COLOCALISATION

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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.

‡ Limitation (best case scenario): optical resolution of the microscope ‡ 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.

http://www.olympusconfocal.com/applications/colocalization.html
Colocalisation: Strategic Planning

Preparing of samples for colocalisation → Acquiring images for colocalisation → Analysing 3D data for colocalisation
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)

![Image of graph showing Image Intensity vs Coverslip Thickness]

![Table showing Performance Reduction with Coverslip Thickness Variation]

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
HOW TO BEST PREPARE YOUR SAMPLES FOR COLOCALISATION

c The last 500 µm are important
   o Coverglas N°1.5 = 0.17 mm (spherical aberrations)
   o Mounting media (avoid bubbles)
   o 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 ($\lambda$ corrected)
**How to Acquire the Images for Co-localisation**

- Use the correct lens
  - Remember, the higher the NA, the better the resolution
  - Aim for a 1.40 NA or above
  - Apochromat lens ($\lambda$ 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
  - 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!

- Better results in colocalisation if you compare green and red fluorophores…
HOW TO ACQUIRE THE IMAGES FOR COLOCALISATION

¢ Use the correct lens
  ó Remember, the higher the NA, the better the resolution
    ‡ Aim for a 1.40 NA or above
  ó Apochromat lens (λ corrected)
  ó Check the PSF (Point Spread Function with multicolored beads)

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

Cross-talk (wrong excitation)  Bleedthrough (wrong emission)

SpectraViewer on https://www.biozentrum.unibas.ch/IMCF-links
HOW TO ACQUIRE THE IMAGES FOR COLOCALISATION

Avoid Cross-talk and Bleedthrough

<table>
<thead>
<tr>
<th>Fluorophore/Filter</th>
<th>580/40</th>
<th>Alexa Fluor 488</th>
<th>13.2%</th>
<th>Alexa Fluor 546</th>
<th>65.1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 488</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
How to acquire the images for colocalisation

- Avoid cross-talk and bleedthrough
- 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 your 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 (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

¢ Example with the LSM700 Zeiss

Fig. 78  Optimize Sectioning and Step: Match Pinhole to Step resulting in equal optical sections for all channels
HOW TO ACQUIRE THE IMAGES FOR COLOCALISATION (LEICA CONFOCAL)

- Match the optical thickness – Oversampling XY
HOW TO ACQUIRE THE IMAGES FOR COLOCALISATION (LEICA CONFOCAL)

¢ Match the optical thickness – Oversampling Z

SP5 Matrix | SP8
## Settings for Deconvolution

### Confocal

<table>
<thead>
<tr>
<th>Microscope and Objective</th>
<th>Required XY</th>
<th>Required Z</th>
<th>Example: Settings to use</th>
</tr>
</thead>
</table>
| 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 |

¢ 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](https://svi.nl/NyquistCalculator)
### Setting for Deconvolution Widefield

<table>
<thead>
<tr>
<th>Microscope and Objective</th>
<th>Required XY</th>
<th>Required Z</th>
<th>Example: Settings to use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deltavision; 100x 1.42 Oil CCD camera</td>
<td>92 nm</td>
<td>279 nm</td>
<td>XY – no Aux Mag: 65 nm Z: 0.2nm</td>
</tr>
<tr>
<td>Deltavision; 60x 1.42 Oil CCD camera</td>
<td>91 nm</td>
<td>264 nm</td>
<td>XY – no Aux Mag: 107 nm Z: 0.2nm</td>
</tr>
<tr>
<td>Nikon Ti2; 100x 1.45 Oil sCMOS camera</td>
<td>89 nm</td>
<td>243 nm</td>
<td>XY – no Aux Mag: 110 nm Z: 0.2 nm</td>
</tr>
<tr>
<td>Nikon Ti2; 60x 1.40 Oil sCMOS camera</td>
<td>92 nm</td>
<td>279 nm</td>
<td>XY – no Aux Mag: 180 nm Z: 0.2 nm</td>
</tr>
<tr>
<td>MORE; 100x 1.4 Oil sCMOS camera</td>
<td>92 nm</td>
<td>279 nm</td>
<td>XY: 65 nm Z: 0.2nm</td>
</tr>
<tr>
<td>MORE; 60x 1.49 Oil sCMOS camera</td>
<td>87 nm</td>
<td>211 nm</td>
<td>XY 109 nm Z: 0.2 nm</td>
</tr>
</tbody>
</table>

- 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 crosstalk 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

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

¢ 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!!!

¢ 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
Ł Only qualitative correlation
HOW TO UNDERSTAND YOUR DATA

Scatter plot (Cytofluorogram)

A. Full colocalisation

B. Colocalisation ≠ intensities

C. Partial colocalisation

D. Exclusive staining

Adapted from Bolte and Cordelière 2006
HOW TO UNDERSTAND YOUR DATA

- Scatter plot (Cytofluorogram)

  A. Full colocalisation
  B. Colocalisation ≠ intensities
  C. Partial colocalisation
  D. Exclusive staining

Effect of Noise and Bleedthrough

Adapted from Bolte and Cordelière 2006
HOW TO UNDERSTAND YOUR DATA

- Pearson coefficient (PCC, noted as $r$)
  - Estimate of the association strength 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
- Not easy to interpret
- Affected by noise
- No perspective of both channels

http://www.svi.nl/ColocalizationCoefficients
† Manders coefficients (M1 and M2)
- Gives the proportion of each protein colocalising with the other
HOW TO UNDERSTAND YOUR DATA

- Manders coefficients (M1 and M2)

😊 Easier to interpret than PCC
😊 Not sensitive to the intensity of the overlapping pixels
 entidad Sensitive to background intensity – Threshold needed!
 entidad Affected by noise
Influence of noise

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

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)

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

Costes et al., 2004
Van Steensel’s approach (Cross-Correlation Function CCF)

A. Full colocalisation

B. Colocalisation ≠ intensities

C. Partial overlap

D. Exclusive staining

δ = pixelshift
HOW TO UNDERSTAND YOUR DATA

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

- Full colocalisation
- Colocalisation ≠ intensities

Effect of Noise

Adapted from Bolte and Cordelière 2006
HOW TO UNDERSTAND YOUR DATA

Li’s approach (Intensity Correlation Analysis ICA)

A. Full colocalisation
B. Colocalisation ≠ intensities
C. Partial colocalisation
D. Exclusion
HOW TO UNDERSTAND YOUR DATA

c Li’s approach (Intensity Correlation Analysis ICA)

A. Full colocalisation
B. Colocalisation ≠ intensities
C. Partial colocalisation
D. Exclusion

Effect of Noise
# How to Understand Your Data

<table>
<thead>
<tr>
<th>Method</th>
<th>Value range</th>
<th>Colocalisation if...</th>
<th>Notes</th>
</tr>
</thead>
</table>
| Pearson’s coef r     | +1 → coloc 0 → random -1 → exclusion                                        | tends to 1           | Insensitive ≠ intensities  
A sensitive intensity offset  
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  
Sensitivity 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 δx=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 colocalised volume, area, centroids...

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

😊 Less dependent on intensities

Objects need segmentability – not for diffuse labelling

Long calculations (3D)

For more info, come to our Wednesday Workshop (open doors – 10:00-12:00)
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

 IOError 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 colocalization 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)
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.


You should read at least this one.
THANKS FOR YOUR ATTENTION!

QUESTIONS?