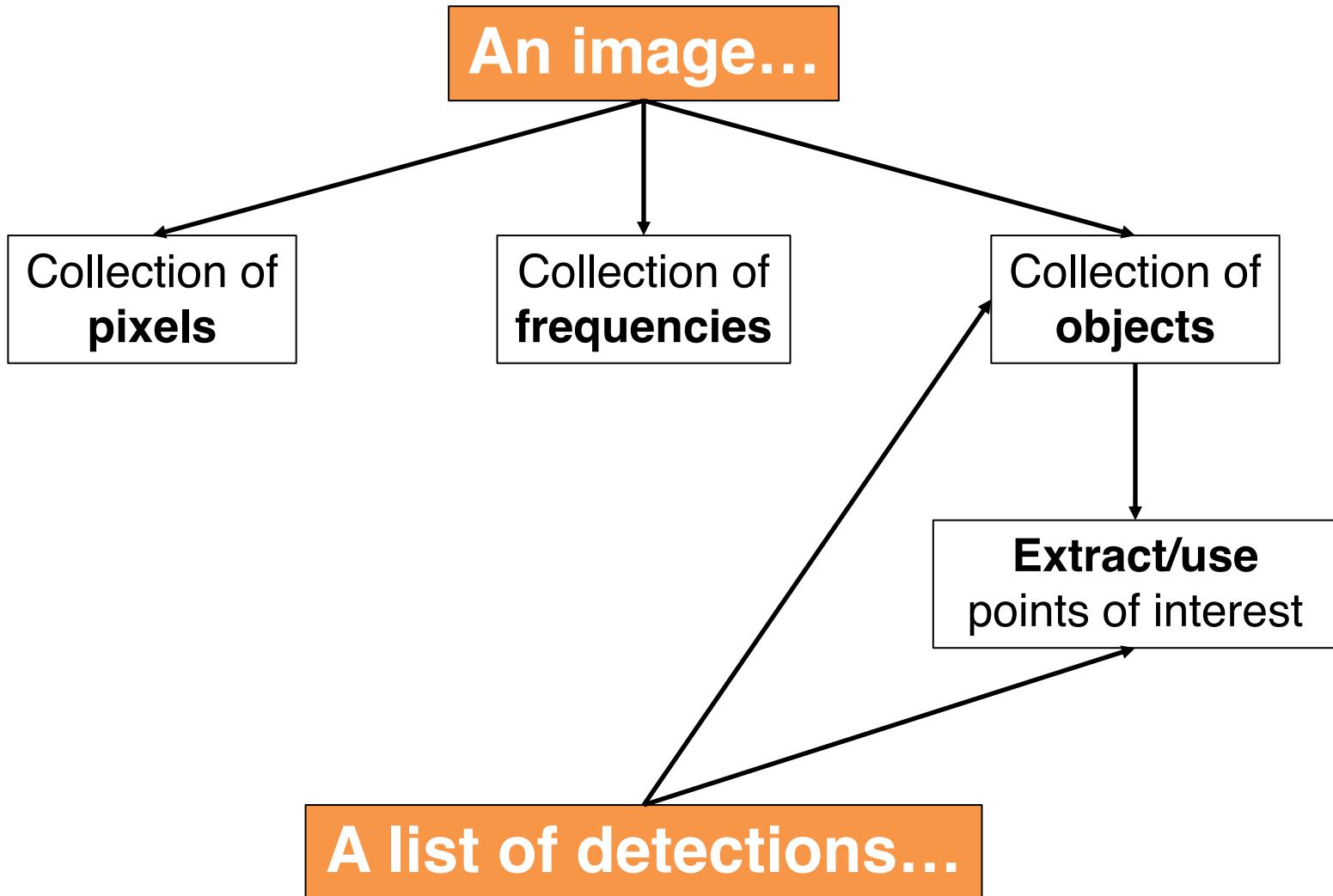
# Choosing a reporter/metric

image  
format  
file  
JPEG



# Co-localisation workflows

## *Data input*



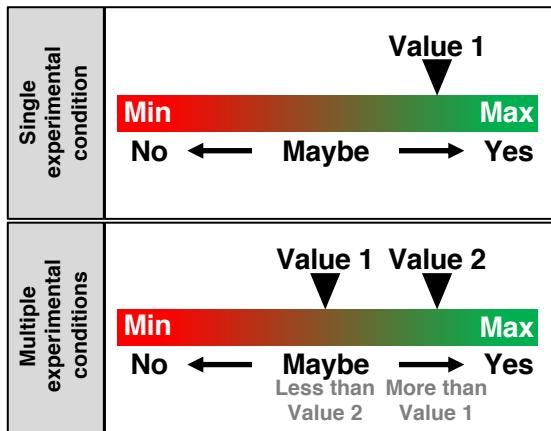
# Co-localisation workflows

## *Choosing a reporter/metric*



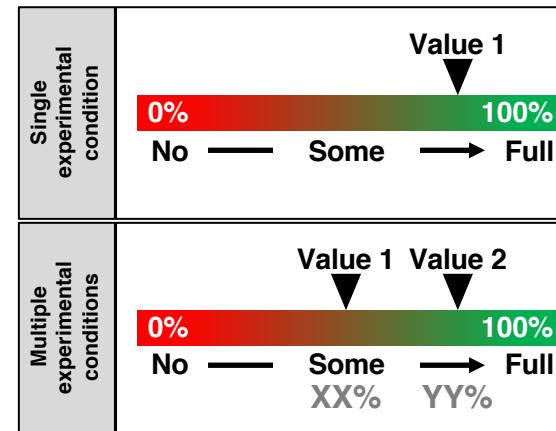
## Indicators

« 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 »

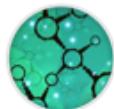


# Quantifiers

« 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. Cordelieres and S. Bolte, *Methods Cell Biol.*, vol. 123, pp. 395–408, Jan. 2014.



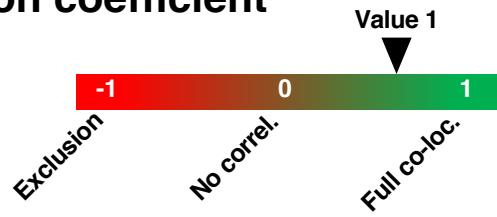
# Co-localisation workflows

## Choosing a reporter/metric



### Pearson's correlation coefficient

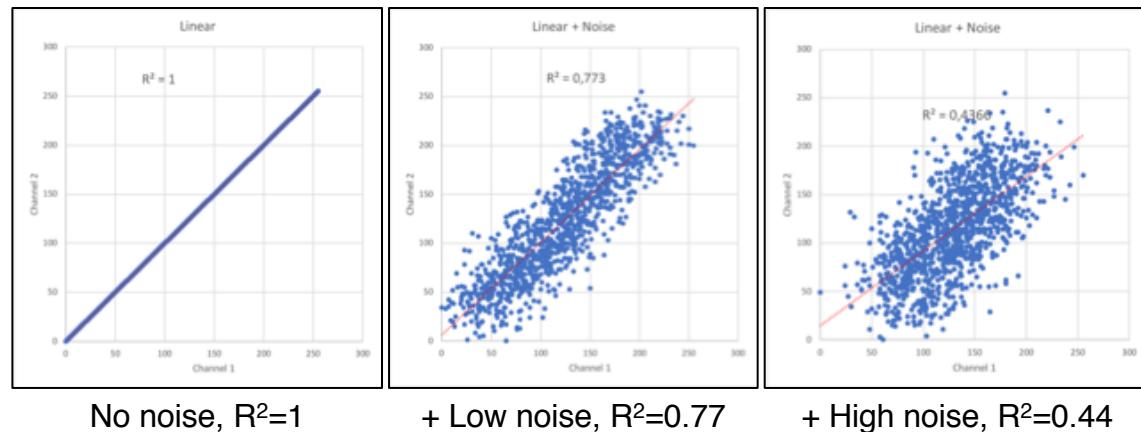
$$r_p = \frac{\sum_i (R_i - R_{aver}) \times (G_i - G_{aver})}{\sqrt{\sum_i (R_i - R_{aver})^2 \times \sum_i (G_i - G_{aver})^2}}$$



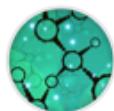
### Indicators

« 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 »

Single experimental condition	
Multiple experimental conditions	



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

Pre-processing

Choosing a reporter/ metric

Comparing  
Interpreting

Assembling a workflow

## Indicators

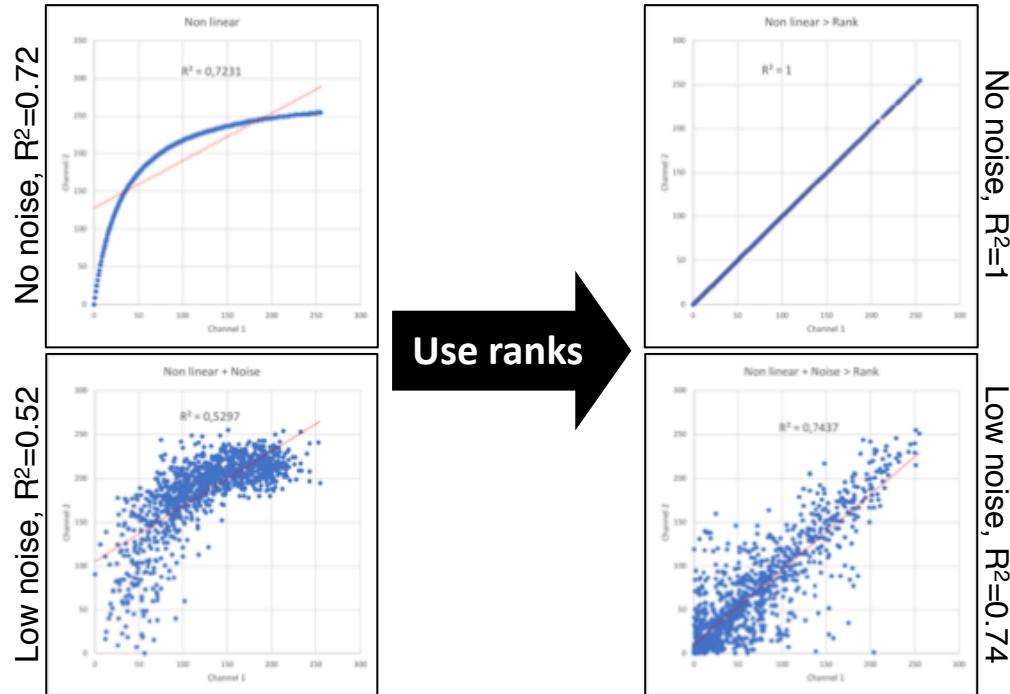
« numerical values that evaluate a degree of signal coincidence over a predefined scale, but are not suitable to calculate a precise amount of overlap. They are suitable for relative comparisons, without being usable for direct quantitative studies »

Single experimental condition	Value 1	
	Min → Maybe → Yes	
	No ← Maybe → Yes	
Multiple experimental conditions	Value 1	Value 2
	Min → 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

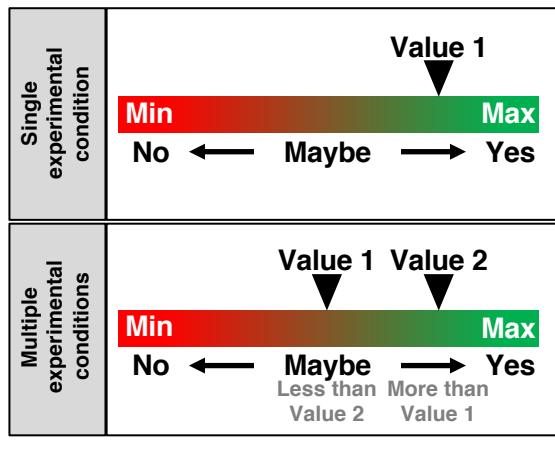
## Choosing a reporter/metric



**Many other indicators exist !**

## Indicators

« 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 »



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

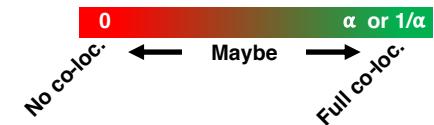
### $k_1$ & $k_2$ coefficients

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

$k_1$ : 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

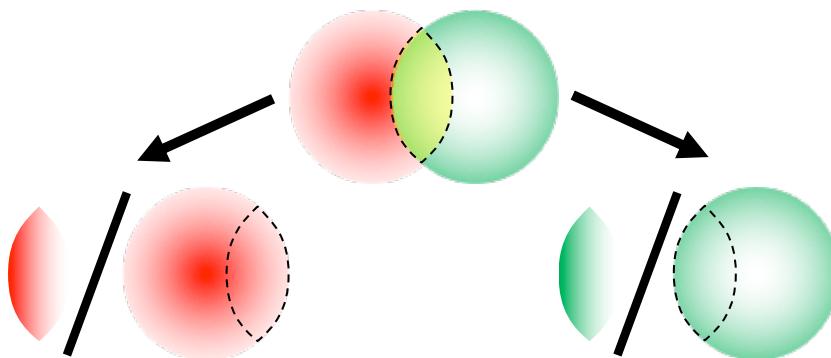
Pre-processing

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

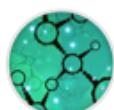
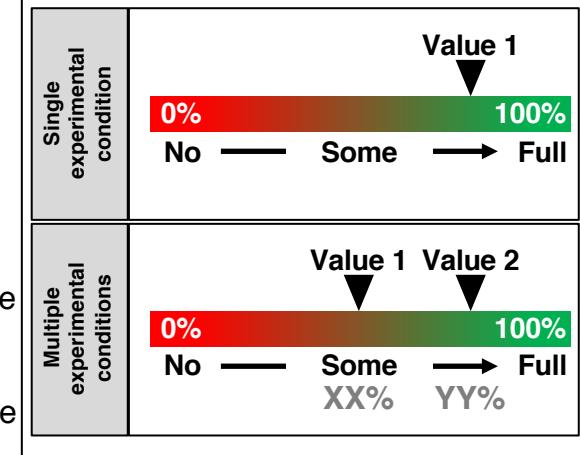
$$M_2 = \frac{\sum_i B_{i,coloc}}{\sum_i B_i}$$

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. »



# Co-localisation workflows

## Choosing a reporter/metric

Checking data integrity

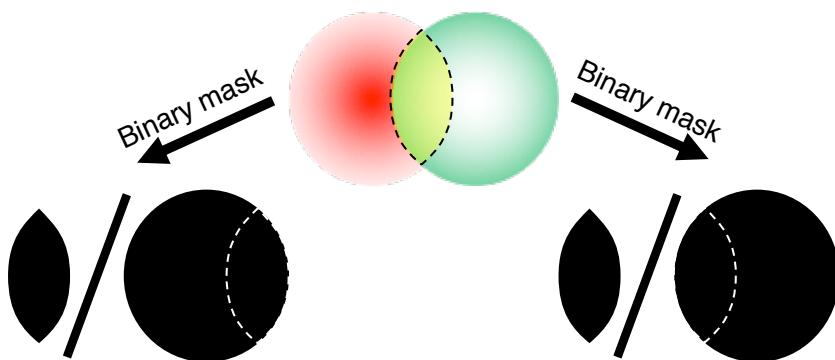
Pre-processing

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  $B_i > \text{Thr}_B$ , 0 otherwise  
and  $A_i=1$  if  $A_i > \text{Thr}_A$ , 0 otherwise

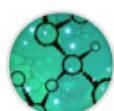
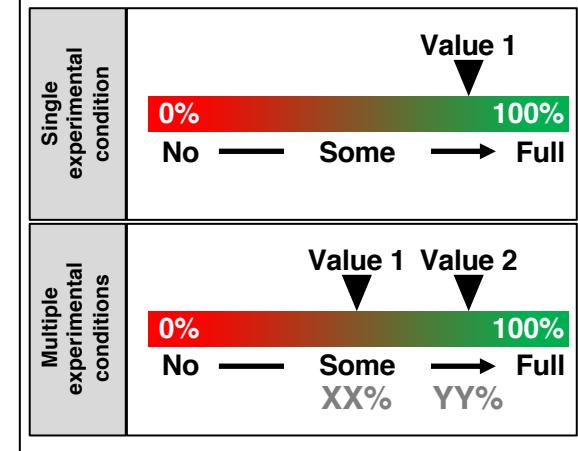
$$M_2 = \frac{\sum_i B_{i,coloc}}{\sum_i B_i}$$

Where  $B_{i,coloc}=1$  if  $A_i > \text{Thr}_A$ , 0 otherwise  
and  $B_i=1$  if  $B_i > \text{Thr}_B$ , 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

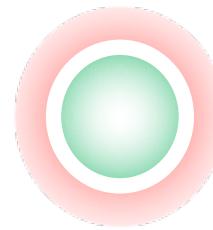
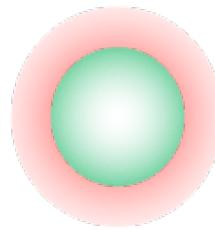
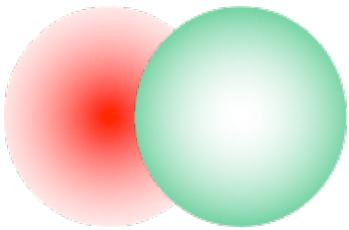
Pre-processing

Choosing a reporter/ metric

Comparing  
Interpreting

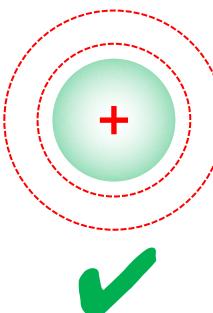
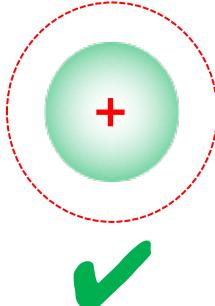
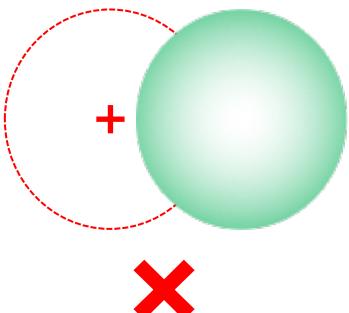
Assembling a workflow

### Based on centre/object overlap



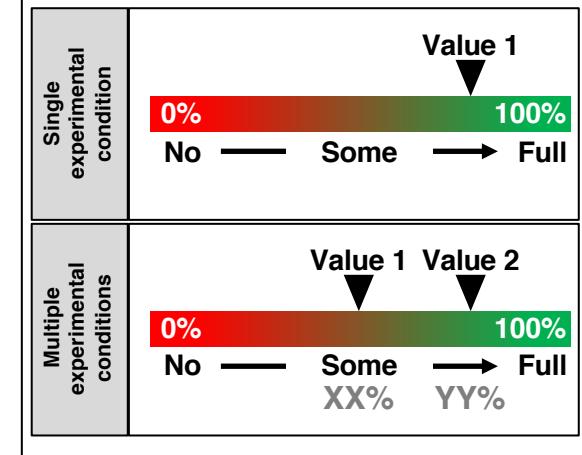
#### Method:

- 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

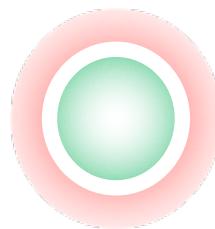
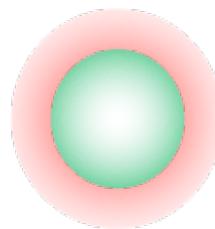
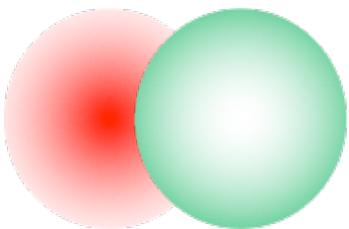
Pre-processing

Choosing a reporter/ metric

Comparing  
Interpreting

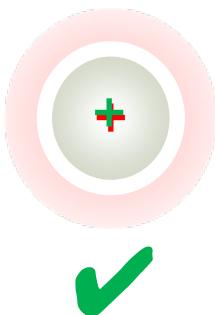
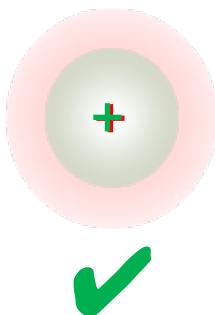
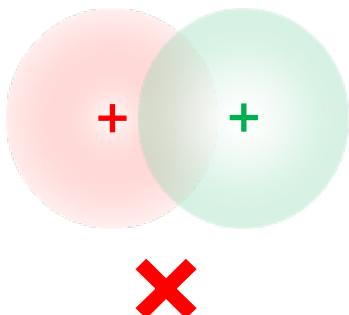
Assembling a workflow

### Based on distances



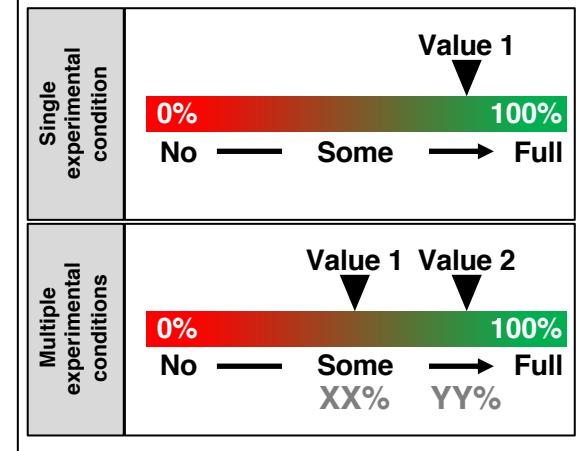
#### Method:

- 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 ?

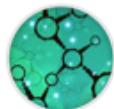


### Quantifiers

« 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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# Comparing/Interpreting



# 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

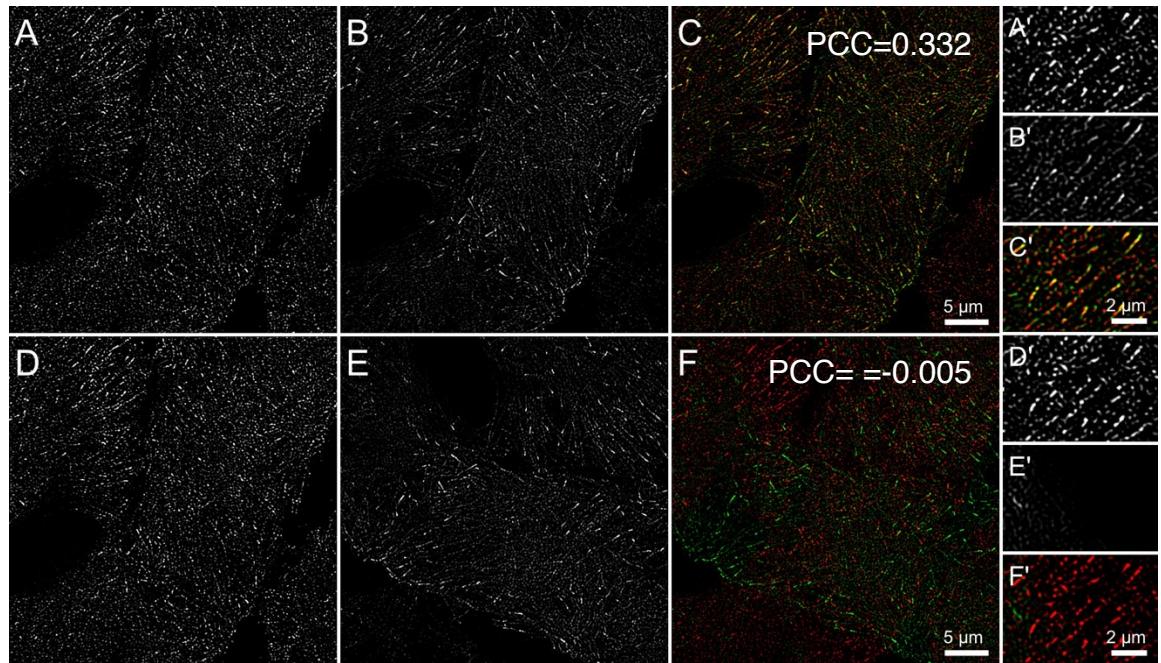
#### Methods:

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

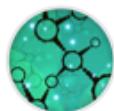
#### In practice:

- 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

Checking data integrity

Pre-processing

Choosing a reporter/ metric

Comparing Interpreting

Assembling a workflow

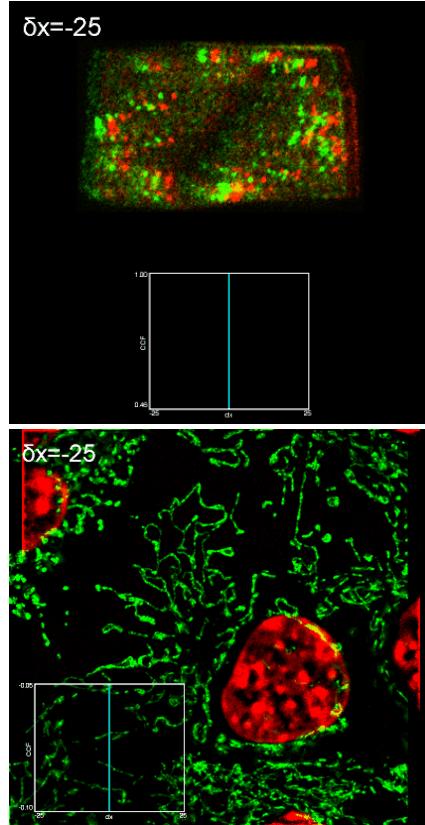
### Getting significance out of a single dataset

#### Methods:

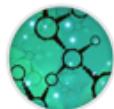
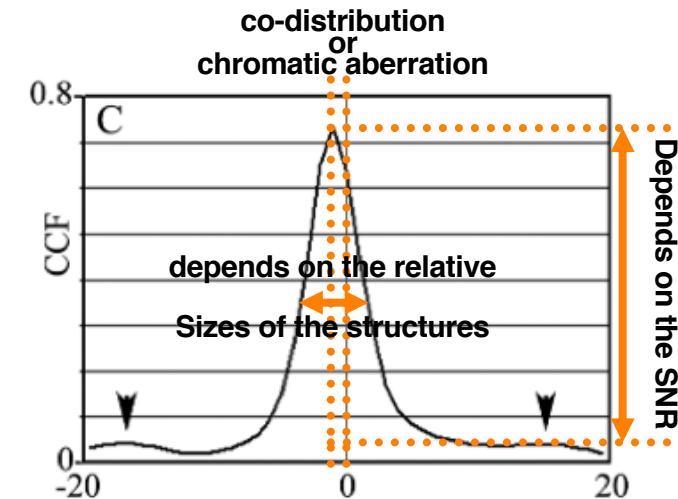
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

#### 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

## Comparing/interpreting

Checking data integrity

Pre-processing

Choosing a reporter/ metric

Comparing  
Interpreting

Assembling a workflow

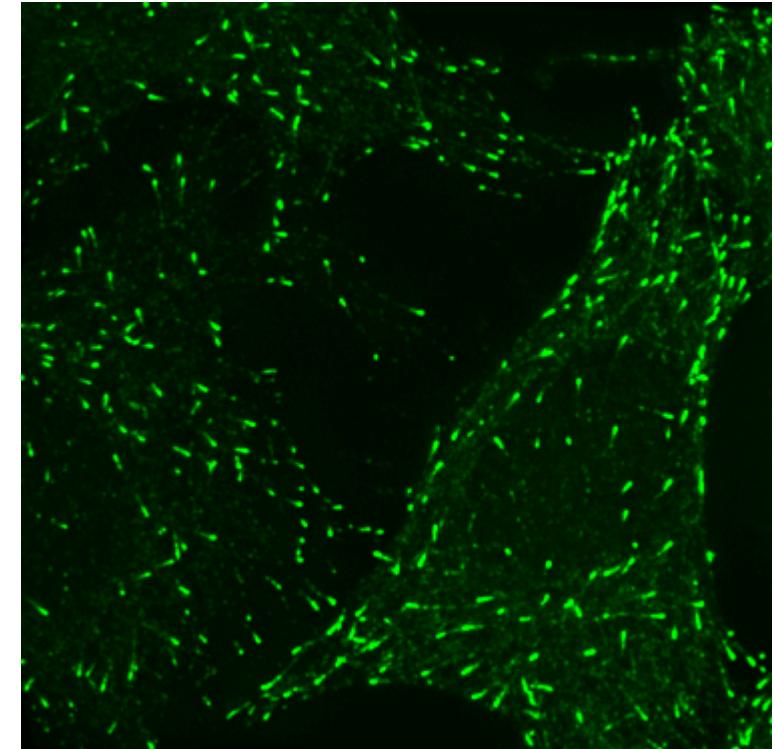
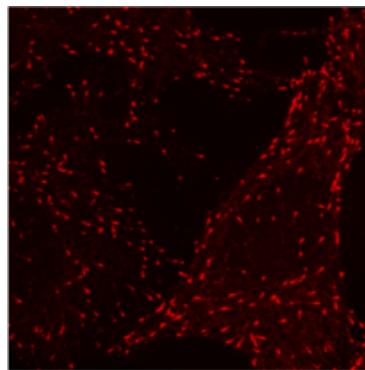
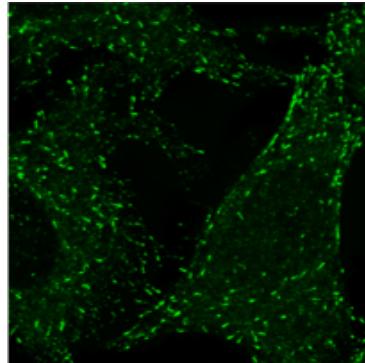
### Getting significance out of a single dataset

#### Methods:

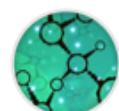
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

#### 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

## Comparing/interpreting

Checking data integrity

Pre-processing

Choosing a reporter/ metric

Comparing  
Interpreting

Assembling a workflow

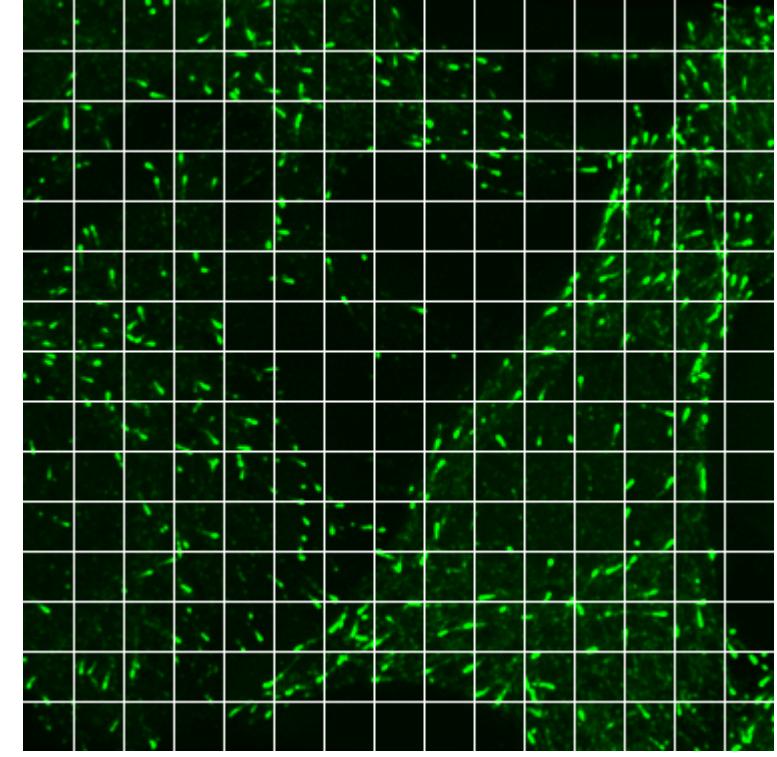
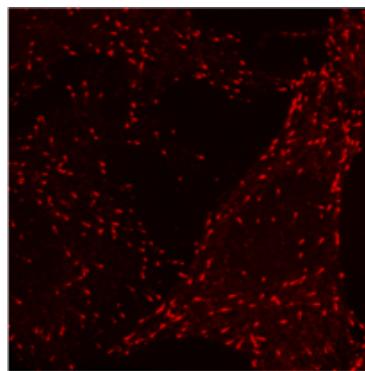
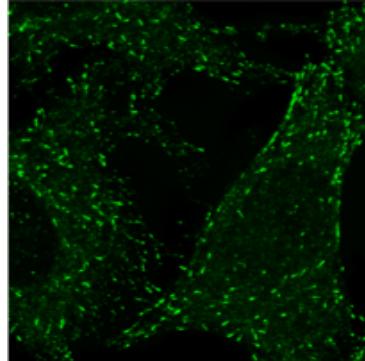
### Getting significance out of a single dataset

#### Methods:

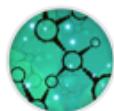
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

#### 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

## Comparing/interpreting

Checking data integrity

Pre-processing

Choosing a reporter/ metric

Comparing  
Interpreting

Assembling a workflow

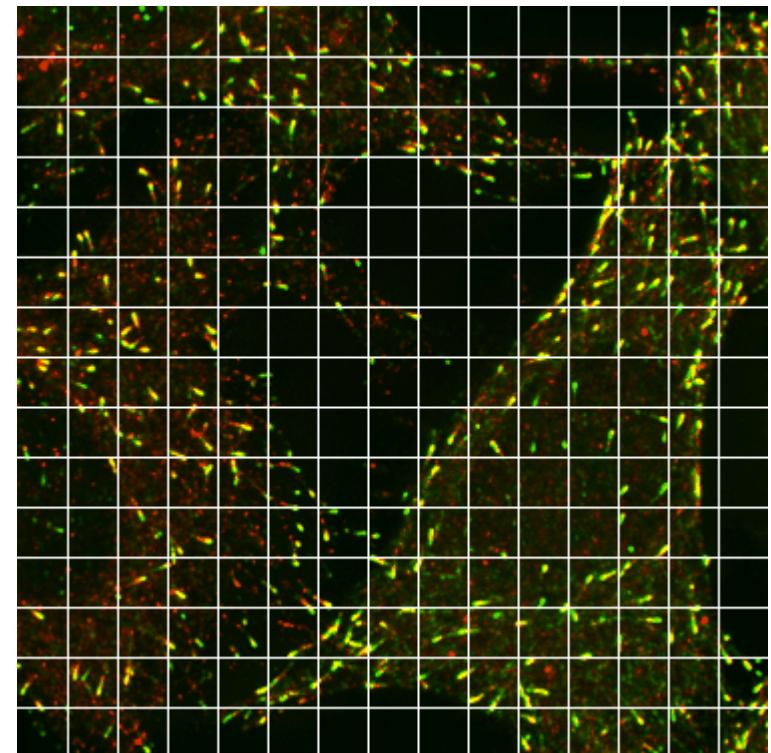
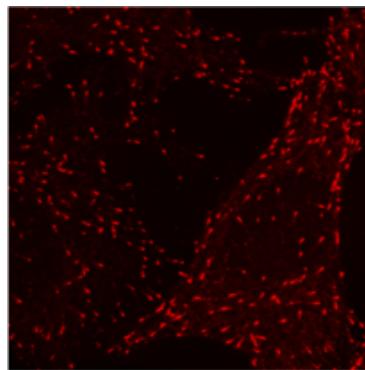
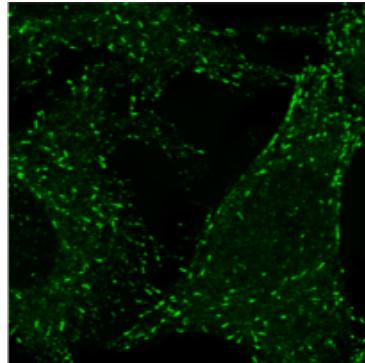
### Getting significance out of a single dataset

#### Methods:

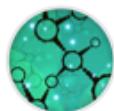
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

#### 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

## Comparing/interpreting

Checking data integrity

Pre-processing

Choosing a reporter/ metric

Comparing Interpreting

Assembling a workflow

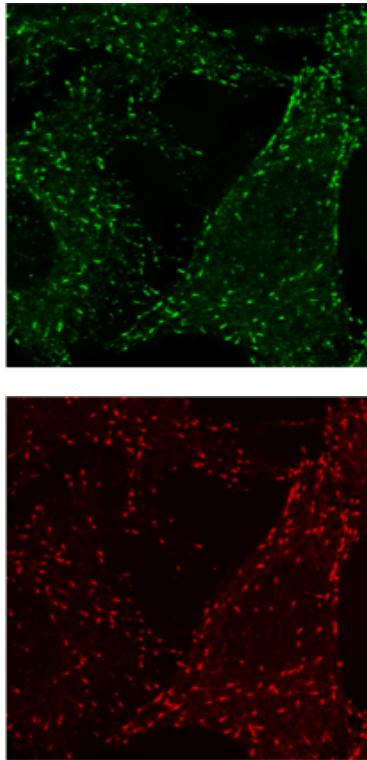
### Getting significance out of a single dataset

#### Methods:

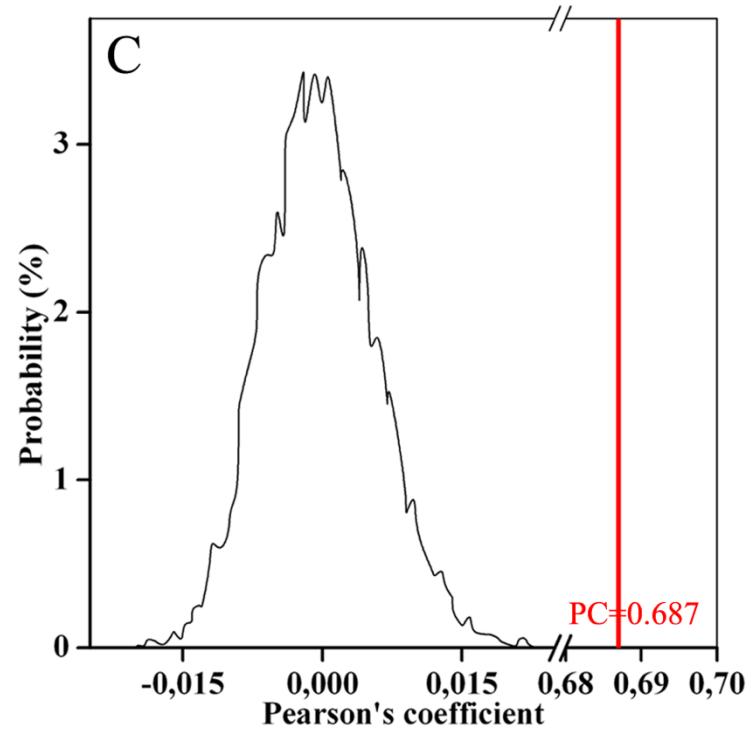
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

#### In practice:

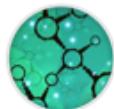
- If image's width and height are the same: rotate one of the two images by 90°
- Translate one image relative to the other
- Randomise the image



### 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.



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# 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

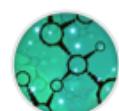
**McDonald & Dunn 2013**

***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:

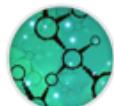
- To repeat the experiment for the same condition
- Calculate the average PCC ( $m_0$ )
- Use the **one-sample one tailed Student's t-test**

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

- **Null hypothesis  $H_0$ :** the difference between the true mean ( $\mu$ ) 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

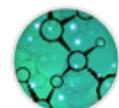
**In practice:**

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.

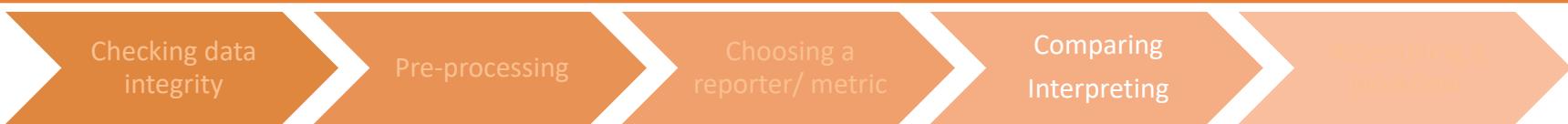
You should read this: J.H. McDonald and K.W. Dunn, Statistical tests for measures of colocalization in biological microscopy., *J. Microsc.*, 252:295–302, 2013.

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

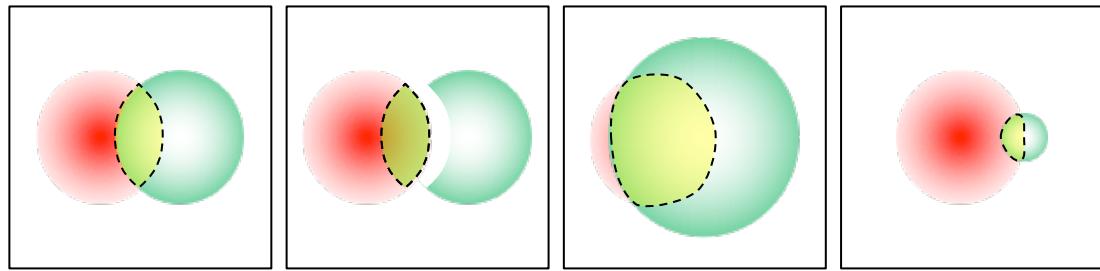
#### In practice:

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:

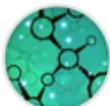


**"This makes MCC values difficult to interpret in isolation:** an MCC<sub>1</sub> of 0.60 would mean **strong colocalization** if only 5% of the image was green, **no association** if 60% of the image was green and **strong anticolocalization** if 95% of the image was green. Evidence for colocalization 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



### 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

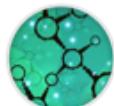
#### In practice:

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>



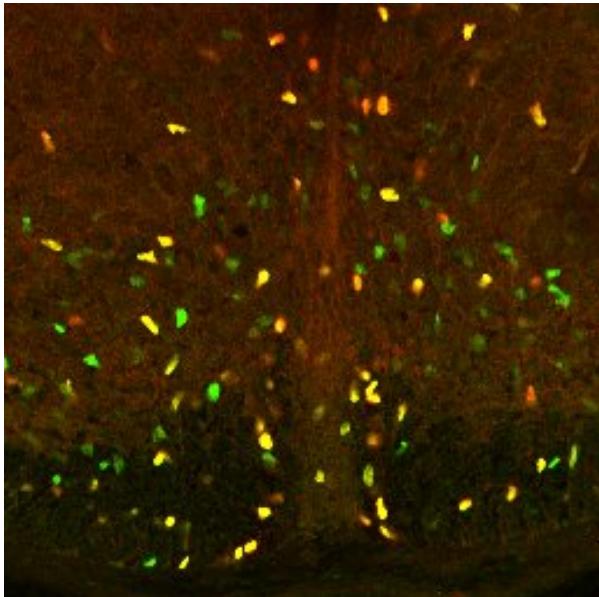
# Assembling a workflow



# 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

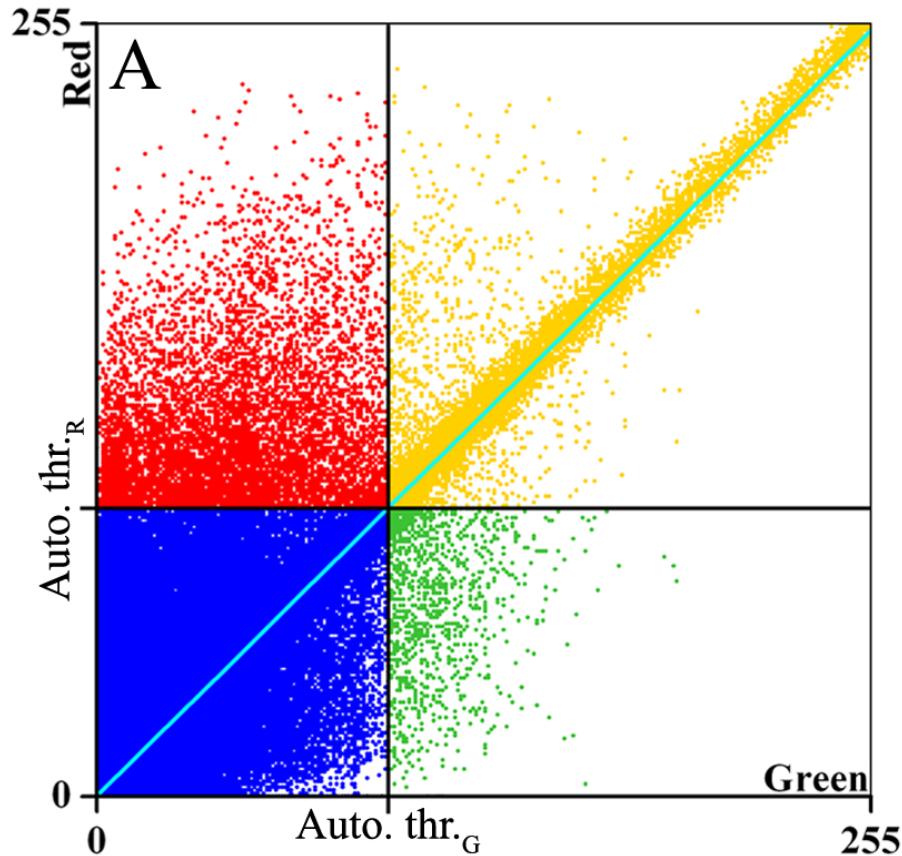
## *Co-expression analysis*

### How to set the threshold ? Costes' automatic threshold

#### Principle:

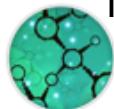
- Set threshold at max of the range
- Compute PCC below thresholds
- If  $PCC > 0$ , lower thresholds
- Stop and get thresholds just before  $PCC \leq 0$

→ 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.



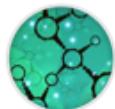
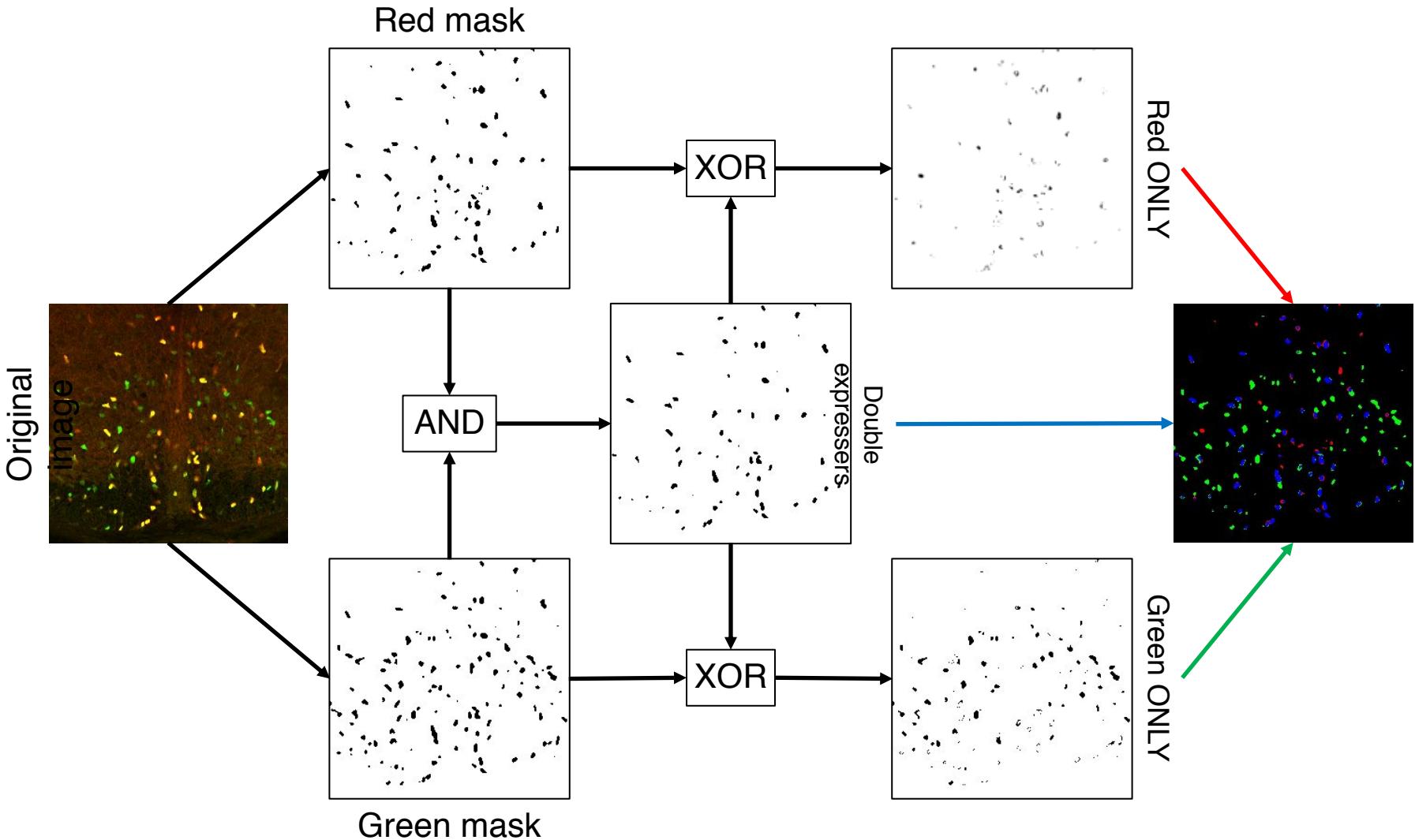
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# Co-localisation workflows

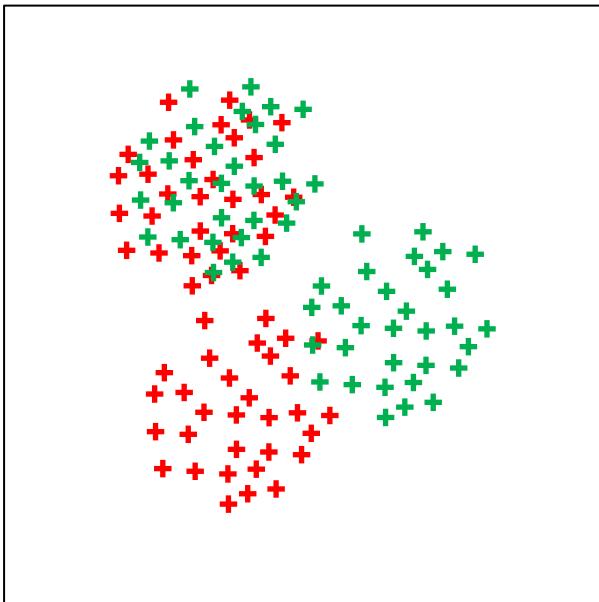
## *Co-expression analysis*



# Co-localisation workflows

## *Working with detections*

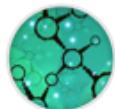
Original image



### 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 ???***



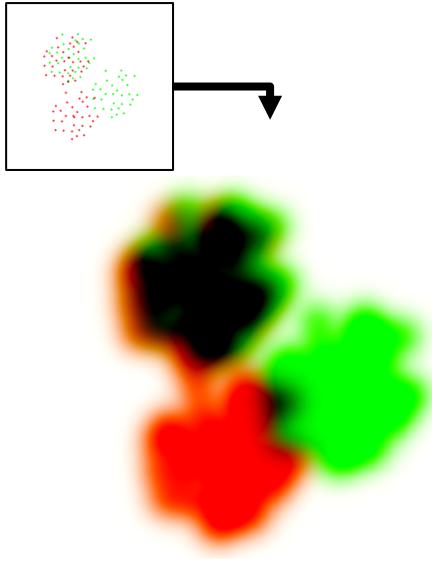
# Co-localisation workflows

## 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

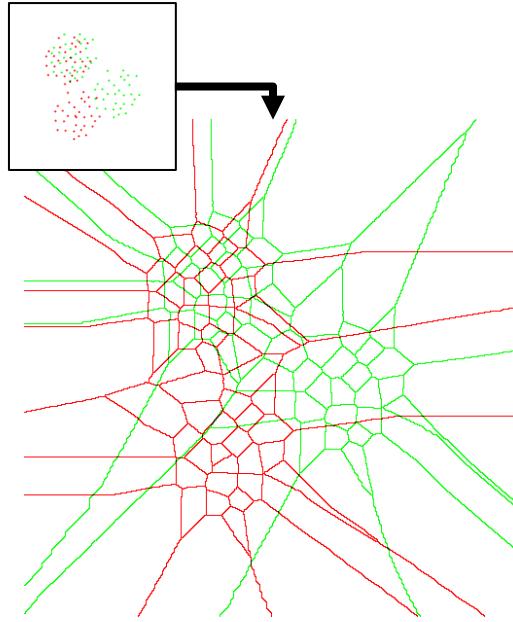


E. Betzig *et al.*, *Science*, 313, 1642–5, 2006.

### 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

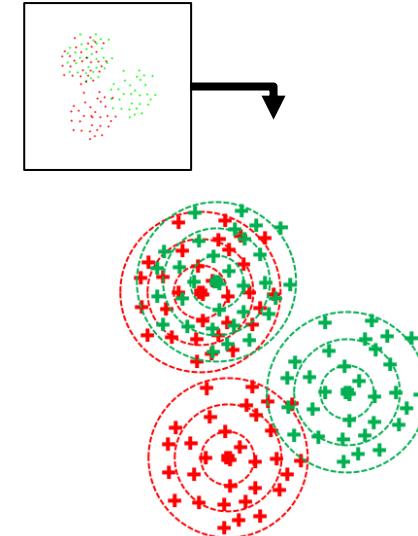


F. Levet *et al.*, *Nat. Methods*, 12, 1065–1071, 2015.  
L. Andronov, *et al.* *Sci. Rep.*, 6, 1–9, 2016.

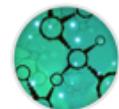
### Working on distances

#### 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.



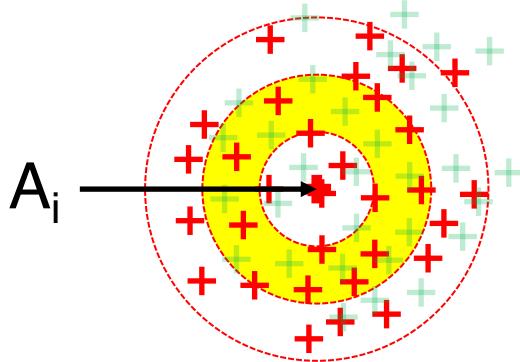
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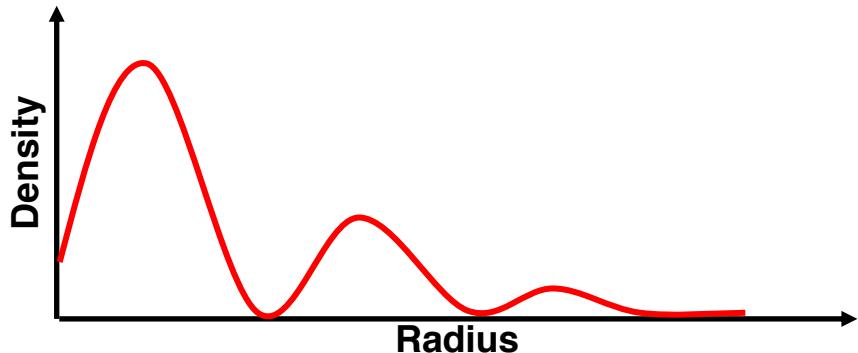
# Co-localisation workflows

## Working with detections



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

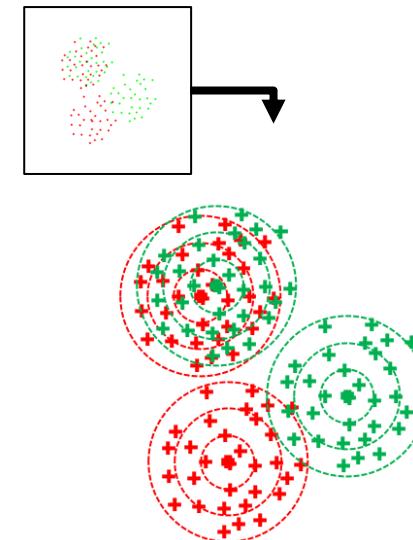
$$D_{A_i,A}(r) = \frac{N_{A_i,A}(r)}{\pi r^2} \times \frac{\pi R_{max}^2}{N_{A_i,A}(R_{max})} = \frac{N_{A_i,A}(r)}{N_{A_i,A}(R_{max})} \times \frac{R_{max}^2}{r^2}$$



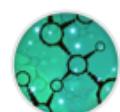
### Working on distances

#### Methods:

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

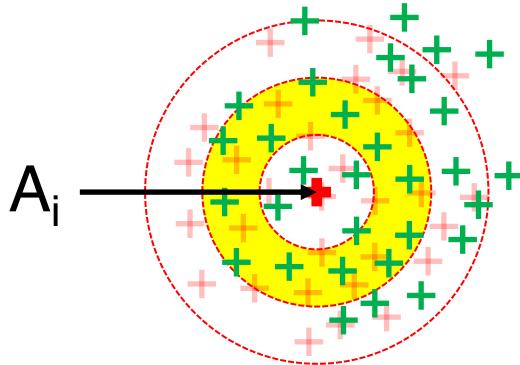


S. Malkusch *et al.*, *Histochem. Cell Biol.*, 137, 1–10, 2012.



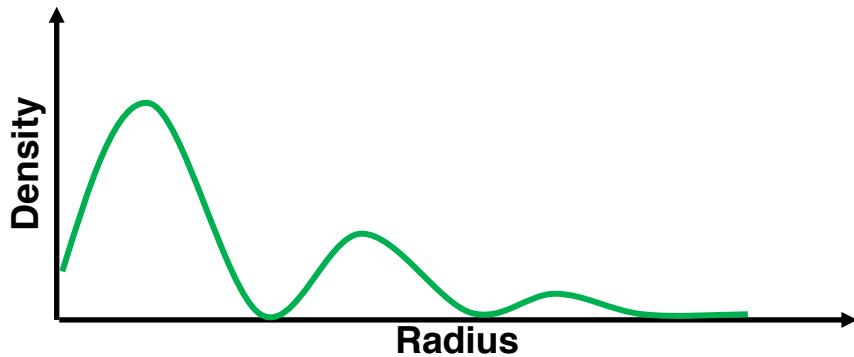
# Co-localisation workflows

## Working with detections



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

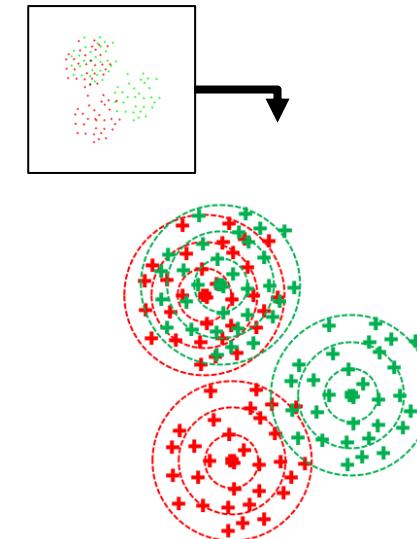
$$D_{A_i,B}(r) = \frac{N_{A_iB}(r)}{N_{A_iB}(R_{max})} \times \frac{R_{max}^2}{r^2}$$



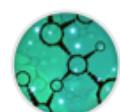
### Working on distances

#### Methods:

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



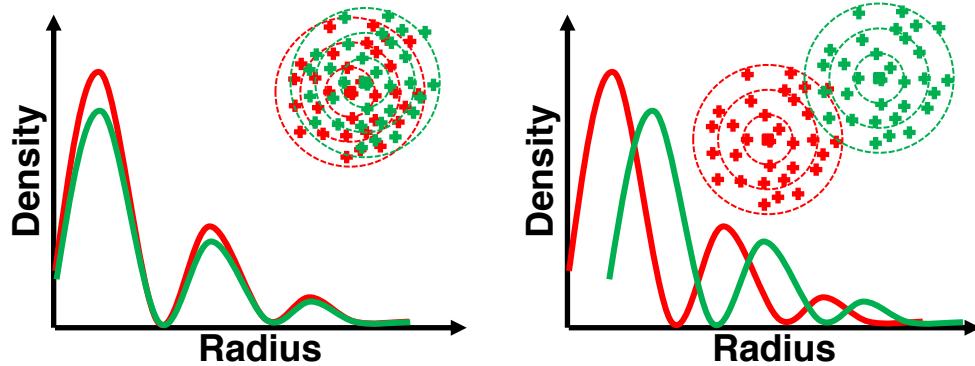
S. Malkusch *et al.*, *Histochem. Cell Biol.*, 137, 1–10, 2012.



# Co-localisation workflows

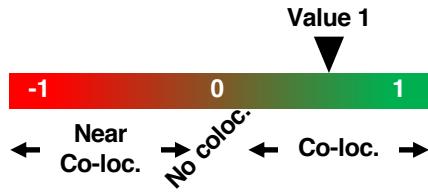
## Working with detections

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

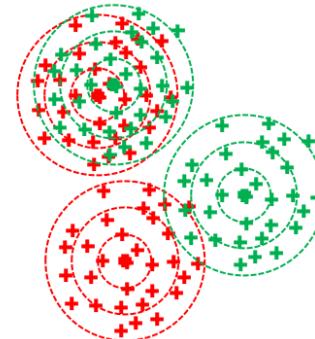
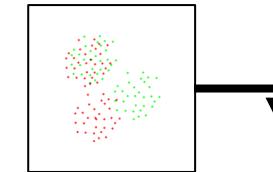
$$C_{Ai} = S_{Ai} \cdot e^{\left( -\frac{E_{Ai,B}}{R_{max}} \right)}$$



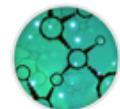
## Working on distances

### Methods:

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

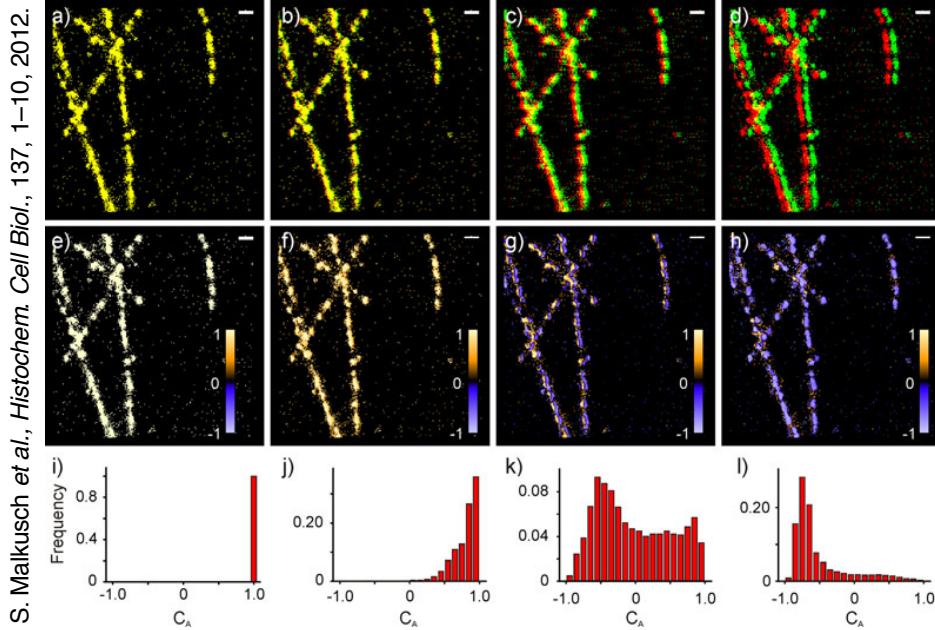


S. Malkusch *et al.*, *Histochem. Cell Biol.*, 137, 1–10, 2012.



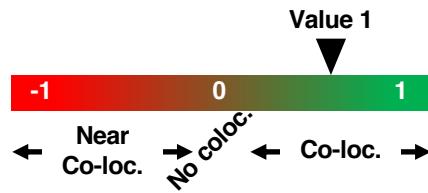
# Co-localisation workflows

## Working with detections



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

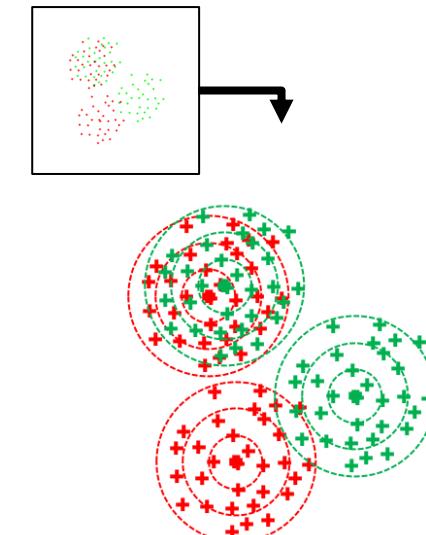
$$C_{A_i} = S_{A_i} \cdot e^{\left( \frac{-E_{A_i, B}}{R_{max}} \right)}$$



## Working on distances

### Methods:

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

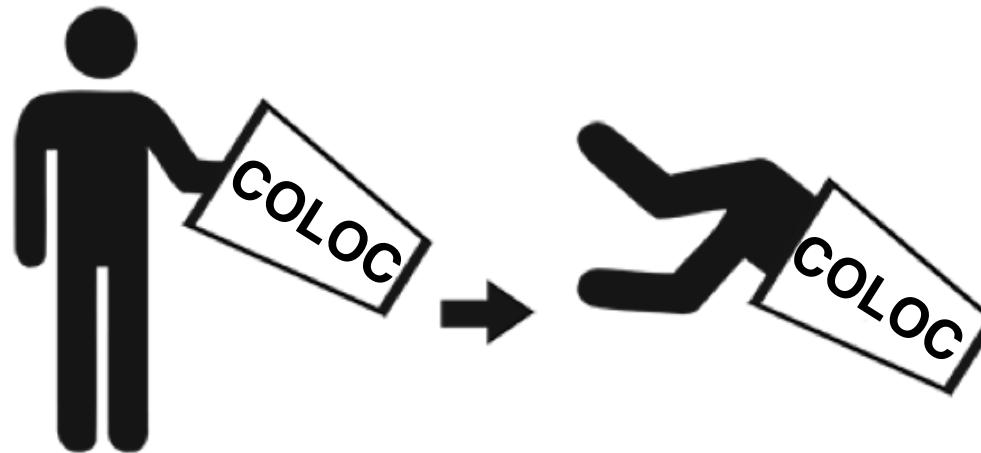


S. Malkusch et al., *Histochem. Cell Biol.*, 137, 1–10, 2012.

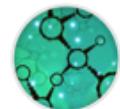
Last advice  
*Think, be creative, test, get help... repeat*

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# DANGER



**THIS MACHINE  
HATES IDIOTS**



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