COLOCALISATION

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The Center for
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BASEL

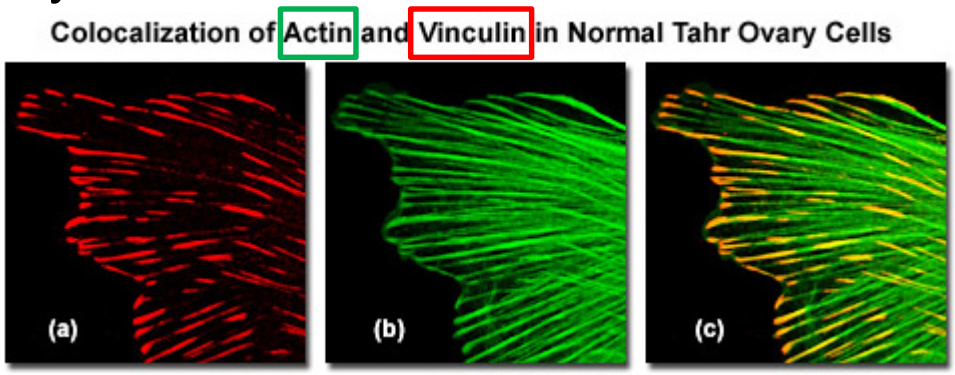
Introduction

- Colocalisation: What does that really mean?

Adapted from Fabrice Cordelières

Introduction

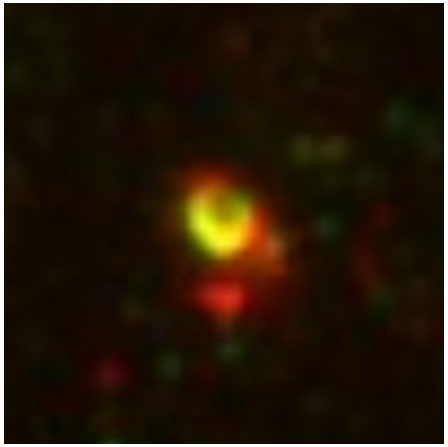
- Colocalisation = Presence of two (or more) structures on the same location
- Colocalisation in fluorescence microscopy at subcellular level = the distance between signal is below the resolution of the imaging system



http://www.olympusconfocal.com/applications/colocalization.html

Introduction

- Colocalisation = Presence of two (or more) structures on the same location
- Colocalisation in fluorescence microscopy at subcellular level = the distance between signal is below the resolution of the imaging system



Deltavision Widefield+Deconvolution

- Limitation (best case scenario): optical resolution of the microscope → XYZ 200 x 200 x 400 nm

Introduction

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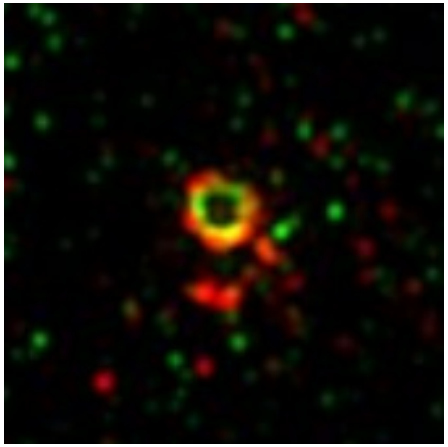
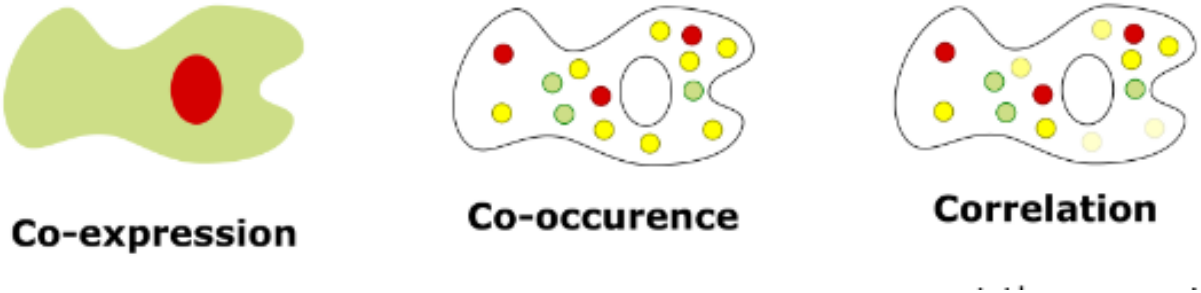


OMX Blaze Super-resolution

- Limitation (3D SIM method): optical resolution of the microscope → XYZ **120 x 120 x 250 nm**
- Colocalisation never measures interaction, it states that 2 dyes are in a close proximity in a defined volume.

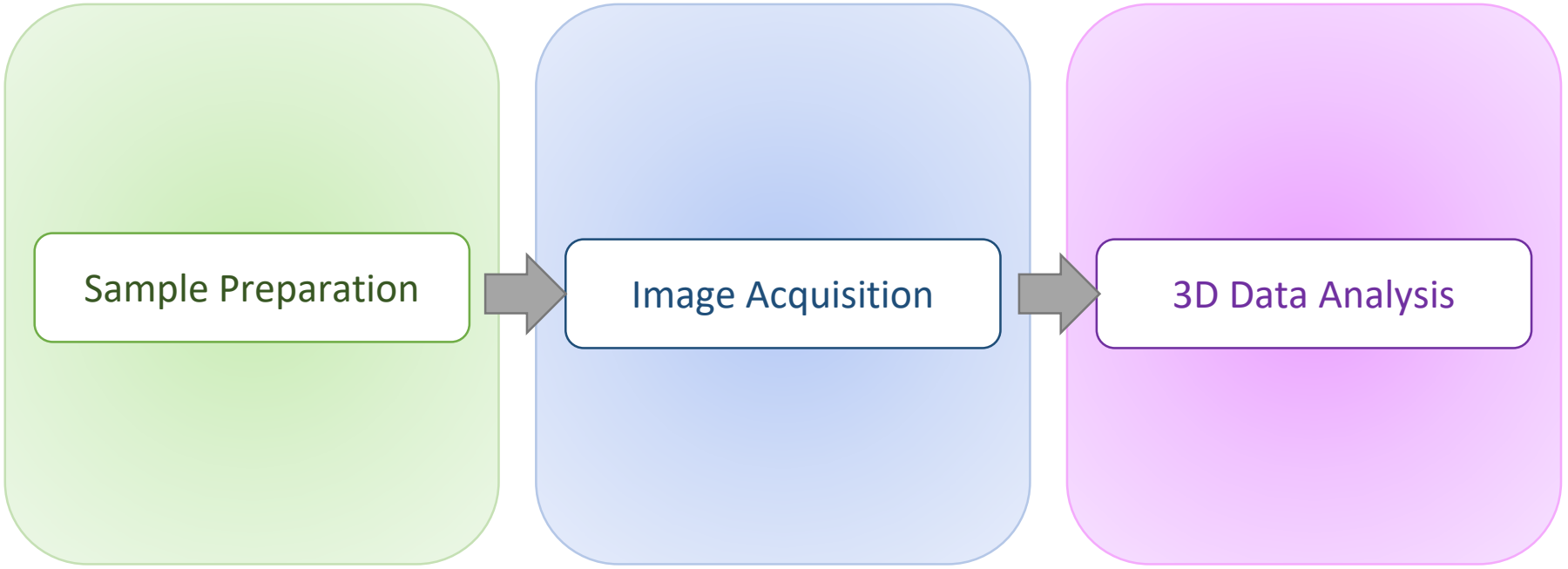
What is your Biological Question?



- Are you looking for Co-Compartmentalisation?
- Are your looking for exclusion / anti-correlation?
- Are you looking for interacting molecules?
  - Then you also need some biochemistry experiments (co-IP, FCS…)
  - FRET / FLIM might be very informative!

Check our webcast 23/06/20

Colocalisation: Strategic Planning

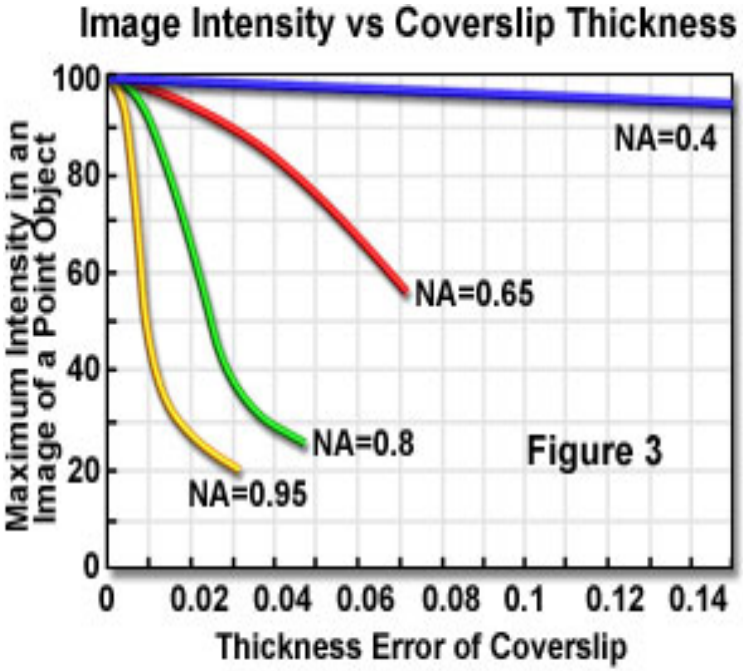


How to best prepare your samples for colocalisation

- It all starts with your experimental design!
- Select stable fluorescent dyes
  - Alexa dyes (Invitrogen/Molecular Probes)
  - Atto dyes
  - Avoid Cyanine dyes, especially Cy2

How to best prepare your samples for colocalisation

- The last 500 µm are important
  - Coverglas N°1.5 = 0.17 mm (spherical aberrations)



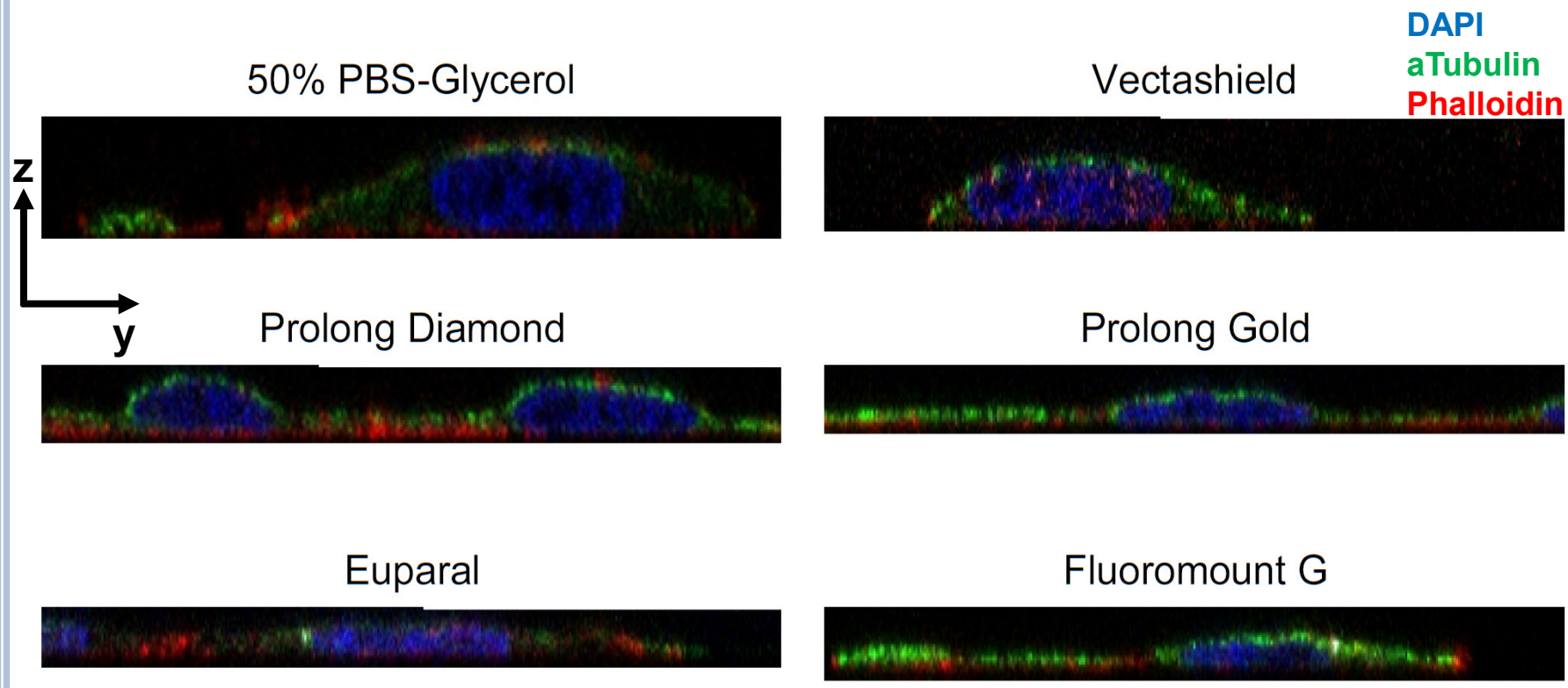
Performance Reduction with Coverslip Thickness Variation

NumericalAperture	0.01 mm Deviation	0.02 mm Deviation
0.30	none	none
0.45	none	none
0.70	2 percent	8 percent
0.85	19 percent	57 percent
0.95	55 percent	71 percent

http://www.olympusmicro.com

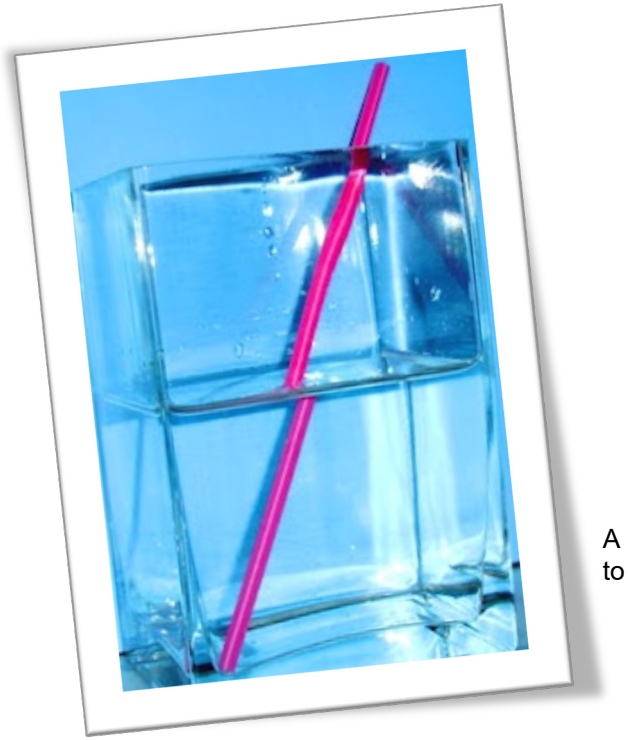
How to best prepare your samples for colocalisation

- The last 500 µm are important
  - Coverglas N°1.5 = 0.17 mm (spherical aberrations)
  - Mounting media (avoid bubbles)

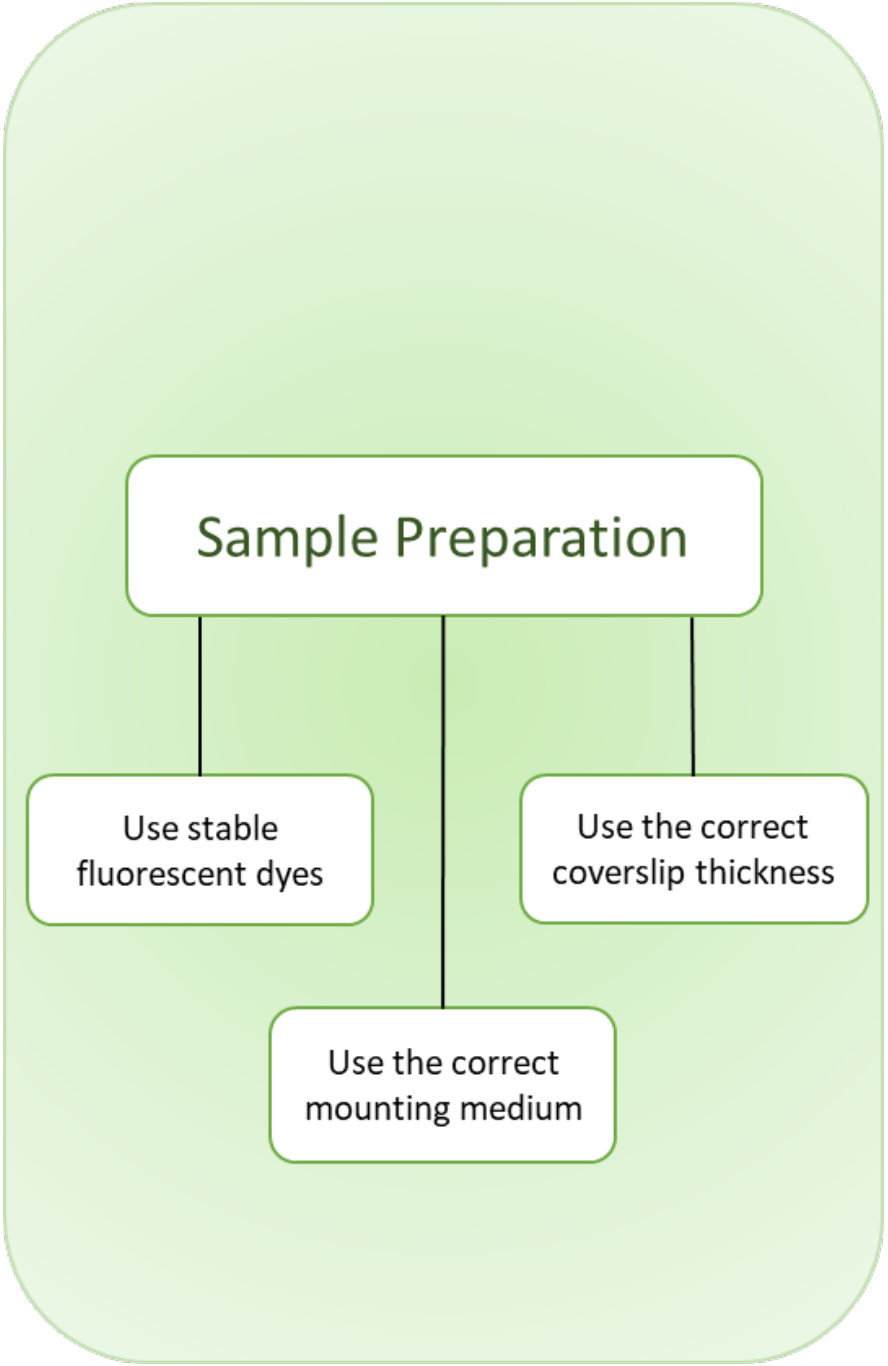


How to best prepare your samples for colocalisation

- The last 500 µm are important
  - Coverglas N°1.5 = 0.17 mm (spherical aberrations)
  - Mounting media (avoid bubbles)
  - Match in the refractive indexes (spherical aberrations)



A refractive index mismatch gives rise to geometrical aberrations.



TAKE HOME MESSAGE PART 1

- Carefully select stable dyes

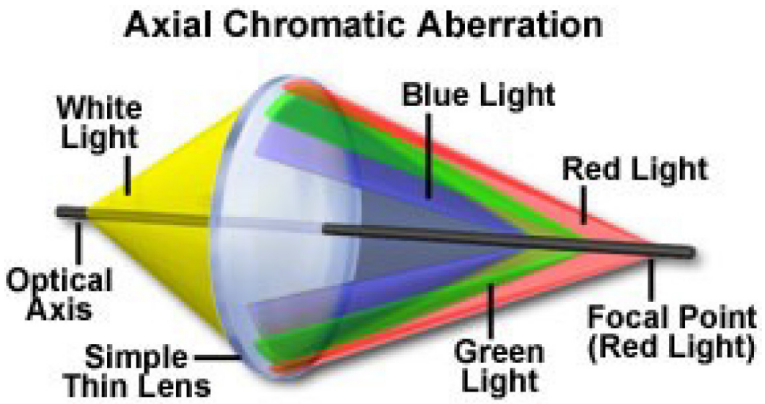
- Make sure that your last "500 µm" are optimal

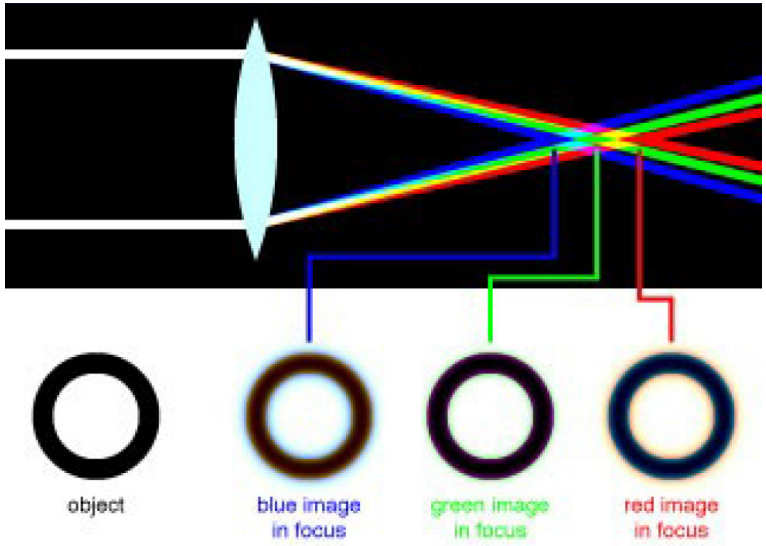
- Don't forget to prepare positive and negative controls

How to acquire the images for colocalisation

- Use the correct lens
  - Remember, the higher the NA, the better the resolution

    →Aim for a 1.40 NA or above
  - Apochromat lens (λ corrected)





How to acquire the images for colocalisation

- Use the correct lens
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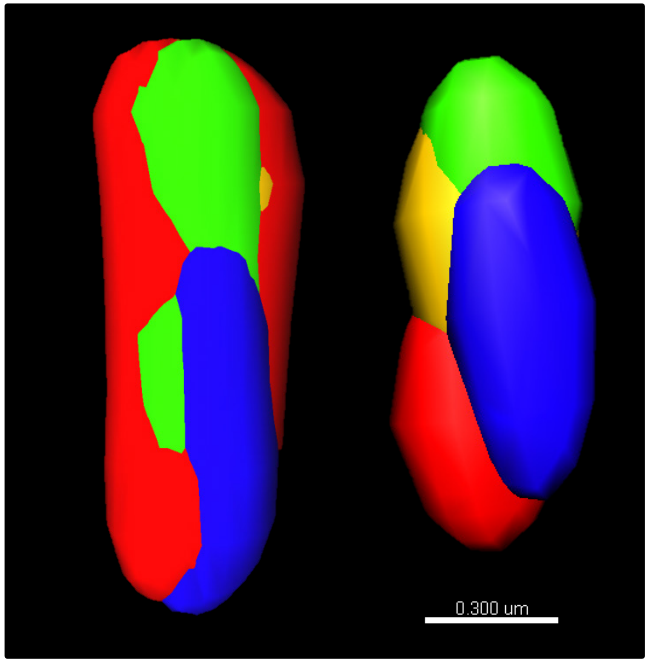
    →Aim for a 1.40 NA or above
  - Apochromat lens (λ corrected)
  - Check the PSF (Point Spread Function with multicolored beads)



Even the best lenses are not perfect!

Left: LSM700 – Confocal, non-deconvolved
Right: DeltaVision – Widefield, deconvolved

How to acquire the images for colocalisation

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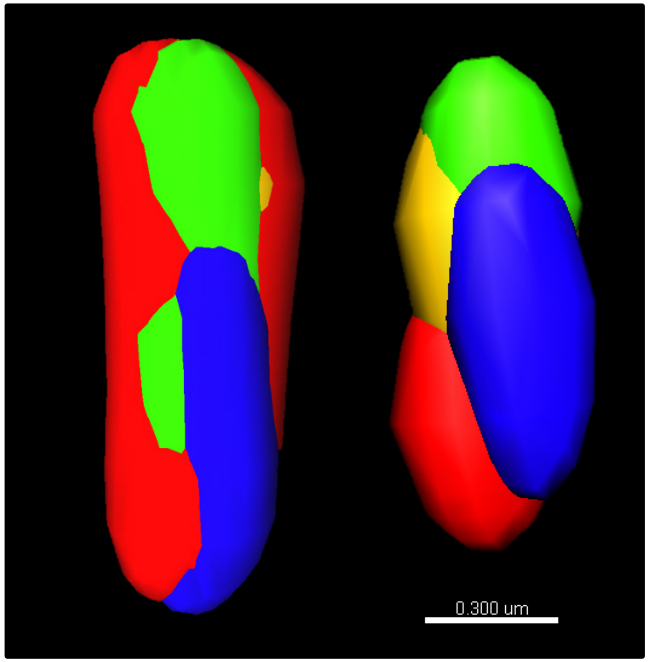

Even the best lenses are not perfect!

→ Better results in colocalisation if you compare green and red fluorophores…

How to acquire the images for colocalisation

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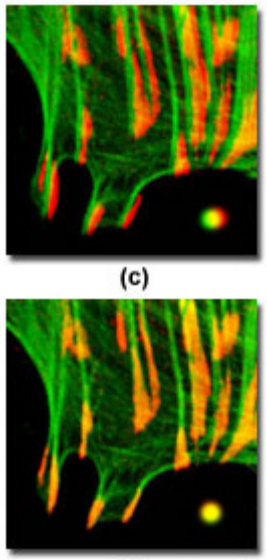


Mis-registration can be corrected afterwards (Post acquisition image processing to restore the image registration)

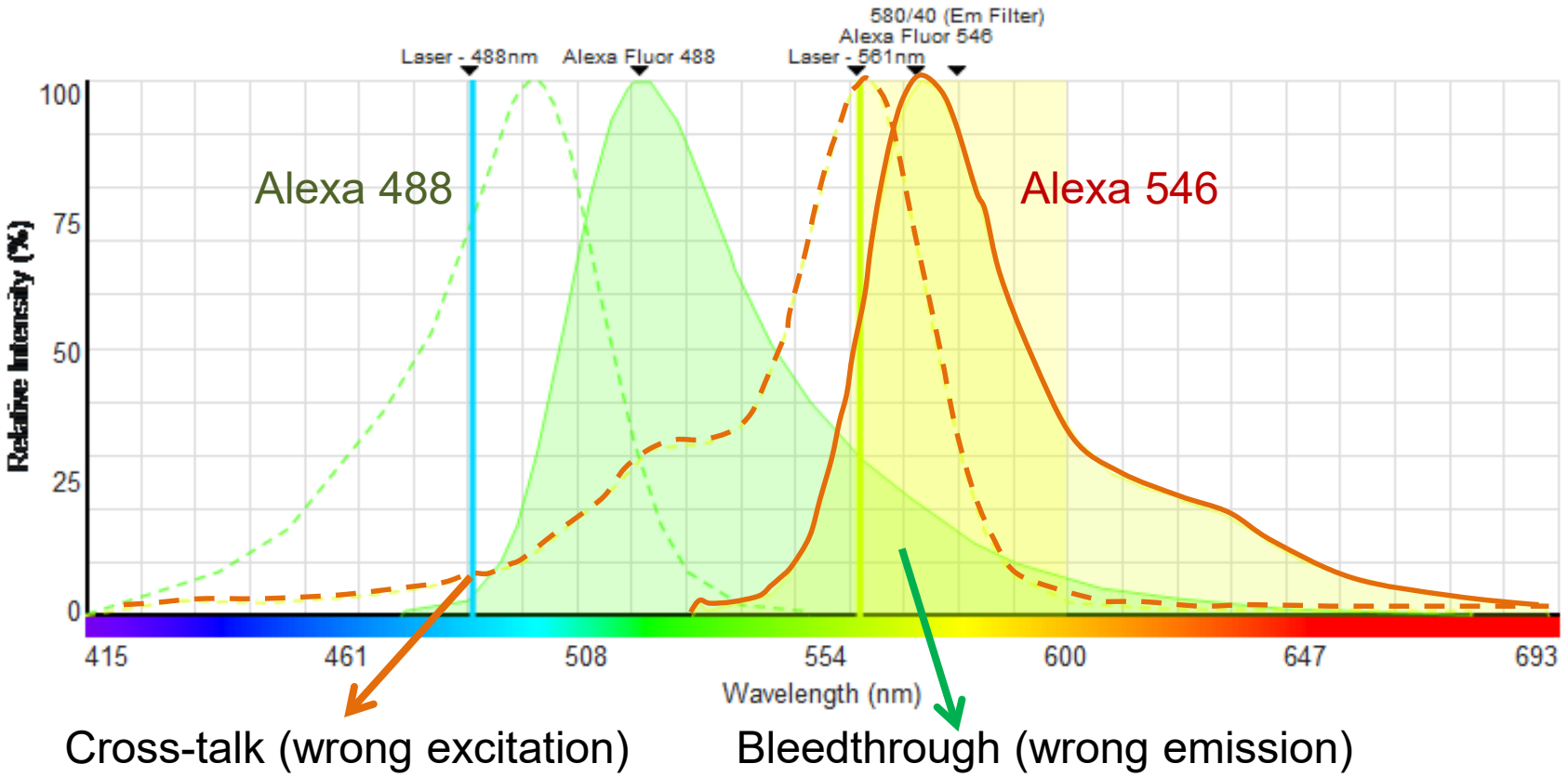
→ needs a reference

→ Mix beads with your samples

http://www.olympusconfocal.com/applications/colocalization.html

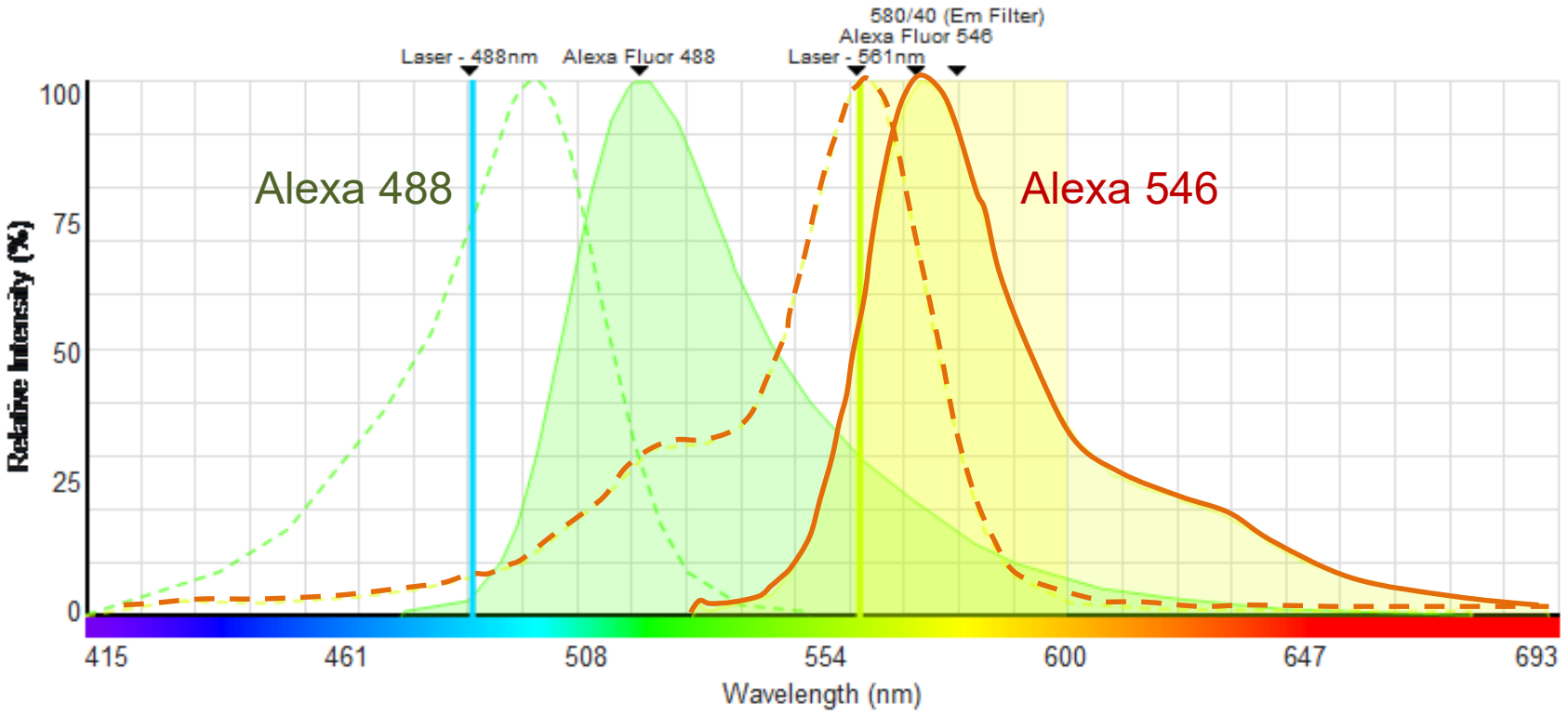
How to acquire the images for colocalisation

- Avoid Cross-talk and Bleedthrough



SpectraViewer on https://www.biozentrum.unibas.ch/IMCF-links

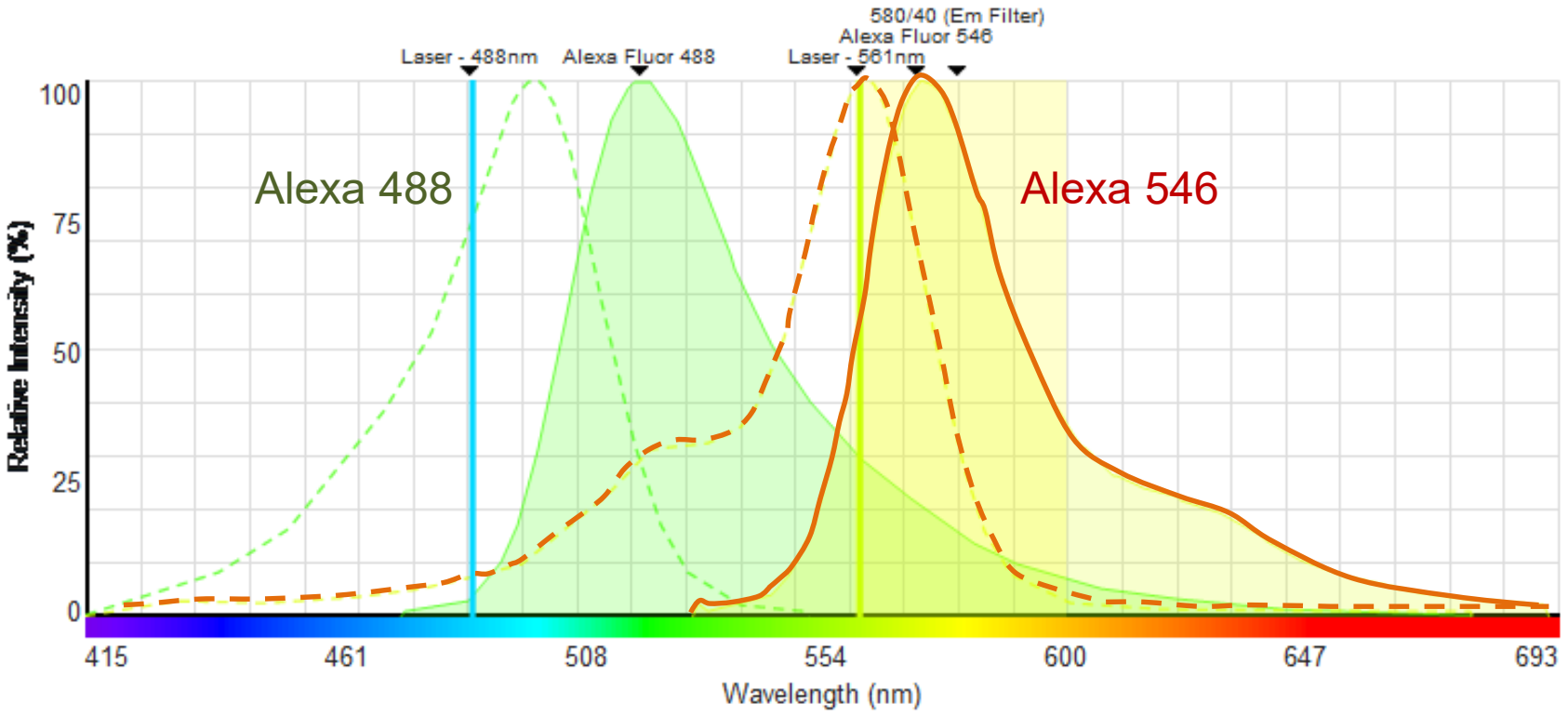
How to acquire the images for colocalisation



Fluorophore/Filter	580/40
Alexa Fluor 488	13.2%
Alexa Fluor 546	65.1%

Bleedthrough

How to acquire the images for colocalisation



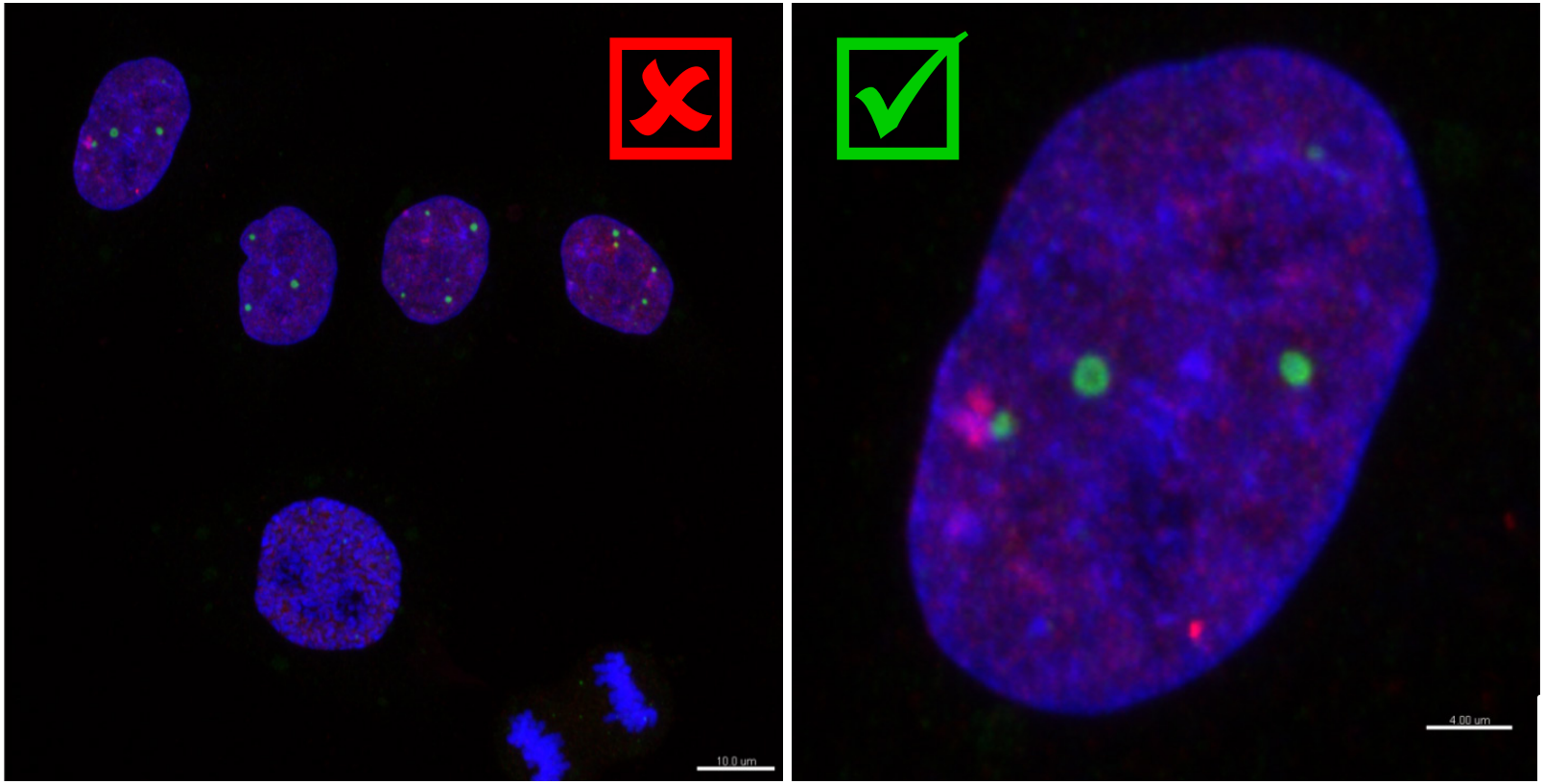
→ Scan with the sequential mode

How to acquire the images for colocalisation

- Avoid bleaching
  - Don't bleach the area before imaging it!
    When possible, use brightfield to find you cells
- Avoid Noise
  - Bleached samples → lower signal/higher noise
- Avoid saturation (use the whole dynamic range)

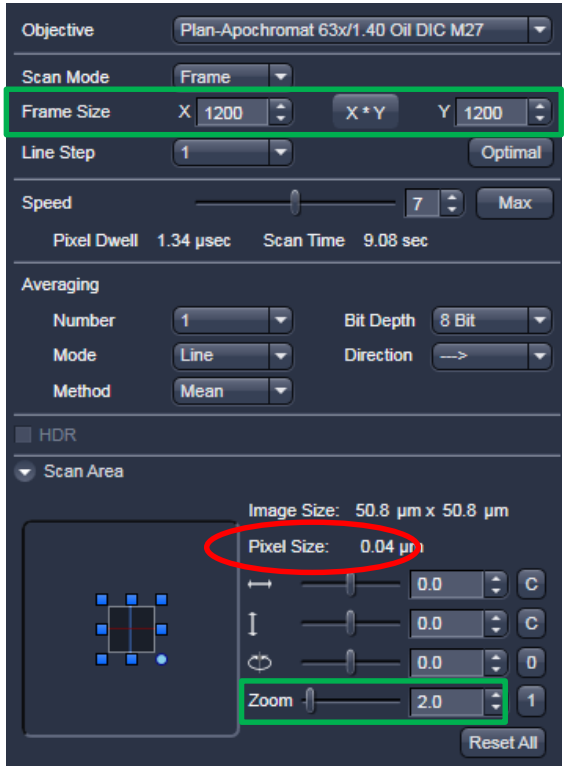
How to acquire the images for colocalisation

- One cell per field of view (pre or post acquisition)

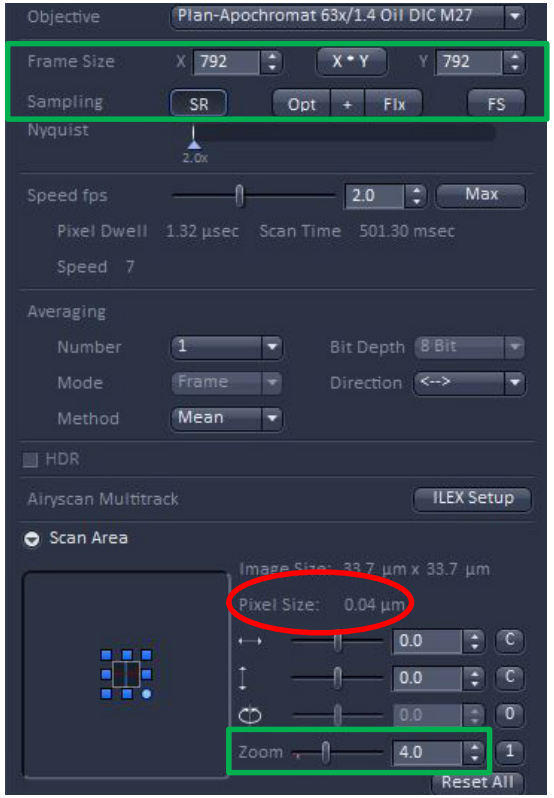


How to acquire the images for colocalisation

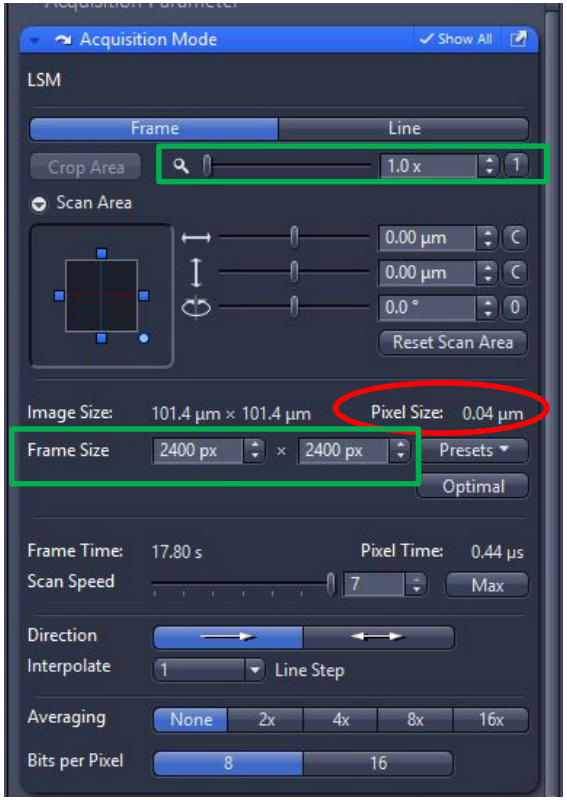
- Match the pixel size – Oversampling XY



LSM700/LSM880



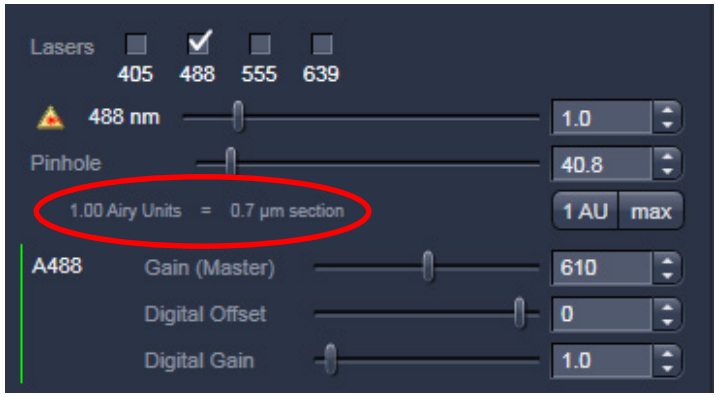
LSM880 (FAST mode)

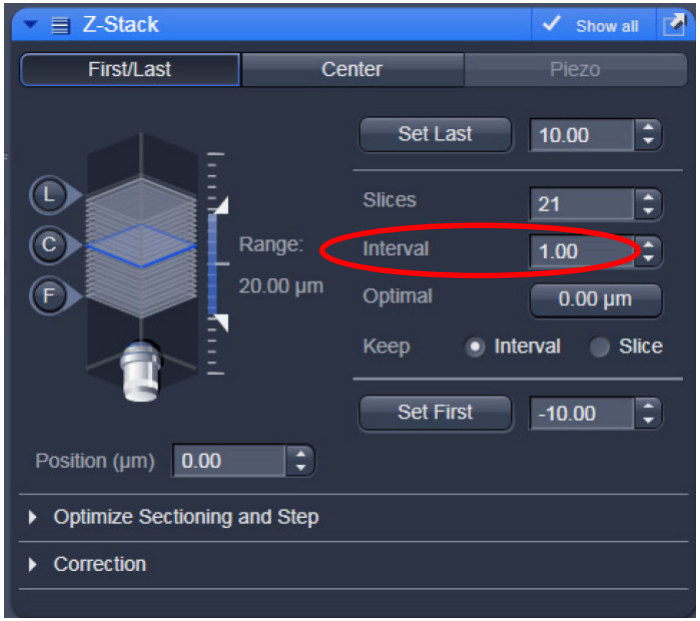


LSM800 II

How to acquire the images for colocalisation

- Match the pixel size – Oversampling Z





LSM700/800/880

How to acquire the images for colocalisation

- Match the optical thickness - pinholes

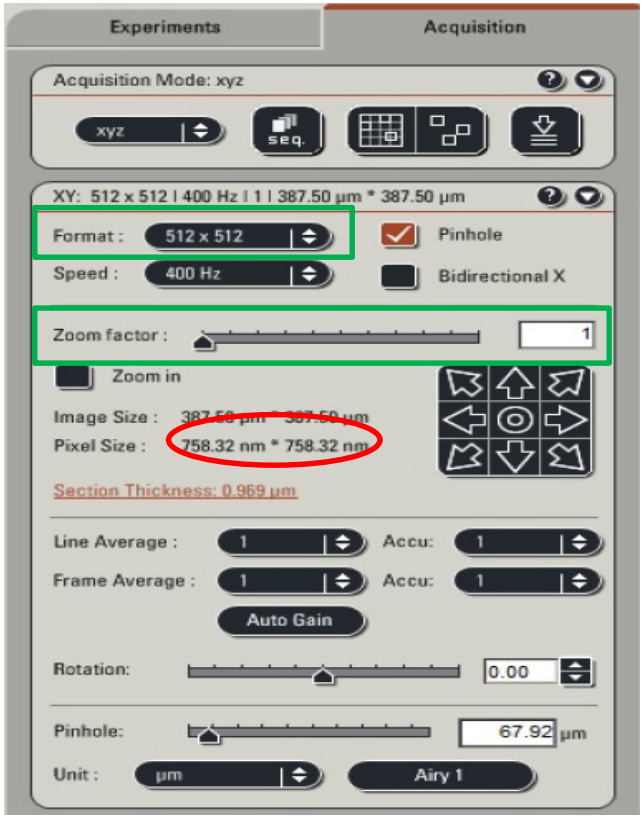
Fig. 77 Optimize Sectioning and Step: Optimal Interval is set starting with one Airy unit for all channels

Fig. 78 Optimize Sectioning and Step: Match Pinhole to Step resulting in equal optical sections for all channels

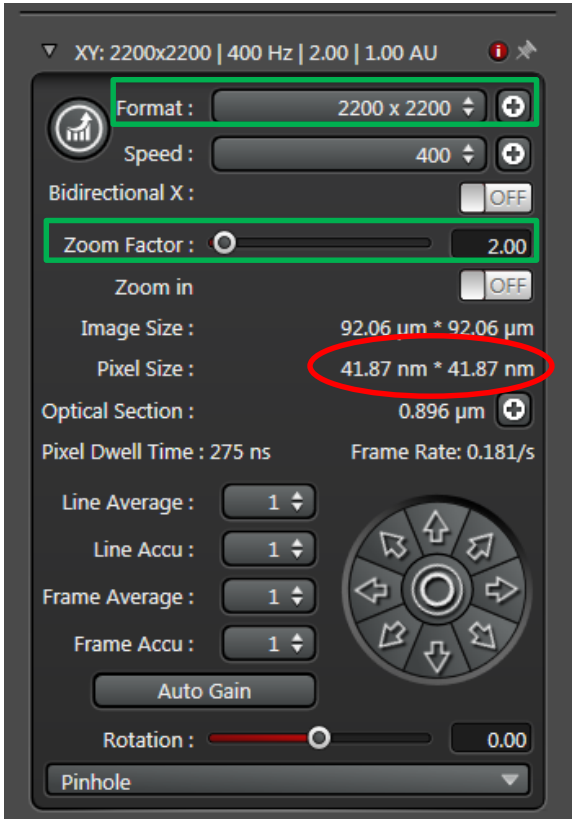
- Example with the LSM700 Zeiss

How to acquire the images for colocalisation

- Match the pixel size – Oversampling XY



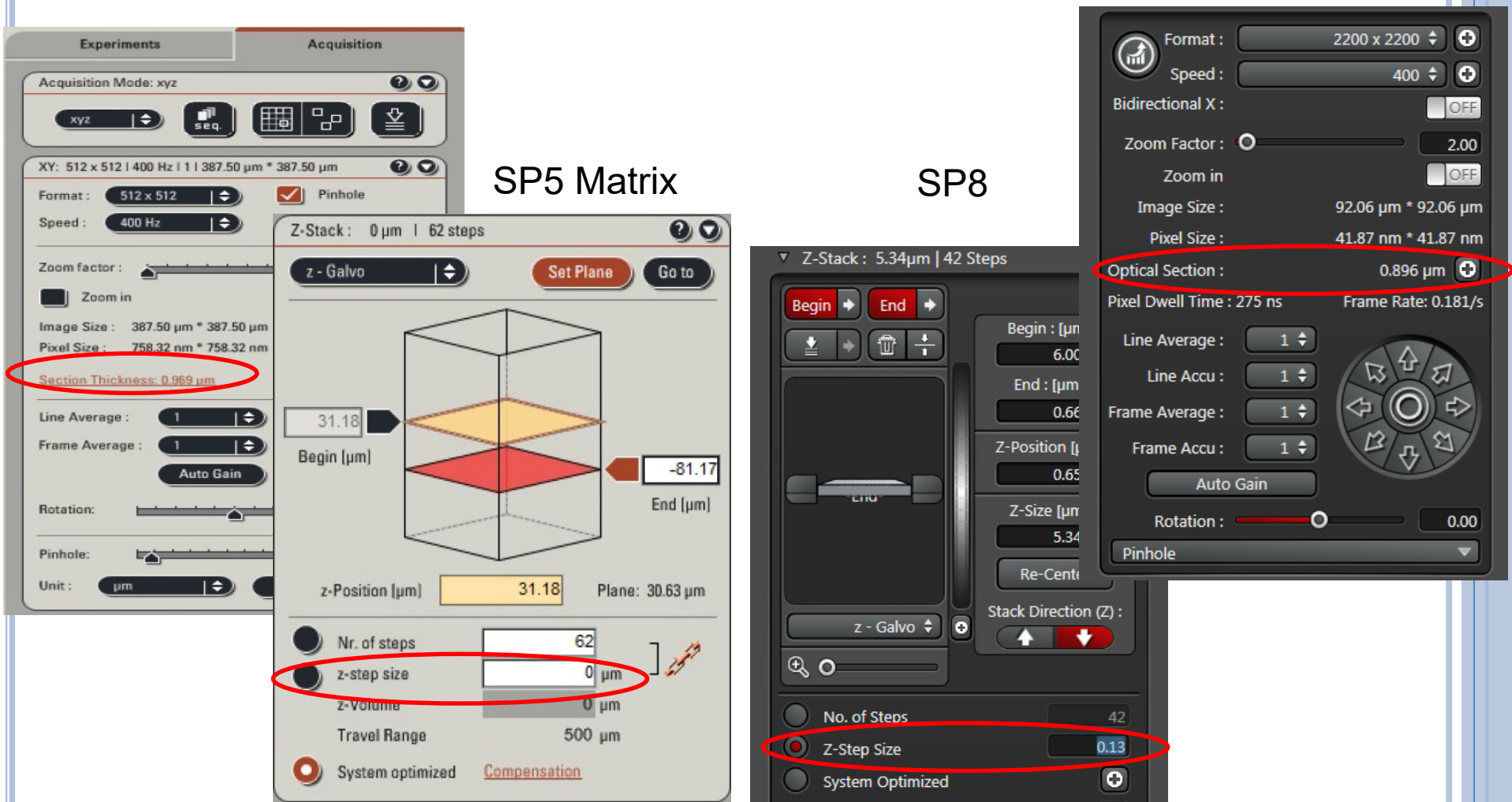
SP5 Matrix



SP8

How to acquire the images for colocalisation

- Match the pixel size – Oversampling Z



Settings for Deconvolution: Confocals

Microscope and Objective	Required XY	Required Z	Example: Settings to use
SP5 II Matrix; 63x 1.4 Oil	43 nm	131 nm	**ZOOM 2** XY: 2800*2800 Z: 0.13 nm **ZOOM 4** XY: 1500*1500 Z: 0.13 nm
SP8; 63x 1.4 Oil	43 nm	131 nm	**ZOOM 2** XY: 2200*2200 Z: 0.13 nm **ZOOM 4** XY: 1080*1080 Z: 0.13 nm
LSM700 Up; 63x 1.4 Oil	43 nm	131 nm	**ZOOM 1** XY: 2048*2048 Z: 0.13 nm **ZOOM 2** XY: 1200*1200 Z: 0.13 nm
LSM800; 63x 1.4 Oil	43 nm	131 nm	**ZOOM 1** XY: 2400*2400 Z: 0.13 nm **ZOOM 2** XY: 1200*1200 Z: 0.13 nm
LSM880; 63x 1.4 Oil	43 nm	131 nm	CONFOCAL - **ZOOM 1.5** XY: 2048*2048 Z: 0.13 nm CONFOCAL - **ZOOM 2.6** XY: 1200*1200 Z: 0.13 nm FAST: Use the SR sampling (2x Nyquist)

- Required values are calculated for a 488-568 nm colocalisation
- Any Zoom/XY frame size is possible, as long as you match the XYZ pixel requirements
- For more information, check https://svi.nl/NyquistCalculator

Settings for Deconvolution: Spinning Disk

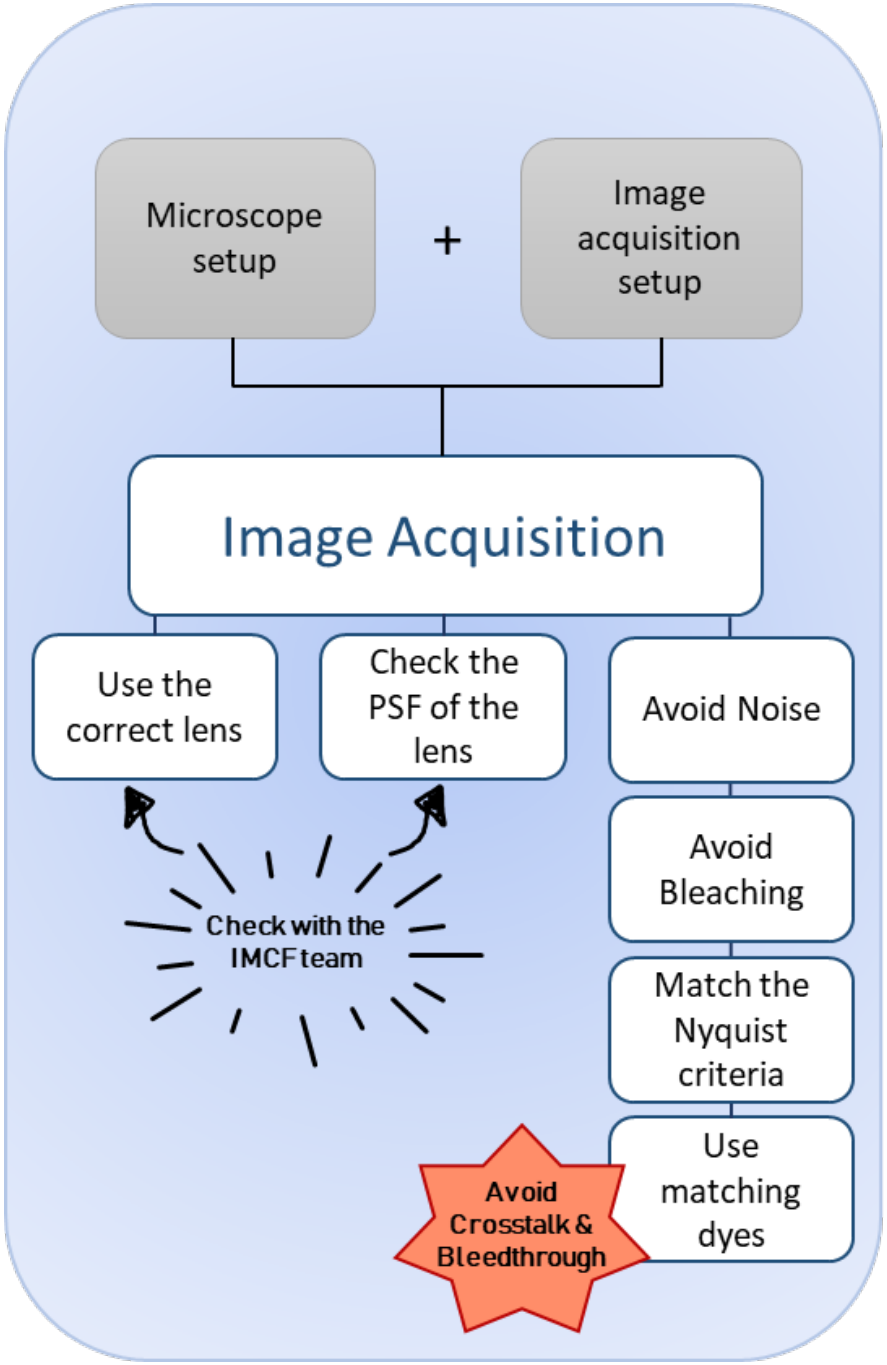
Microscope and Objective	Required XY	Required Z	Example: Settings to use
SpinSR; 60x 1.5 Oil	40 nm	93 nm	**Check the camera installed on the system***
SpinSR; 100x 1.5 Oil	40 nm	93 nm	**Check the camera installed on the system***

- Required values are calculated for a 488-568 nm colocalisation
- XY pixel size is determined by the camera and the use or not of an extra auxiliary lens
- * the final camera choice is under testing at the IMCF
- Any Zoom/XY frame size is possible, as long as you match the XYZ pixel requirements
- For more information, check https://svi.nl/NyquistCalculator

Settings for Deconvolution: Widefields

Microscope and Objective	Required XY	Required Z	Example: Settings to use
Deltavision; 100x 1.40 Oil CCD camera	92 nm	279 nm	**XY – no Aux Mag: 65 nm Z: 0.2nm**
Deltavision; 60x 1.42 Oil CCD camera	91 nm	264 nm	XY – no Aux Mag: 107 nm Z: 0.2nm **XY – with Aux Mag: 65 nm Z: 0.2nm**
Nikon Ti2; 100x 1.45 Oil sCMOS camera	89 nm	243 nm	XY - no Aux Mag: 110 nm Z: 0.2 nm **XY - with Aux Mag: 73 nm Z: 0.2 nm**
Nikon Ti2; 60x 1.40 Oil sCMOS camera	92 nm	279 nm	XY - no Aux Mag: 180 nm Z: 0.2 nm **XY - with Aux Mag: 120 nm Z: 0.2 nm**
MORE; 100x 1.4 Oil sCMOS camera	92 nm	279 nm	XY: 65 nm Z: 0.2nm
MORE; 60x 1.49 Oil sCMOS camera	87 nm	211 nm	XY 109 nm Z: 0.2nm

- Required values are calculated for a 488-568 nm colocalisation
- XY pixel size is determined by the camera and the use or not of the extra auxiliary lens (1.5x for Nikon Ti2, 1.6x for Deltavision)
- Changing the XY px size with the extra lens does NOT change the acquisition time
- Changing the frame size can speed up the acquisition (MORE and Nikon Ti2)



TAKE HOME MESSAGE PART 2

- Make sure that you are aware of the PSF of the lens (beads)

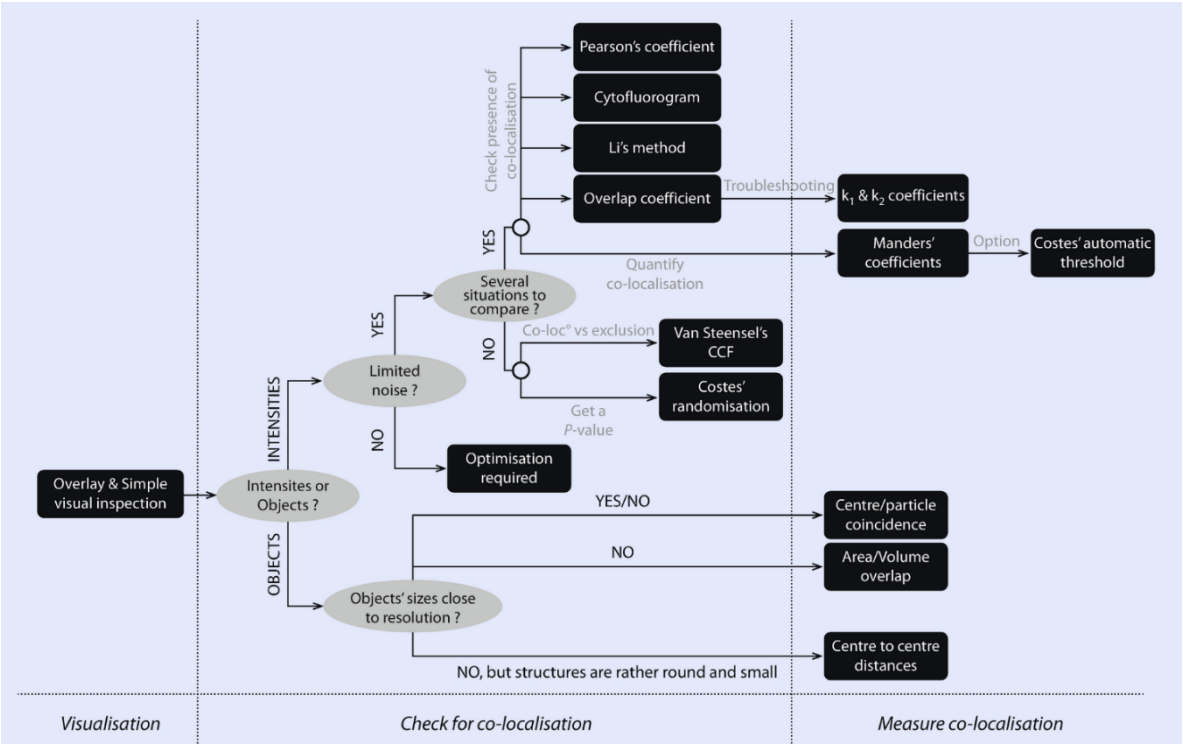
- Avoid noise/ bleaching/ saturation when you acquire your images (good SNR)

- Make sure you match pinholes and the oversampling (Nyquist)

- Make sure that you use sequential acquisition if you suspect crosstalk and/or bleedthrough

How to analyse your Colocalisation Data

- Colocalisation is 3D
- Colocalisation should be more thought in terms of correlation
- Colocalisation needs Quantification & Statistics



[3D Quantitative Colocalisation Analysis (Cordelières & Zhang, 2019)]

How to analyse your Colocalisation Data

(1) Intensity/pixel-based

Correlation of the strength of linear relation between two channels
→ *no spatial exploration of the colocalisation signal*

(2) Object-based

Structure identification and determination of overlap of objects (for discrete structures)
→ *Segmentation*

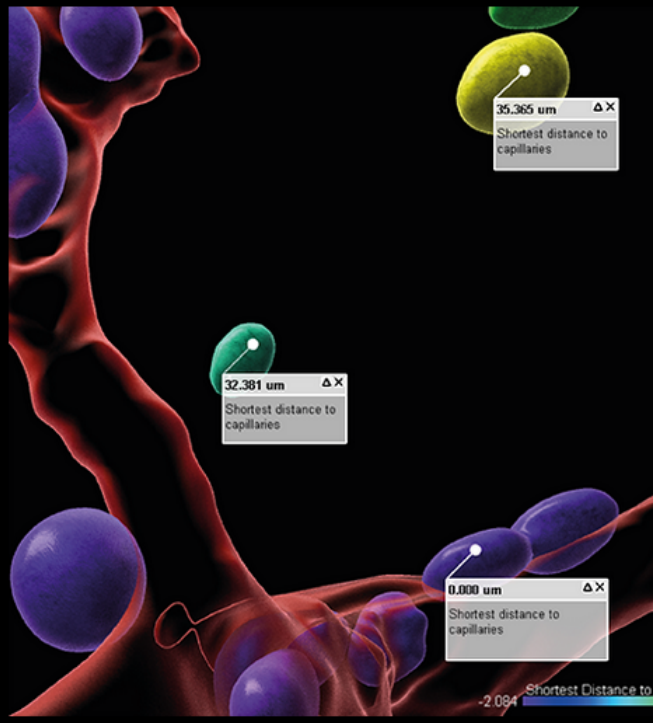
- Many tools available
  See Bioimage Informatics Search Engine (BISE) – colocalisation
- Object-based analysis: Please contact our IMCF Image Analysis experts (Wednesday Workshops)

How to analyse your Data: Proximity – Imaris

Native Distance Measurements

For every Surfaces and Spots object Imaris 9.5 **natively calculates** the **shortest distance** to any other Surfaces or Spots. The edge of surfaces and the center of spots are used for these calculations. The computation is fast and capable of running on **large images**. Calculations can also be performed on multiple datasets at once in **Batch mode**

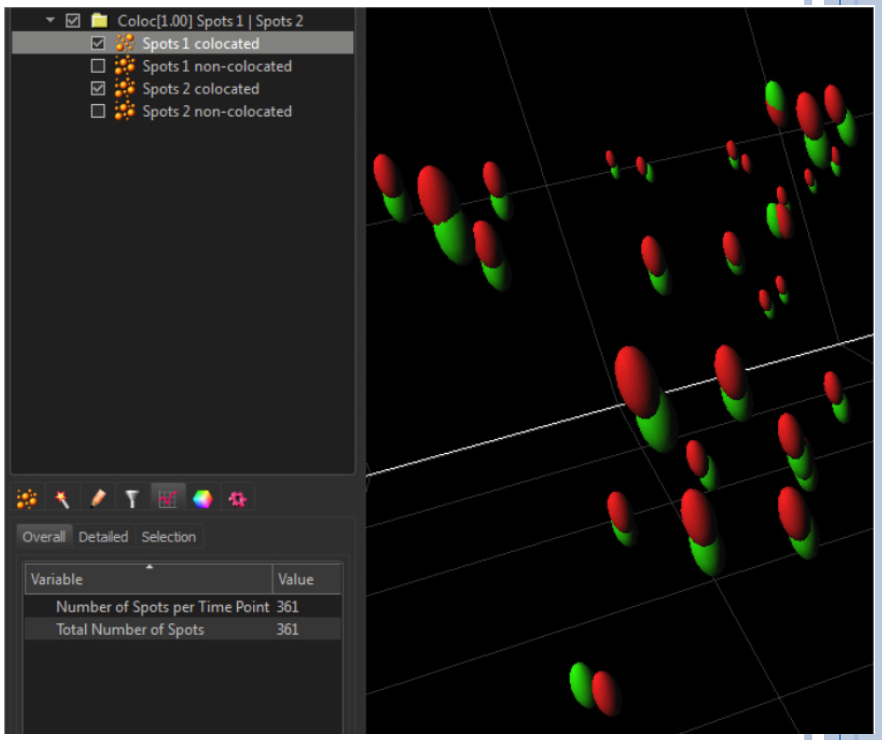
- Color code Spots or Surfaces by Distance from Surfaces (**view movie**).
- Filter Spots inside or outside of Surfaces (**view movie**)
- Count Spots inside the ROI (**view movie**)
- Filter Spots in a specific distance band outside or inside Surfaces. (**view movie**)
- Filter tracks of objects that "go inside" Surfaces (**view movie**)
- Calculate Shortest Distance for multiple datasets at once in the Batch mode **view movie**



Caution! No statistics, this is NOT real colocalisation!!!

How to analyse your Data: Proximity – Imaris

- Matlab extension for Imaris
- See dedicated wiki page : https://wiki.biozentrum.unibas.ch/pages/viewpage.action?spaceKey=IMCF&title=Imaris+-+colocalize+spots



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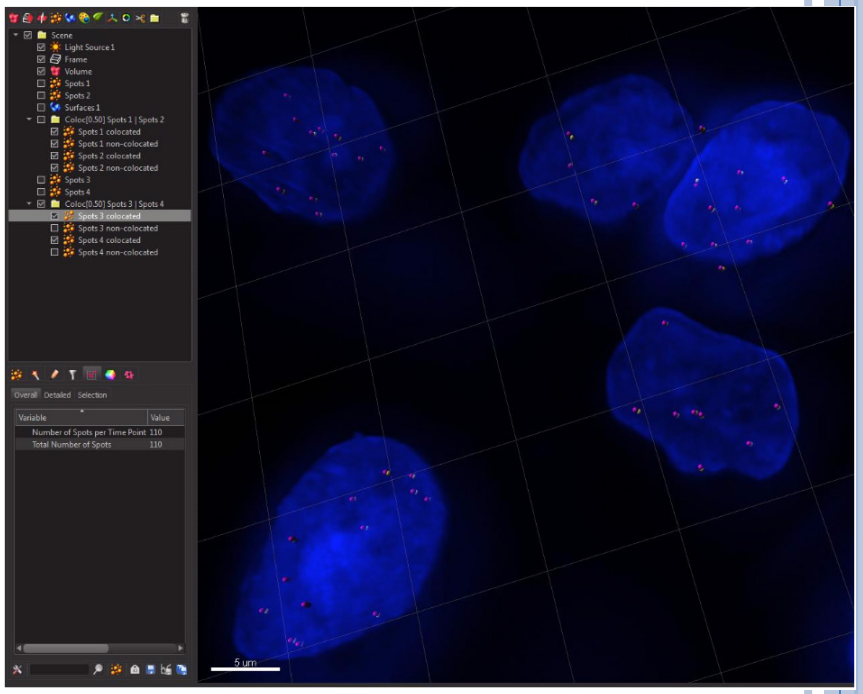
How to analyse your Data: Proximity – Imaris

- Matlab extension for Imaris
- See dedicated wiki page : https://wiki.biozentrum.unibas.ch/pages/viewpage.action?spaceKey=IMCF&title=Imaris+-+colocalize+spots
- Can be limited to spots within a certain region (ie. DAPI here)



Caution! No statistics, this is NOT real colocalisation!!!

JACoP Just Another Colocalisation Plugin

Journal of Microscopy, Vol. 224, Pt 3 December 2006, pp. 213–232
Received 13 April 2006; accepted 28 June 2006

TUTORIAL REVIEW

A guided tour into subcellular colocalization analysis in light microscopy

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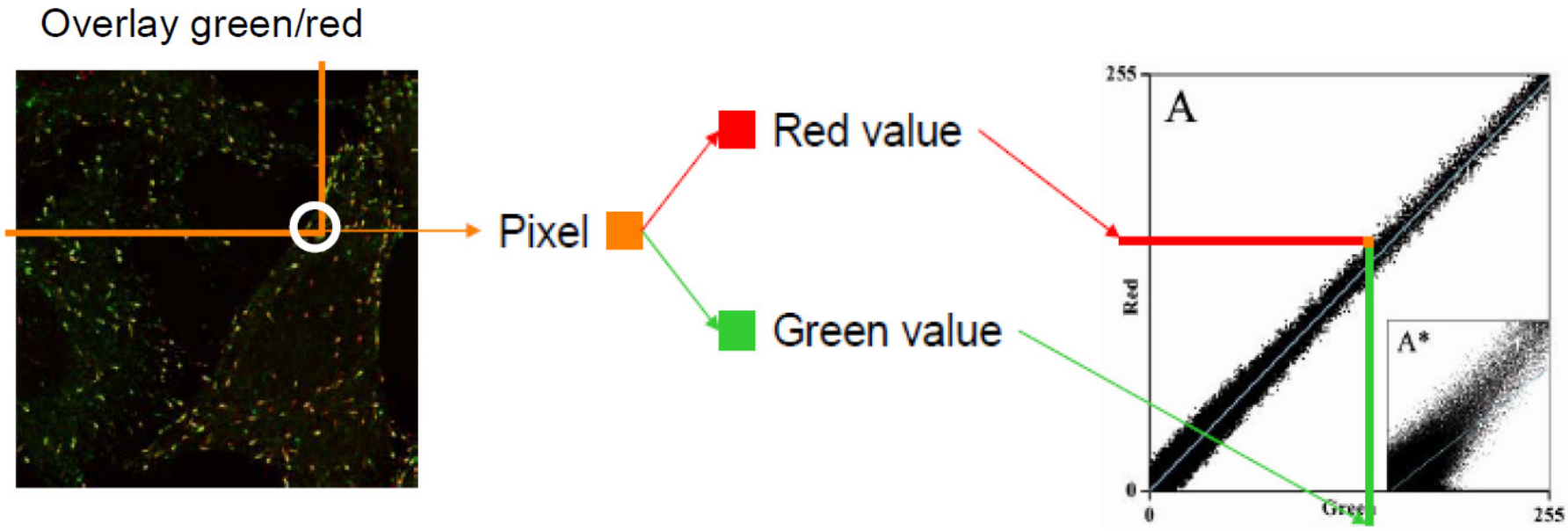
**Plateforme d'Imagerie et de Biologie Cellulaire, IFR 87 'la Plante et son Environnement', Institut des Sciences du Végétal, Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France*
†Institut Curie, CNRS UMR 146, Plateforme d'Imagerie Cellulaire et Tissulaire, Bâtiment 112, Centre Universitaire, 91405 Orsay Cedex, France

Key words. Colocalization, confocal microscopy, fluorescence microscopy, image analysis, wide-field microscopy.

Must Read

https://doi.org/10.1111/j.1365-2818.2006.01706.x

JACoP: Scatter plot/Cytofluorogram

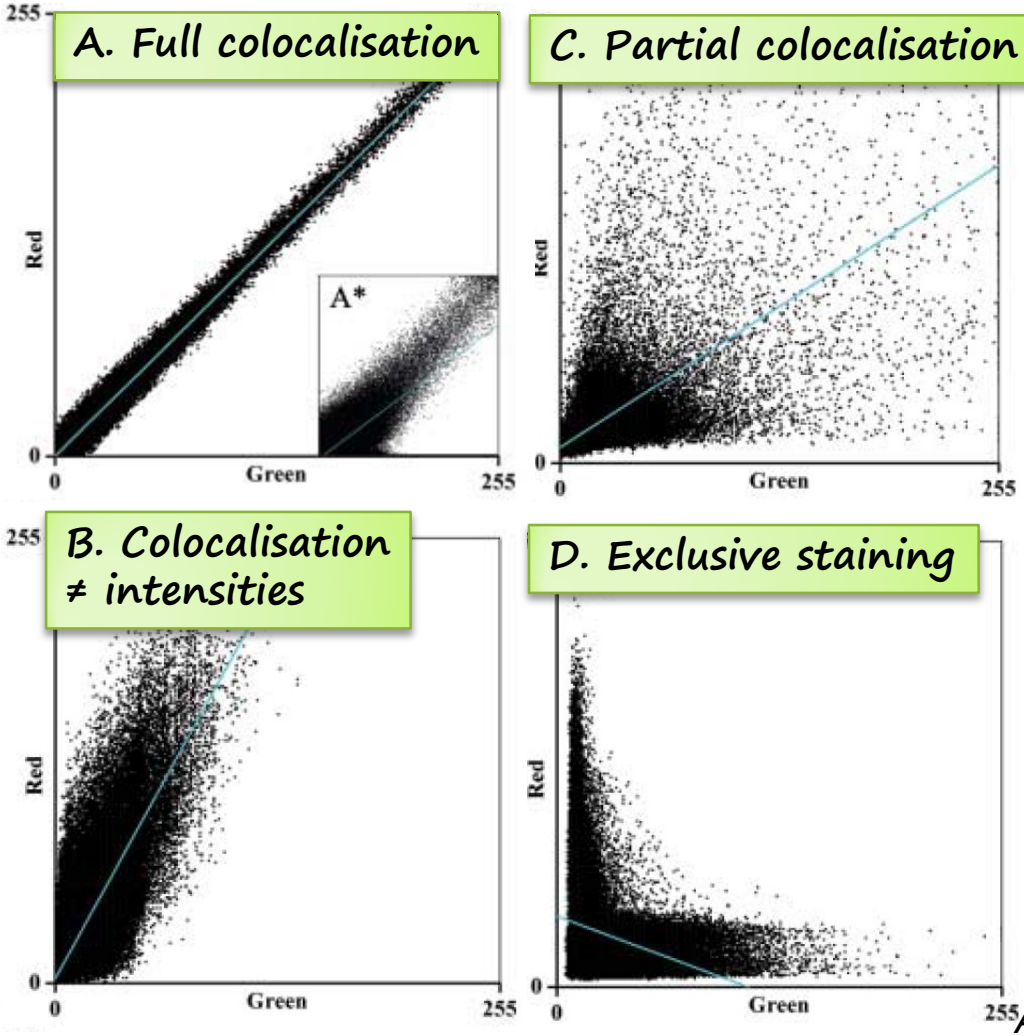


☺ Good first visual estimate of colocalisation

☺Information about the image quality

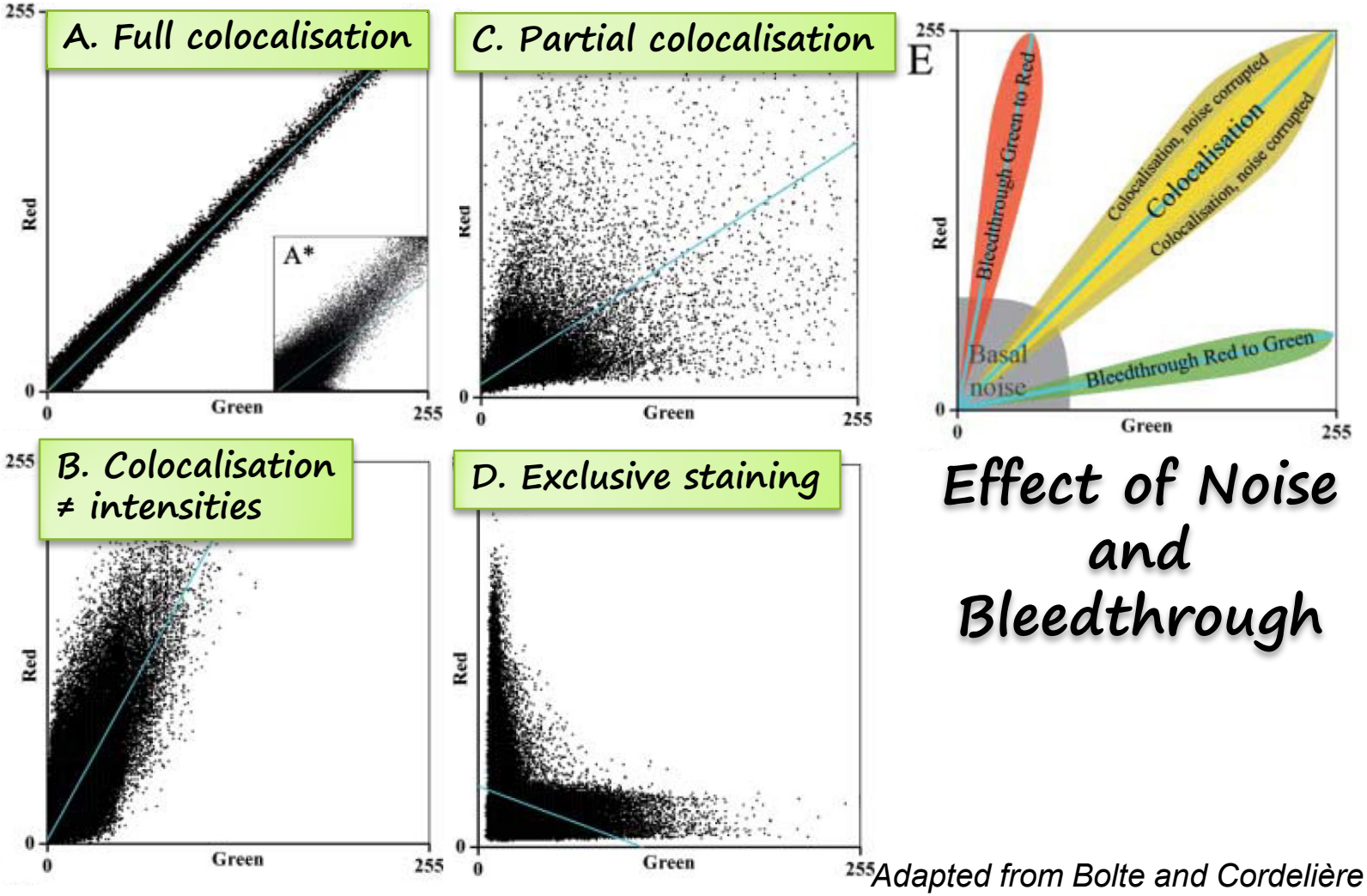
☹ Only qualitative correlation

JACoP: Scatter plot/Cytofluorogram



Adapted from Bolte and Cordelière 2006

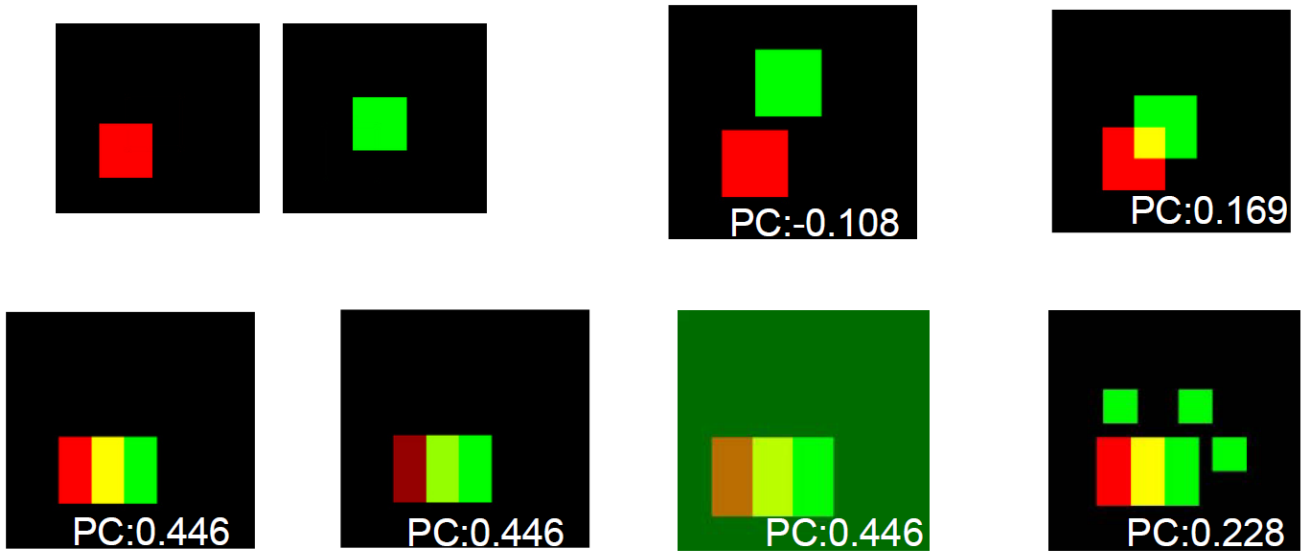
JACoP: Scatter plot/Cytofluorogram



Effect of Noise and Bleedthrough

Adapted from Bolte and Cordelière 2006

Pearson's coefficient

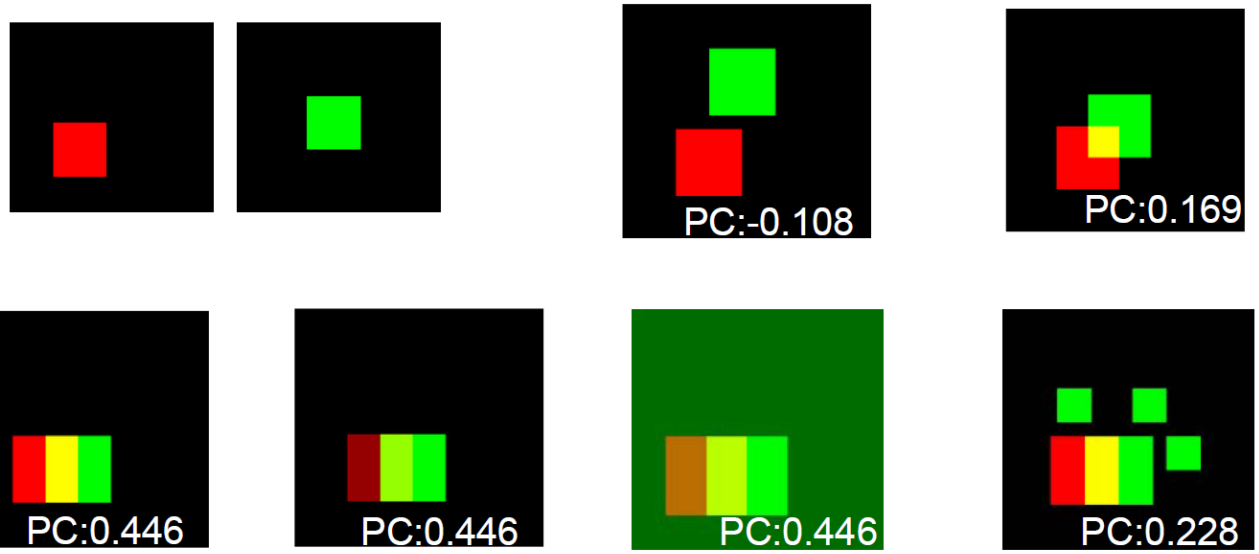


- PCC or « r »

Estimate of the association strength between 2 proteins

http://www.svi.nl/ColocalizationCoefficients

Pearson's coefficient



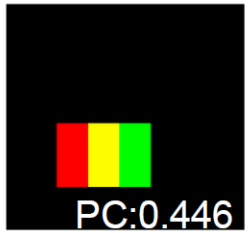
☺ Not sensitive on ≠ intensity of the overlapping pixels
☺ Not sensitive on background intensity
☹ Not easy to interpret
☹ Affected by noise
☹ No perspective of both channels

http://www.svi.nl/ColocalizationCoefficients

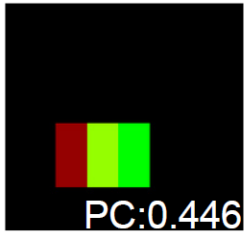
MANDERS COEFFICIENTS

M1:0.000 M2:0.000

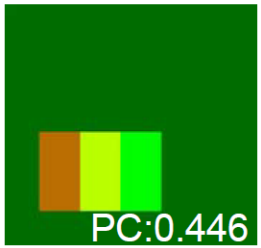
M1:0.250 M2:0.250



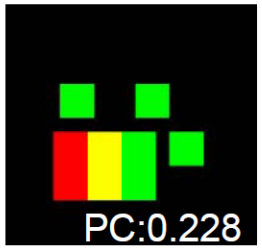
M1:0.500 M2:0.500



M1:0.500 M2:0.500



M1:1.000 M2:0.163



M1:0.500 M2:0.286

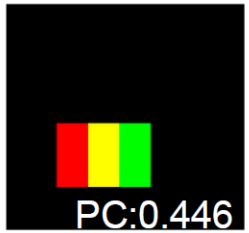
- M1 and M2

Gives the proportion of each protein colocalising with the other

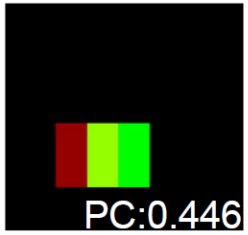
How to analyse your Colocalisation Data

M1:0.000 M2:0.000

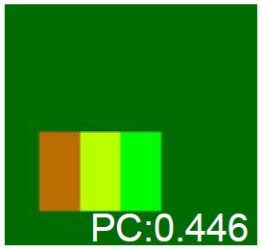
M1:0.250 M2:0.250



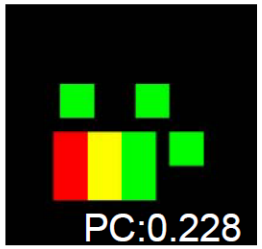
M1:0.500 M2:0.500



M1:0.500 M2:0.500



M1:1.000 M2:0.163



M1:0.500 M2:0.286

☺ Easier to interpret that PCC

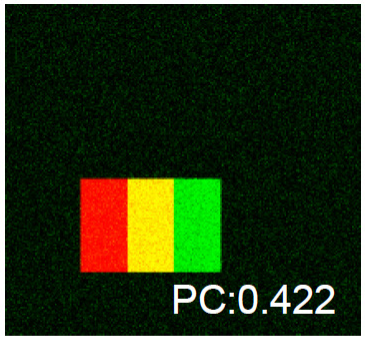
☺ Not sensitive to the intensity of the overlapping pixels

☹ Sensitive to background intensity – Threshold needed!

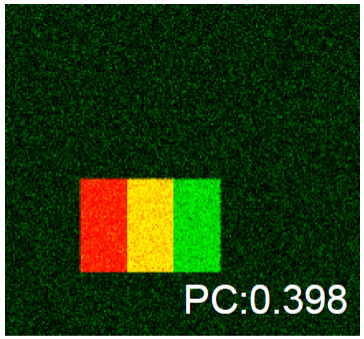
☹ Affected by noise

Influence of Noise

M1:0.500 M2:0.500



M1:0.490 M2:0.260



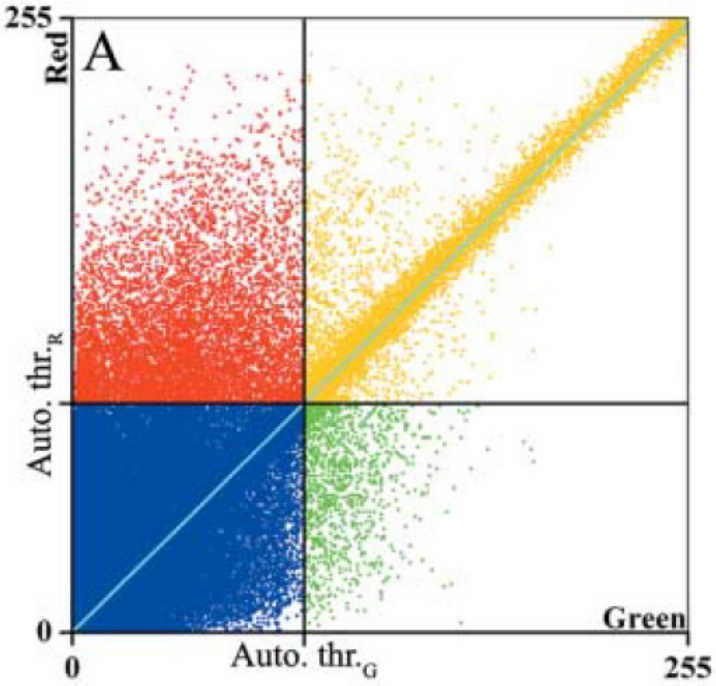
M1:0.490 M2:0.180

These coefficients are influenced by noise
Minimize noise during the acquisition, and deconvolve your datasets prior analysis

Deconvolution improves colocalization analysis of multiple fluorochromes in 3D confocal data sets more than filtering techniques. L. Landmann. Journal of Microscopy **208**:2, 134 (2002).

Costes' Approach

- Estimation of an automatic threshold
- Test of the statistical significance (Costes' P-value)



Costes' Approach

- Estimation of an automatic threshold
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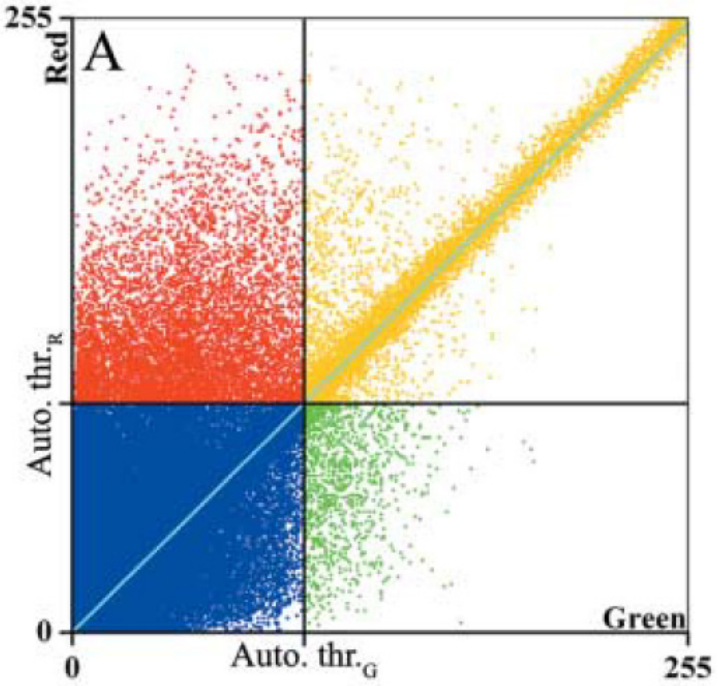
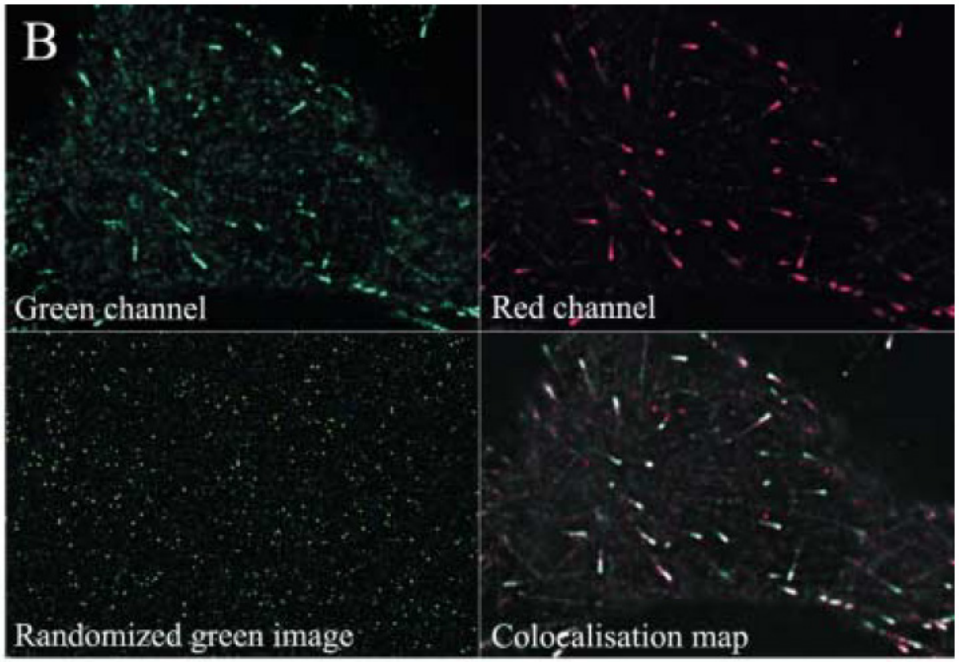
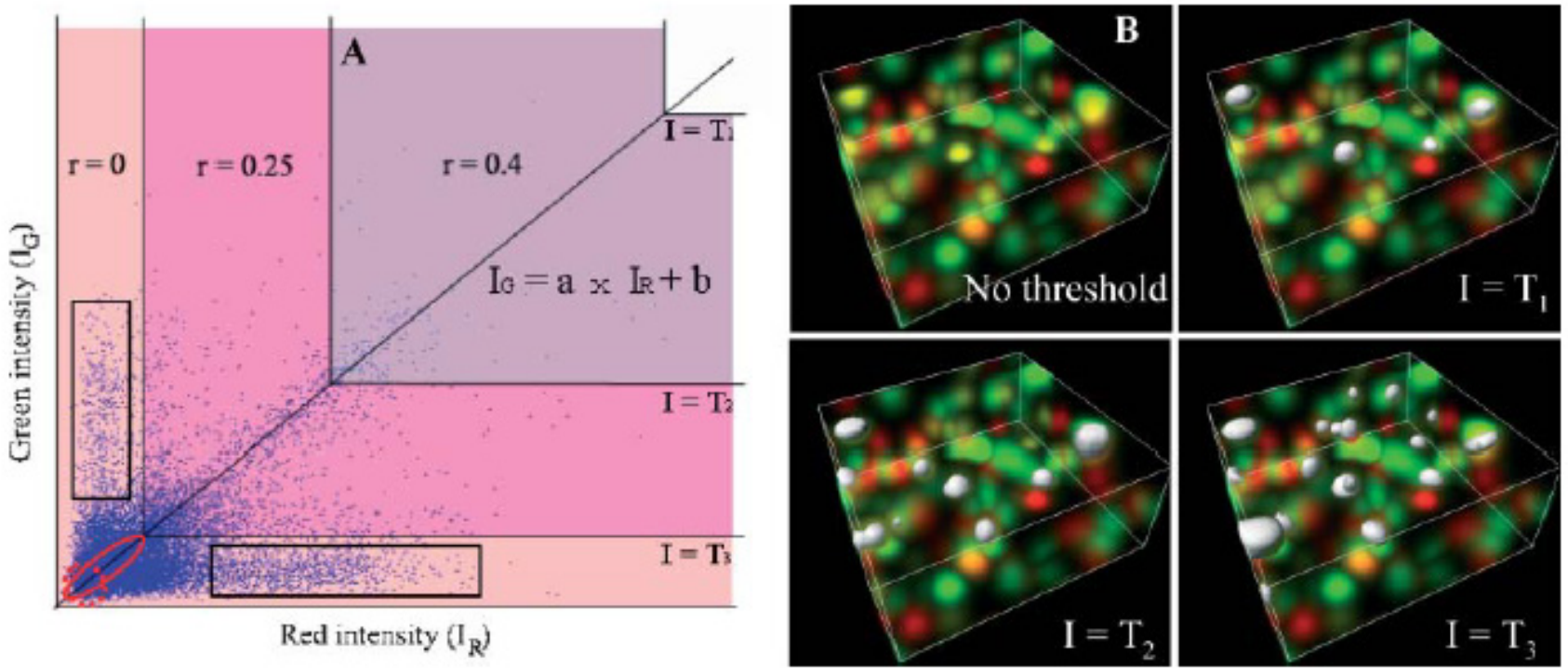




If > 95% of the random images correlate (PCC) worse than the real image, then you can trust the correlation coefficient

Costes' Approach



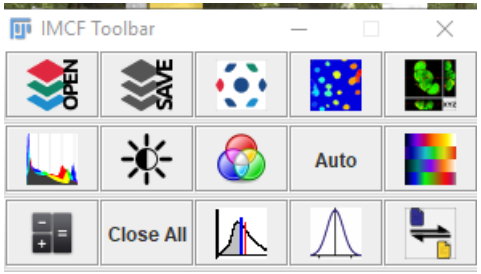
Costes et al., 2004

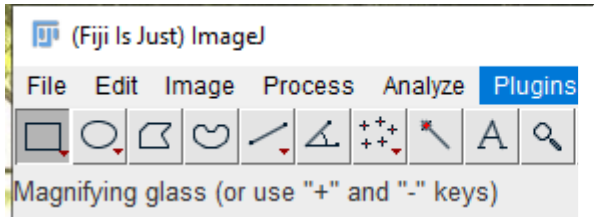
☺ Statistical approach
☺ Minimises the influence of noise
☹ Long calculations (3D)

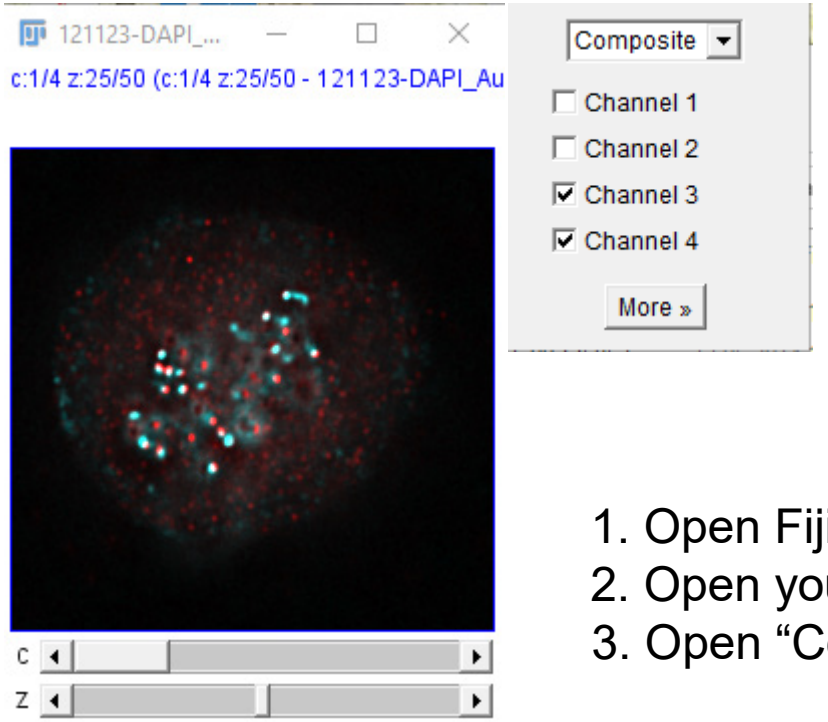
How to understand your data

	Value range	Colocalisation if…	Notes
Pearson's coef r	+1 → coloc 0 → random -1 → exclusion	tends to 1	Insensitive ≠ intensities Insensitive intensity offset Affected by noise Not robust for Bioimages
Manders' coef M1 (or M2)	0 → 0% of Ch1 colocalize with Ch2 1 → 100% of Ch1 colocalize with Ch2	tends to 1	Insensitive ≠ intensities Sensitive intensity offset Affected by noise Biologically meaningful
Costes (P-value)	P<95% → no coloc P≥95% → coloc	≥95%	Automated thresholds Statistical approach Minimises influence of noise

Colocalisation Map





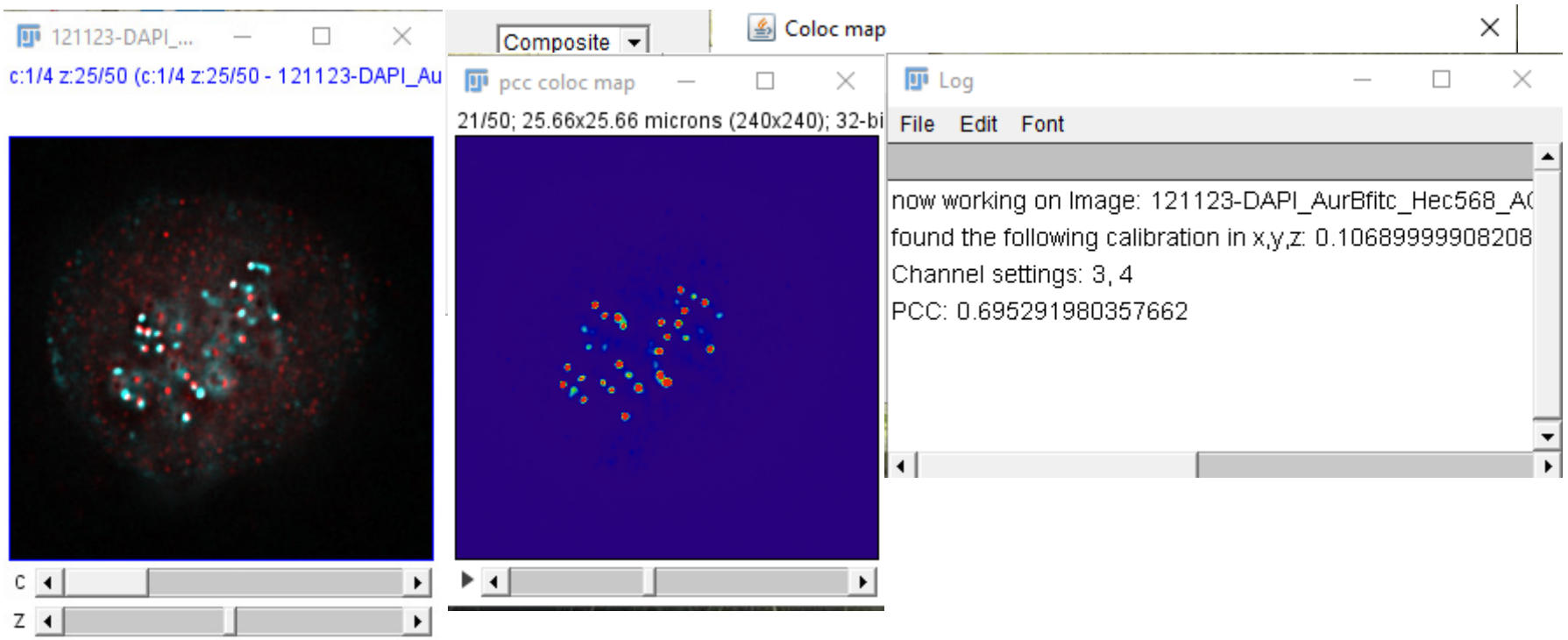


1. Open Fiji
2. Open your image
3. Open “Coloc_Map”

Kai Schleicher, script upon request

Colocalisation Map

4. Select the 2 channels of interest
5. The Pearson Correlation Coefficient Map is generated
    - *Color coded for correlated pixels*
    - *PCC value is summed*



Analysis Pipeline

1- Deconvolution (better resolution, lesser noise)

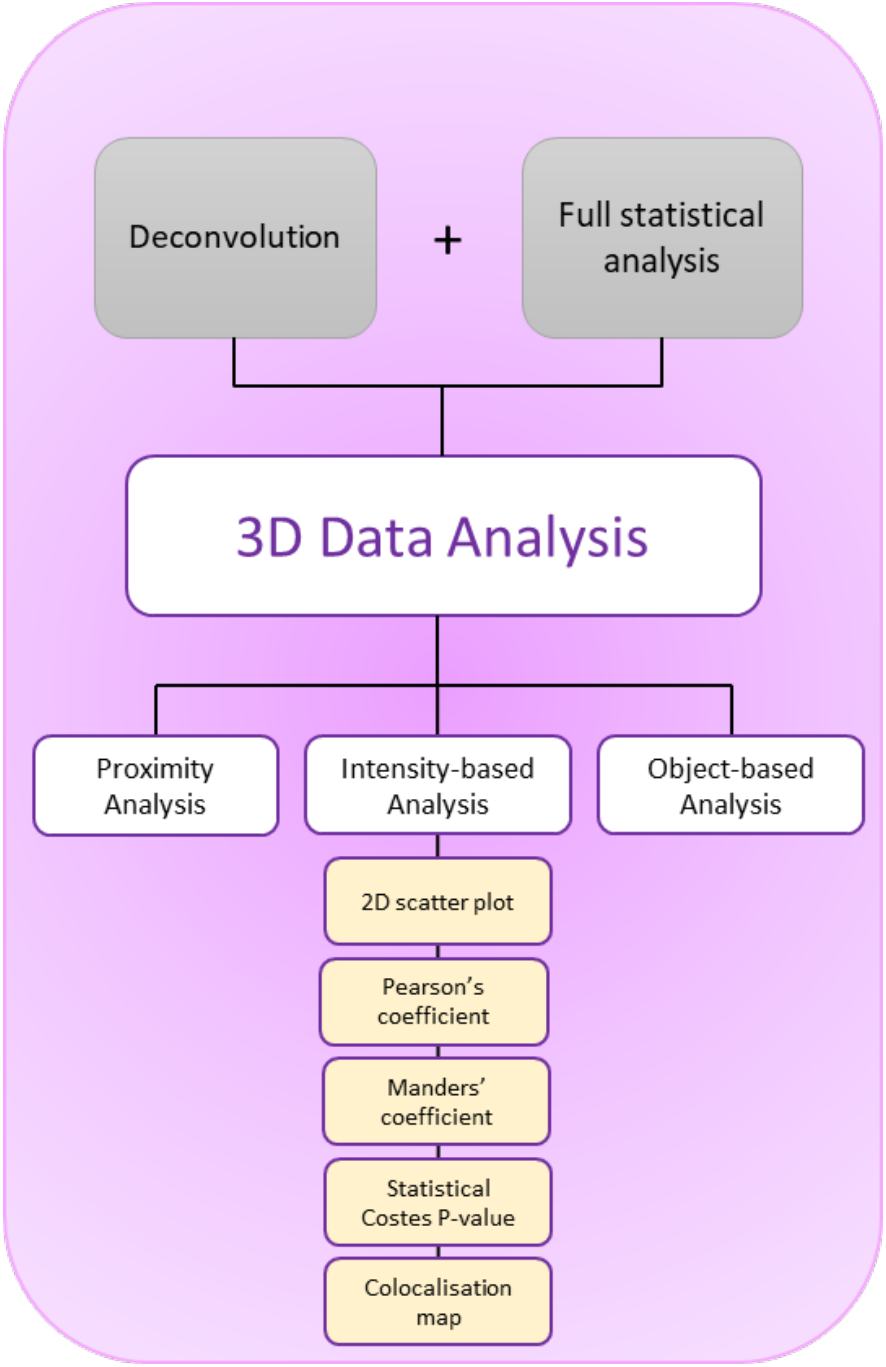
- Softworx (DeltaVision widefield images)
- Huygens (Confocals + Widefield)

2a- Colocalisation analysis (method 1, intensity-based)

- JACoP
- Colocalisation map

2b- Colocalisation analysis (method 2, object-based)

- Check the IMCF team to find the best solution



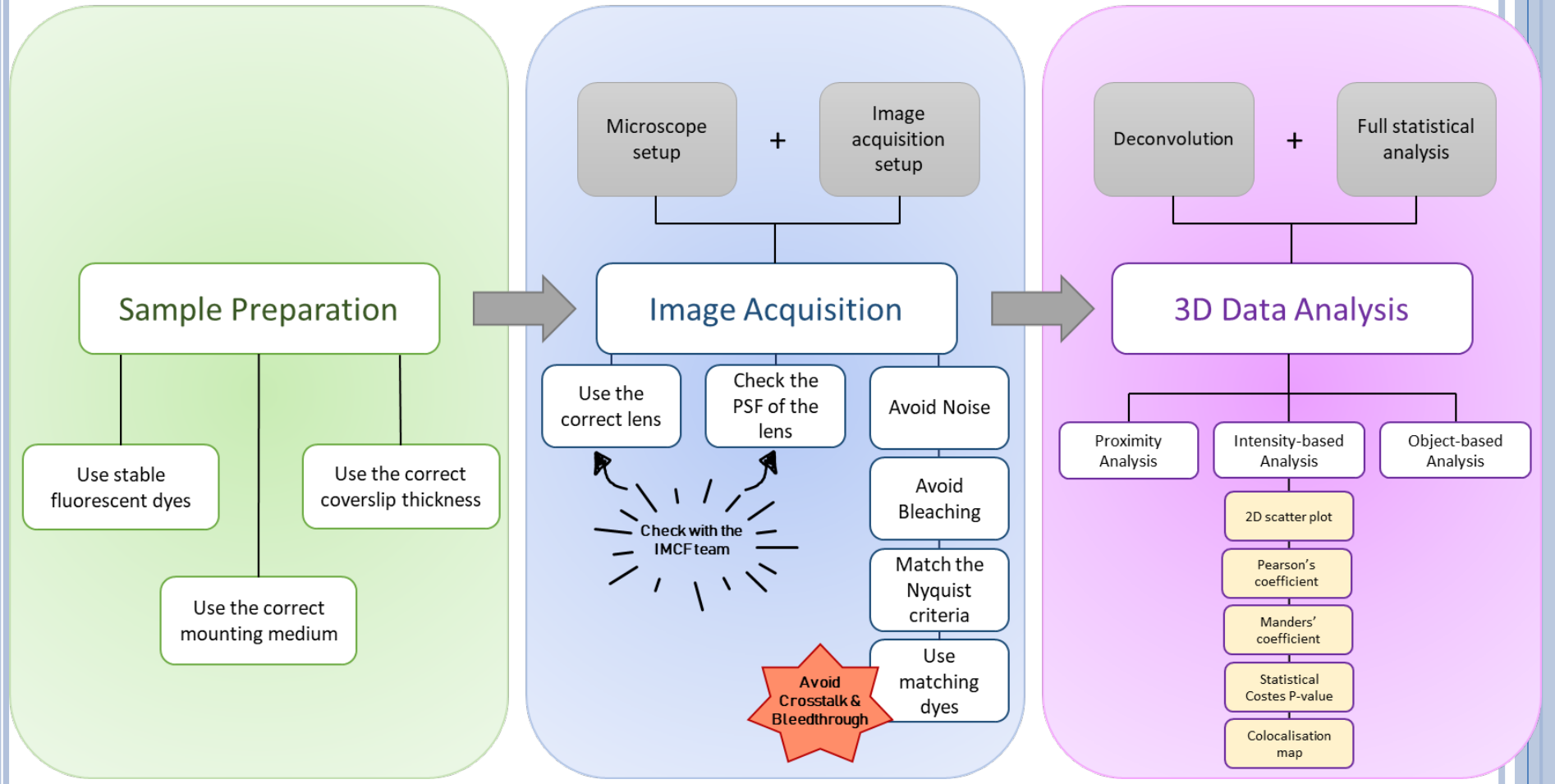
TAKE HOME MESSAGE PART 3

- Work on deconvolved images

- Perform a full colocalisation analysis
  - Intensity-based (no defined structure)
  - Object-based (defined structure)

- For publications, indicate:
  - Pearson's coefficient
  - thresholded Manders' coefficients
  - colocalisation map

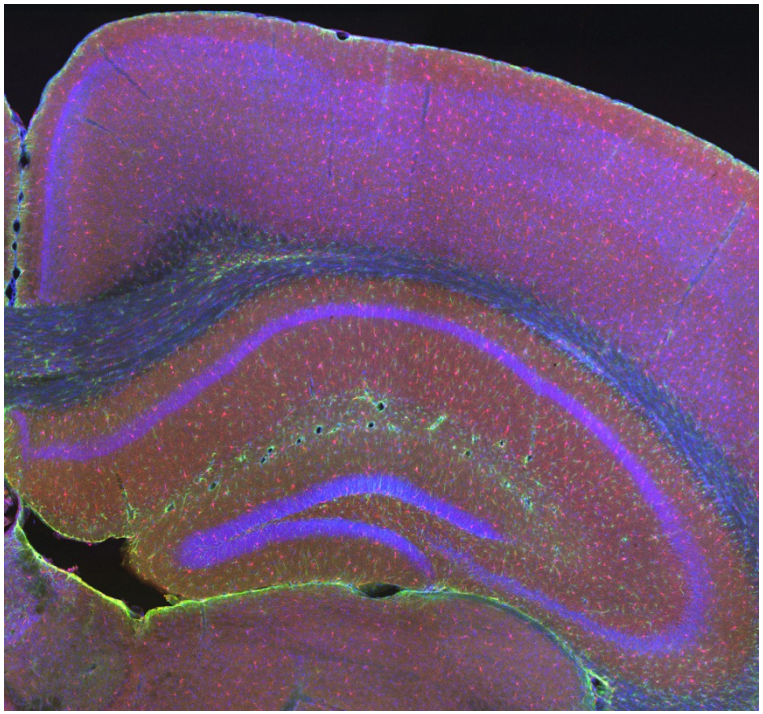
Colocalisation: Strategic Planning



Colocalisation is always relative to the resolution!

Thanks for your attention!

UNI BASEL



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