Colocalisation

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Universität Basel
The Center for
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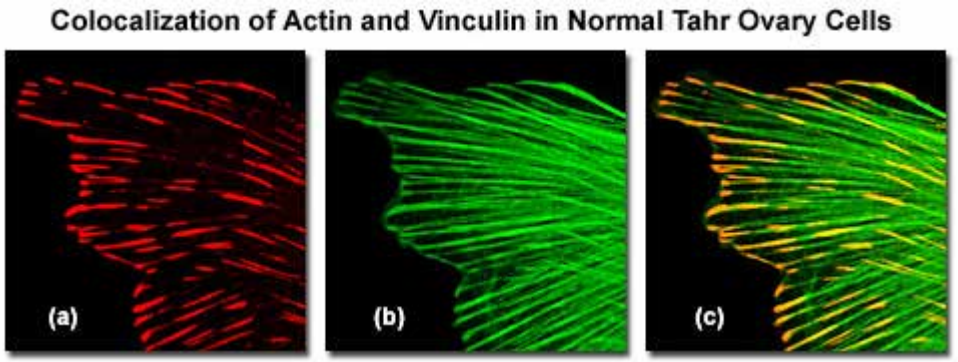
INTRODUCTION

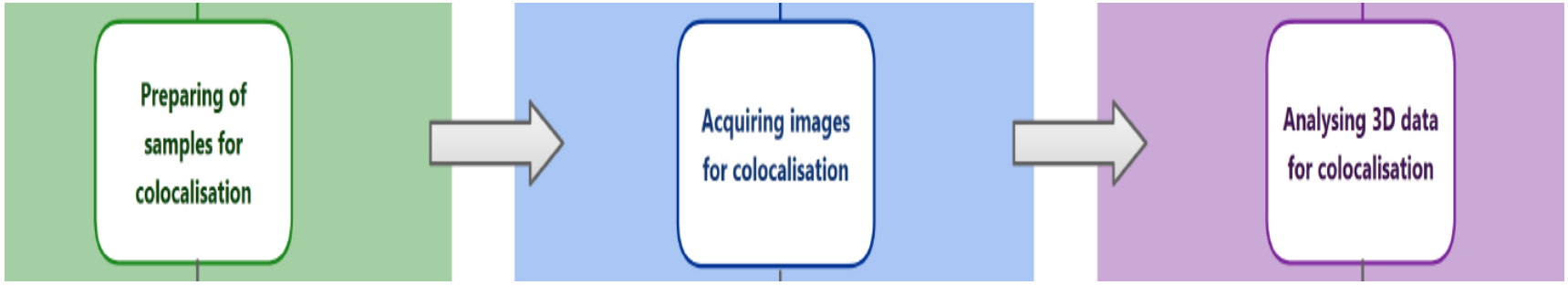
- Colocalisation = Presence of two (or more) structures on the same location
- Colocalisation in fluorescence microscopy at subcellular level = Presence of two (or more) different fluorophores at the same structure in a cell.



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

- Limitation (best case scenario): optical resolution of the microscope à XYZ 200 x 200 x 400 nm
- Colocalisation never measures interaction, it states that 2 dyes are in a close proximity in a defined volume.

Colocalisation: Strategic Planning

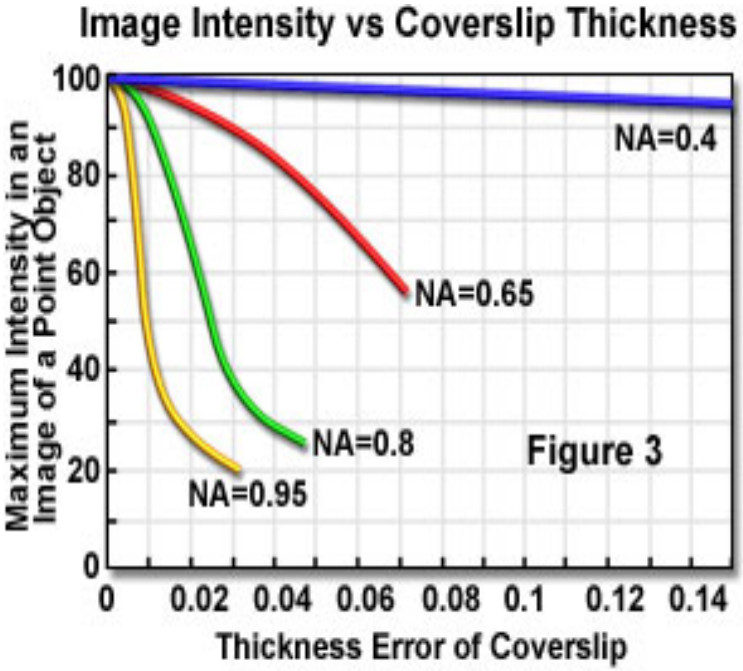


How to best prepare your samples for colocalisation

- 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)



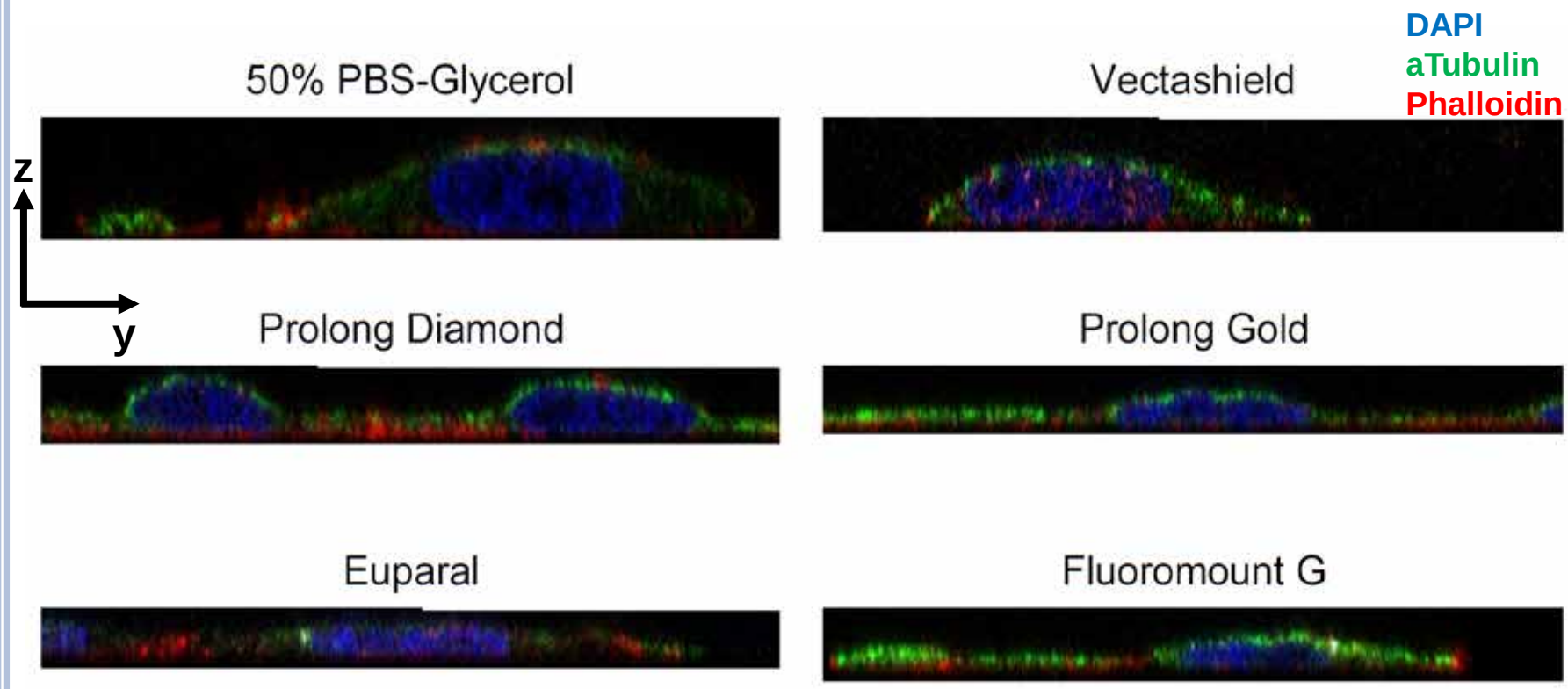
Performance Reduction with Coverslip Thickness Variation

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)



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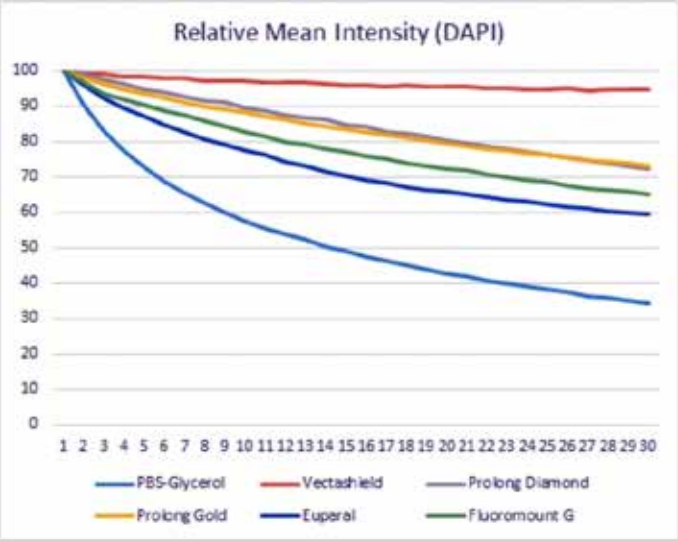
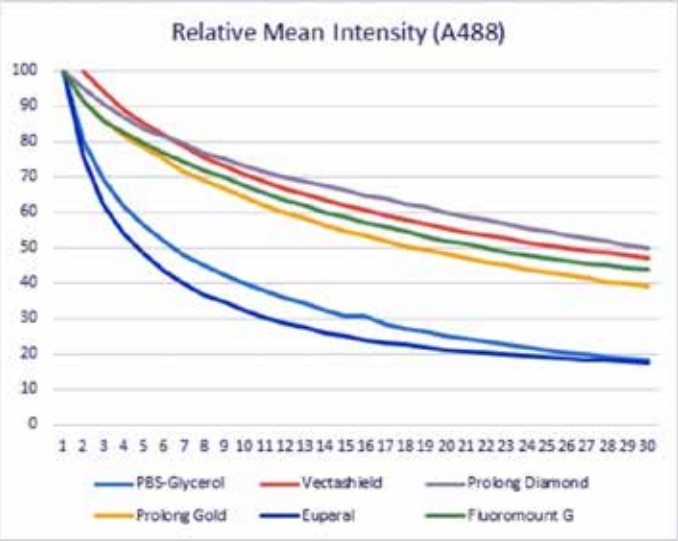
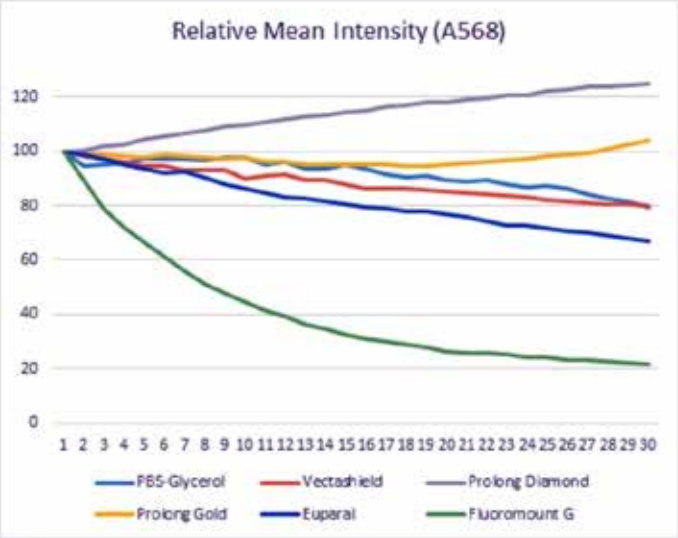
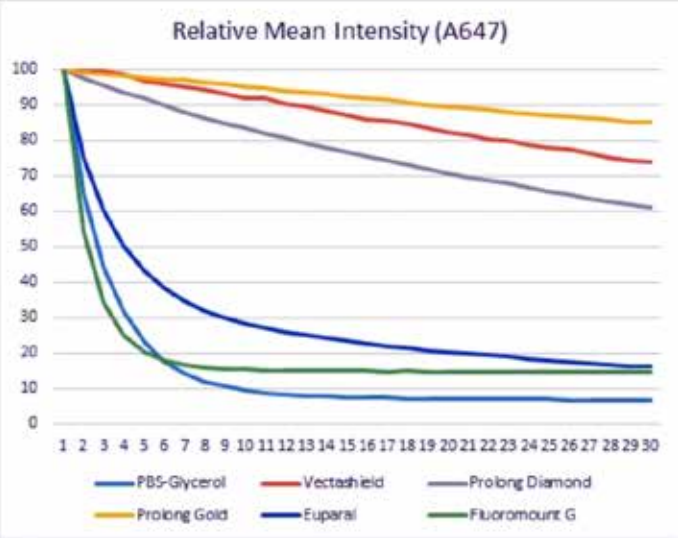




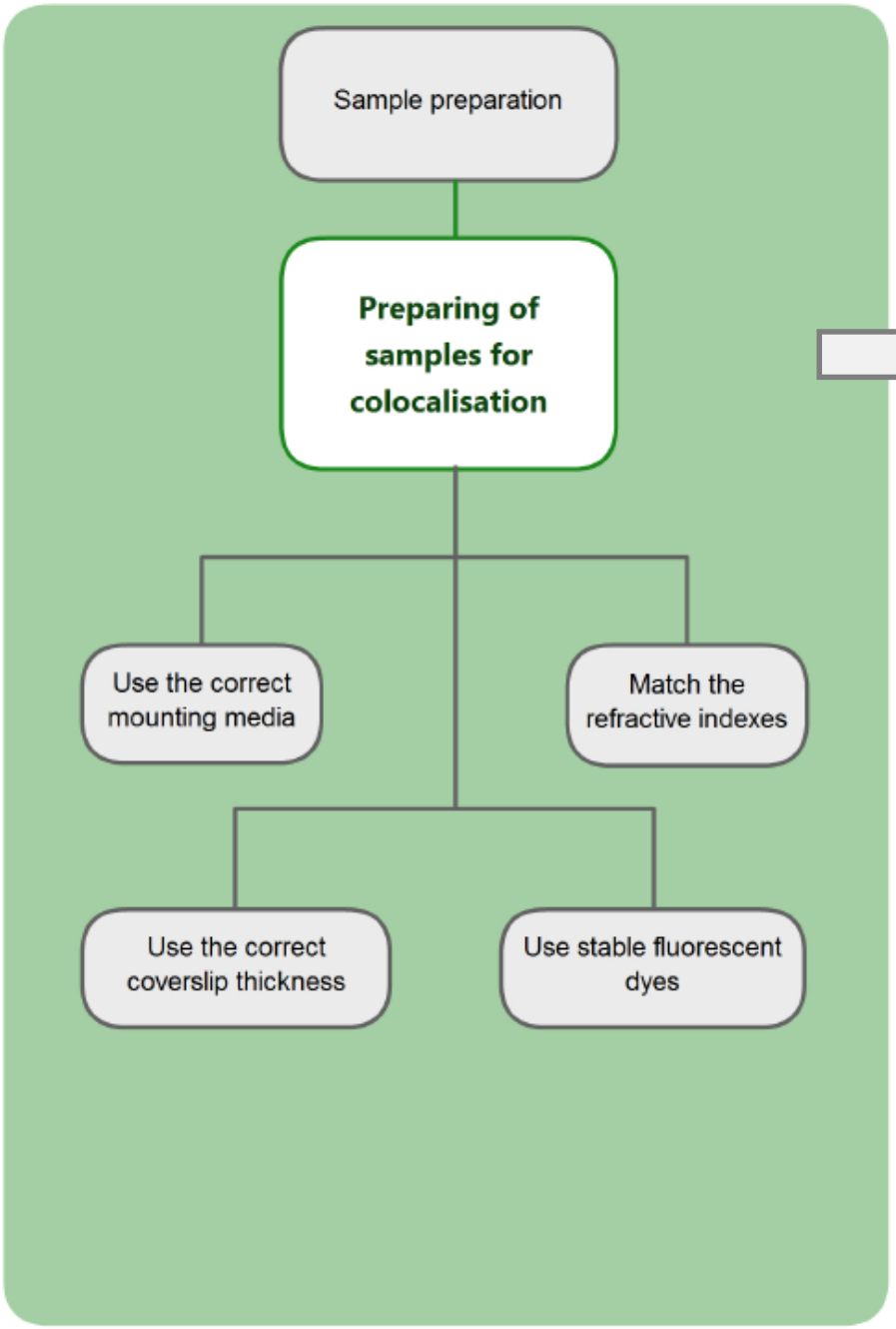




How to best prepare your samples for colocalisation

- 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

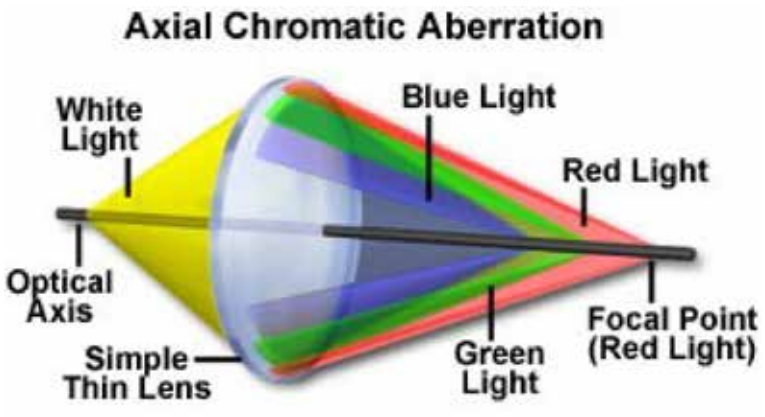
- Make sure that your last “500 µm” are optimal

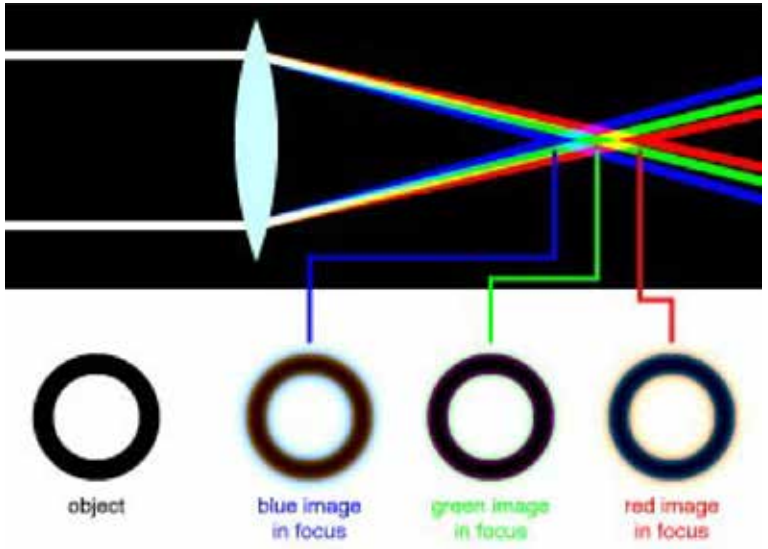
- Don’t forget to prepare positive and negative controls

How to acquire the images for colocalisation

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

    à Aim for a 1.40 NA or above
  - Apochromat lens (l corrected)

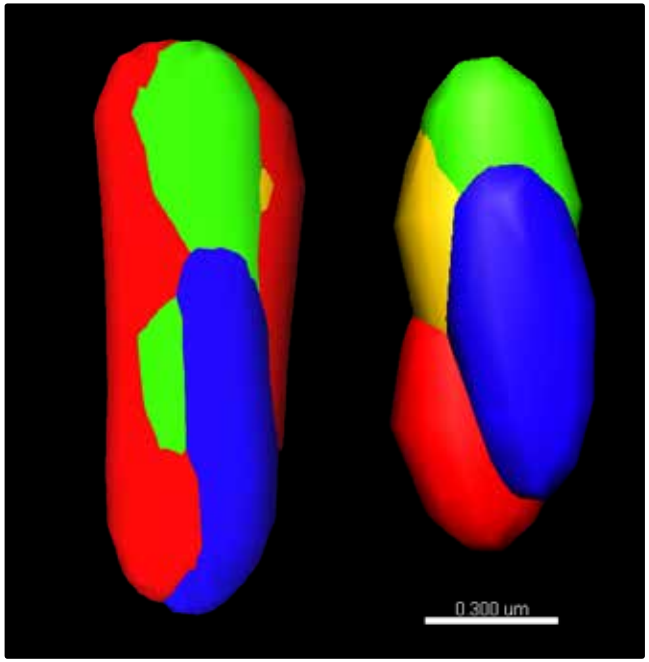




How to acquire the images for colocalisation

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

    à Aim for a 1.40 NA or above
  - Apochromat lens (l corrected)
  - Check the PSF (Point Spread Function with multicolored beads)



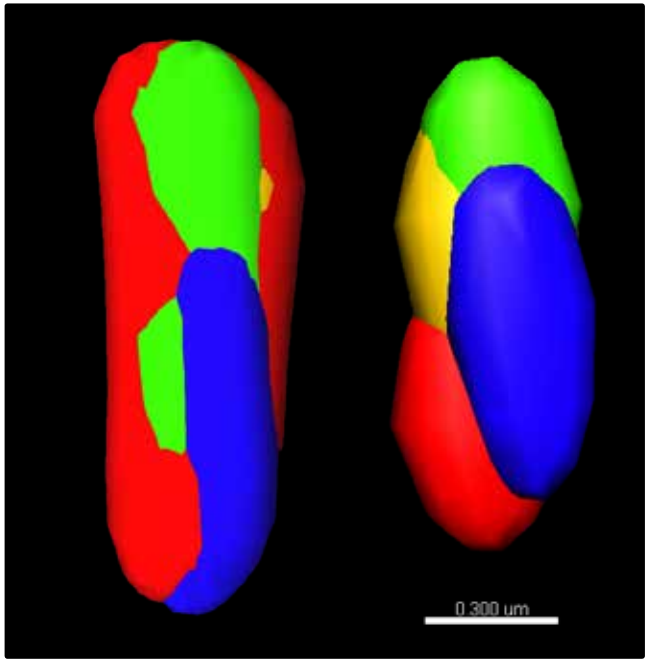
Even the best lenses are not perfect!

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

How to acquire the images for colocalisation

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

    à Aim for a 1.40 NA or above
  - Apochromat lens (l corrected)
  - Check the PSF (Point Spread Function with multicolored beads)



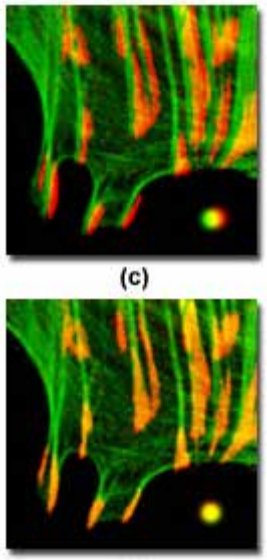
Even the best lenses are not perfect!

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

How to acquire the images for colocalisation

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

    à Aim for a 1.40 NA or above
  - Apochromat lens (l corrected)
  - Check the PSF (Point Spread Function with multicolored beads)



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

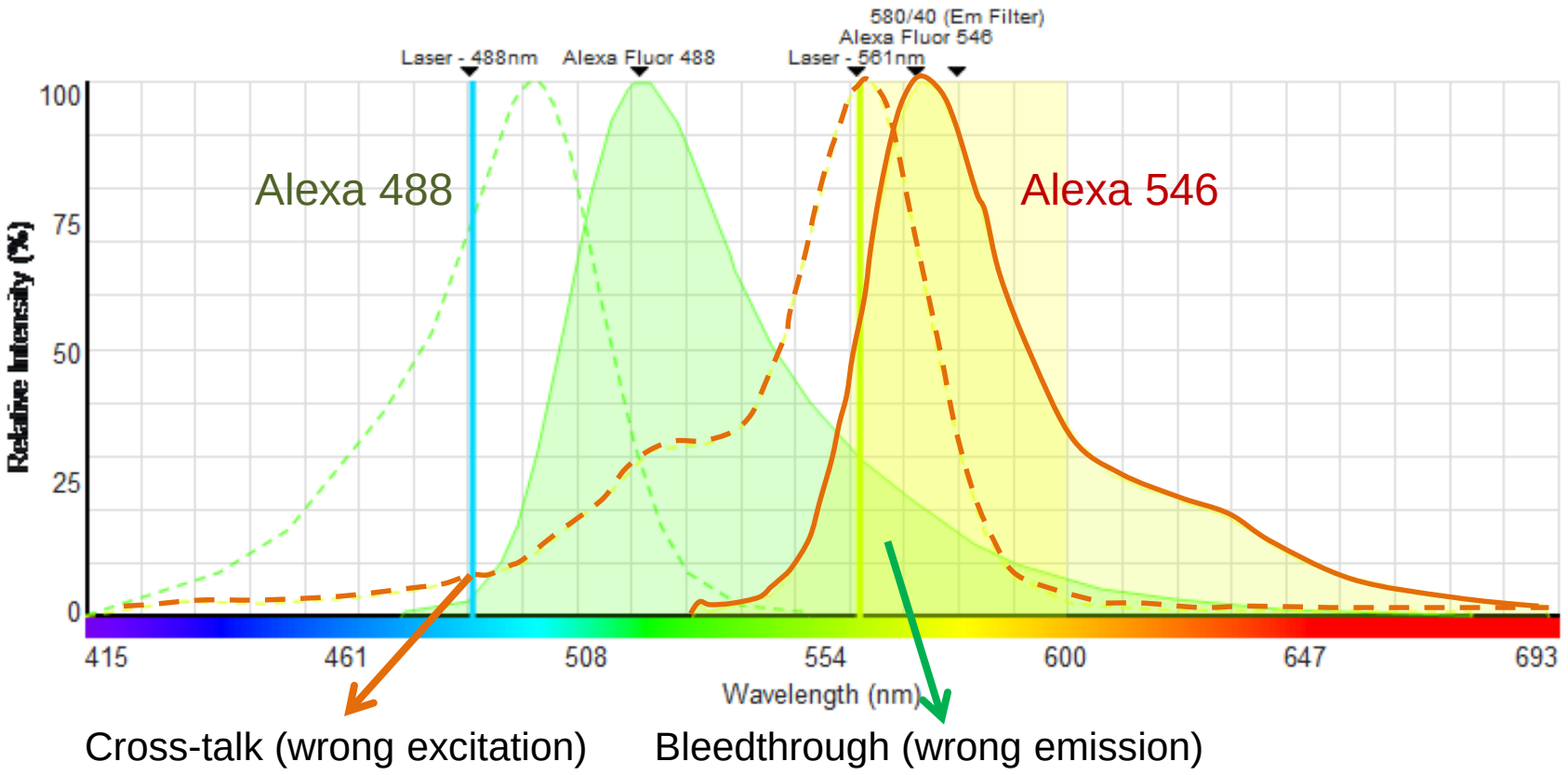
à needs a reference

à Mix beads with your samples

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

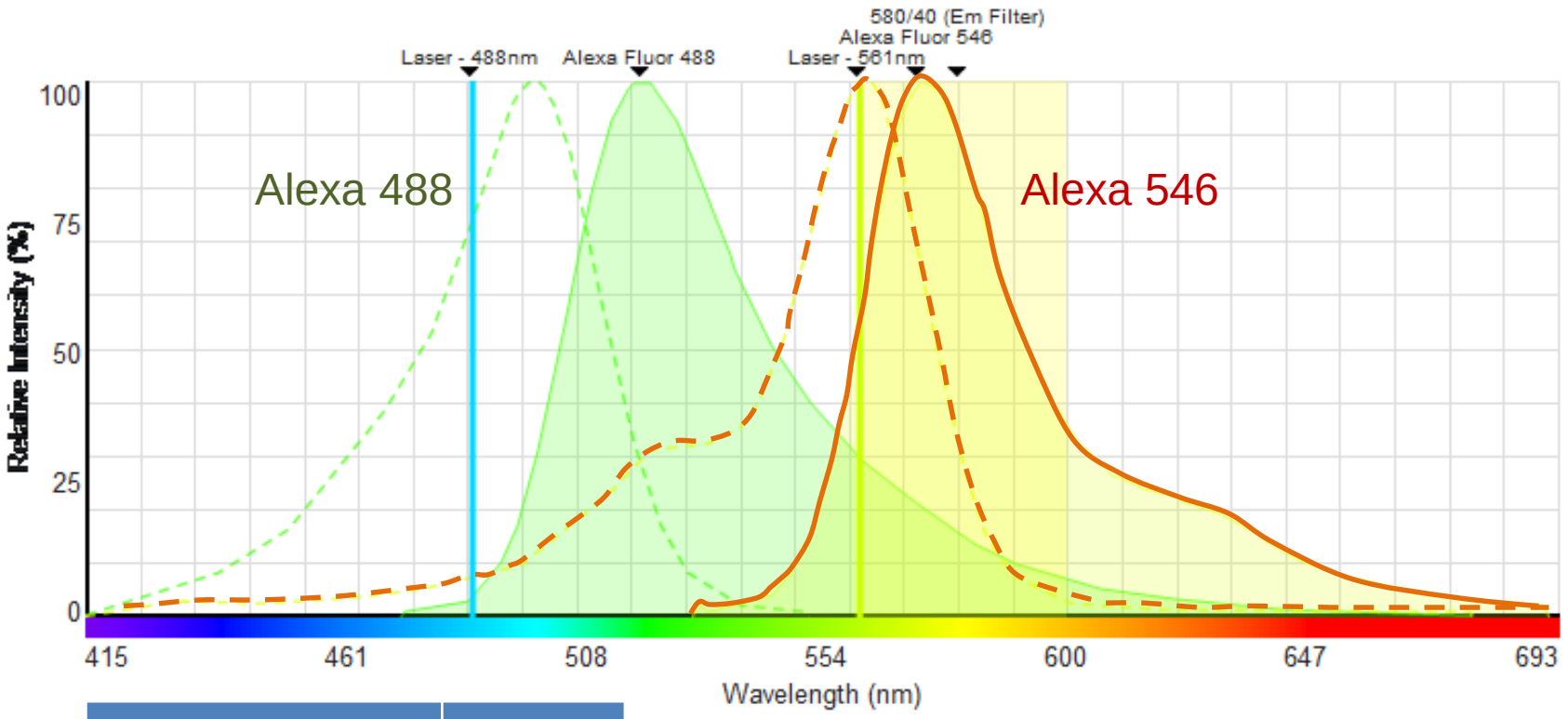
How to acquire the images for colocalisation

- Avoid Cross-talk and Bleedthrough



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

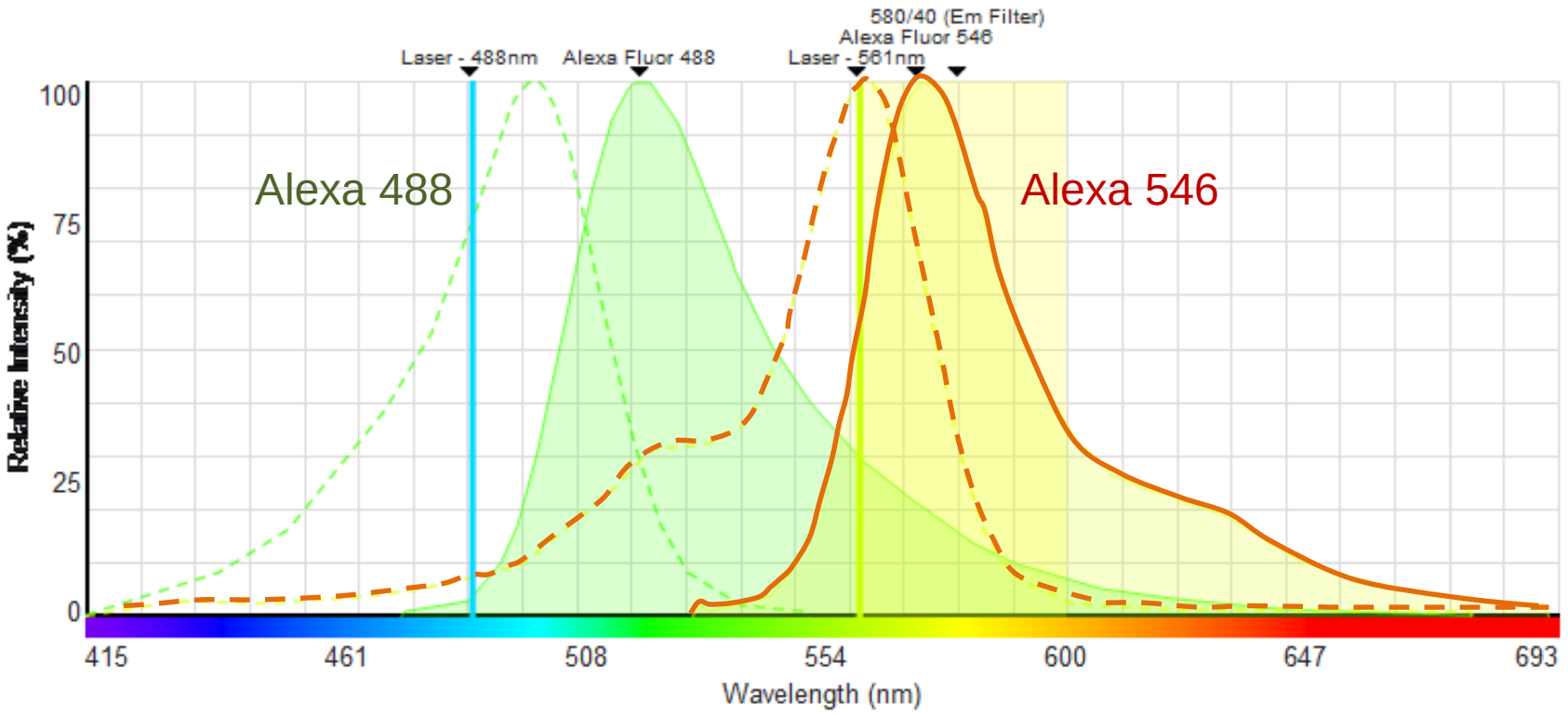
How to acquire the images for colocalisation



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

Bleedthrough

How to acquire the images for colocalisation



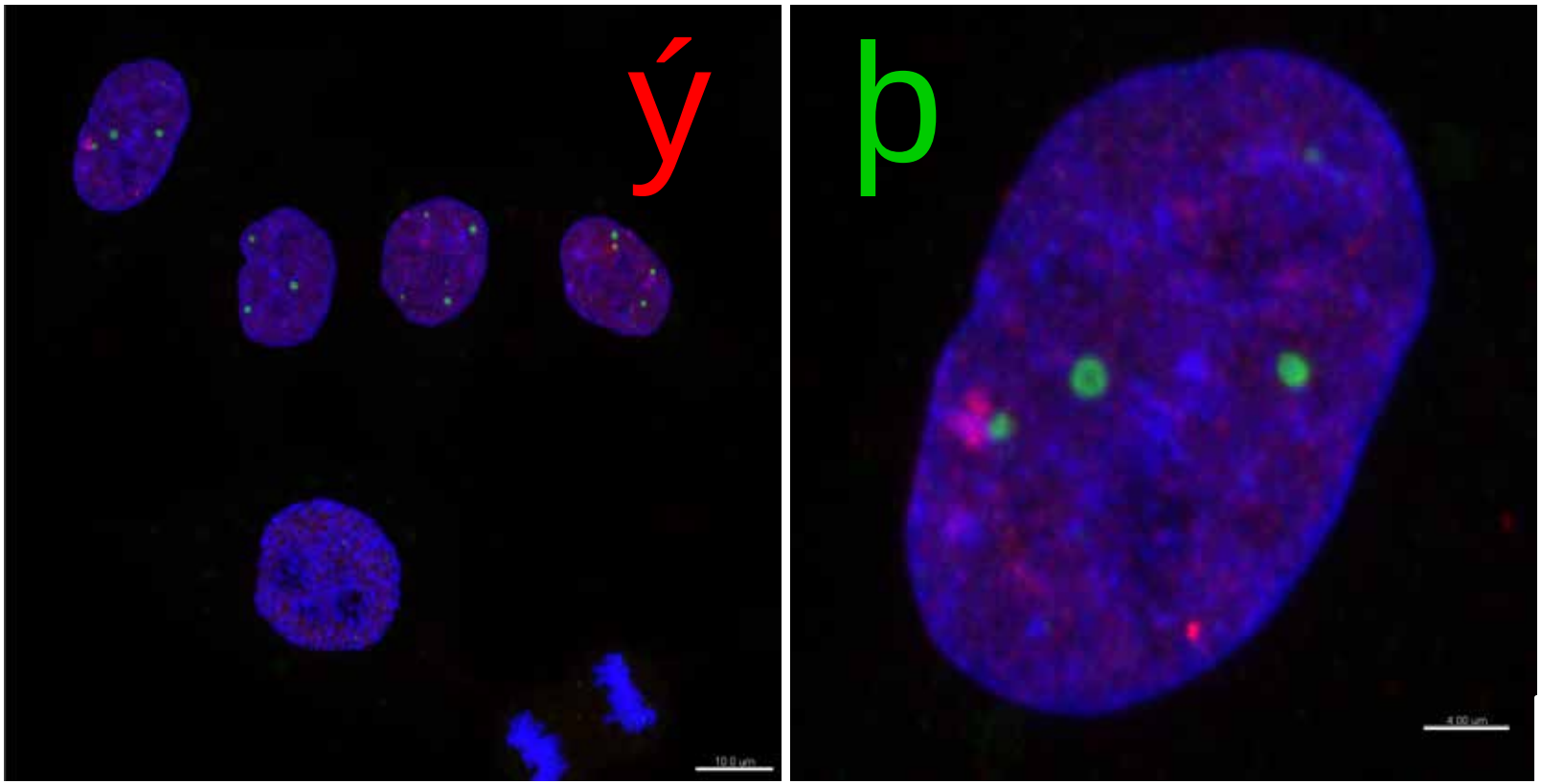
à Scan with the sequential mode

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 à lower signal/higher noise
- Avoid saturation (use the whole dynamic range)

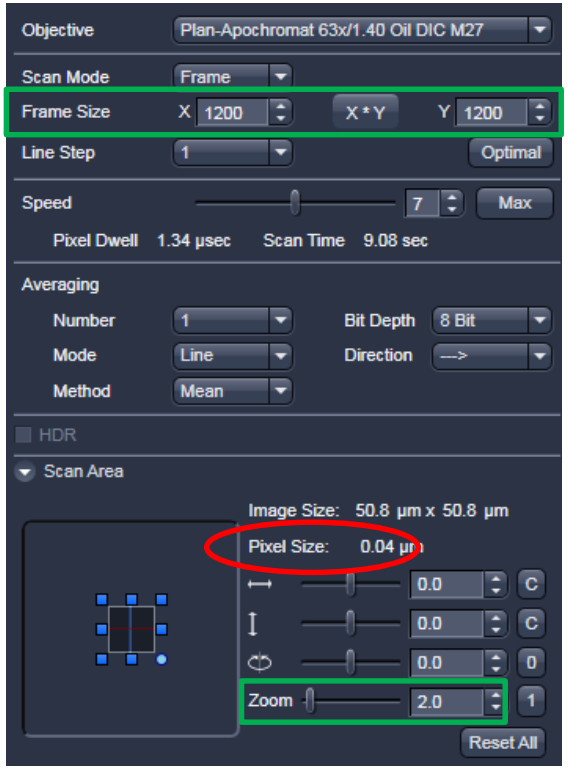
How to acquire the images for colocalisation

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

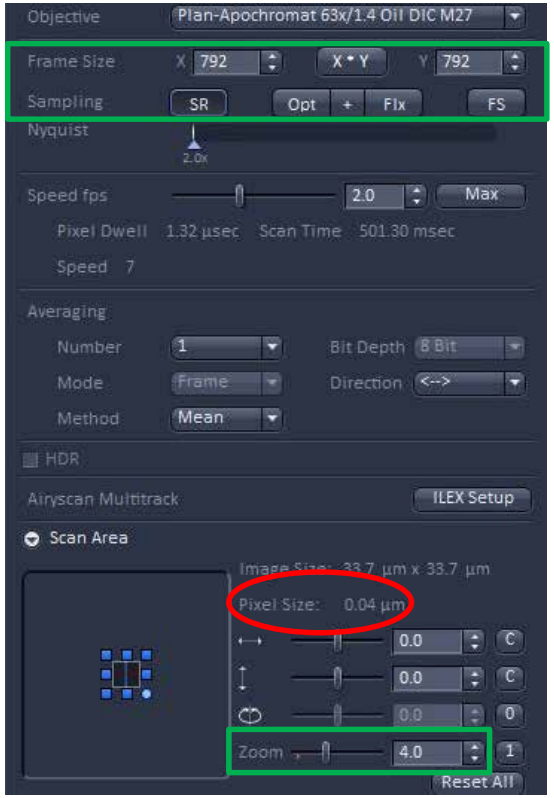


How to acquire the images for colocalisation (Zeiss Confocal)

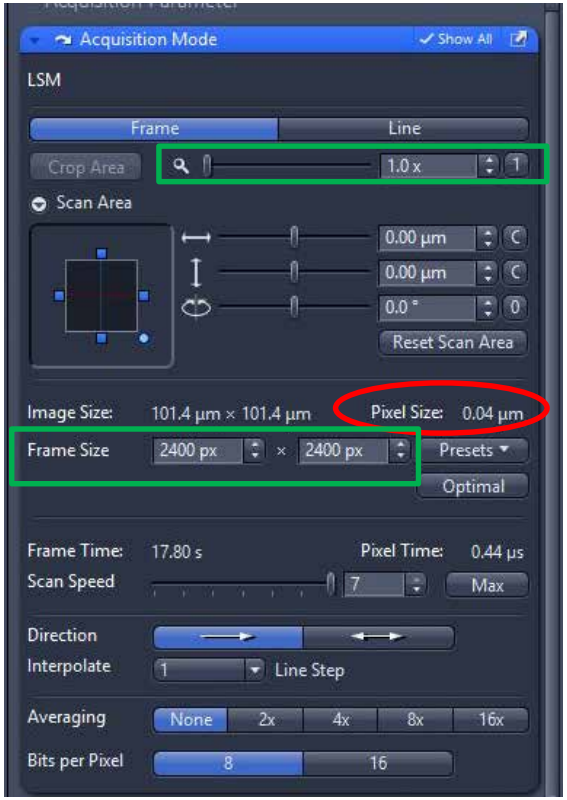
- Match the optical thickness – Oversampling XY



LSM700/LSM880



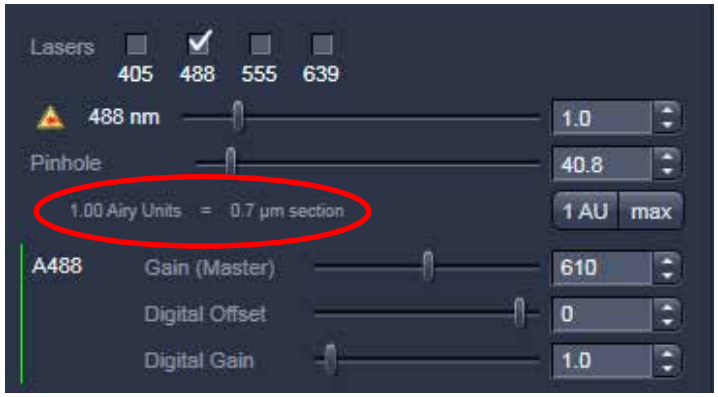
LSM880 (FAST mode)

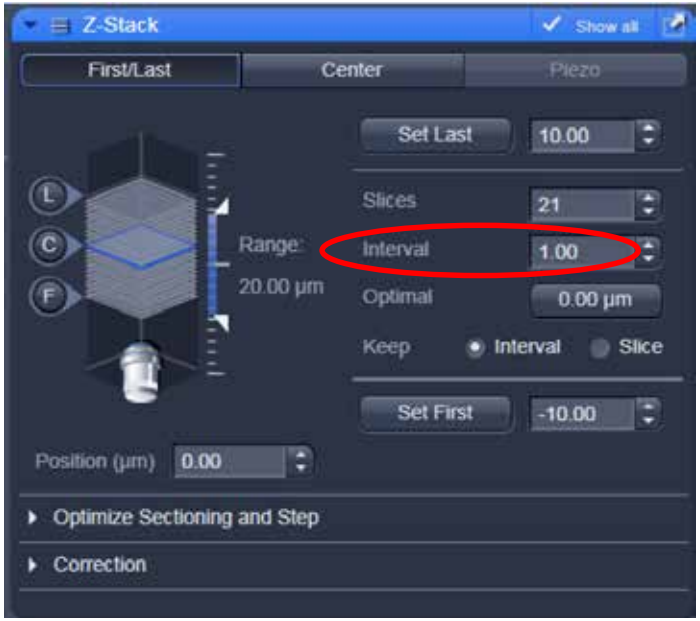


LSM800 II

How to acquire the images for colocalisation (Zeiss Confocal)

- Match the optical thickness – Oversampling Z





LSM700/800/880

How to acquire the images for colocalisation (Zeiss Confocal)

- Match the optical thickness - pinholes

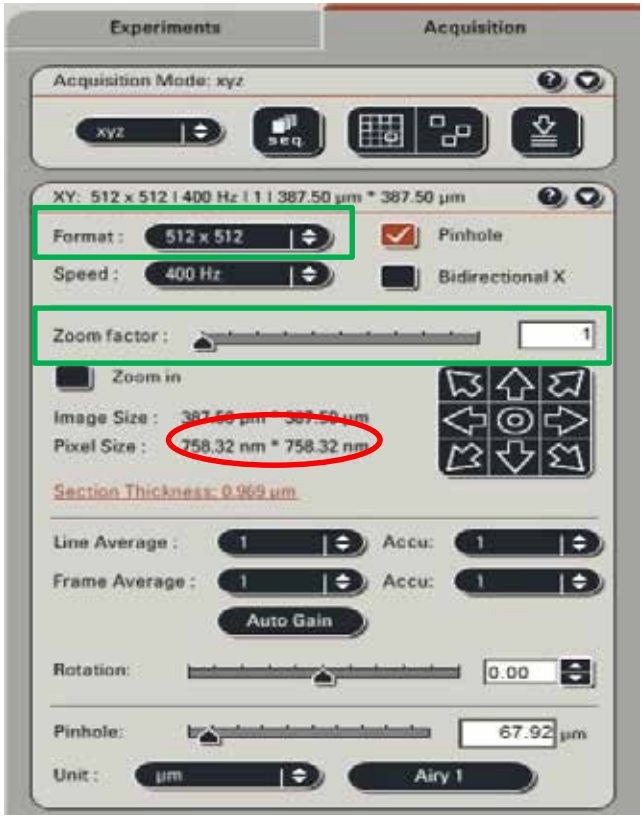
Fig. 77 Optimize Sectioning and Step: Optimal Interval is set starting with one Airy unit for all channels

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

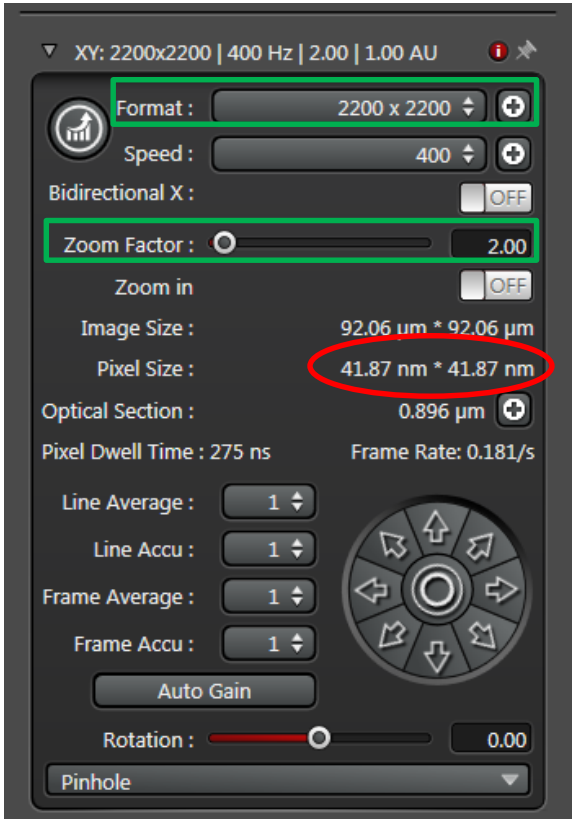
- Example with the LSM700 Zeiss

How to acquire the images for colocalisation (Leica Confocal)

- Match the optical thickness – Oversampling XY



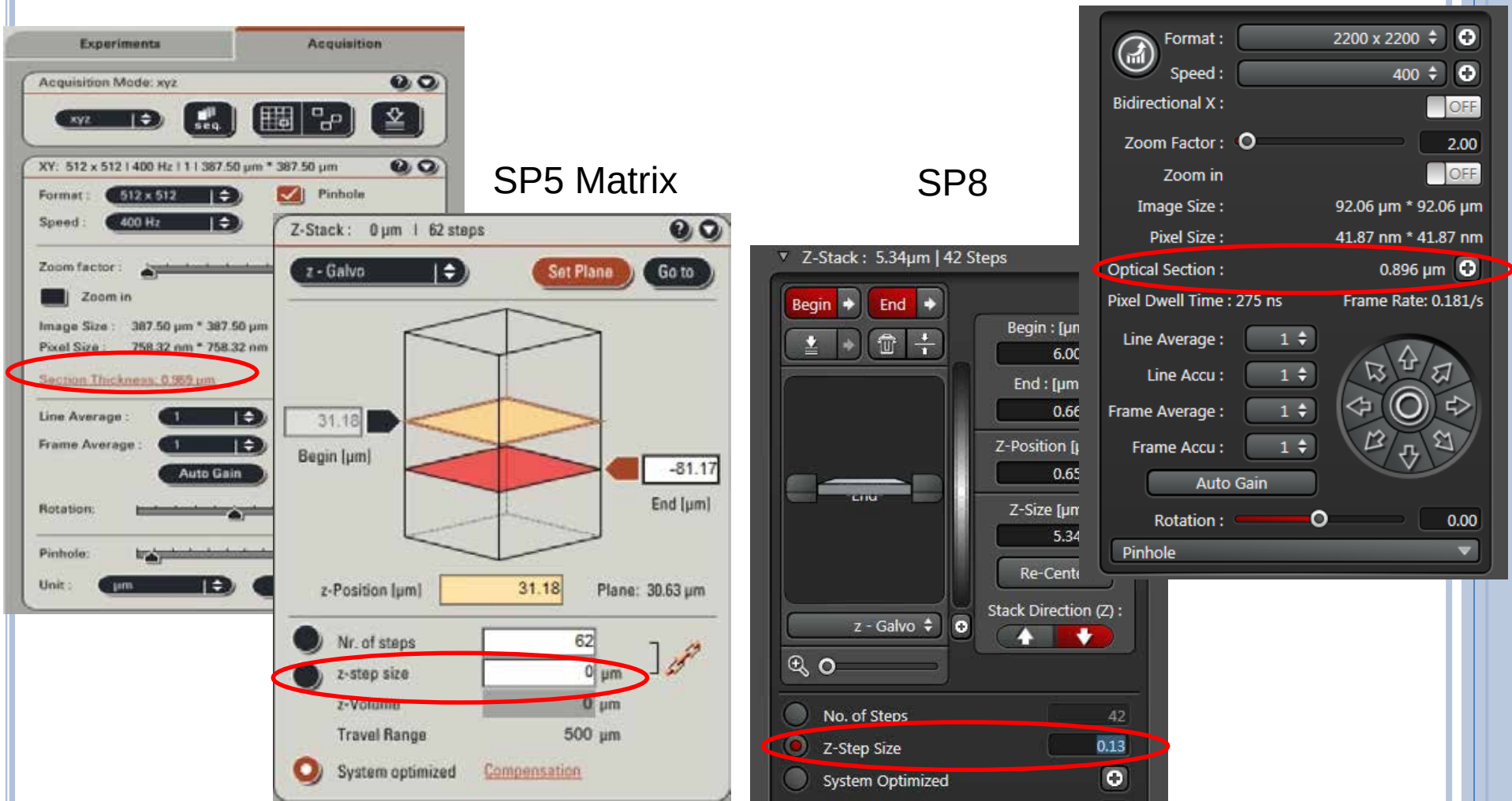
SP5 Matrix



SP8

How to acquire the images for colocalisation (Leica Confocal)

- Match the optical thickness – Oversampling Z



Settings for Deconvolution Confocal

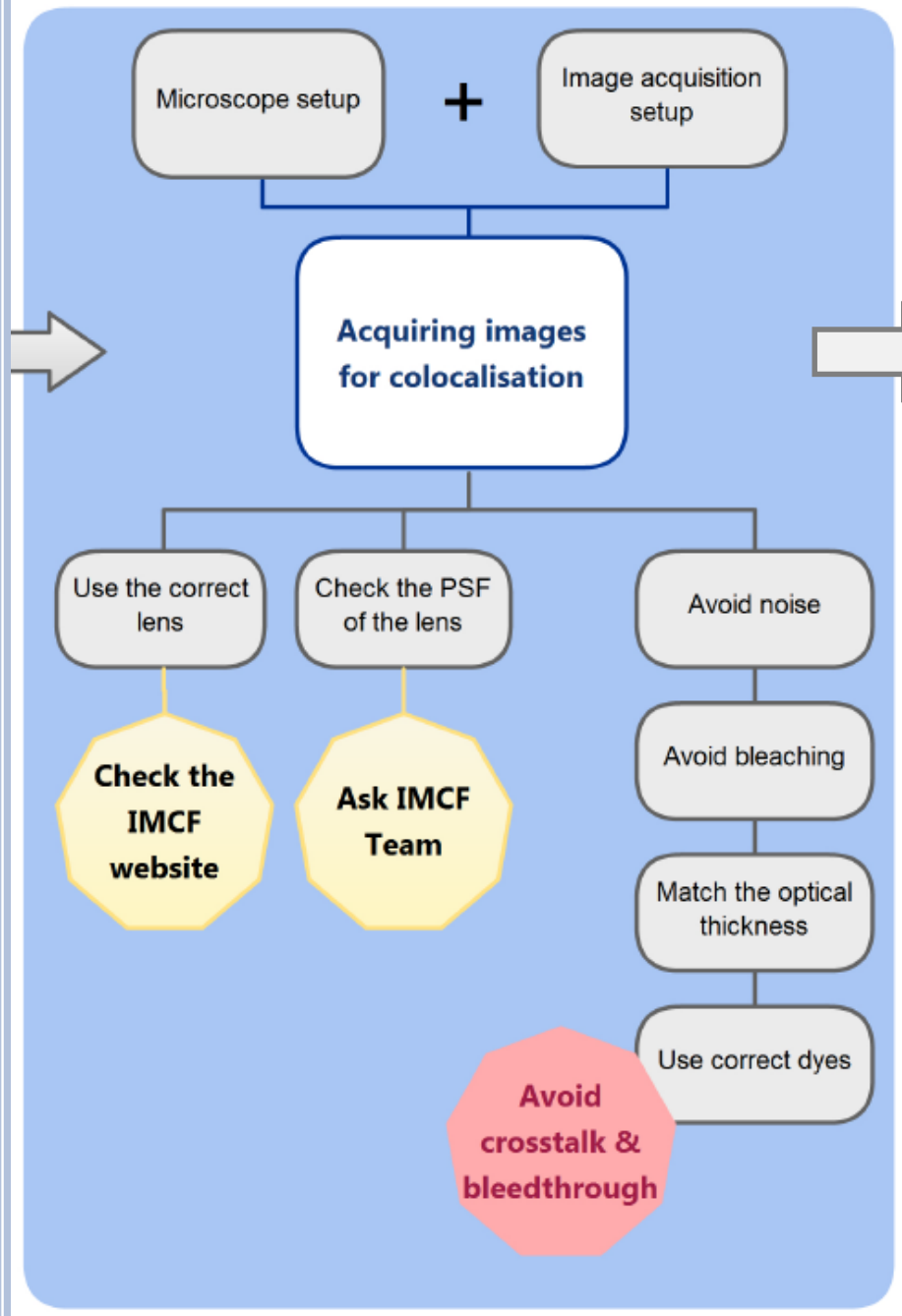
Microscope and Objective	Required XY	Required Z	Example: Settings to use
SP5 II Matrix; 63x 1.4 Oil	43 nm	131 nm	**ZOOM 2** XY: 2800*2800 Z: 0.13 nm **ZOOM 4** XY: 1500*1500 Z: 0.13 nm
SP8; 63x 1.4 Oil	43 nm	131 nm	**ZOOM 2** XY: 2200*2200 Z: 0.13 nm **ZOOM 4** XY: 1080*1080 Z: 0.13 nm
LSM700 Up; 63x 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

Setting for Deconvolution Widefield

Microscope and Objective	Required XY	Required Z	Example: Settings to use
Deltavision; 100x 1.42 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 that you use sequential acquisition if you suspect cross-talk and/or bleedthrough

- Make sure you match pinholes and the oversampling

- Make sure you deconvolve your images (widefield AND confocal)

How to analyse your data

- **Things to keep in mind**

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

How to analyse your data

- **Things to keep in mind**

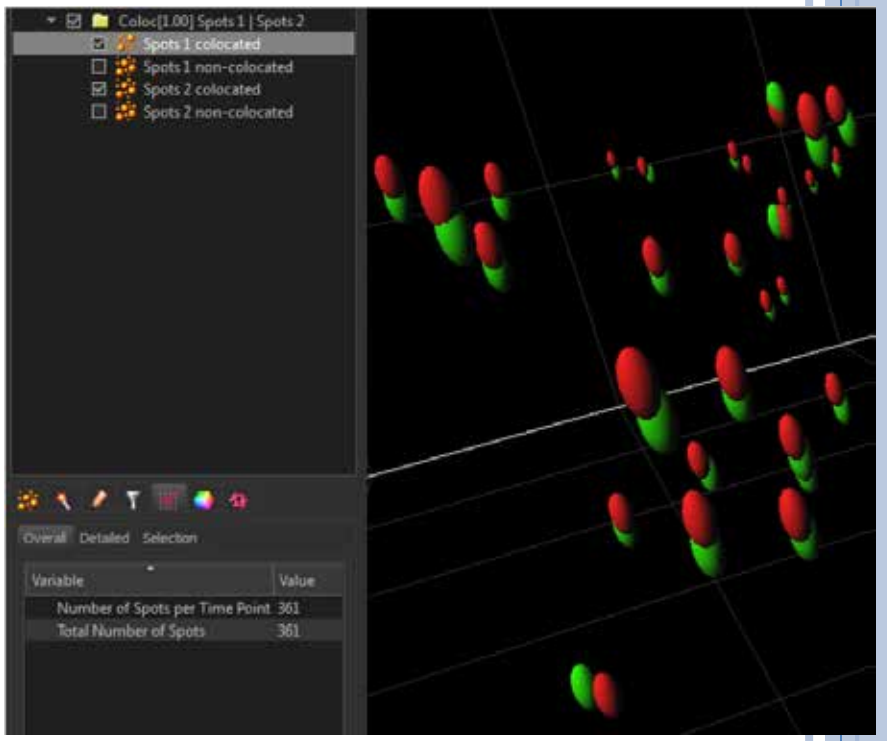
- Colocalization is 3D
- Colocalization should be more thought in terms of correlation
- Colocalization needs Quantification & Statistics

(1) Intensity-based	**(2) Object-based**	**(3) Pixel-based**
Correlation of the strength of linear relation between two channels (no spatial exploration of the colocalisation signal)	Structure identification and determination of overlap of objects (for discrete structures)	Measures correlation between 2 channels. Compared to (1), offers more options to identify objects

How to analyse your data

- **Spot proximity visualisation**

- Matlab extension for Imaris
- See dedicated wiki page : https://wiki.biozentrum.unibas.ch/pages/viewpage.action?spaceKey=IMCF&title=Imaris+-+colocalize+spots
- Caution! No statistics, this is NOT real colocalisation!!!



How to analyse your data

- **Spot proximity visualisation**

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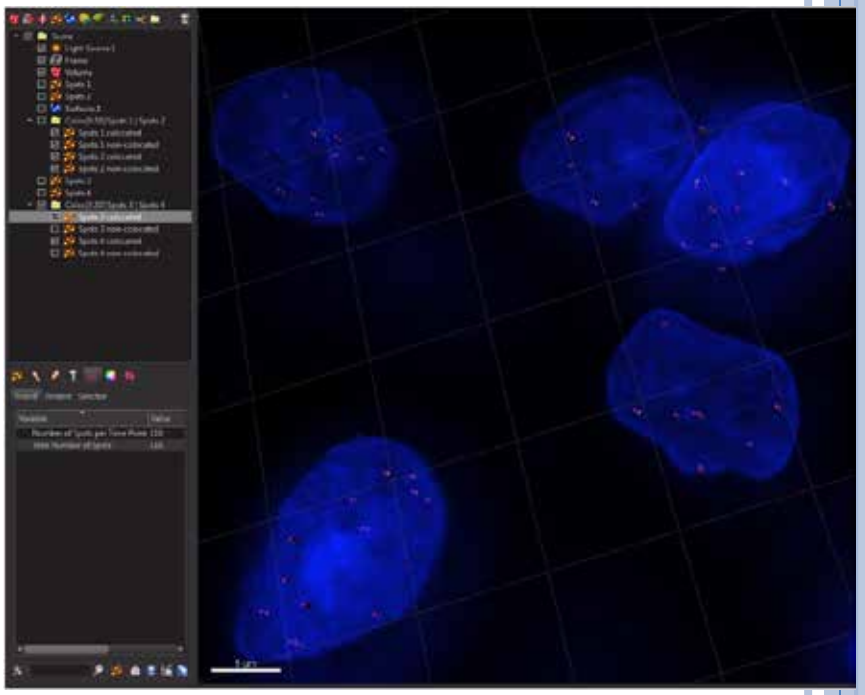
- Caution! No statistics, this is NOT real colocalisation!!!
- Can be limited to spots within a certain region, (DAPI in the example of the PDF)

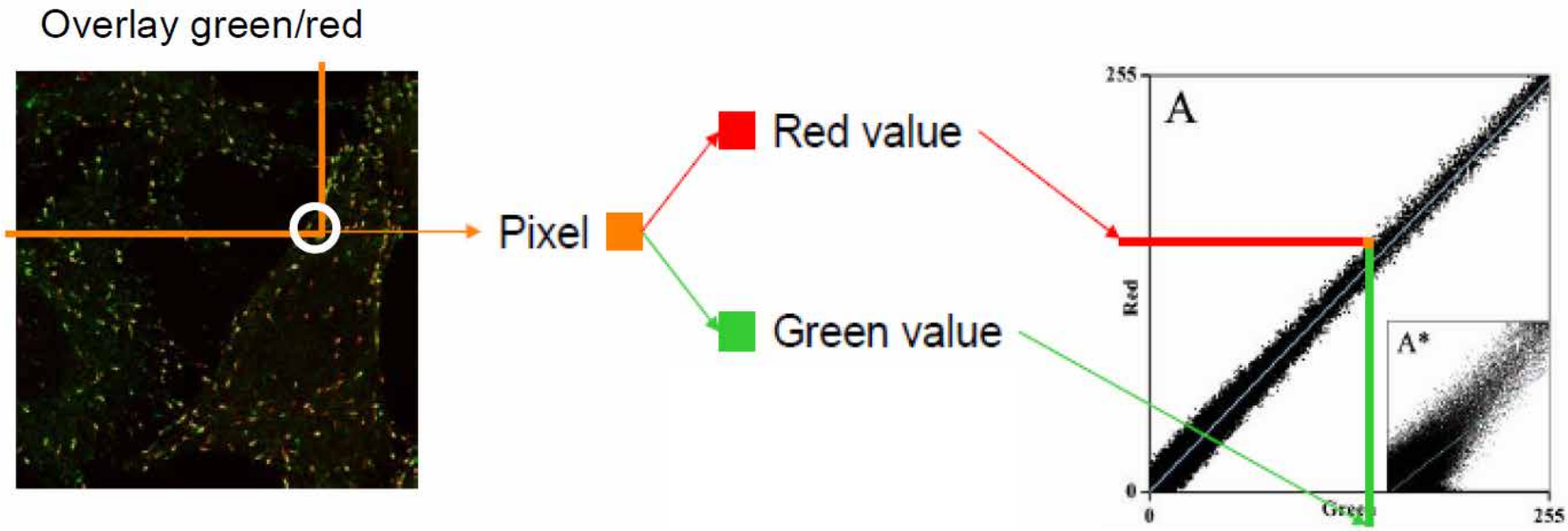


How to analyse your data

- **Criteria to use**

- « Colocalization Analysis » should include
  - Scatter plots
  - Pearson's coefficient
  - Manders coefficients
  - Costes' approach
  - (Van Steensel's approach)
  - (Li's approach)
  - Object-based analysis when discrete structures

How to understand your data



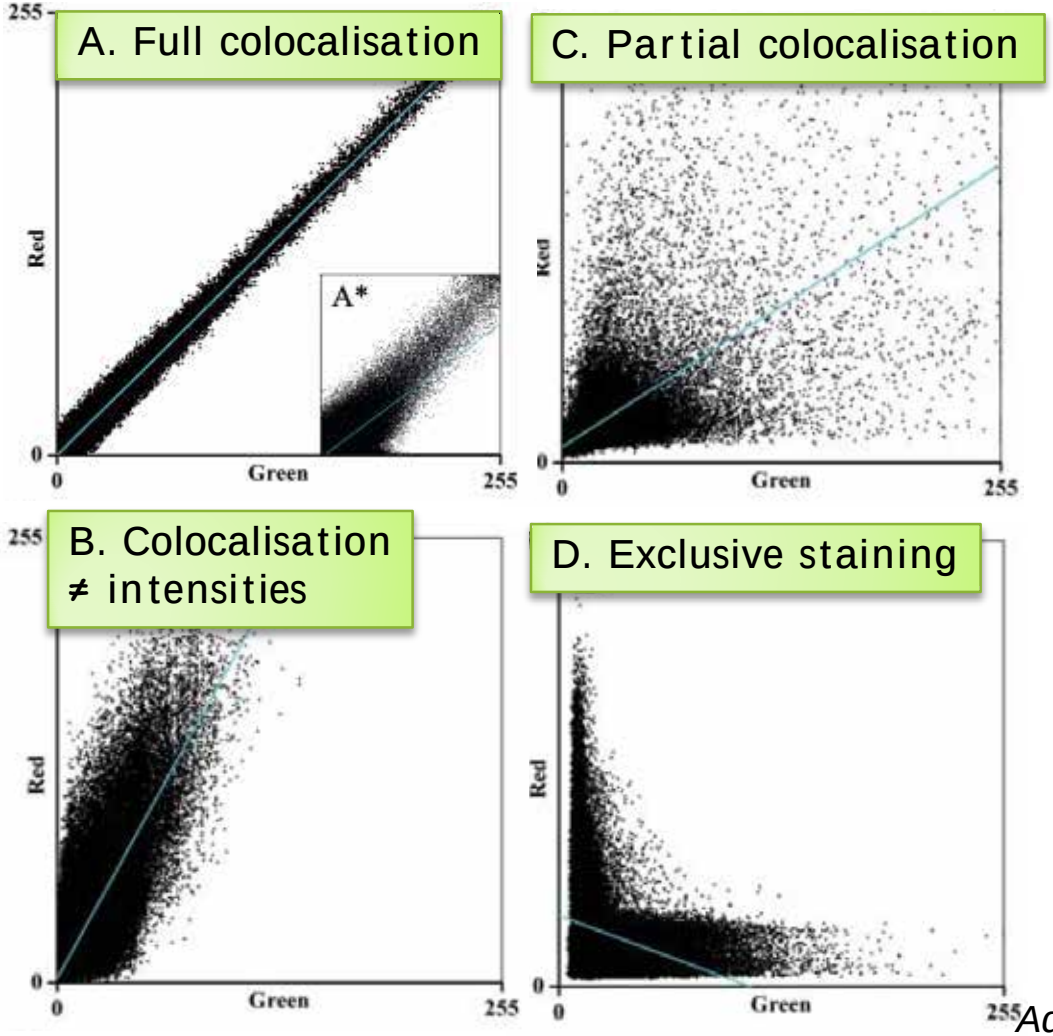
☺ Good first visual estimate of colocalisation

☺Information about the image quality

L Only qualitative correlation

How to understand your data

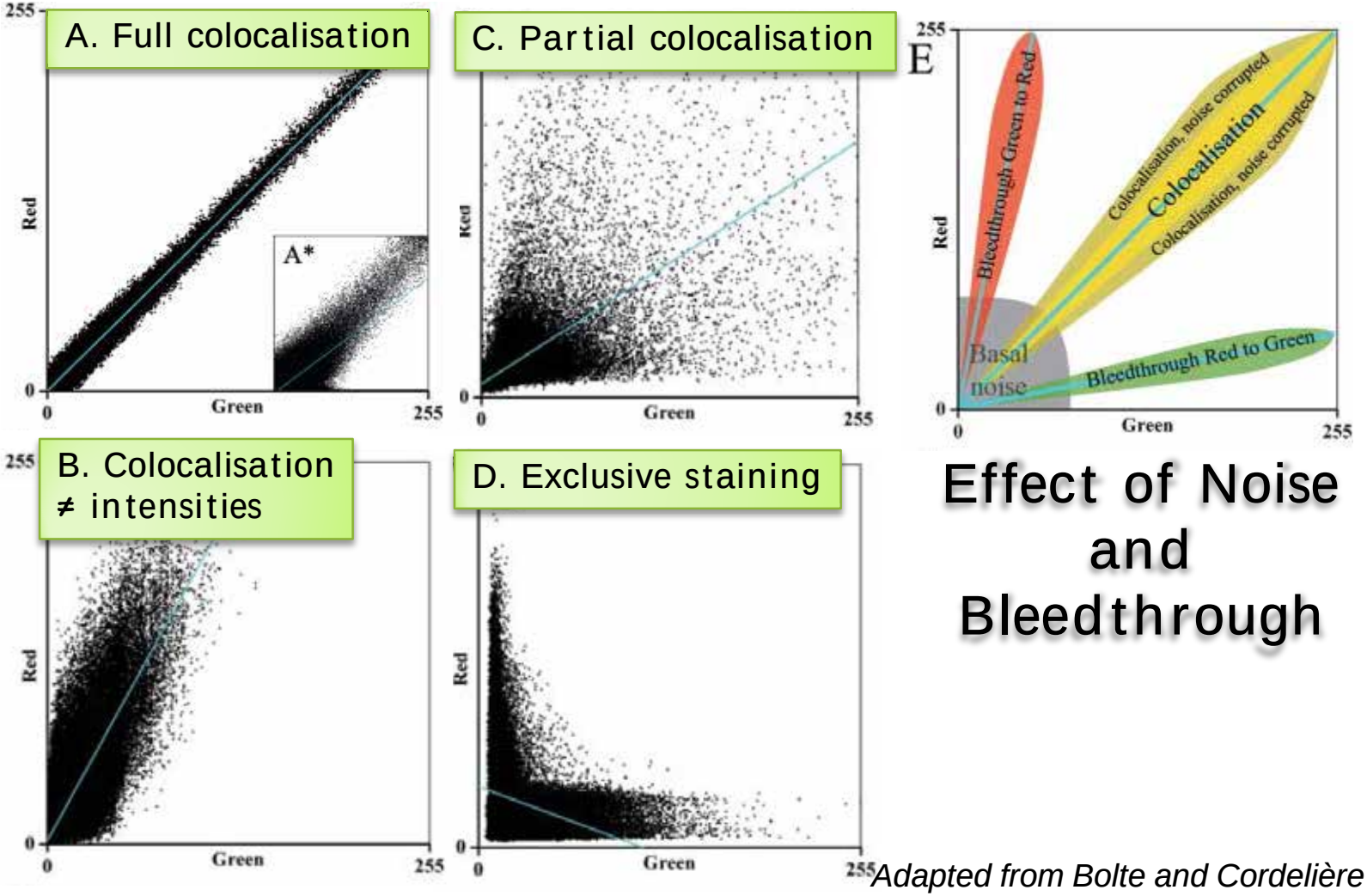
- Scatter plot (Cytofluorogram)



Adapted from Bolte and Cordelière 2006

How to understand your data

- Scatter plot (Cytofluorogram)

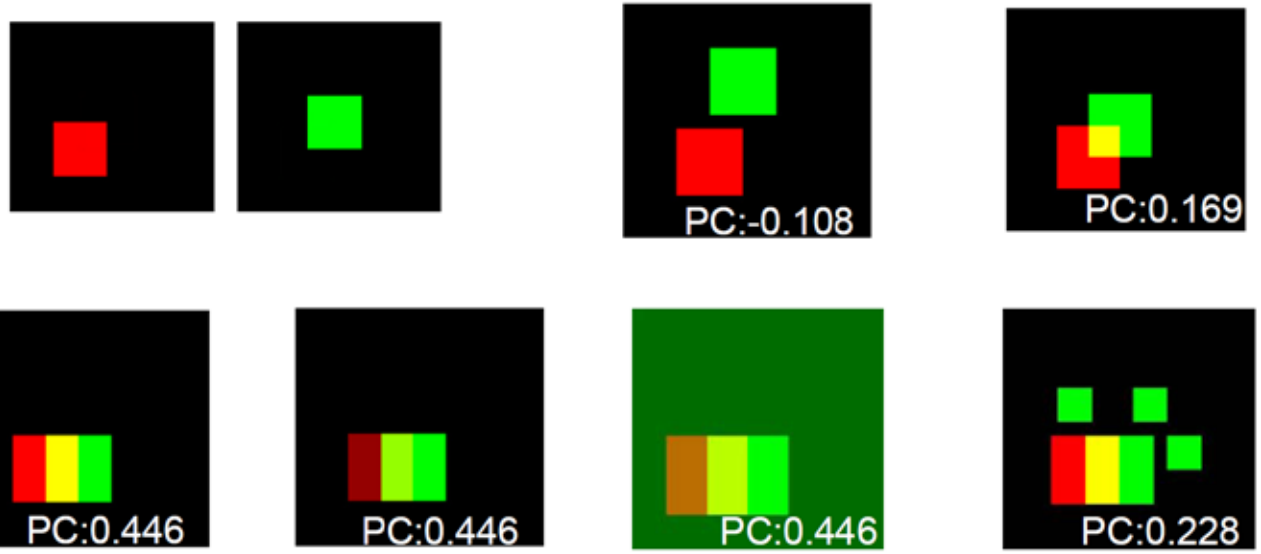


Effect of Noise and Bleedthrough

Adapted from Bolte and Cordelière 2006

How to understand your data

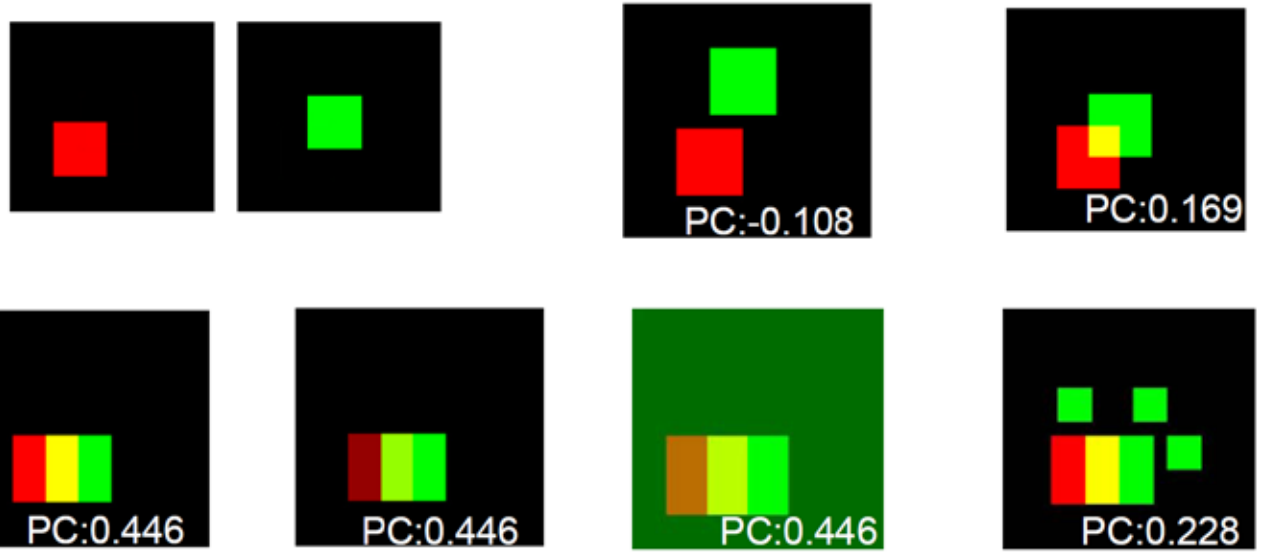
- Pearson coefficient (PCC, noted « r »)
- Estimate of the association strengh between 2 proteins



http://www.svi.nl/ColocalizationCoefficients

How to understand your data

- Pearson coefficient (PCC, noted « r »)



☺ Not sensitive on background intensity

☺ Not sensitive on ≠ intensity of the overlapping pixels

L Not easy to interpret

L Affected by noise

L No perspective of both channels

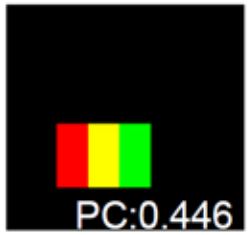
http://www.svi.nl/ColocalizationCoefficients

How to understand your data

- Manders coefficients (M1 and M2)
- Gives the proportion of each protein colocalising with the other

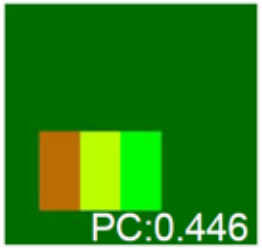
M1:0.000 M2:0.000

M1:0.250 M2:0.250



M1:0.500 M2:0.500

M1:0.500 M2:0.500

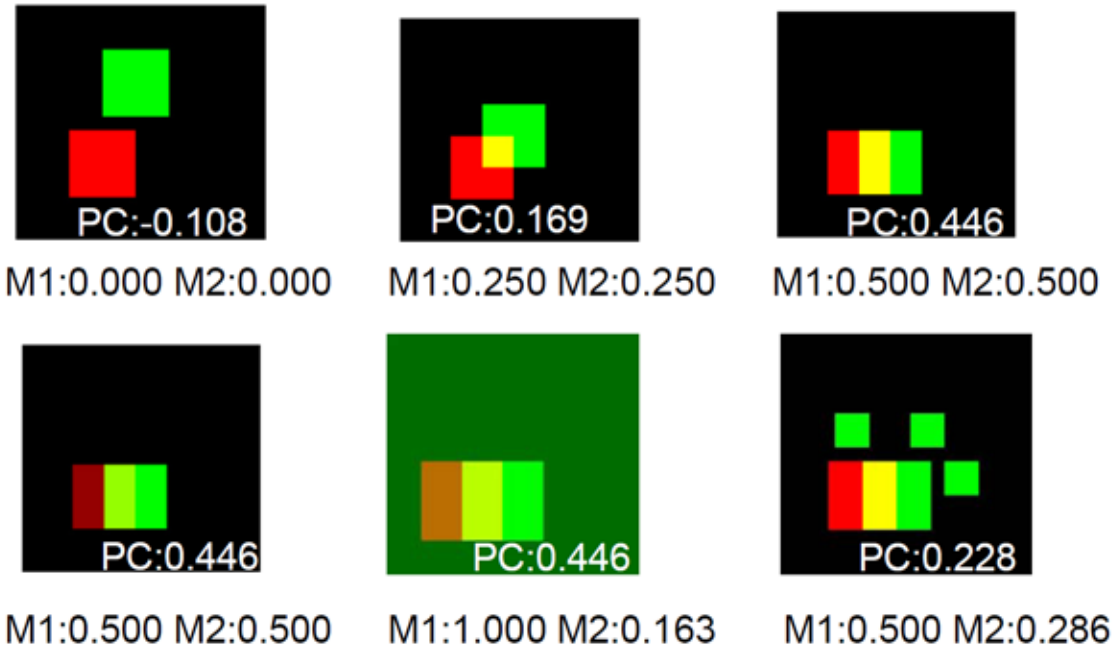


M1:1.000 M2:0.163

M1:0.500 M2:0.286

How to understand your data

- Manders coefficients (M1 and M2)



☺ Easier to interpret that PCC

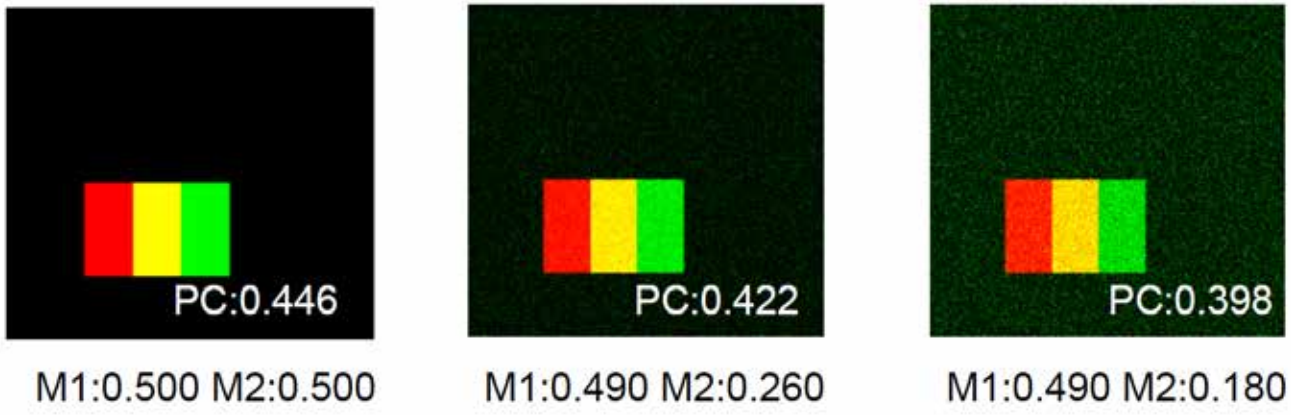
☺ Not sensitive to the intensity of the overlapping pixels

L Sensitive to background intensity – Threshold needed!

L Affected by noise

How to understand your data

- Influence of noise

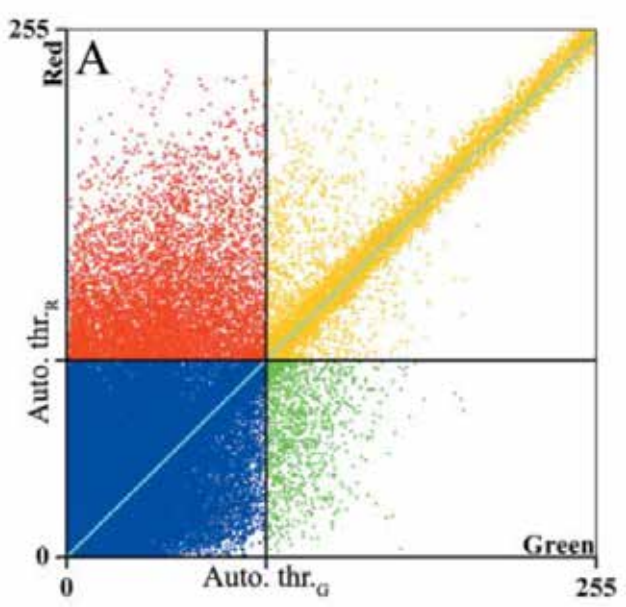


These coefficients are influenced by noise
Minimize noise during the acquisition, and deconvolve your datasets prior analysis

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).

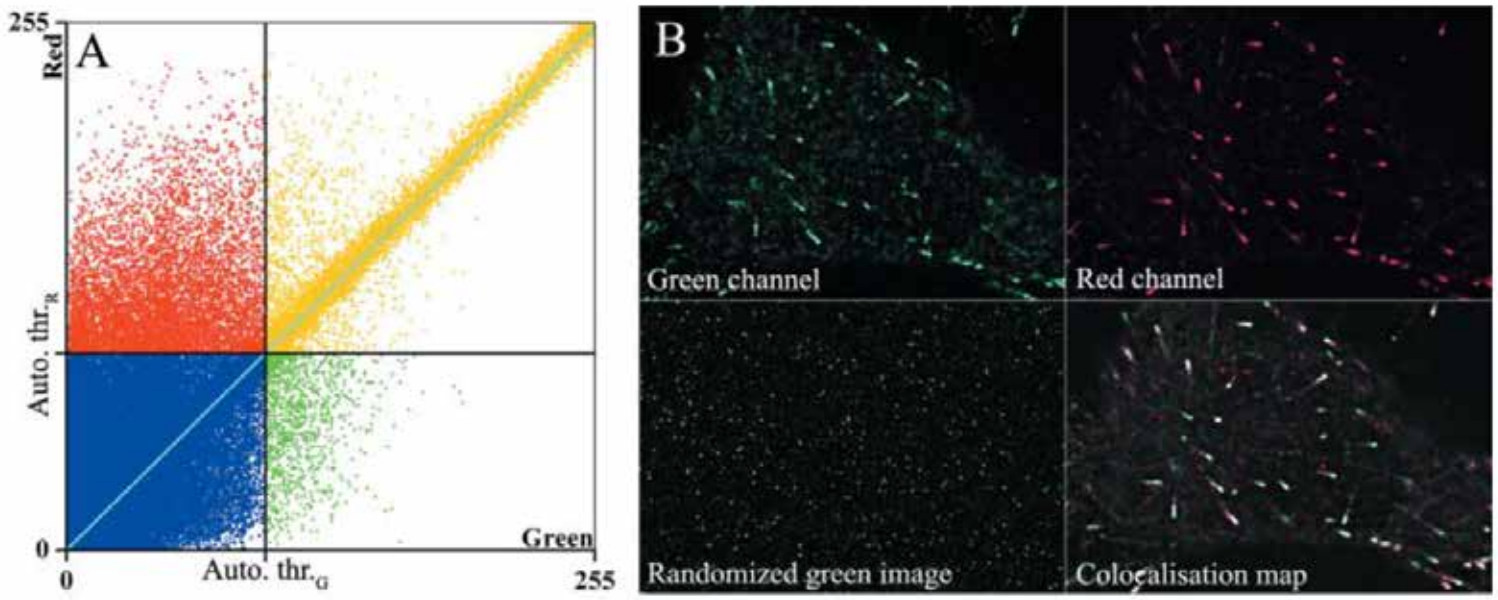
How to understand your data

- Costes' approach
  - Estimation of an automatic threshold



How to understand your data

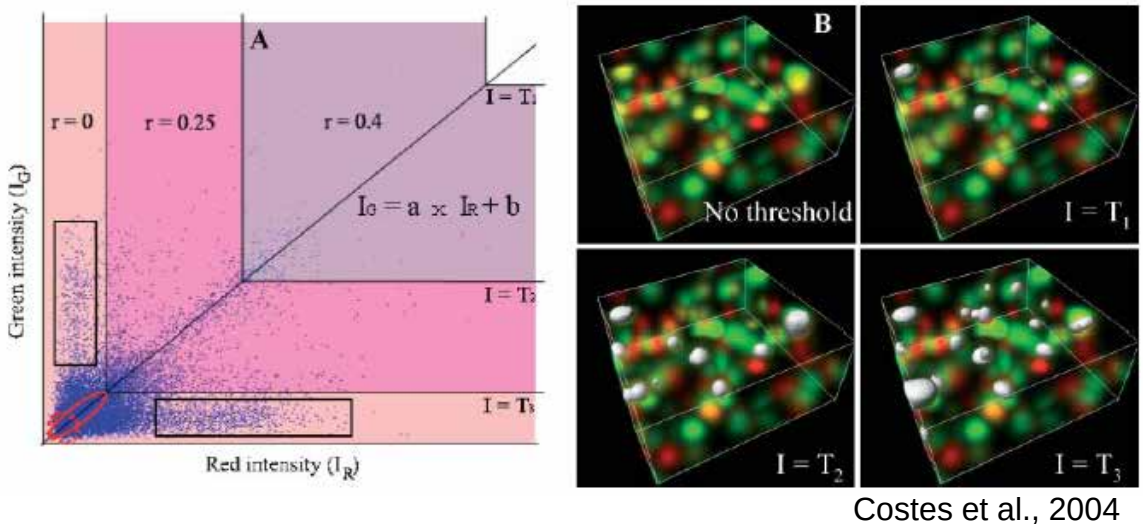
- Costes' approach
- Estimation of an automatic threshold
- Test of the statistical significance (Costes' P-value)



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

How to understand your data

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



Costes et al., 2004

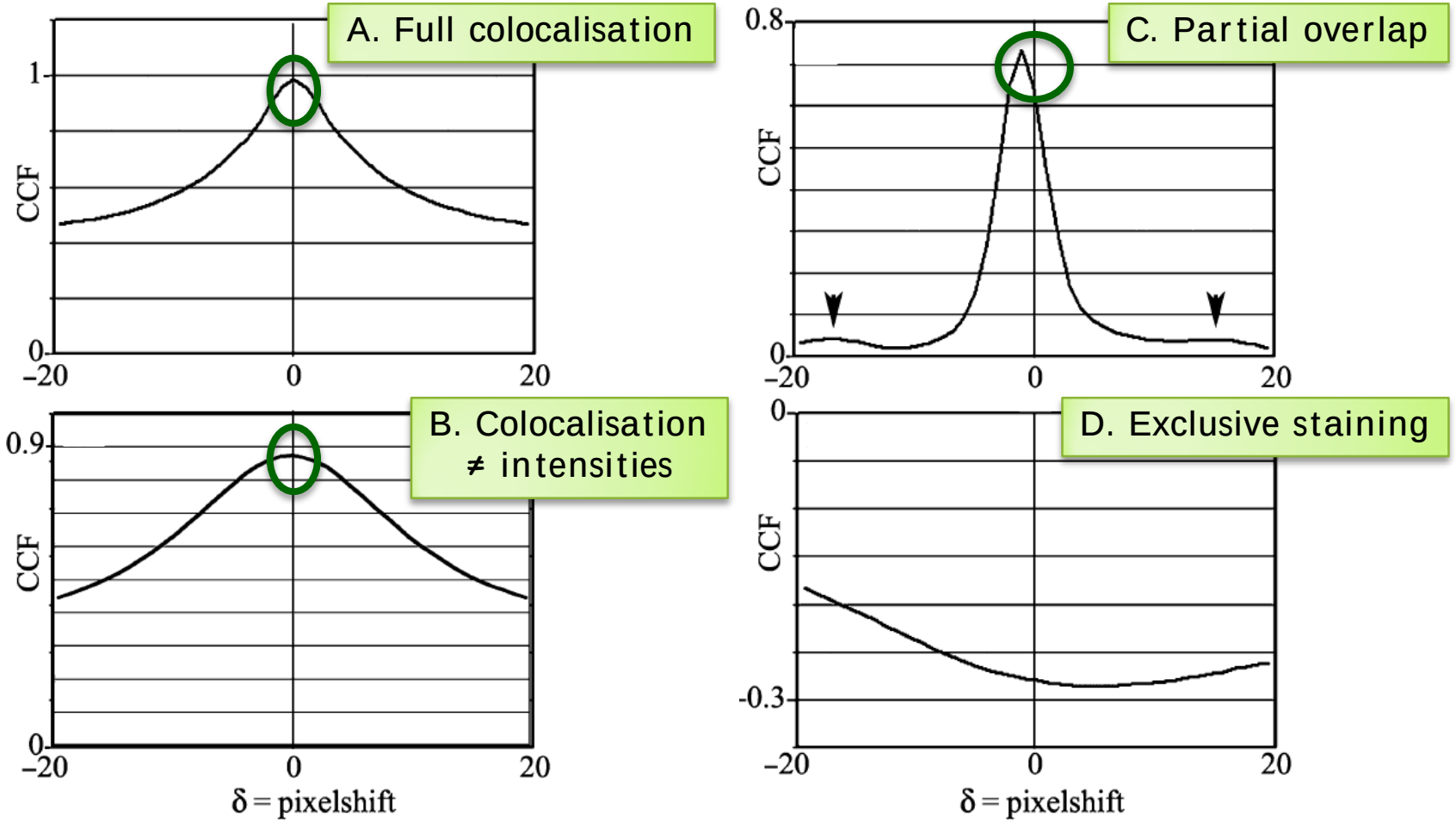
☺ Statistical approach

☺ Minimises the influence of noise

L Long calculations (3D)

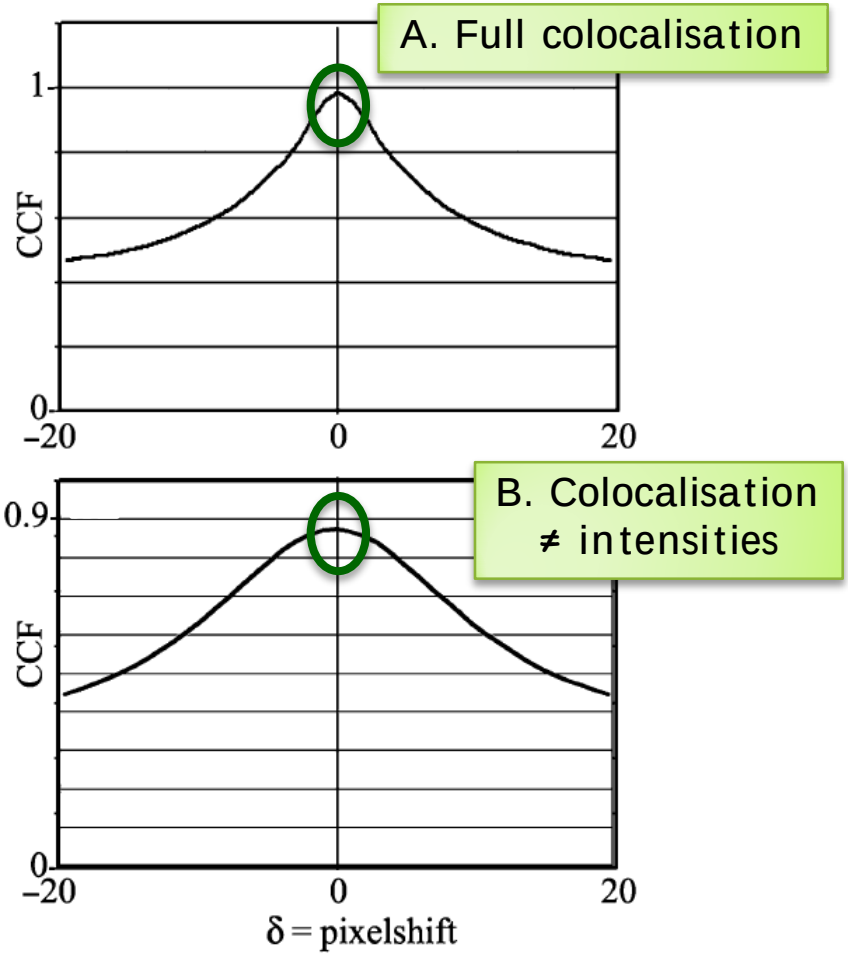
How to understand your data

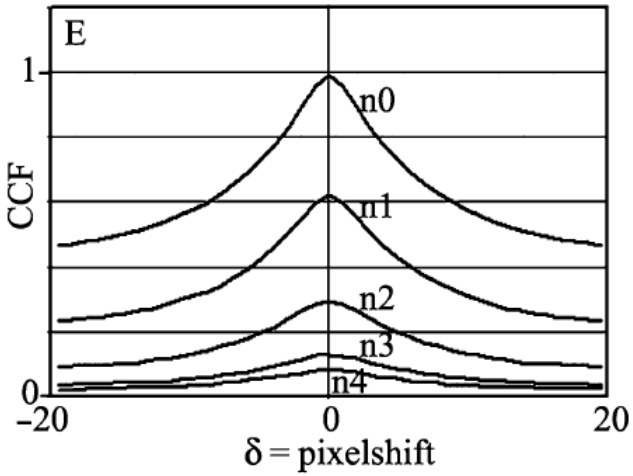
- Van Steensel's approach (Cross-Correlation Function CCF)



How to understand your data

- Van Steensel's approach (Cross-Correlation Function CCF)



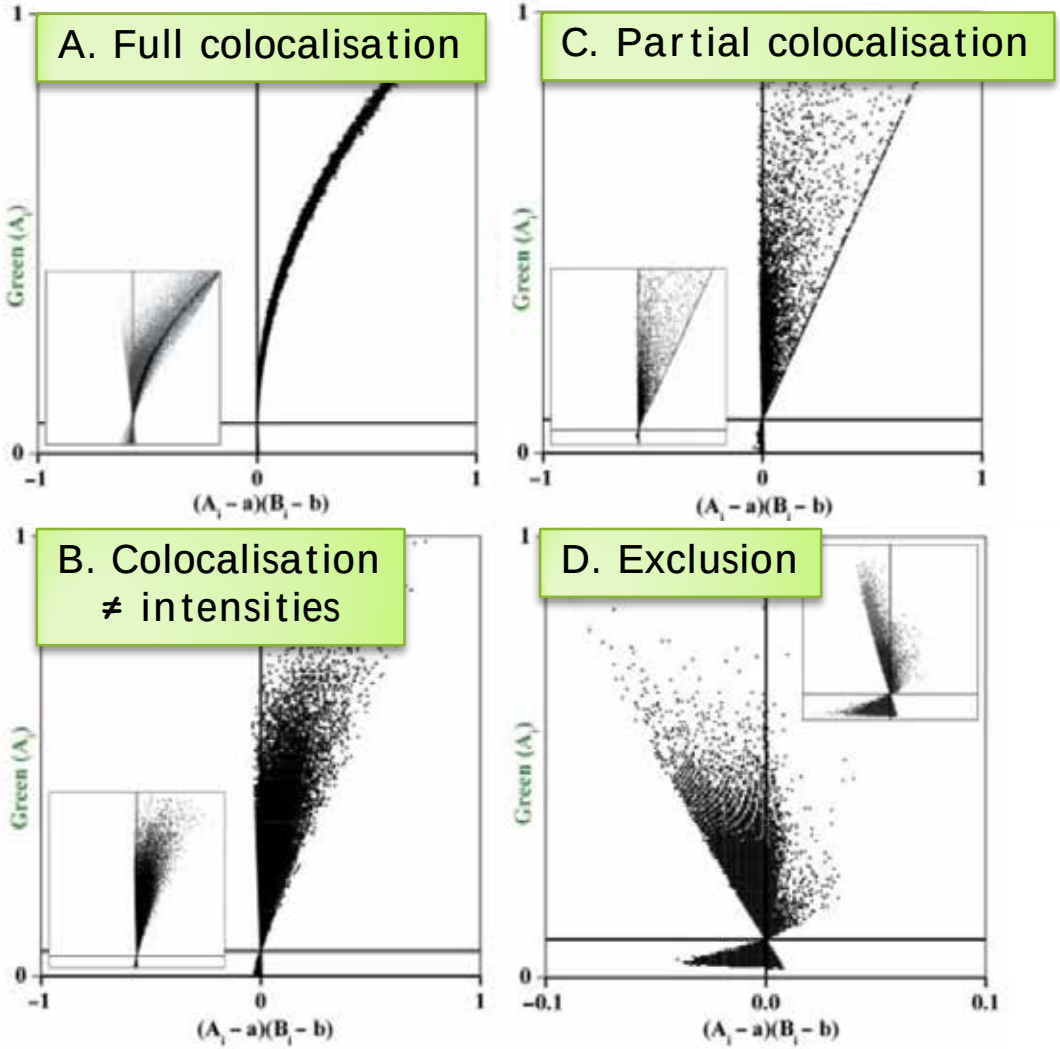


Effect of Noise

Adapted from Bolte and Cordelière 2006

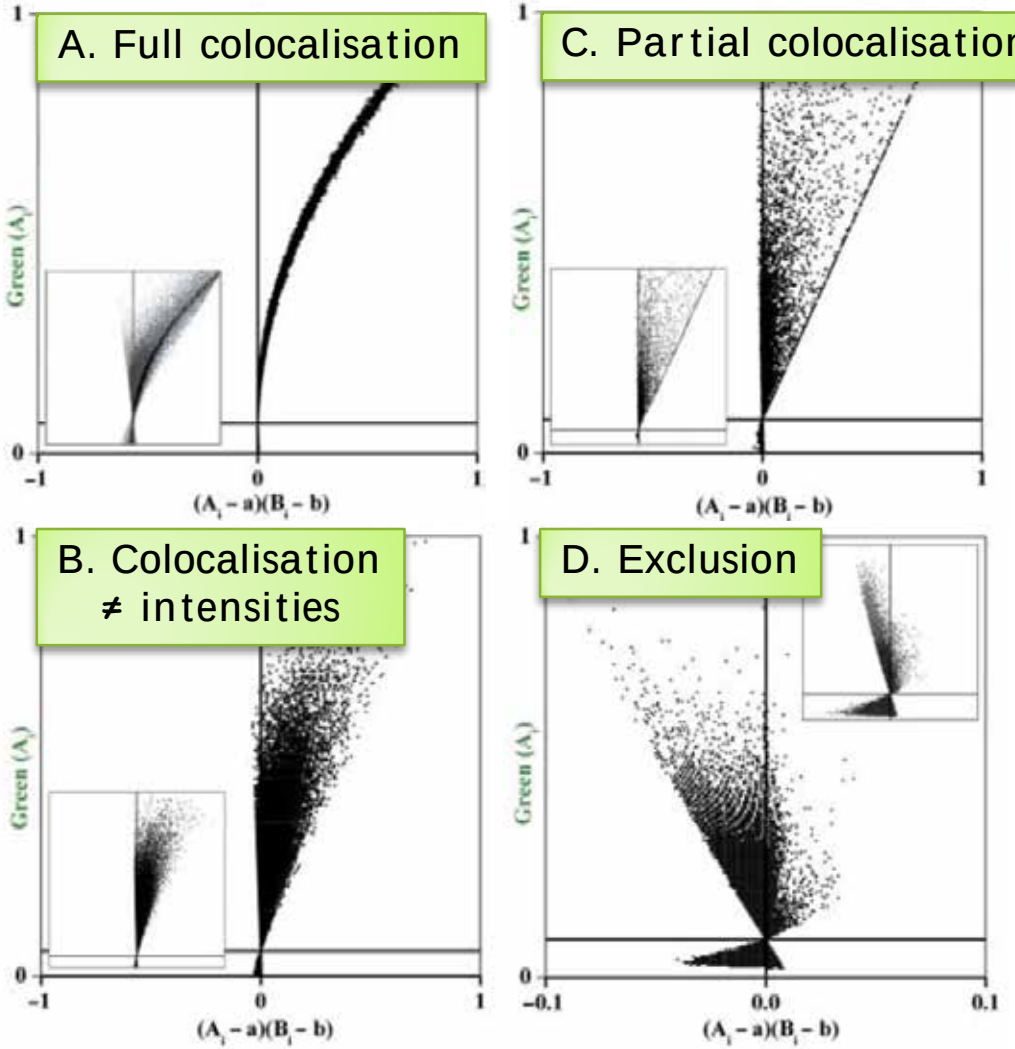
How to understand your data

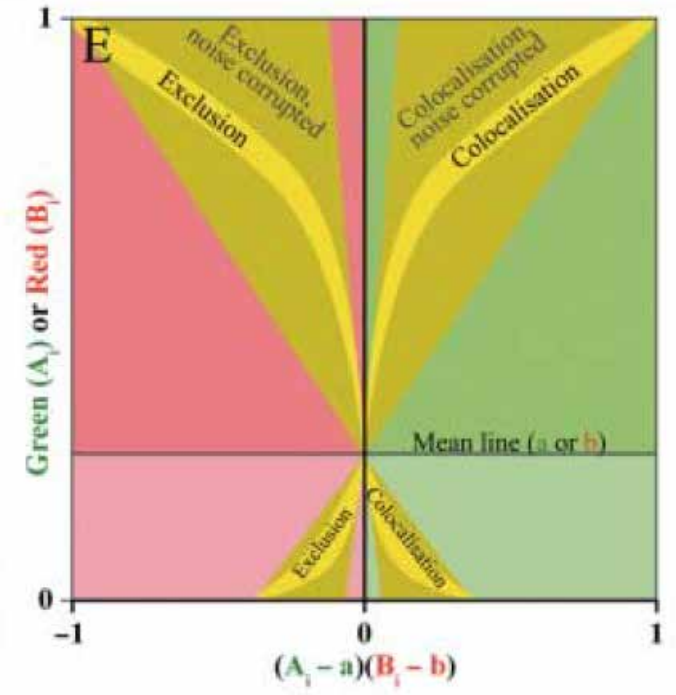
- Li's approach (Intensity Correlation Analysis ICA)



How to understand your data

- Li's approach (Intensity Correlation Analysis ICA)





Effect of Noise

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
Van Steensel (CCF)	Min and Max Range from 0 to 1	Max tends to 1 Bell-shaped curve centered on $\delta x=0$	Affected by noise Needs regularly shaped objects as orientation can be a problem
Li (ICQ)	0.5 → coloc 0 → random -0.5 → exclusion	Tends to 0.5	Affected by noise ICA graphs: Dot cloud on the right side means coloc

How to understand your data

- Object-based analysis

1. Segmentation: Object / Background
2. Connexity analysis: definition of objects
3. Calculation of colocalis

☺ Less depe

L Objects need elling

L Long calc

- Pixel-based analysis

1. Gives Pearson and Manders stats
2. Option when the intensity-based method is limited

How to analyse your data

- **Software we advise you to use**

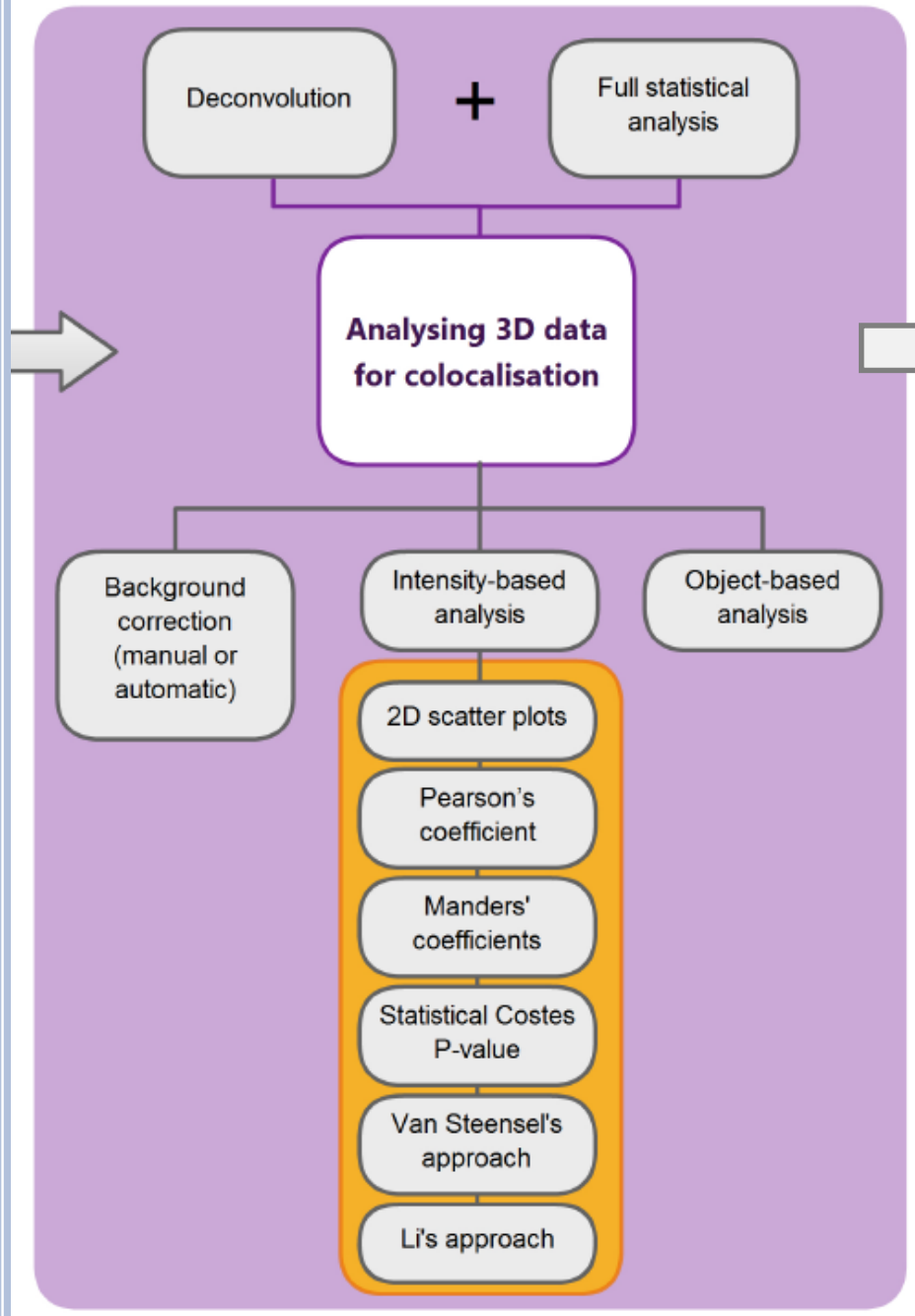
- Deconvolution
  - Softworx (DeltaVision widefield images)
  - Huygens (Confocals + MORE/Nikon Ti2 widefield)

How to analyse your data

- **Software we advise you to use**

- Deconvolution
  - Softworx (DeltaVision widefield images)
  - Huygens (Confocals + MORE/Nikon Ti2 widefield)

- Colocalisation analysis (method 1, intensity-based)
  - JACoP
  - (Huygens)



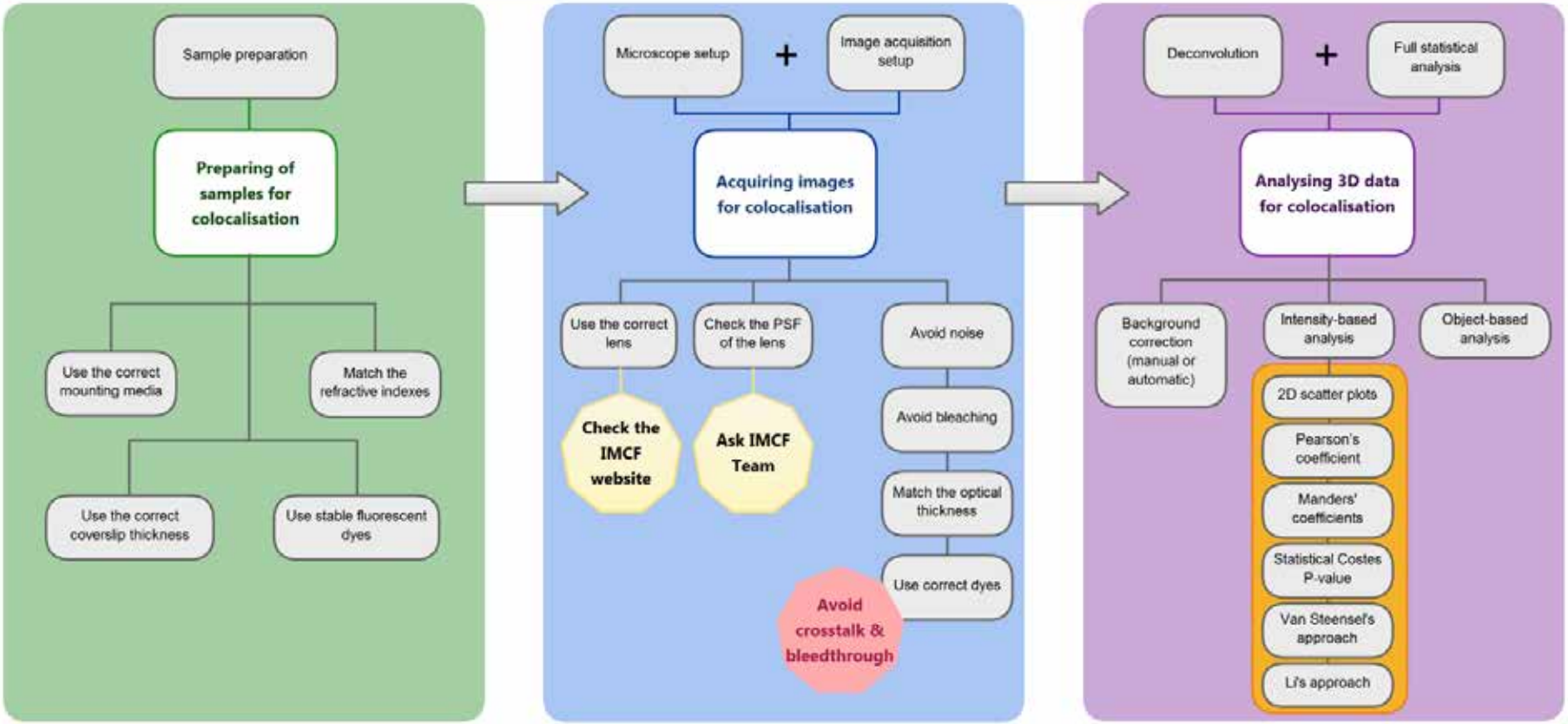
TAKE HOME MESSAGE PART 3

- Start with deconvolved images

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

- For publications, report Pearson's coefficient, threshold Manders' coefficients and a colocalisation image
(but do not forget to look at the other indicators mentioned earlier)

Colocalisation: Strategic Planning



Note: Colocalisation is always relative to the resolution, and it has to be stated.

Literature

- Manders et al. (1992). Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy.
- Manders et al. (1993). Measurement of co-localisation of objects in dual-colour confocal images. *Journal of Microscopy*.
- Costes et al. (2004). Automatic and quantitative measurements of protein-protein colocalization in live-cells. *Biophysical Journal*.

You should read at least this one

- Bolte and Cordelières (2006). A guided tour into subcellular colocalization analysis in light microscopy. *Journal of Microscopy.*
- Comeau et al. (2006). A guide to accurate fluorescence microscopy colocalization measurements. *Biophysical Journal*.

Thanks for your attention!

Questions?