Deconstructing co-localisation workflows:

A journey into the black boxes

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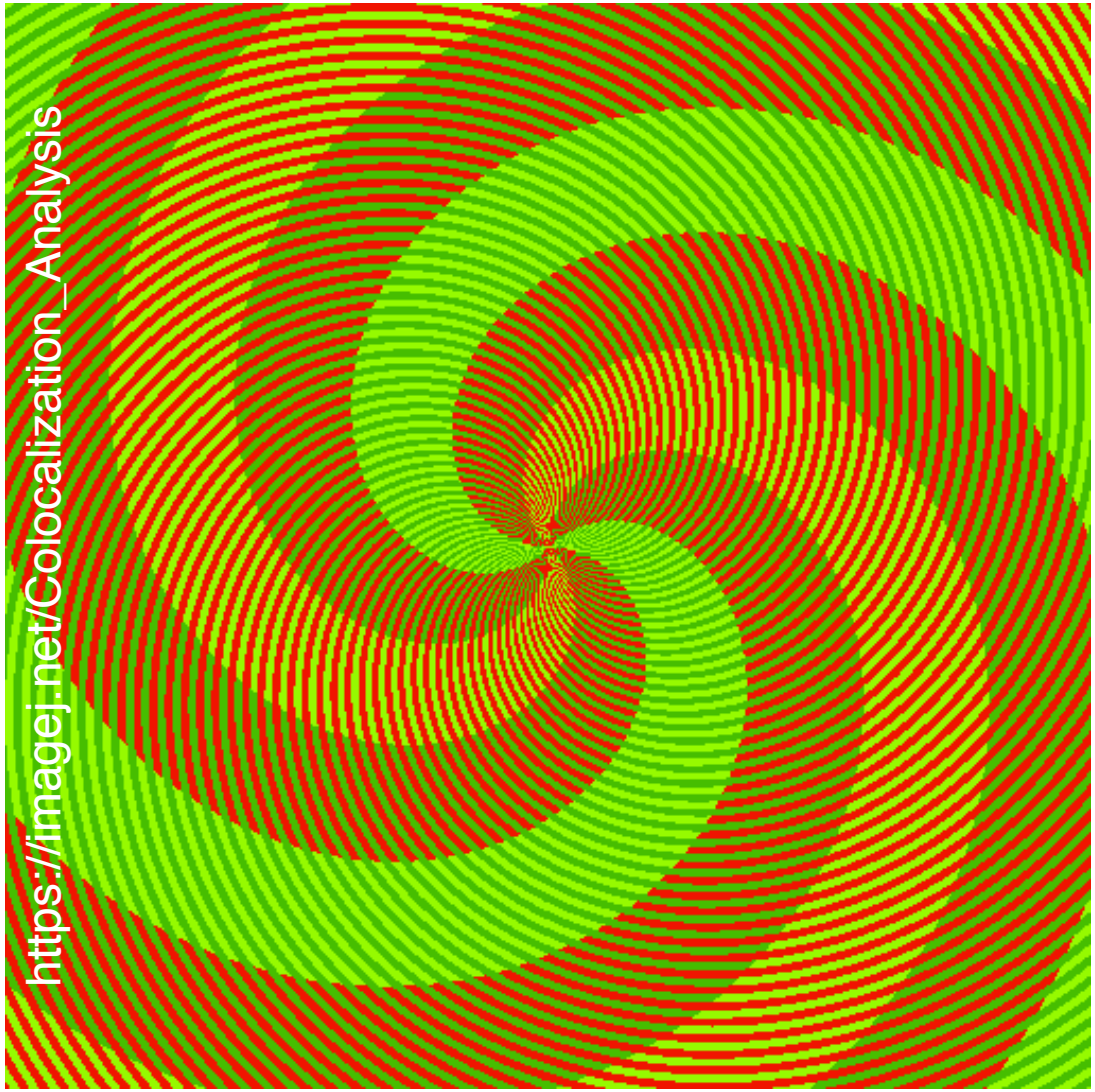
FRANCE-BIOIMAGING

Bordeaux Imaging Center

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



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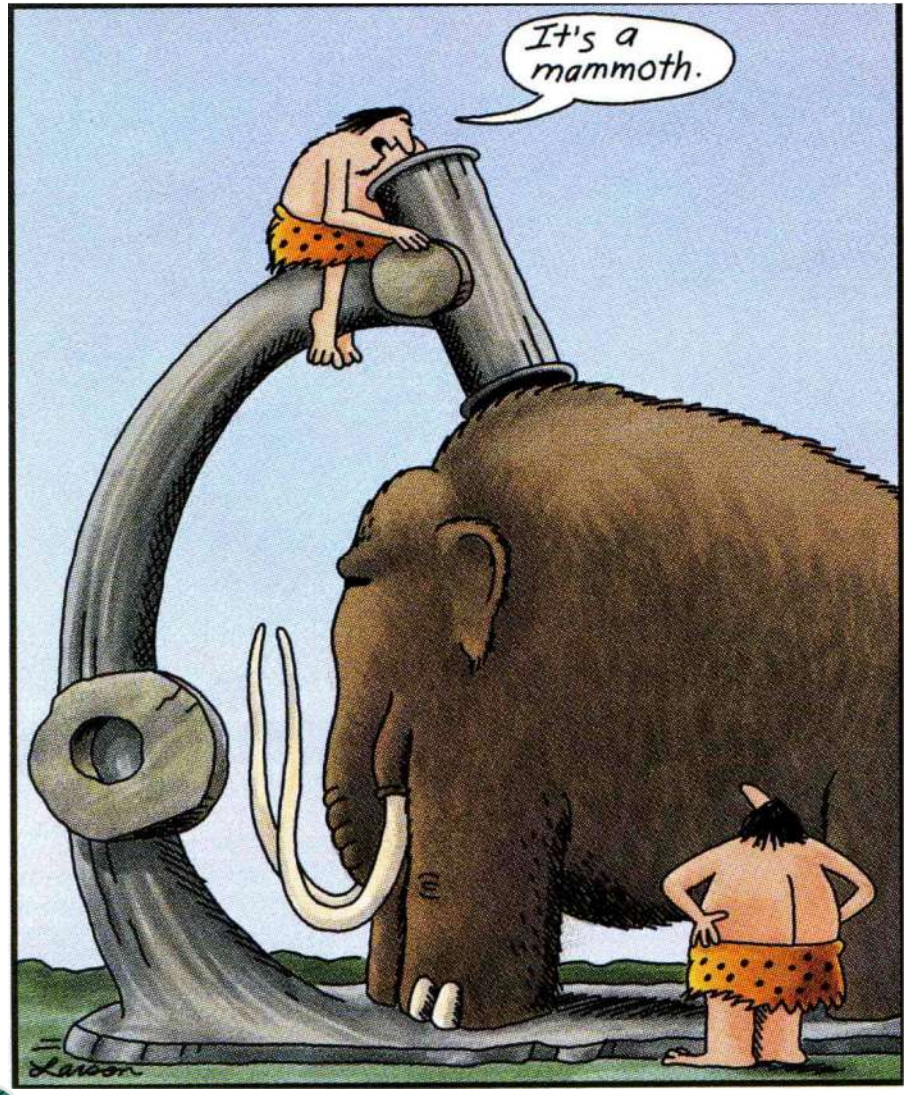

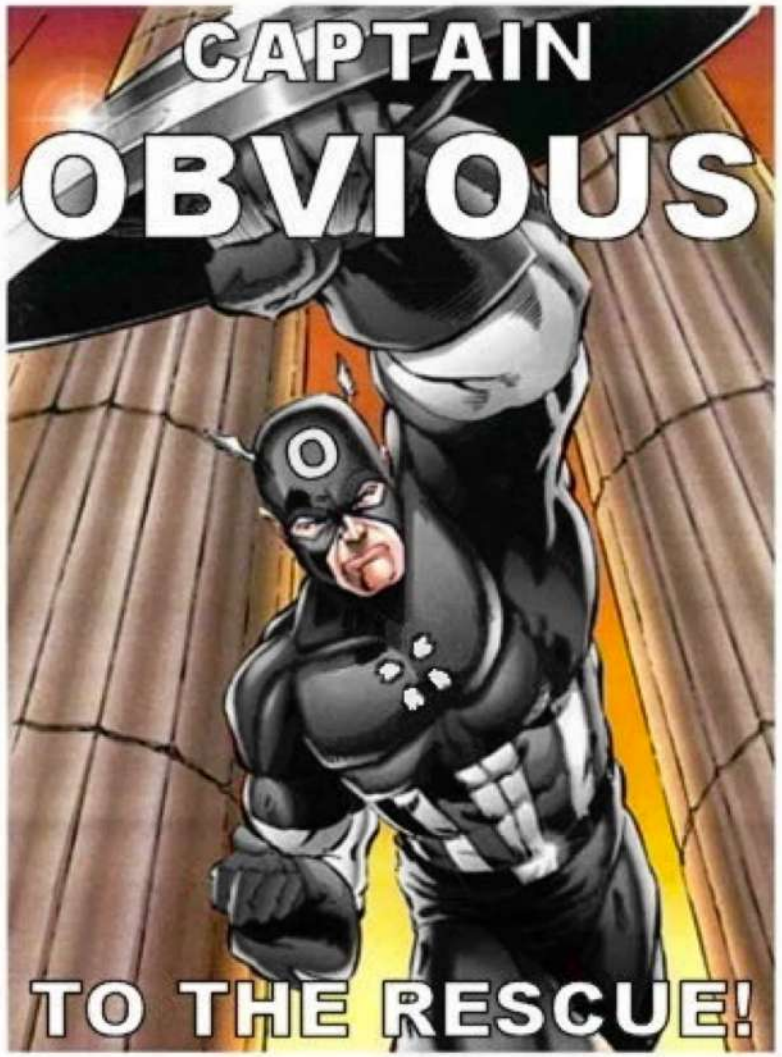

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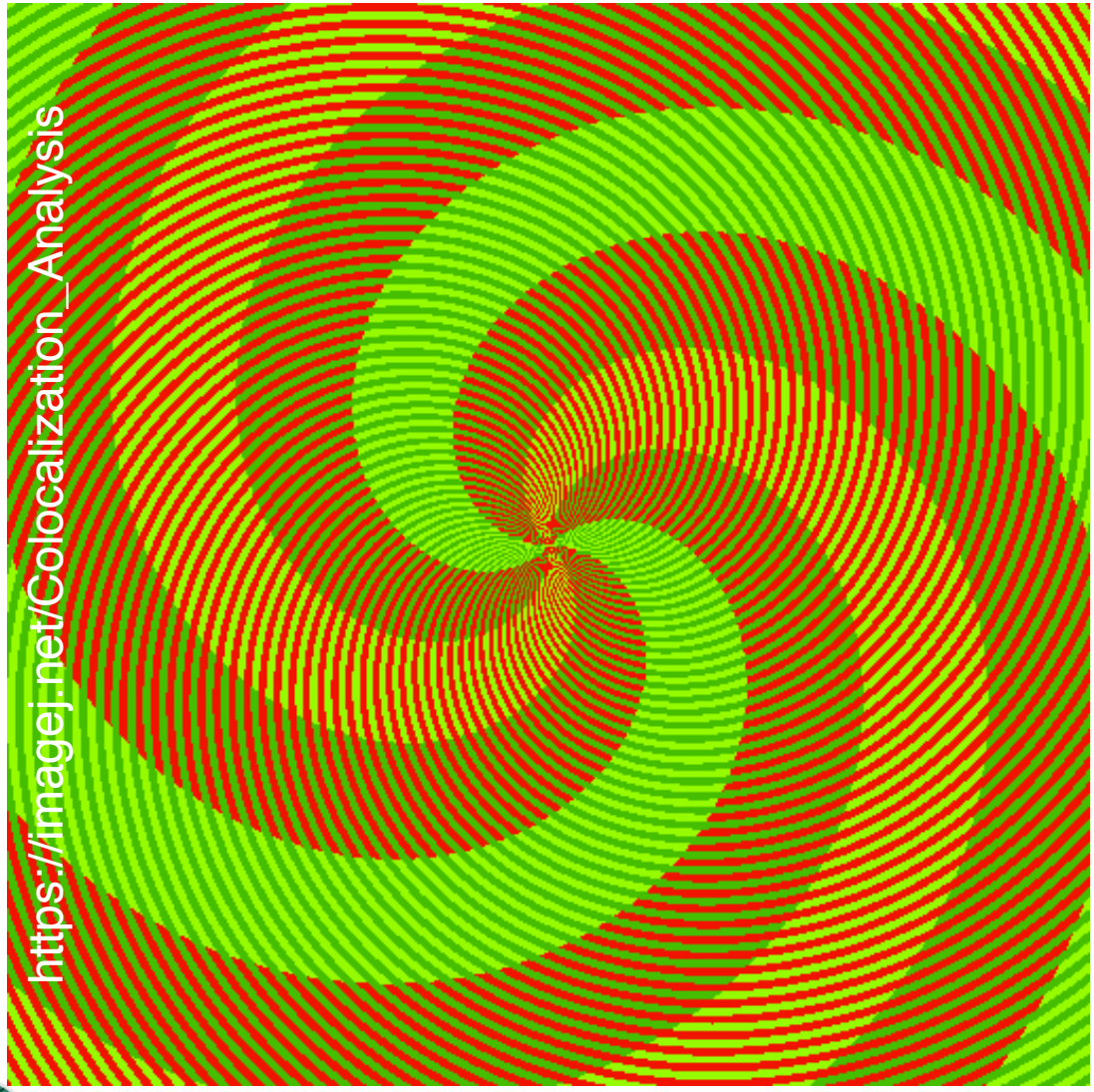
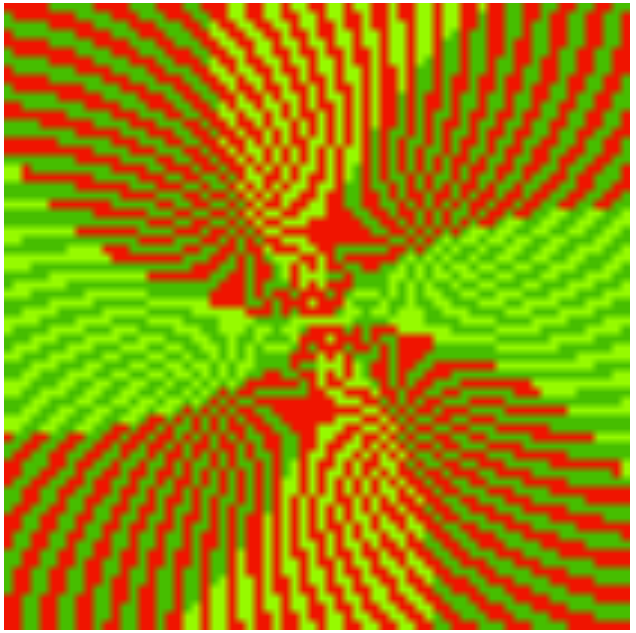


Obvious, was it ?



File ▸ Open Samples ▸ Spirals (Macro) in Fiji

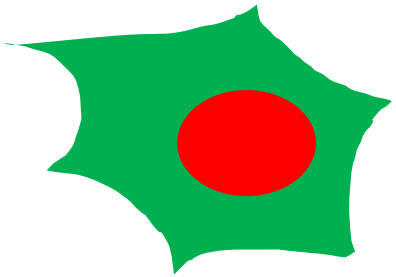
Co-localisation

One word, many meanings

Co-localisation

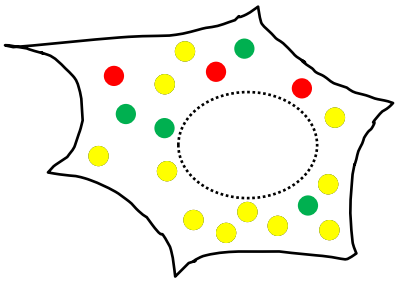
Co-expression

Two proteins are located within the same structure/cell



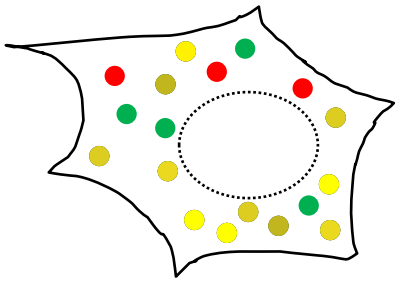
Co-occurence

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



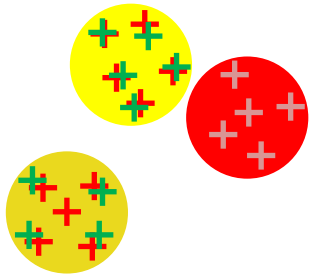
Correlation

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

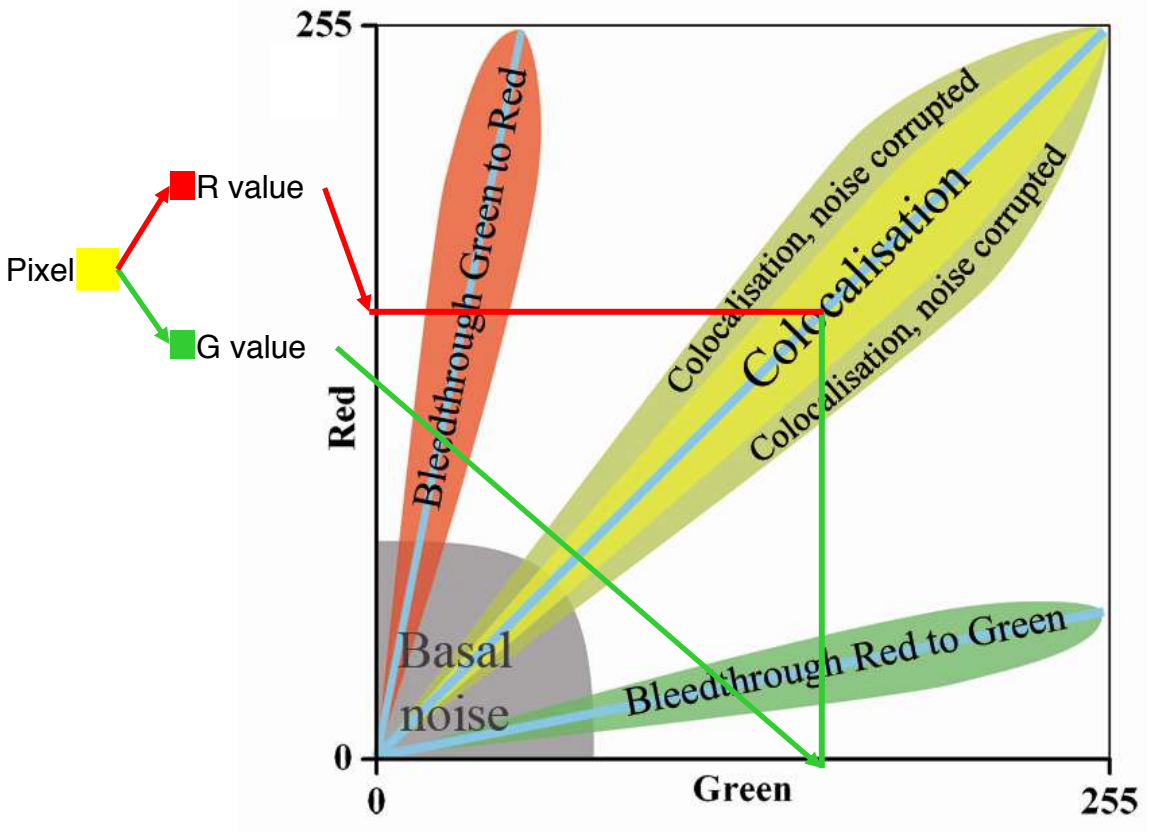
Co-localisation workflows

Checking data integrity

Dyes

Check for bleethrough and/or cross-talk

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

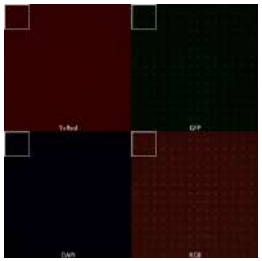


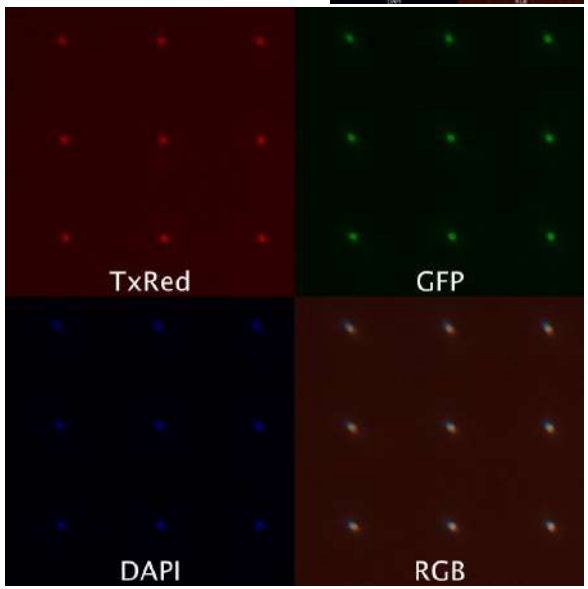
Co-localisation workflows

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



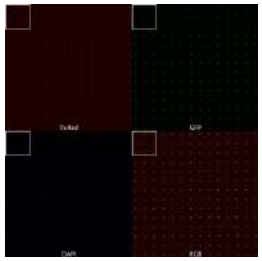


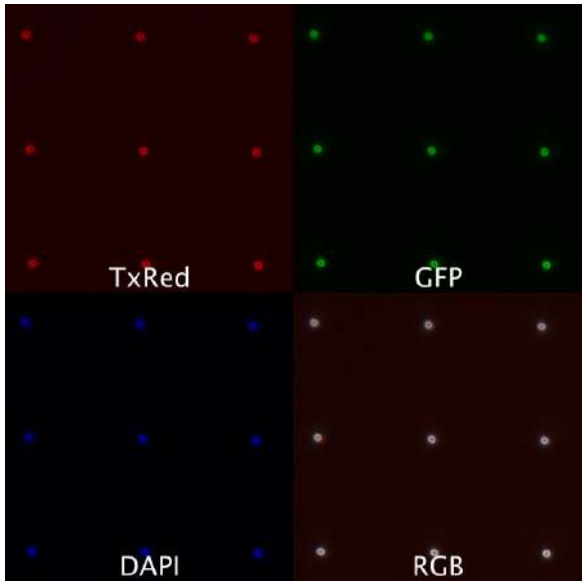
Co-registration

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

Use reference slides (fluorescent beads, Argolight slide) to check for mis-registration and aberrations

Have a look at the MetroloJ plugin !





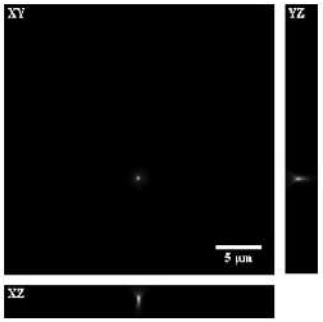
Leica 63x/1.4
Hamamatsu Flash 4.0
sCMOS c-mount

Co-localisation workflows

Checking data integrity

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

Profile view:



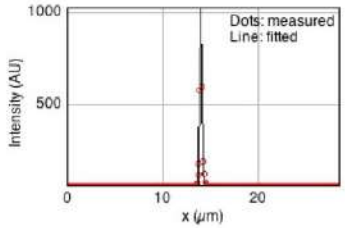
Microscope infos:

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

Resolution table:

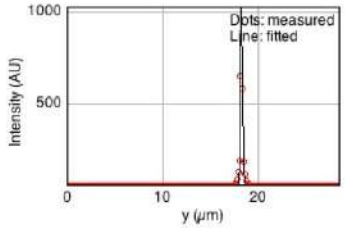
	FWHM	Theoretical resolution
x	0.251 µm	0.166 µm
y	0.26 µm	0.166 µm
z	0.753 µm	0.414 µm

X profile & fitting parameters:



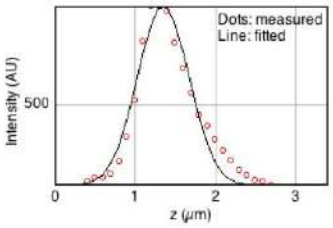
Fitted on y = a + (b-a)*exp(-(x-c)^2/(2*d^2))
Number of iterations: 485 (8000)
Number of restarts: 2 (2)
Sum of residuals squared: 11530.2429
Standard deviation: 6.7243
R^2: 0.9927
Parameters:
a = 56.7427
b = 1024.0978
c = 14.1057
d = 0.1066

Y profile & fitting parameters:



Fitted on y = a + (b-a)*exp(-(x-c)^2/(2*d^2))
Number of iterations: 532 (8000)
Number of restarts: 2 (2)
Sum of residuals squared: 12035.9240
Standard deviation: 6.8702
R^2: 0.9927
Parameters:
a = 56.9917
b = 1031.3087
c = 18.3514
d = 0.1105

Z profile & fitting parameters:



Fitted on y = a + (b-a)*exp(-(x-c)^2/(2*d^2))
Number of iterations: 454 (8000)
Number of restarts: 2 (2)
Sum of residuals squared: 116116.2975
Standard deviation: 58.4396
R^2: 0.9617
Parameters:
a = 111.0574
b = 983.7723
c = 1.3568
d = 0.3198

Resolution

Know your limits

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

Have a look at the MetroloJ plugin !

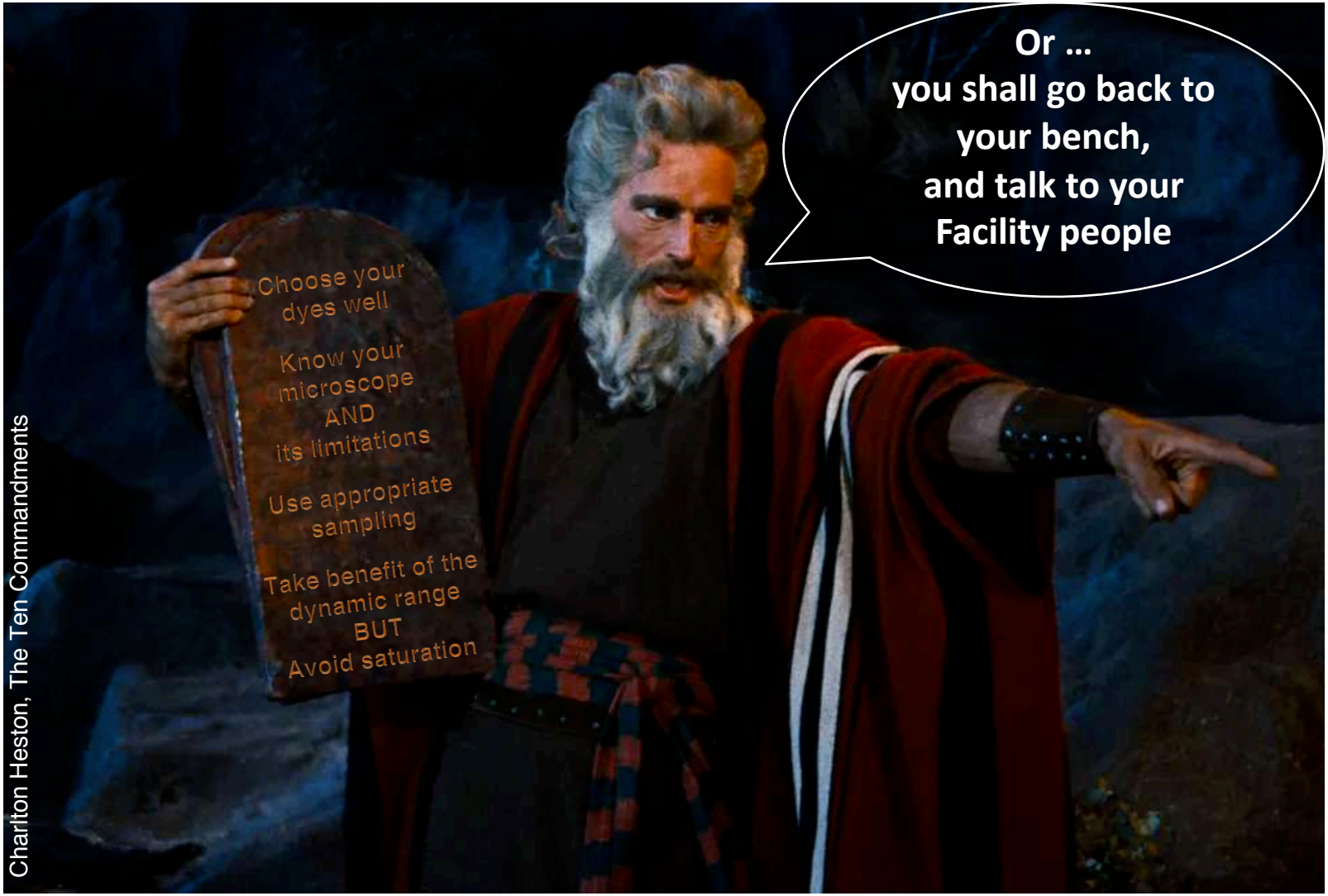
Co-localisation workflows

Checking data integrity

Checking data integrity → Pre-processing → Choosing a reporter/ metric → Comparing Interpreting → Assembling a workflow

Dyes	Co-registration	Resolution
Check for bleethrough and/or cross-talk	*Make sure what should be co-localised is co-localised*	*Know your limits*
One possible way is to use the cytofluorogram, looking for dots clouds close to the axis	Use reference slides (fluorescent beads, Argolight slide) to check for mis-registration and aberrations Have a look at the MetroloJ plugin !	Use reference slides (fluorescent beads, Argolight slide) to measure resolution Have a look at the MetroloJ plugin !

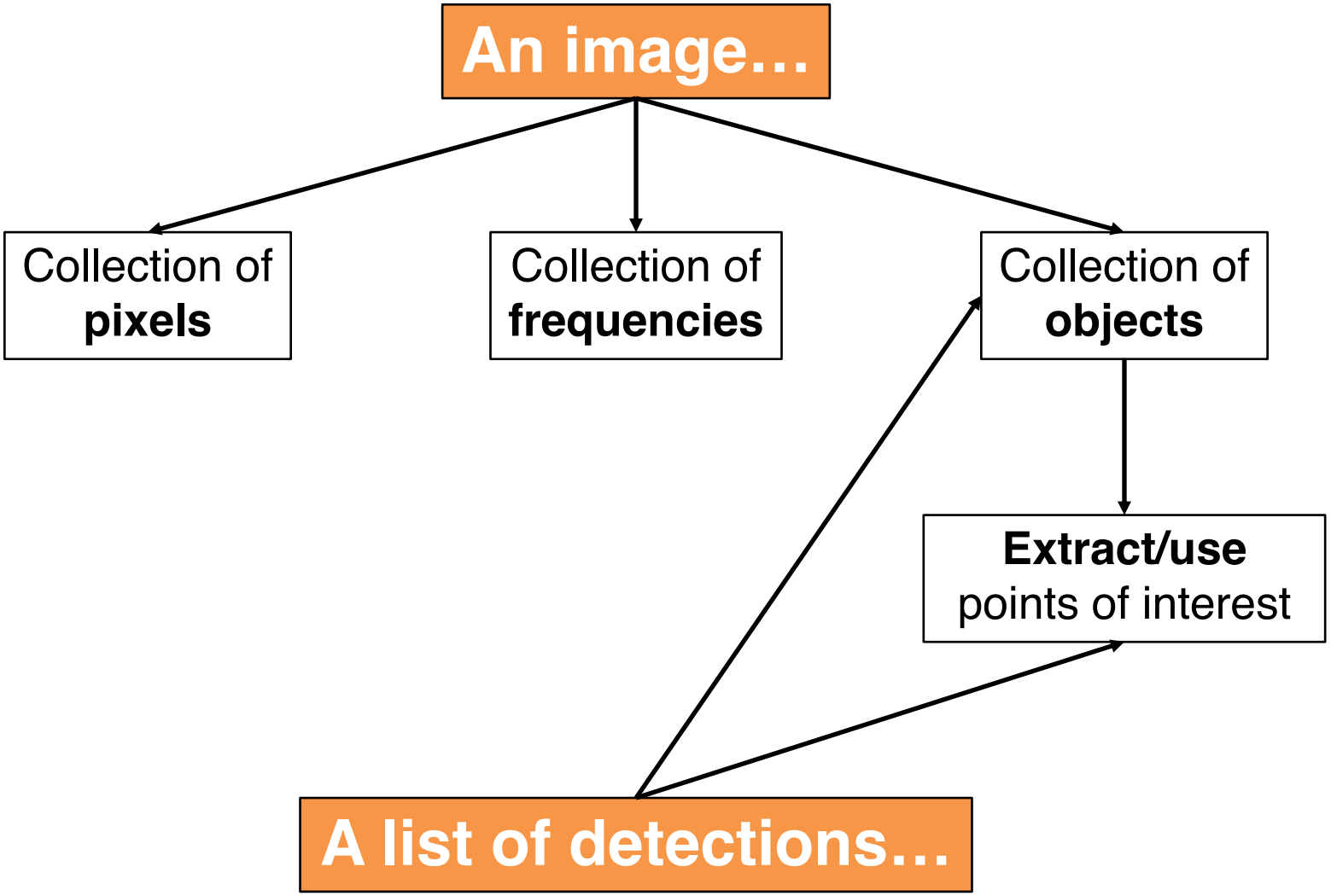
Without good images, there is no point going further !




Pre-processing

Co-localisation workflows

Data input



Co-localisation workflows

Pre-processing

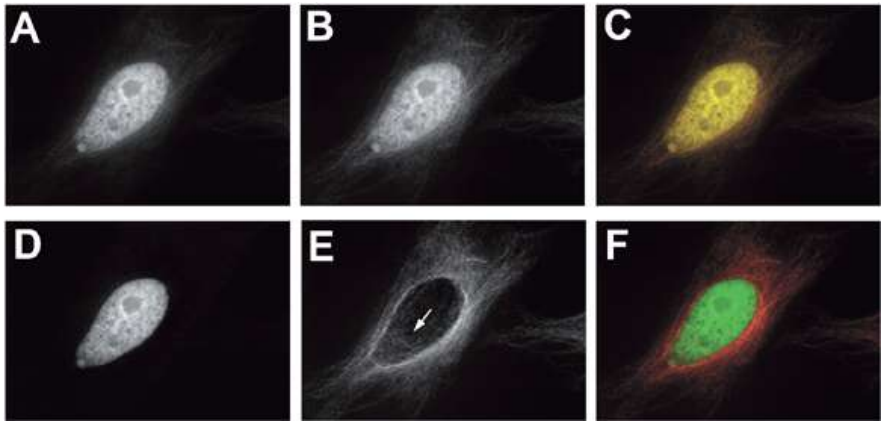
Corrections

Image acquisition-related

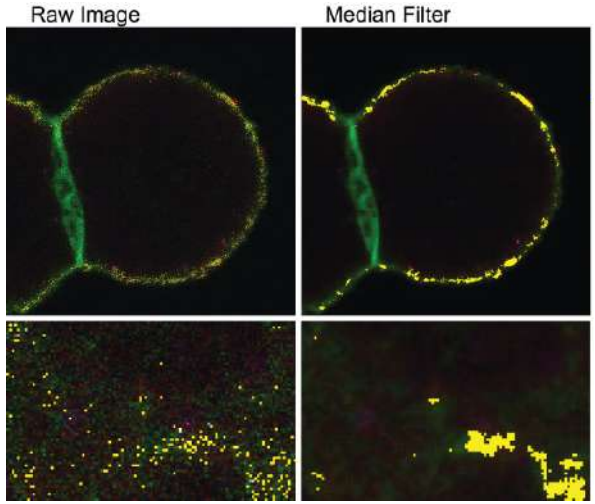
- Bleedthrough/crosstalk: better go back to the microscope before trying unmixing
- Chromatic shift: better be corrected on the microscope before trying to compensate by translation

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.

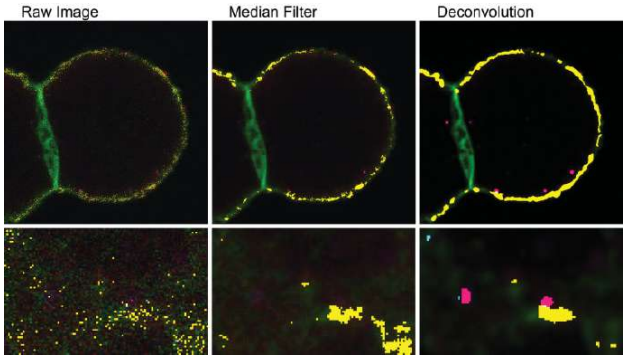


L. Landmann and P. Marbet, *Microsc. Res. Tech.*, vol. 64, pp. 103–112, 2004.

Co-localisation workflows

Pre-processing

Checking data integrity → Pre-processing → Choosing a reporter/ metric → Comparing Interpreting → Assembling a workflow



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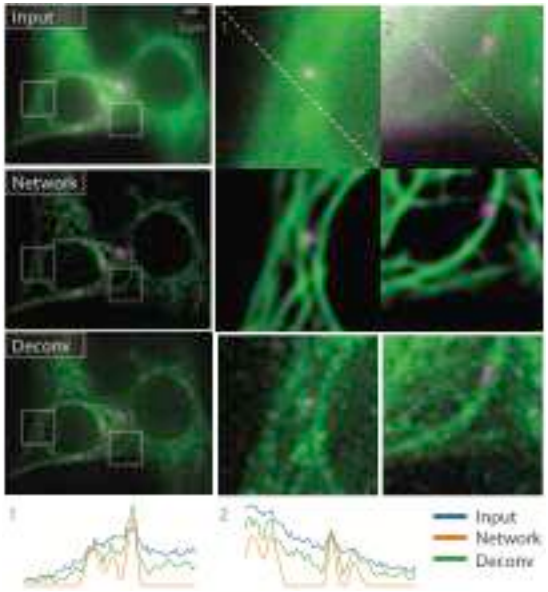
Restoration

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.

Co-localisation workflows

Pre-processing

Checking data integrity | **Pre-processing** | Choosing a reporter/ metric | Comparing Interpreting | Assembling a workflow

Alternatively, you may ask a friend…

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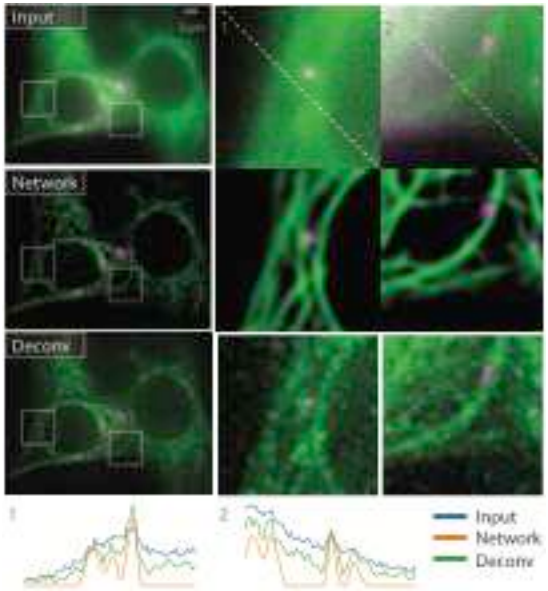

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Co-localisation workflows

Pre-processing

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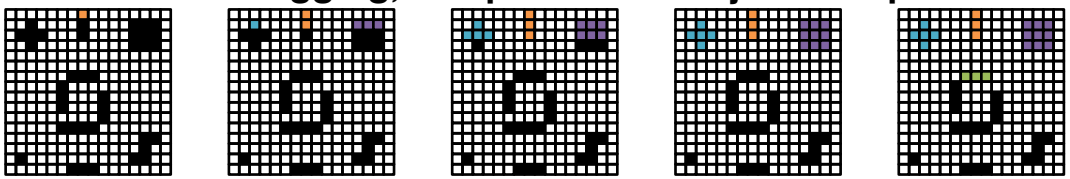
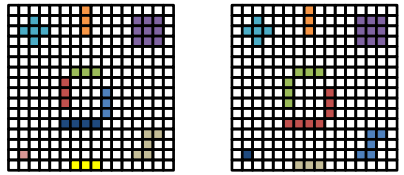
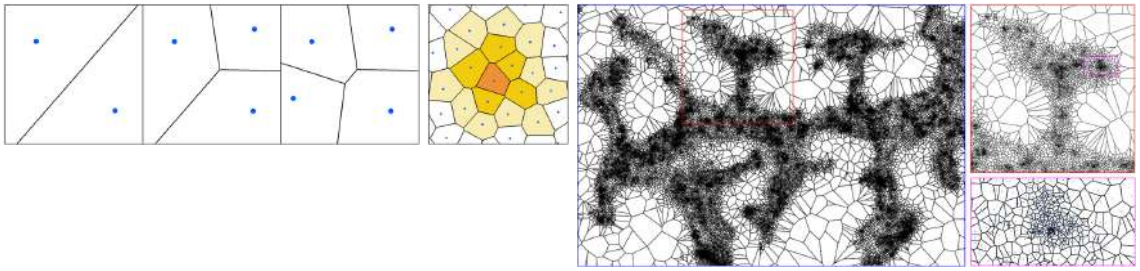
Pixel tagging, line per line ➔ Objects' map



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.

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.

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

Choosing a reporter/metric

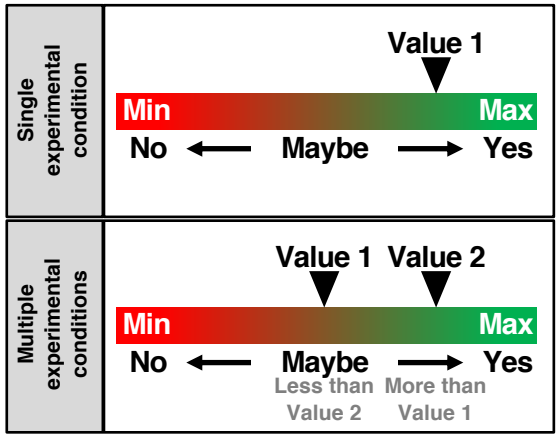
Co-localisation workflows

Choosing a reporter/metric

Checking data integrity → Pre-processing → **Choosing a reporter/ metric** → Comparing Interpreting → Assembling a workflow

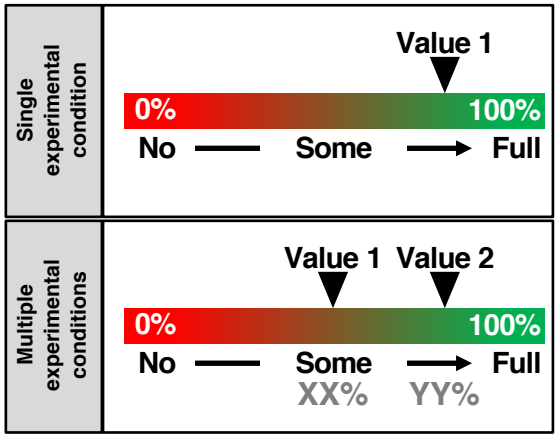
Indicators

« 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.

Co-localisation workflows

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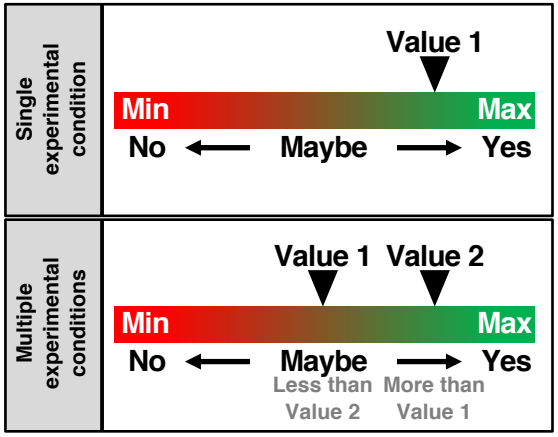
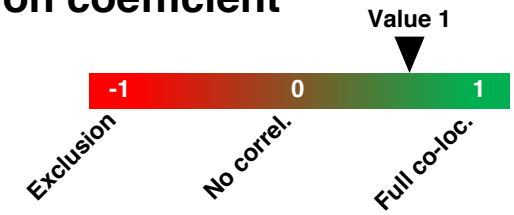
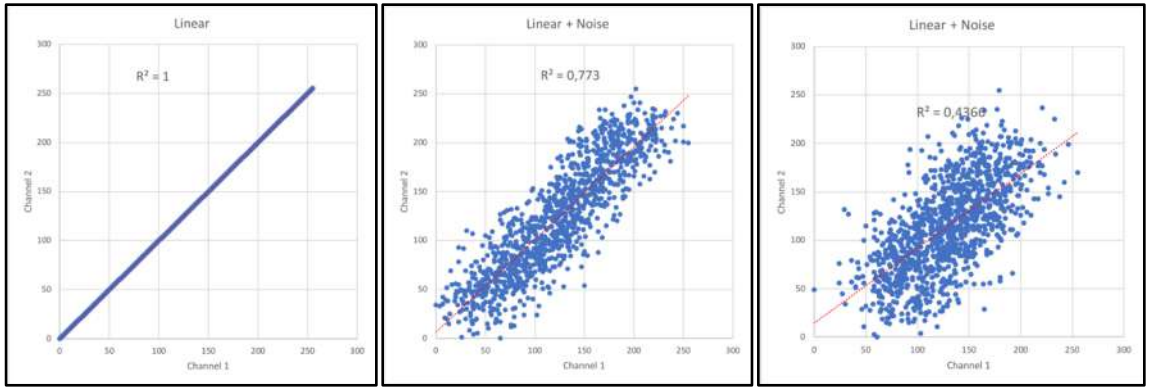


Pearson's correlation coefficient

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



« 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 »



No noise, R^2=1 | + Low noise, R^2=0.77 | + High noise, R^2=0.44

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

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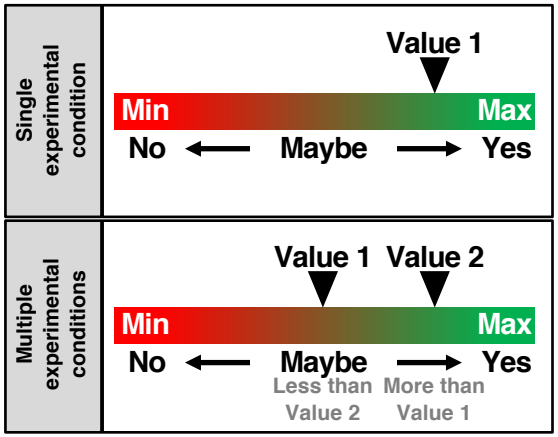
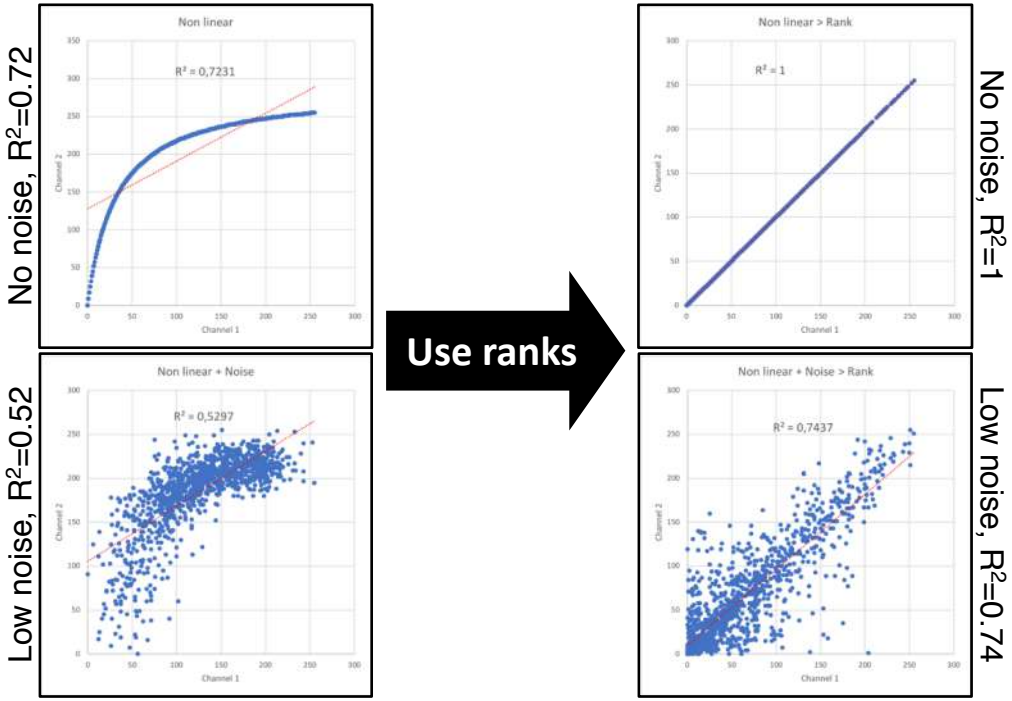


Spearman's correlation coefficient

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

It's already a workflow !!!

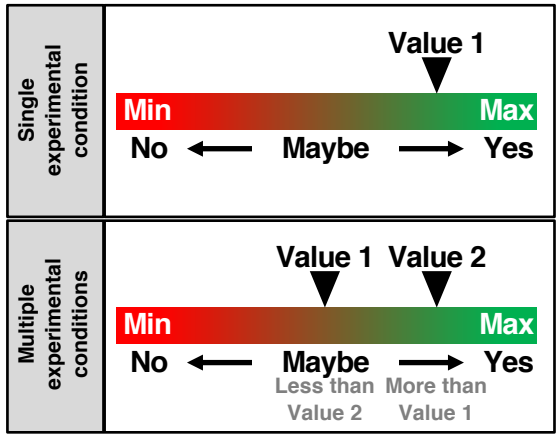


C. Spearman, *Am. J. Psychol.*, 1904 / J. Adler, et al., *J. Microsc.*, 2008 / A. P. French, et al., *Nat. Protoc.*, 2008.

Co-localisation workflows
Choosing a reporter/metric

Indicators

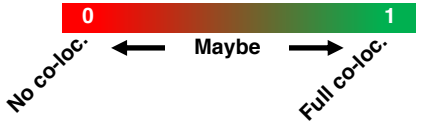
« numerical values that evaluate a degree of signal coincidence over a predefined scale, but are not suitable to calculate a precise amount of overlap. They are suitable for relative comparisons, without being usable for direct quantitative studies »



Many other indicators exist !

Overlap coefficient

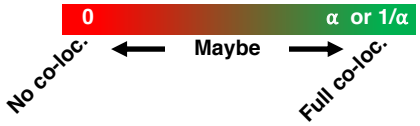
$$r = \frac{\sum_i R_i \times G_i}{\sqrt{\sum_i (R_i)^2 \times \sum_i (G_i)^2}}$$



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

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



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*

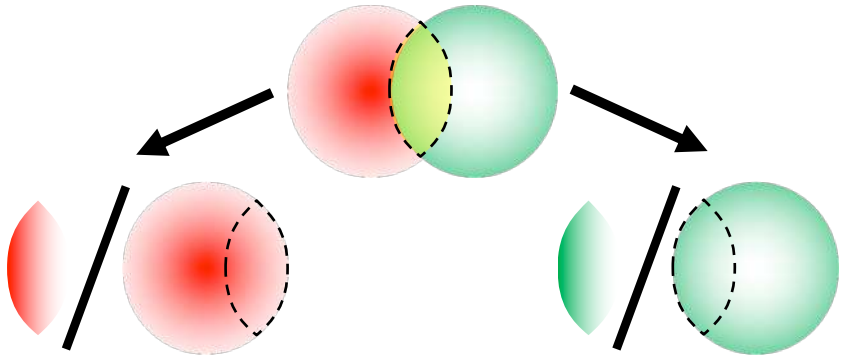
E. M. Manders, *et al.*, *J. Cell Sci.*, vol. 103, pp. 857–62, Nov. 1992.

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

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

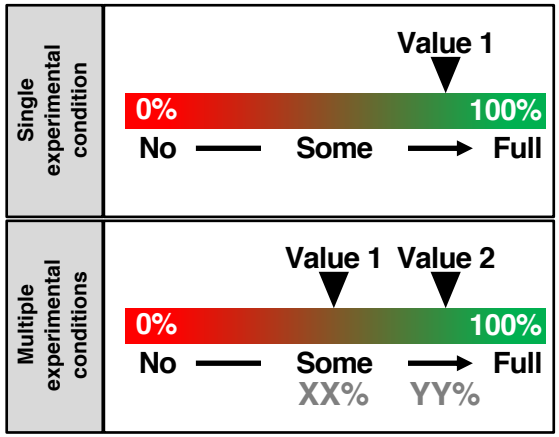
Original paper: where $A_{i,coloc}=A_i$ if $B_i>0$, 0 otherwise
Modified: tM_1, where $A_{i,coloc}=A_i$ if $B_i>Thr_B$, 0 otherwise

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

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

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. »

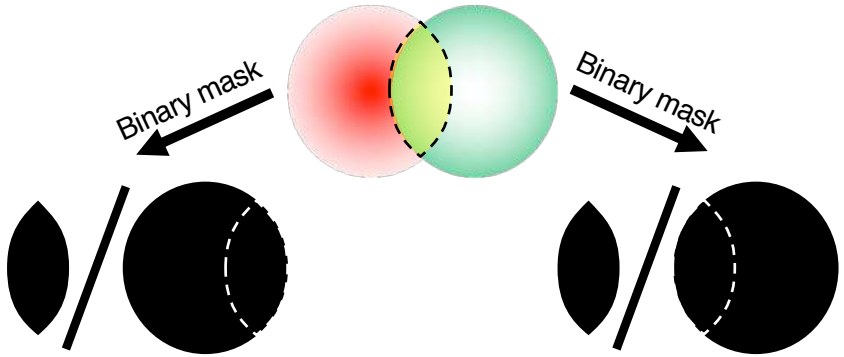


E. M. Manders, *et al.*, *J. Cell Sci.*, vol. 103, pp. 857–62, Nov. 1992.

Co-localisation workflows

Choosing a reporter/metric

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

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

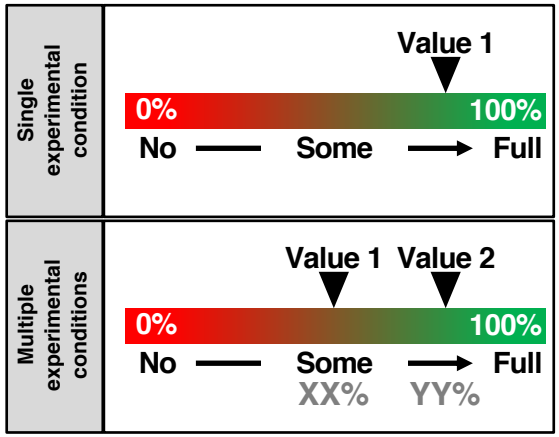
Where $A_{i,coloc}$=1 if B_i>Thr_B, 0 otherwise
and A_i=1 if A_i>Thr_A , 0 otherwise

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

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

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. »



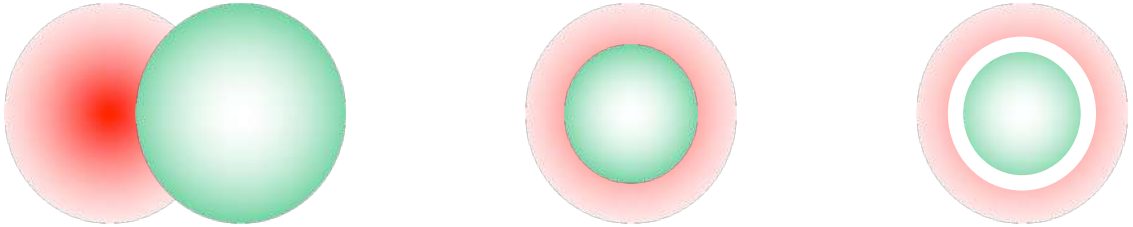
Modified from the original definitions found in: E. M. Manders, *et al.*, *J. Cell Sci.*, vol. 103, pp. 857–62, Nov. 1992.

Co-localisation workflows

Choosing a reporter/metric

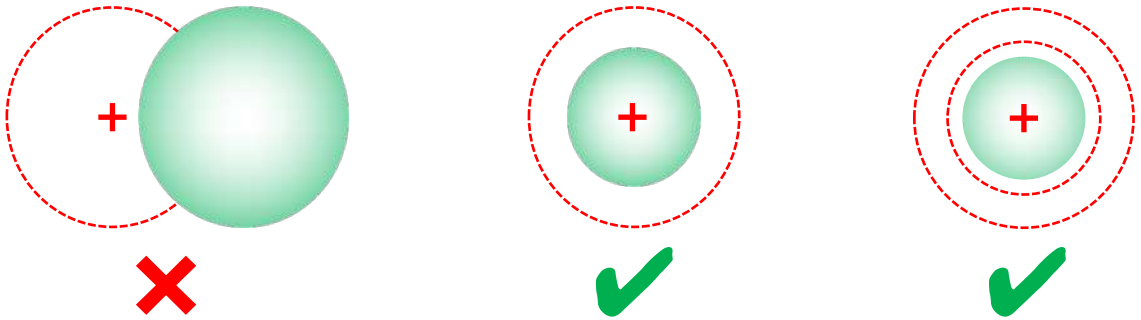
Checking data integrity → Pre-processing → Choosing a reporter/ metric → Comparing Interpreting → Assembling a workflow

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

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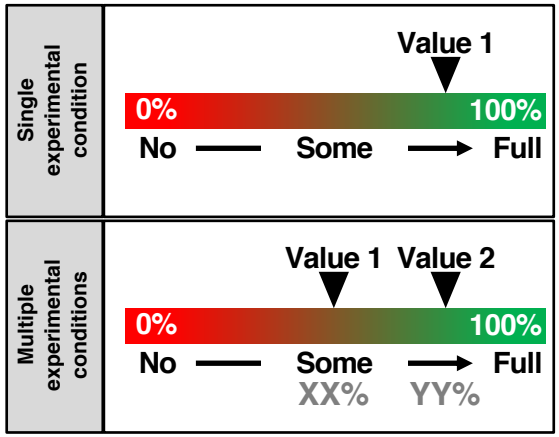


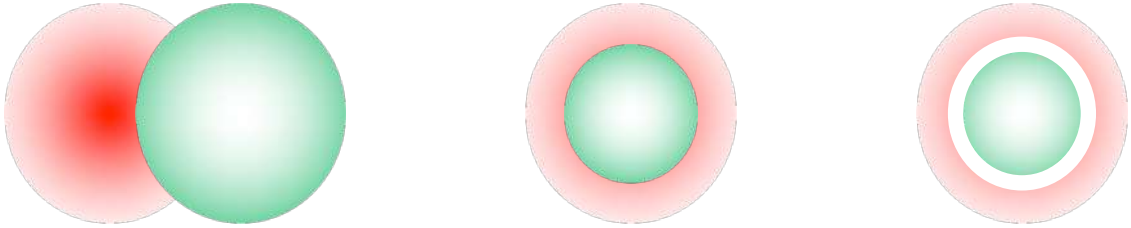
E. Lachmanovich, et al., *J. Microsc.*, vol. 212, pp. 122–31, 2003.

Co-localisation workflows

Choosing a reporter/metric

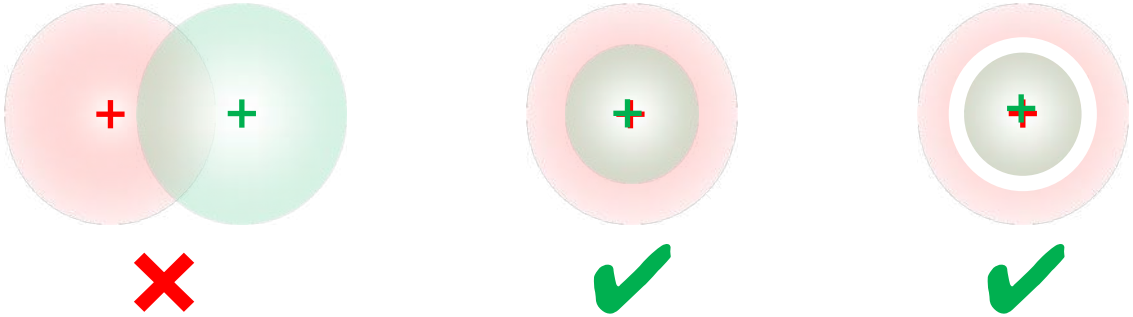
Checking data integrity → Pre-processing → Choosing a reporter/ metric → Comparing Interpreting → Assembling a workflow

Based on distances



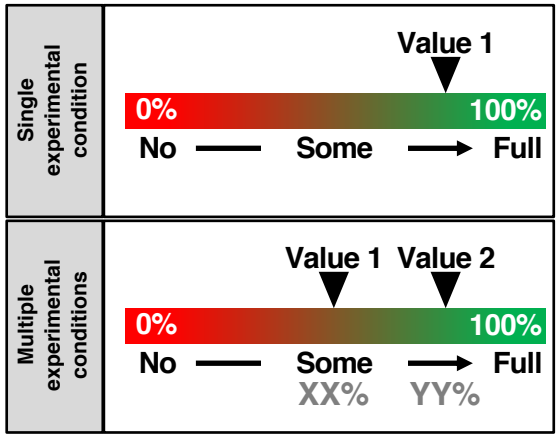
Method:

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.

Comparing/Interpreting

Co-localisation workflows

Comparing/interpreting

Checking data integrity → Pre-processing → Choosing a reporter/ metric → Comparing Interpreting → Assembling a workflow

Strategy 1: Rotate

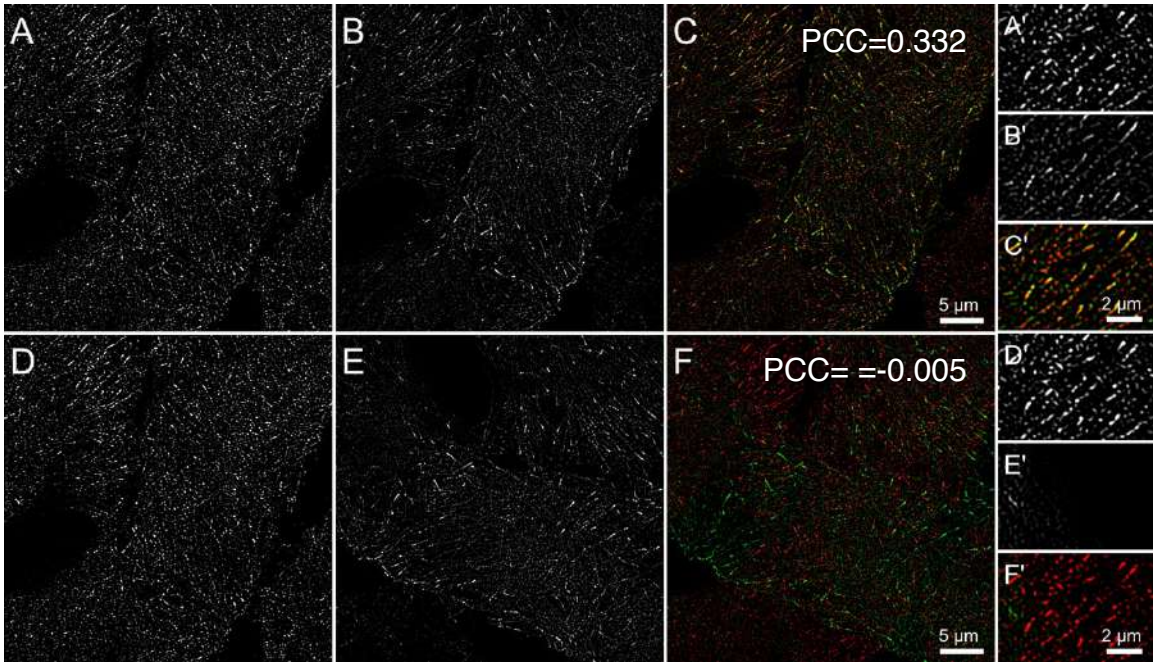
Getting significance out of a single dataset

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°



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.

Co-localisation workflows

Comparing/interpreting

Strategy 2: Translate

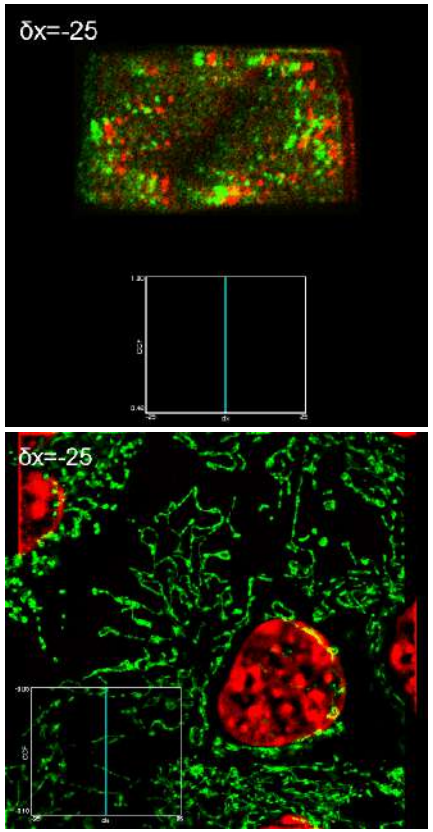
Getting significance out of a single dataset

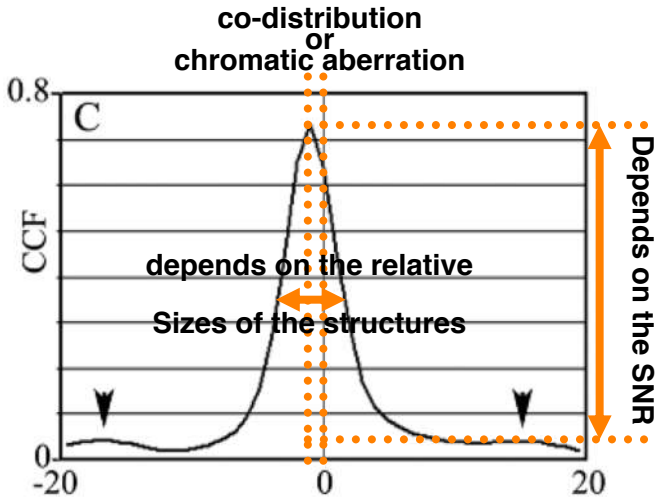
Methods:

- 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.

Co-localisation workflows

Comparing/interpreting

Checking data integrity → Pre-processing → Choosing a reporter/ metric → Comparing Interpreting → Assembling a workflow

Strategy 3: Randomise

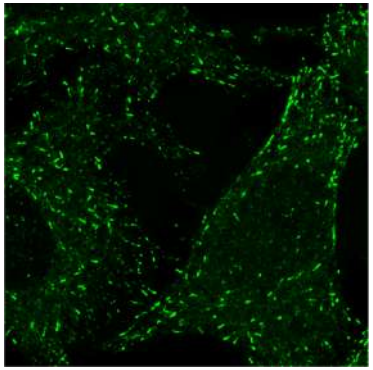
Getting significance out of a single dataset

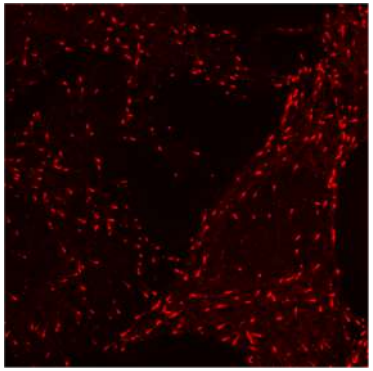
Methods:

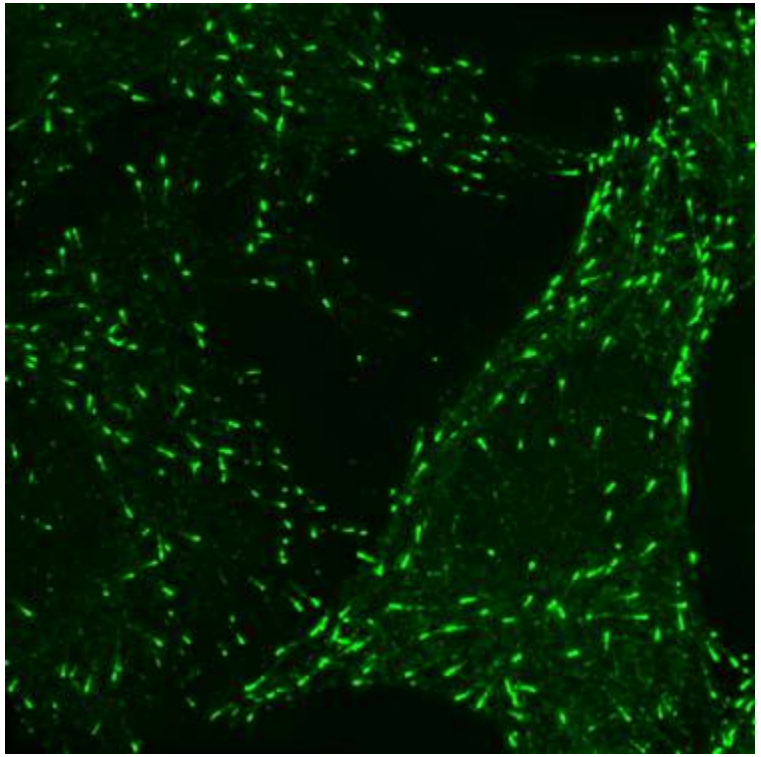
- Generating a dataset where the data content is the same, but localised differently
- Compare to original dataset

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.

Co-localisation workflows

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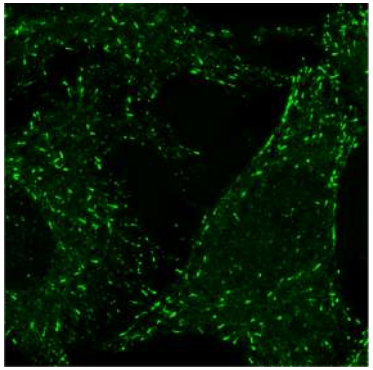

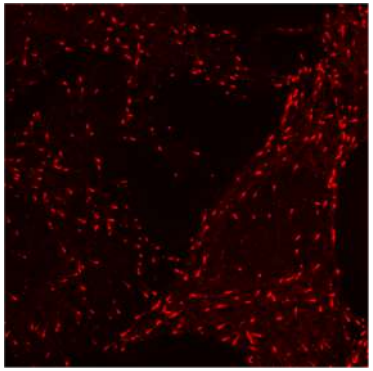

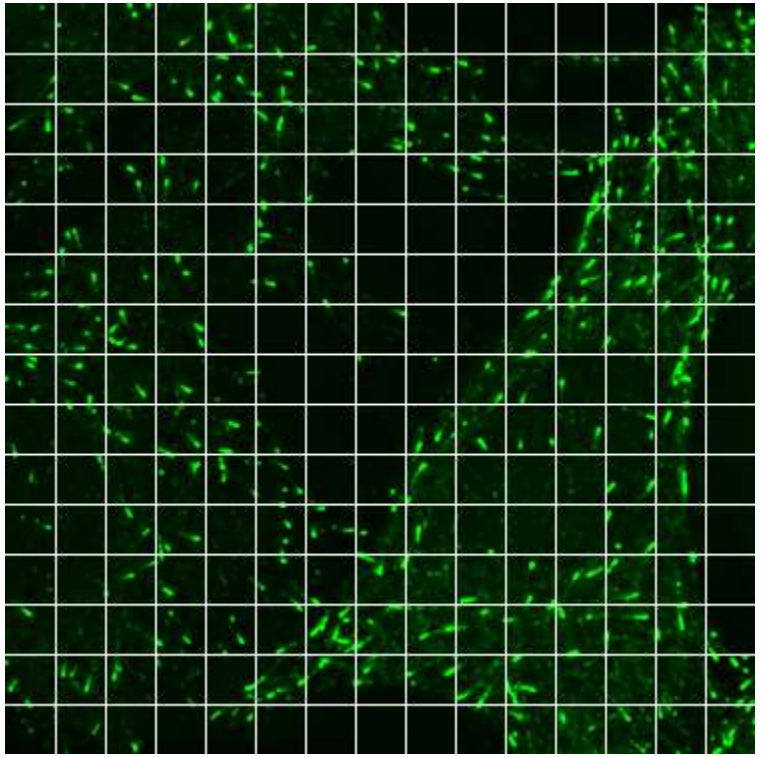

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Co-localisation workflows

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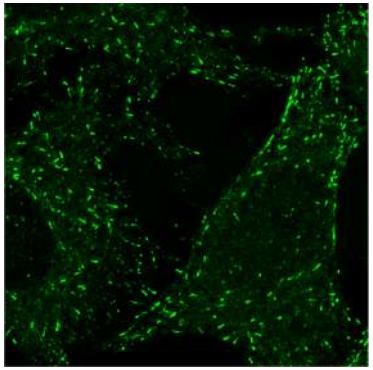

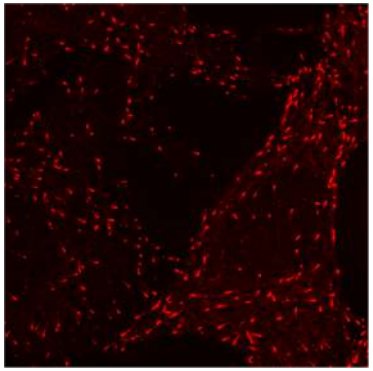

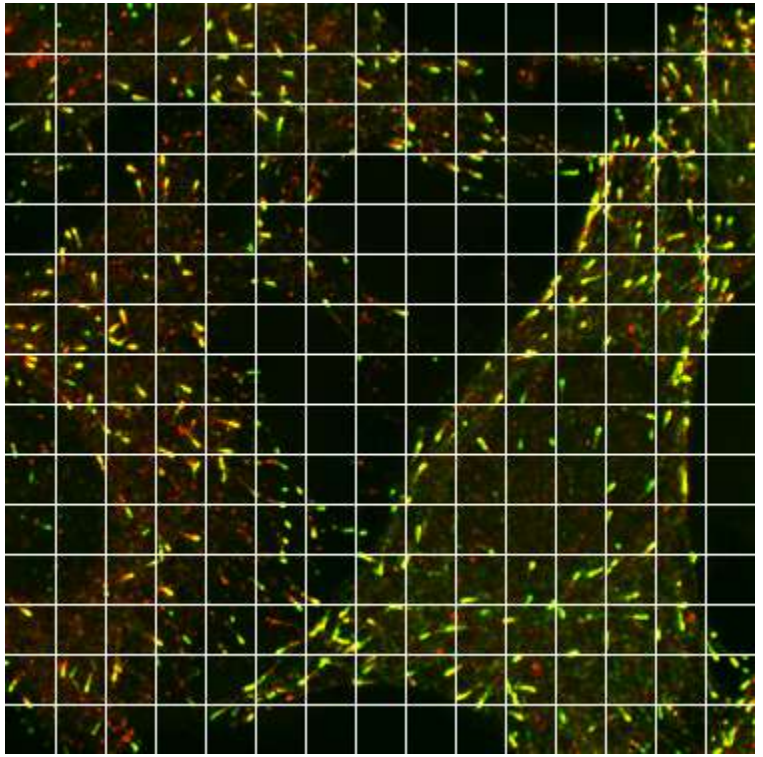

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Co-localisation workflows

Comparing/interpreting

Checking data integrity → Pre-processing → Choosing a reporter/ metric → Comparing Interpreting → Assembling a workflow

Strategy 3: Randomise

Getting significance out of a single dataset

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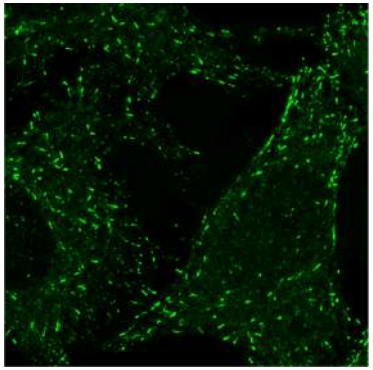
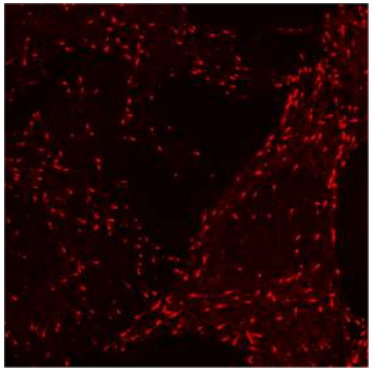
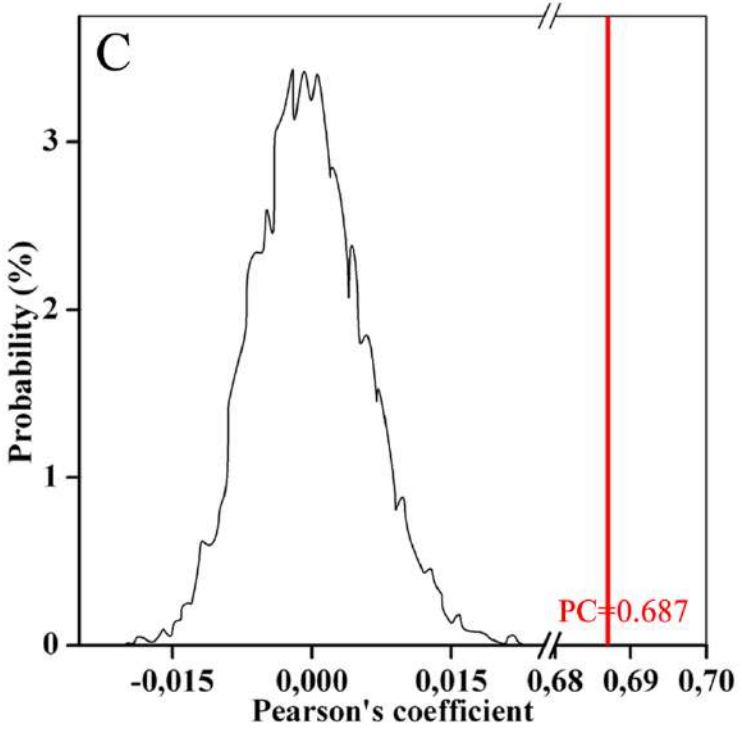
- Randomise the image







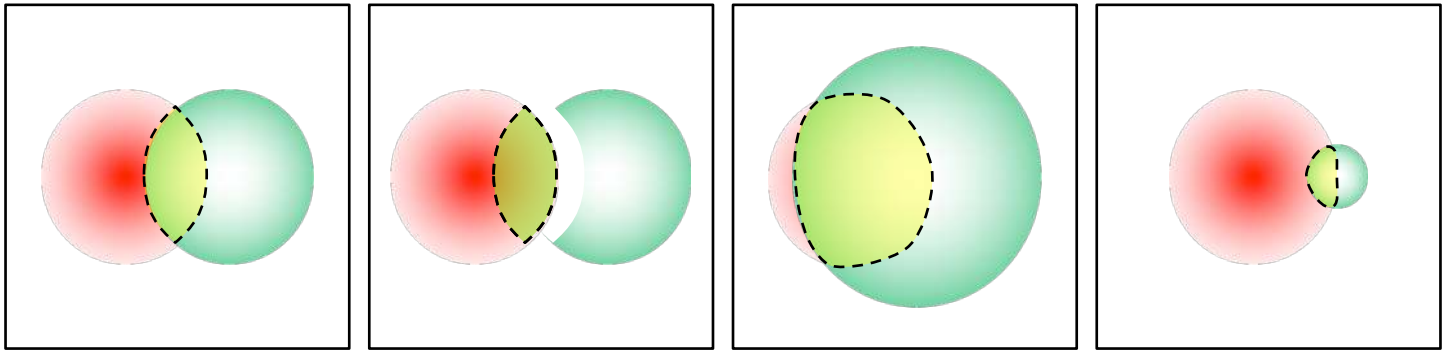
S. V Costes, D. Daelemans, E. H. Cho, Z. Dobbin, G. Pavlakis, and S. Lockett, *Biophys. J.*, vol. 86, no. 6, pp. 3993–4003, Jun. 2004.

Co-localisation workflows

Comparing/interpreting

Checking data integrity → Pre-processing → Choosing a reporter/ metric → Comparing Interpreting → Assembling a workflow

Warning ! Same values may not reflect the same experimental situation !!!



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.

Co-localisation workflows

Questions to adrdess, metrics and software to use

Checking data integrity → Pre-processing → Choosing a reporter/ metric → Comparing Interpreting → Assembling a workflow

Questions to address:

- What type of co-localisation method is the most appropriate for YOUR problematic ?
- Are the published methods adapted to you problematic ?

Depending on your answers:

- Nope: be creative, build your how metric, caracterize it, use it !
- Yes: find the tool that will make your life easier !

Chapter 10

Which Elements to Build Co-localization Workflows? From Metrology to Analysis

Patrice Mascalchi and Fabrice P. Cordelières

Abstract

Co-localization analysis is one of the main interests of users entering a facility with slides in hands and nice analysis perspectives in mind. While being available through most, if not all, analysis software, co-localization tools are mainly perceived as black boxes, fed with images, that will, hopefully, return (the expected) numbers.

In this chapter, we will aim at deconstructing existing generic co-localization workflows, extracting elementary tools that may be reused and recombined to generate new workflows. By differentiating work cases, identifying co-localization reporters and the metrics others have been using, we aim at providing the audience with the elementary bricks and methods to build their really own co-localization workflows. A special emphasis is given on the preparatory phase where the acquisition system is assessed, using basic metrological tests.

Key words Co-localization, Co-expression, Co-occurrence, Correlation, Co-distribution, Elements, Workflow, Image processing, Image analysis

1 Introduction

1.1 Co-localization or Co-localizations: One Word, Many Meanings

From the biologist perspective, co-localization often appears as a word conveying several meanings. Its precise definition is highly linked to the phenomenon the experimenter is trying to characterize (Fig. 1).

When dealing with large-scale samples, such as slices of tissues, the word "co-localization" is generally used in the sense "co-expression." In this case, the aim is to determine whether a same set of cells are positive for two proteins of interest. This experimental situation does not presuppose the two molecular actors to be at the same location. One could expect "co-localization" while, for example, working on a nuclear transcription factor and the product

Electronic supplementary material: The online version of this chapter (https://doi.org/10.1007/978-1-4939-9686-5_10) contains supplementary material, which is available to authorized users.

Elena Rebollo and Marwi Bosch (eds.), Computer Optimized Microscopy: Methods and Protocols, Methods in Molecular Biology, vol. 2040, https://doi.org/10.1007/978-1-4939-9686-5_10, © Springer Science+Business Media, LLC, part of Springer Nature 2019

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Mascalchi P., Cordelières F.P. (2019) In: Rebollo E., Bosch M. (eds) Computer Optimized Microscopy. Methods in Molecular Biology, vol 2040.

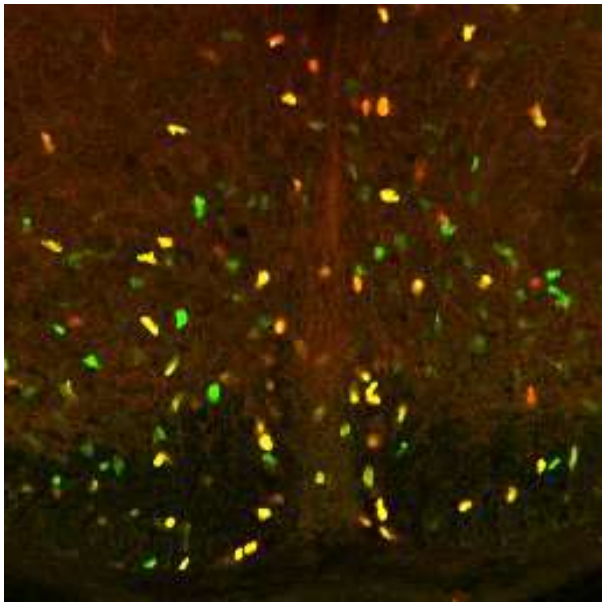
Assembling a workflow

Co-localisation workflows

Co-expression analysis

Checking data integrity | Pre-processing | Choosing a reporter/ metric | Comparing Interpreting | Assembling a workflow

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

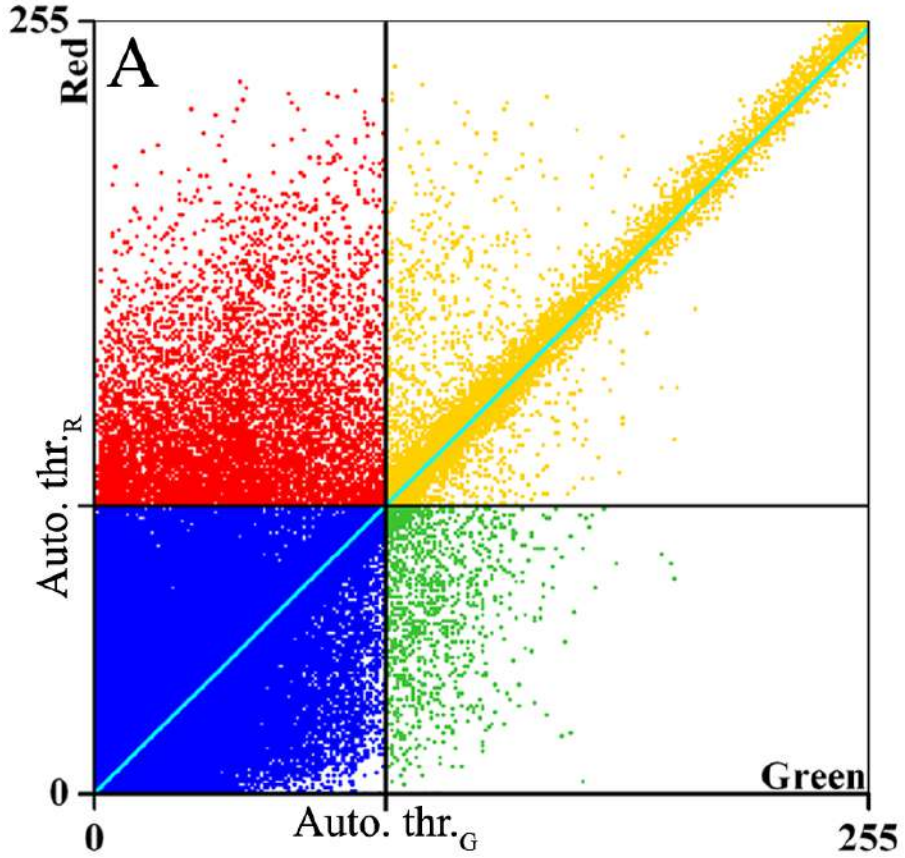
Co-expression analysis

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≤0

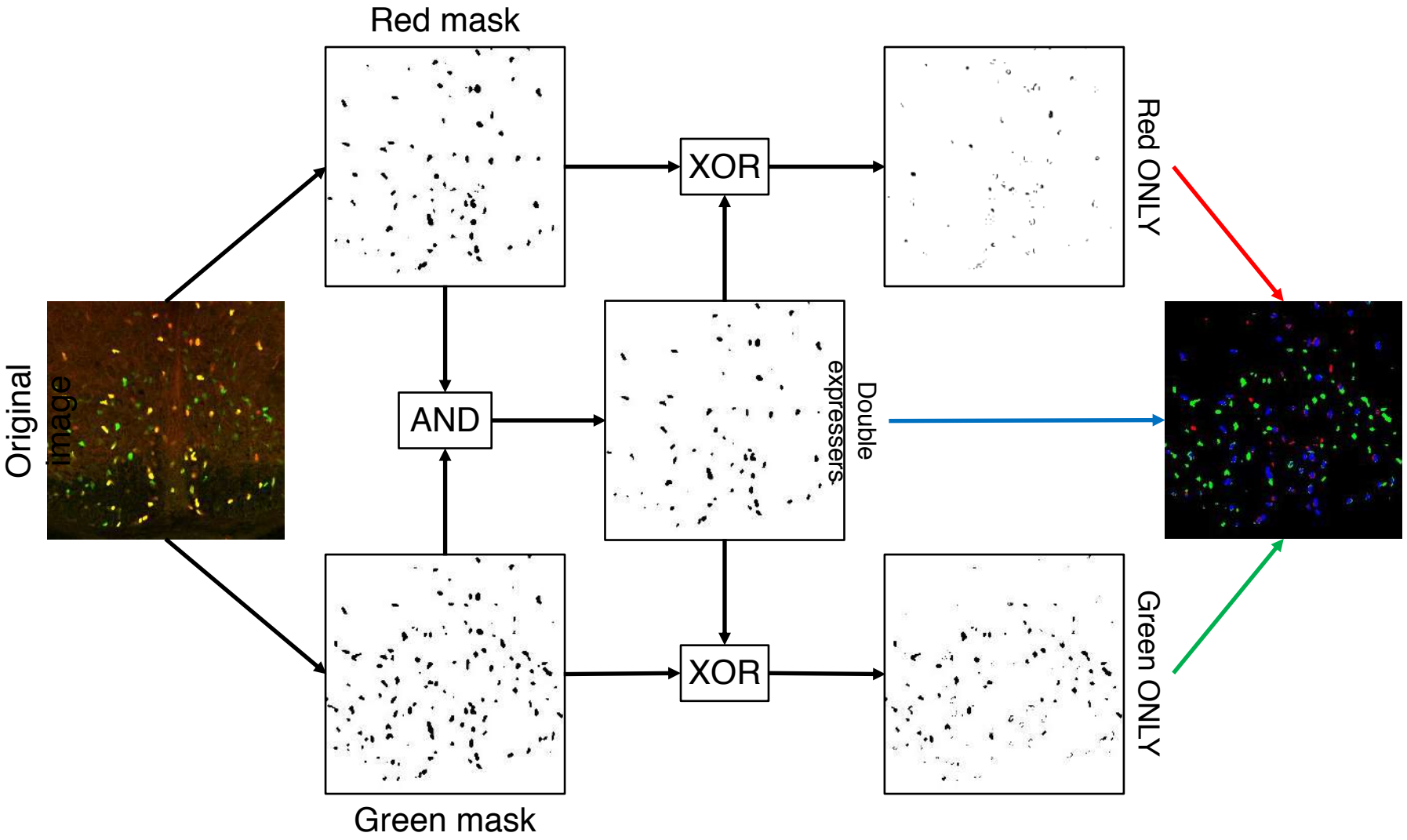
➔ ***Sets thresholds by maximising the number of pixels with correlated intensities / minimising the number of pixels with uncorrelated pixels***



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.

Co-localisation workflows

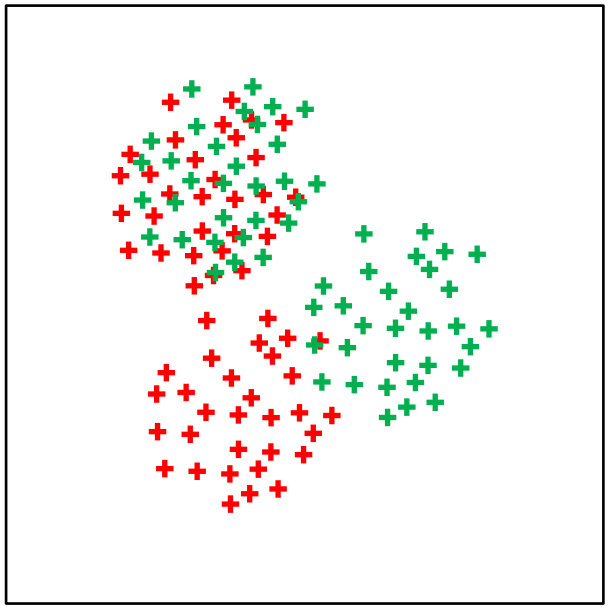
Co-expression analysis



Co-localisation workflows

Working with detections

Original image



Synopsis:

- The image presents two populations 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 ???

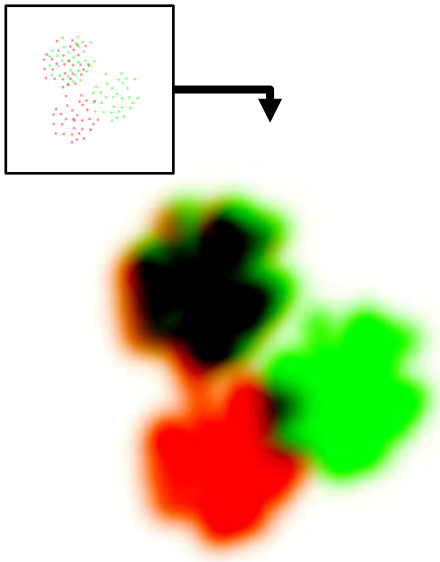
Co-localisation workflows

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

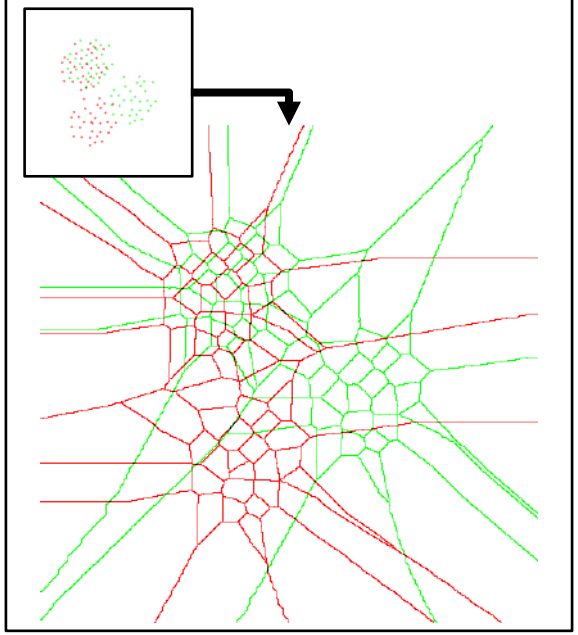


E. Betzig *et al.*, *Science*, 313, 1642–5, 2006.

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

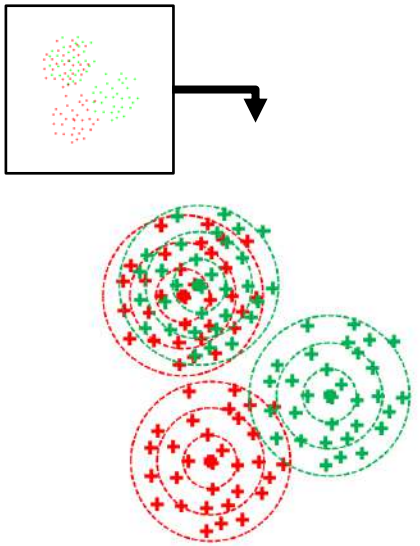


F. Levet *et al.*, *Nat. Methods*, 12, 1065–1071, 2015.
L. Andronov, *et al. Sci. Rep.*, 6, 1–9, 2016.

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.

Don’t miss co-localisation, part 2 next week !!!

Live Webinar

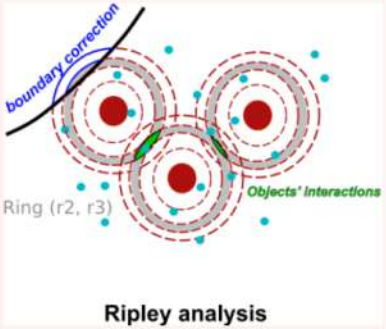
Advanced Learning, Demo, Q&As

Open-source Software

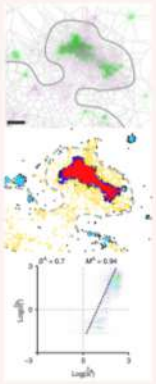
Advanced colocalization methods for SMLM and object-based spatial distribution

6 October, 2020, 15h30-17h00 CEST (Brussels Time)

Kindly hosted by the Crick Advanced Light Microscopy (CALM)



Ripley analysis



TARGET AUDIENCE

★★★★★

Bioimage Analysts, Facility Staff, Early career Investigators

Ideal if you want to learn how to compute colocalization estimators for SMLM data and between objects.

— Format

The Webinar will be broadcasted live with Zoom, in the form of an interactive webinar with Questions&Answers. Attendance will be limited to 3000 participants.

Questions will be live-moderated, Q&As will be further reported in a note file shared with attendants. Registered participants will receive a link to connect live. The event will be recorded for further viewing and stored on NEUBIAS Youtube Channel.

Last advice

Think, be creative, test, get help… repeat