COLOCALISATION

Alexia Loynton-Ferrand

Imaging Core Facility
Biozentrum 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

![Colocalization of Actin and Vinculin in Normal Tahr Ovary Cells](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
- Limitation (best case scenario): optical resolution of the microscope → XYZ 200 x 200 x 400 nm
**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

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 you 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

- Sample Preparation
- Image Acquisition
- 3D Data Analysis
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
The last 500 µm are important
- Coverglass N°1.5 = 0.17 mm (spherical aberrations)

![Image](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)

- 50% PBS-Glycerol
- Vectashield
- Prolong Diamond
- Prolong Gold
- Euparal
- Fluoromount G

DAPI
aTubulin
Phalloidin
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)
- 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 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!

Left: LSM700 – Confocal, non-deconvolved
Right: DeltaVision – Widefield, deconvolved
HOW TO ACQUIRE THE IMAGES FOR COLOCALISATION

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

[Diagram showing emission spectra for Alexa 488 and Alexa 546, indicating cross-talk and bleedthrough wavelengths.]

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

<table>
<thead>
<tr>
<th>Fluorophore/Filter</th>
<th>580/40</th>
<th>Alexa Fluor 488</th>
<th>Alexa Fluor 546</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>13.2%</td>
<td>65.1%</td>
</tr>
</tbody>
</table>
HOW TO ACQUIRE THE IMAGES FOR COLOCALISATION

→ Scan with the sequential mode
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

- Match the pixel size – Oversampling XY
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

SP5 Matrix

SP8
## Settings for Deconvolution: Confocals

<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  
ZOOM 4  
XY: 2800*2800  
Z: 0.13 nm  
XY: 1500*1500  
Z: 0.13 nm |
| SP8; 63x 1.4 Oil          | 43 nm       | 131 nm     | ZOOM 2  
ZOOM 4  
XY: 2200*2200  
Z: 0.13 nm  
XY: 1080*1080  
Z: 0.13 nm |
| LSM700 Up; 63x 1.4 Oil    | 43 nm       | 131 nm     | ZOOM 1  
ZOOM 2  
XY: 2048*2048  
Z: 0.13 nm  
XY: 1200*1200  
Z: 0.13 nm |
| LSM800; 63x 1.4 Oil       | 43 nm       | 131 nm     | ZOOM 1  
ZOOM 2  
XY: 2400*2400  
Z: 0.13 nm  
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](https://svi.nl/NyquistCalculator)
### SETTINGS FOR DECONVOLUTION: SPINNING DISK

<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>SpinSR; 60x 1.5 Oil</td>
<td>40 nm</td>
<td>93 nm</td>
<td>Check the camera installed on the system*</td>
</tr>
<tr>
<td>SpinSR; 100x 1.5 Oil</td>
<td>40 nm</td>
<td>93 nm</td>
<td>Check the camera installed on the system*</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 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](https://svi.nl/NyquistCalculator)
## Settings for Deconvolution: Widefields

<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 1.40 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 XY – with Aux Mag: 65 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 XY - with Aux Mag: 73 nm Z: 0.2 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 XY - with Aux Mag: 120 nm Z: 0.2 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: 0.2nm</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 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!!!
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!!!
Matlab extension for Imaris

See dedicated wiki page:
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Can be limited to spots within a certain region (ie. DAPI here)

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

A guided tour into subcellular colocalization analysis in light microscopy

S. BOLTE* & F. P. CORDELIÈRES†
*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.
Good first visual estimate of colocalisation

Information about the image quality

Only qualitative correlation

Overlay green/red

JACoP: SCATTER PLOT/CYTOFLUOROGRAM
JACoP: SCATTER PLOT/CYTOFLUOROGRAM

A. Full colocalisation

C. Partial colocalisation

B. Colocalisation ≠ intensities

D. Exclusive staining

Adapted from Bolte and Cordelière 2006
JACoP: Scatter plot/Cytofluorogram

A. Full colocalisation

C. Partial colocalisation

B. Colocalisation ≠ intensities

D. Exclusive staining

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 and M2**
  - Gives the proportion of each protein colocalising with the other
HOW TO ANALYSE YOUR COLOCALISATION DATA

😊 Easier to interpret than PCC
😊 Not sensitive to the intensity of the overlapping pixels
😊 Sensitive to background intensity – Threshold needed!
😊 Affected by noise
These coefficients are influenced by noise. Minimize noise during the acquisition, and deconvolve your datasets prior analysis.

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.
Costes’ Approach

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

Costes et al., 2004
<table>
<thead>
<tr>
<th><strong>Value range</strong></th>
<th><strong>Colocalisation if...</strong></th>
<th><strong>Notes</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pearson’s coef r</strong></td>
<td>+1 → coloc 0 → random -1 → exclusion</td>
<td>tends to 1</td>
</tr>
<tr>
<td><strong>Manders’ coef M1 (or M2)</strong></td>
<td>0 → 0% of Ch1 colocalize with Ch2 1 → 100% of Ch1 colocalize with Ch2</td>
<td>tends to 1</td>
</tr>
<tr>
<td><strong>Costes (P-value)</strong></td>
<td>P&lt;95% → no coloc P≥95% → coloc</td>
<td>≥95%</td>
</tr>
</tbody>
</table>
1. Open Fiji
2. Open your image
3. Open “Coloc_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*
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 is always relative to the resolution!
THANKS FOR YOUR ATTENTION!

Imaging Core Facility
Biozentrum
University of Basel
imcf@unibas.ch
www.biozentrum.unibas.ch/imcf