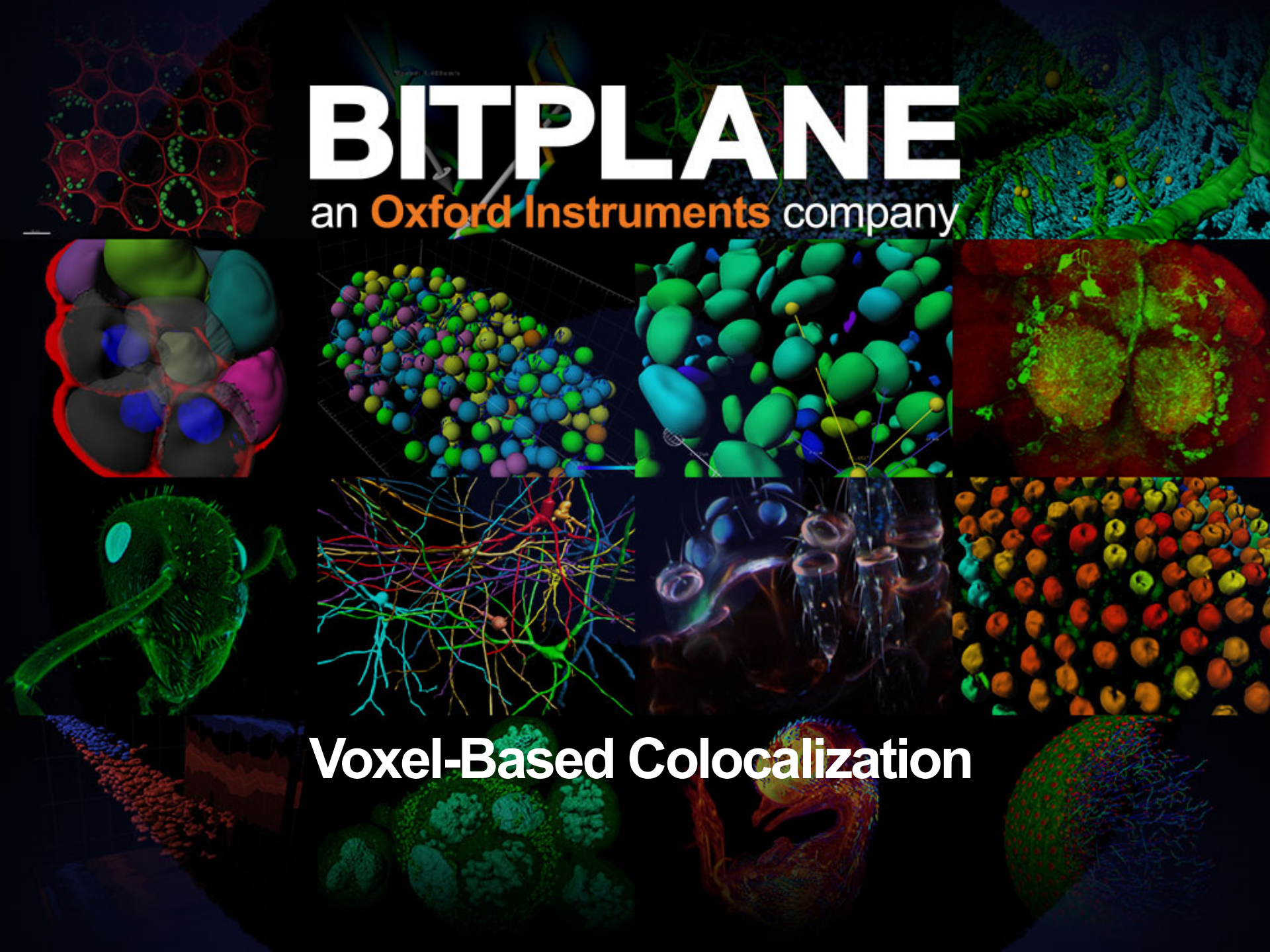
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Voxel-Based Colocalization

# Colocalization as a Tool for Multichannel Analysis

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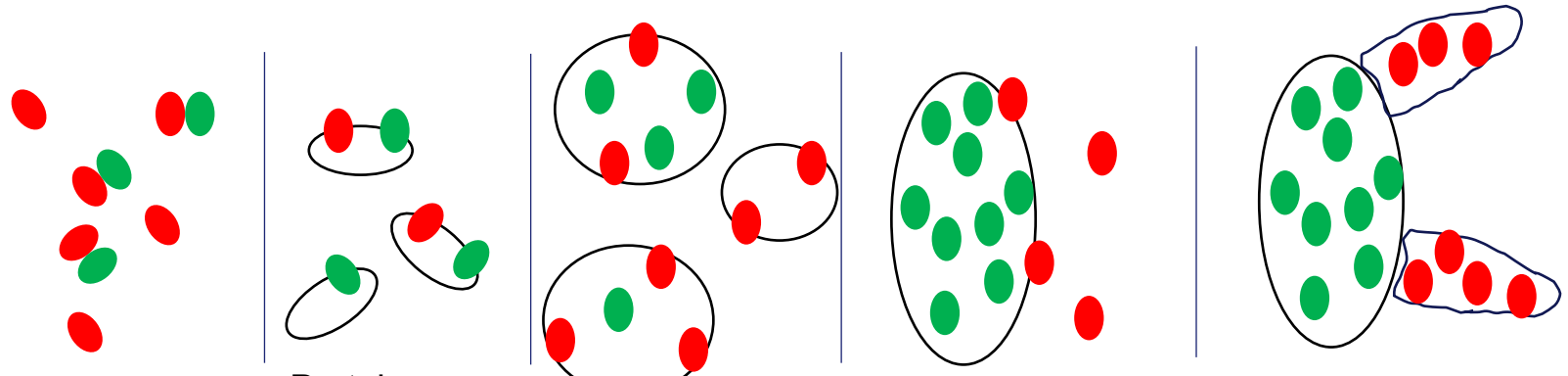
- Imaris has several tools to quantify and visualize colocalization:
  - Intensity/voxel-based (ImarisColoc)
  - Object-based (Spots, Surfaces, Filaments)
- Which method is right for you?
  - Fluorescent labels form distinct objects or diffused distribution?
  - What is the underlying biological hypothesis?
  - Interaction? Co-expression? Proximity? Volume overlap?
  - A practical guide to evaluating colocalization in biological microscopy. Dunn et al. 2011 *Am J Physioil Cell Physiol*



# ImarisColoc: Intensity-Based Colocalization

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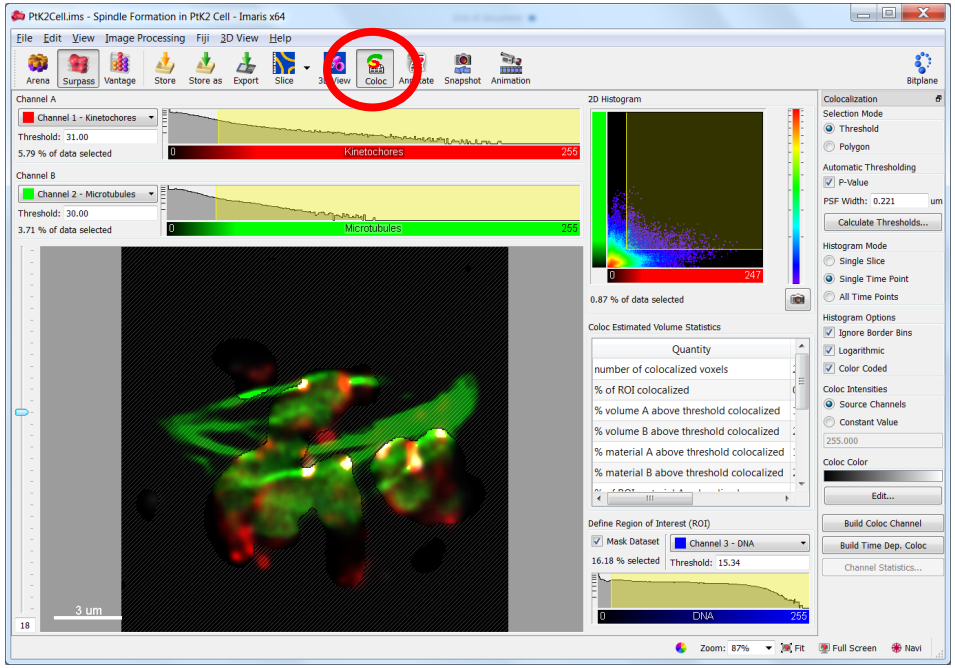


- Automated, standardized determination of colocalized voxels.
- Processes 2D, 3D, and 4D images.
- Real-time feedback on changes in selection.
- Display of co-localized voxels with original channels as defined in channel visibility editor.
- Output of co-localized voxels into new channel allows maximal flexibility with respect to display and analysis using all other Imaris functions.

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# ImarisColoc: Quantitative Outputs

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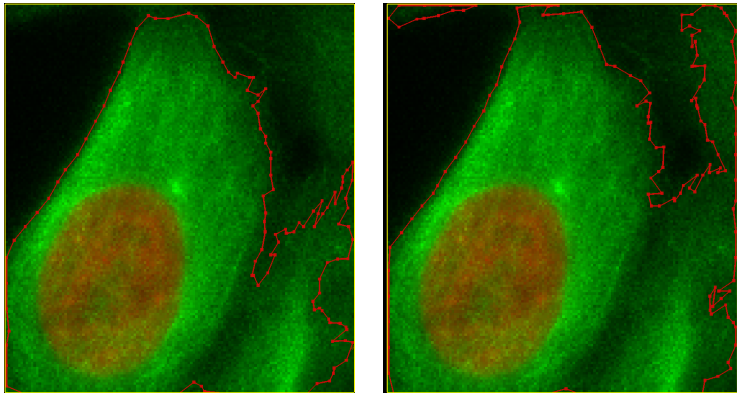
Base Color | Mapped Color | Coloc Estimated Statistics

| | |
|---|---|
| time frame | 1 |
| threshold A | 20.000 |
| threshold B | 18.000 |
| number of colocalized voxels | 449211 |
| % of dataset colocalized | 5.16 |
| % of ROI colocalized | 19.32 |
| % of volume A above threshold colocalized | 57.51 |
| % of volume B above threshold colocalized | 48.85 |
| % of material A above threshold colocalized | 63.22 |
| % of material B above threshold colocalized | 56.99 |
| % of ROI material A colocalized | 42.48 |
| % of ROI material B colocalized | 42.86 |
| Pearson's coefficient in dataset volume | 0.5808 |
| Pearson's coefficient in ROI volume | 0.3271 |
| Pearson's coefficient in colocalized volume | -0.0990 |
| original Mander's coefficient A | 0.9694 |
| original Mander's coefficient B | 0.9351 |
| thresholded Mander's coefficient A | 0.4988 |
| thresholded Mander's coefficient B | 0.3783 |

- Colocalization statistics are calculated within the ROI and determined by intensity threshold.
  - ROI can be defined by any channel.
  - ImarisColoc provides both automatic and manual Threshold selection.



Which threshold value is right?

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# Quantitative Outputs in a Nutshell

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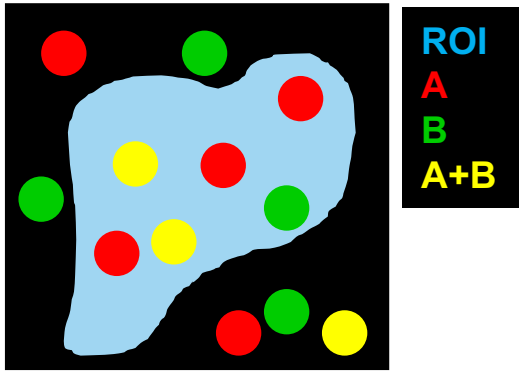
number of colocalized voxels
% of dataset colocalized
% of ROI colocalized
% of volume A above threshold colocalized
% of volume B above threshold colocalized
% of material A above threshold colocalized
% of material B above threshold colocalized
% of ROI material A colocalized
% of ROI material B colocalized
Pearson's coefficient in dataset volume
Pearson's coefficient in ROI volume
Pearson's coefficient in colocalized volume
original Mander's coefficient A
original Mander's coefficient B
thresholded Mander's coefficient A
thresholded Mander's coefficient B

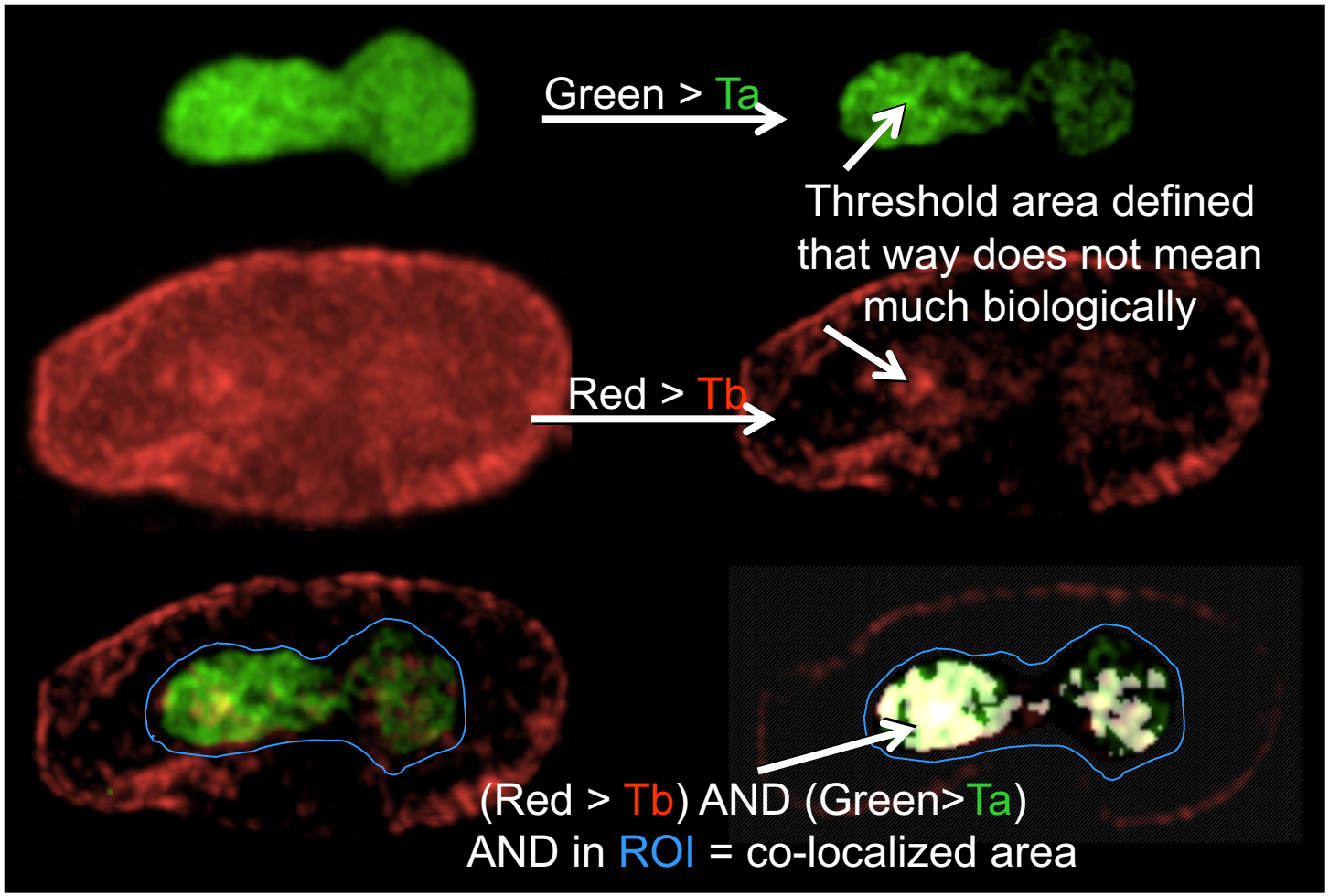



- Colocalized volume: number of voxels.
- Colocalized material: intensity sum.

- Pearson's coefficient (r):
  - Pixel-by-pixel covariance.
  - r ~ 0 → no correlation; r~1 → high correlation.
- Mender's coefficient ($M_A$, $M_B$):
  - Measures co-occurrence independent of signal proportionality between 2 channels.

- ROI, thresholds, and background intensity can affect all these numbers.

# Real Image Illustration




- Blue shows ROI
- Top row: Green pixels before and after Ta.
- Middle row: Red pixels before and after Tb
- Bottom row left: Red and green pixels after thresholds
- Bottom row right: White = overlap pixels from left that are above thresholds and also in the ROI

# Step 0: Optimize Acquisition

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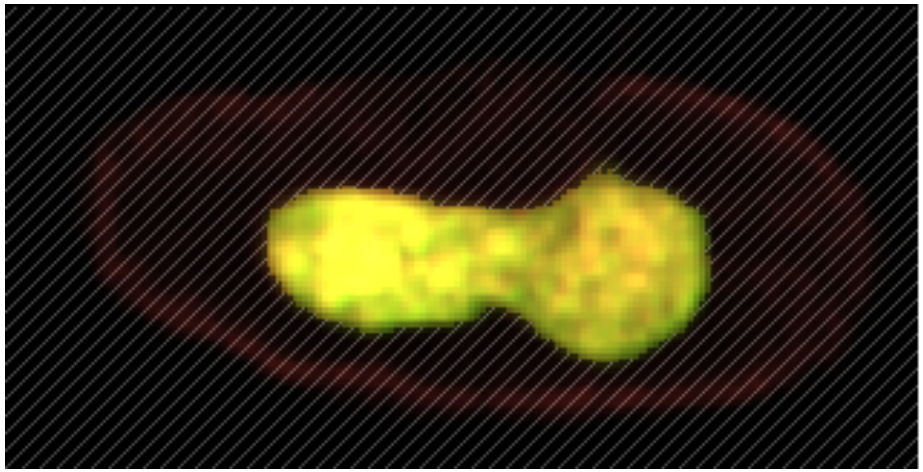
- **Signal consistency:**
  - Consistent microscope settings (laser power, PMT/camera gain, exposure time, etc.).
  - Adequate signal to noise ratio.
  - Avoid saturated pixels.
  - Enough intensity dynamic range with higher bit depth (12/16 bit).

- **Multi-channel acquisition:**
  - No channel bleed through (check single labeled probes).
  - Similar Point Spread function for each color (check PSF of the channels with multicolor beads).
  - No registration errors between channels.
  - Minimize chromatic aberration.

# Step 1: Region of Interest



- The automated colocalization analysis takes into account the whole image.
- For accurate results, a region of interest should be defined in order to ignore the background and to focus on biologically meaningful region for analysis.



# Step 1: Region of Interest

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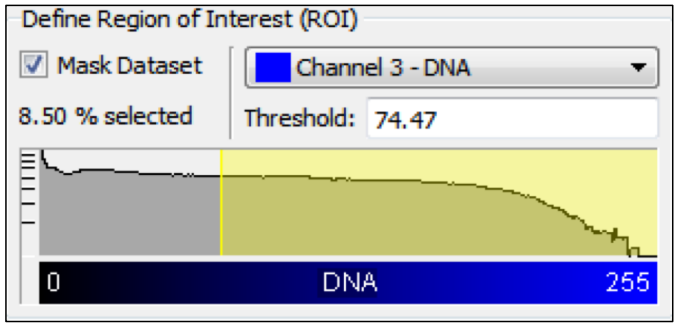
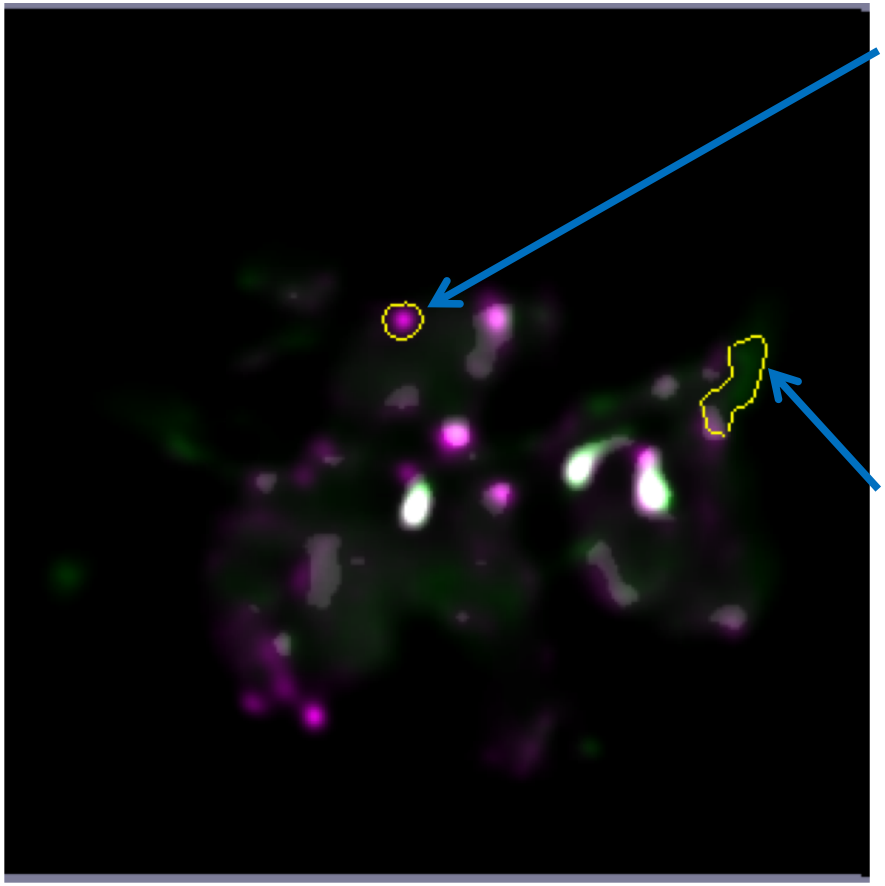
- Options:
  - Acquire a 3rd channel in which the labelling represents the ROI.
  - Use Surface > Mask to generate an ROI channel.
    - Draw a manual Contour Surface, or
    - Use existing channel to create Surface.
    - For masking, set outside to 0, inside to any mid-range value.
  - Use Image Processing > Channel Arithmetics to merge the two channels into one mask channel.

The resulting channels from above can be used to create an ROI in Coloc:



# Step 2.1: Manual Thresholding





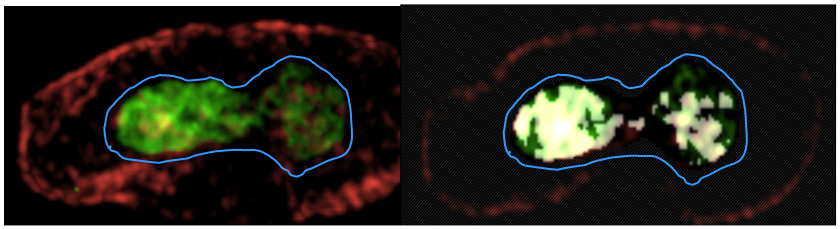
1) Click on a bright area and drag toward a dark region to set Channel A threshold.

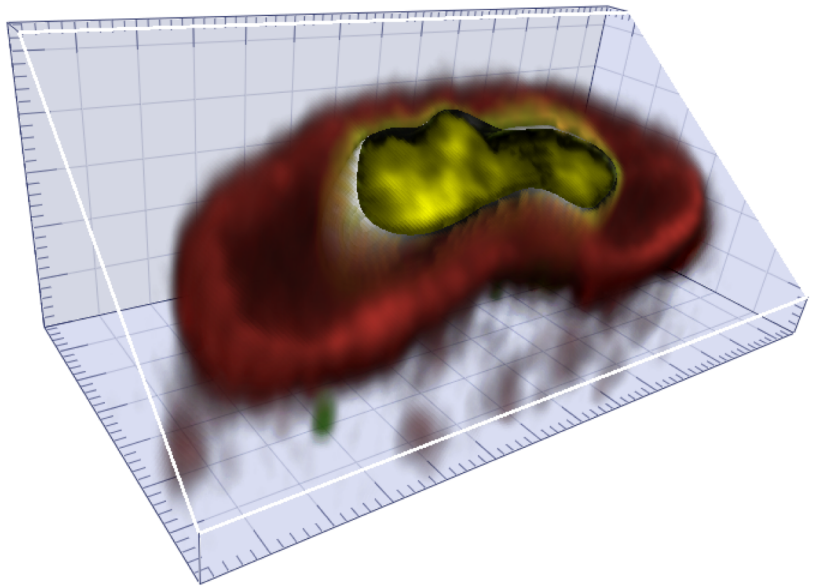
2) An IsoLine tool is used to visualize the area above the threshold next to the mouse.

3) Hold Shift Key and do same to set Channel B threshold.

- For 3D image, the optimal slice to perform manual thresholding for each channel may not be the same.
- For complex images, manual thresholding can be subjective.

# Step 2.2: Automatic Thresholding





- ImarisColoc – especially the implementation of the automated thresholding – was developed together with Dr. Sylvain Costes, today working at the Lawrence Berkeley National Laboratory.

- Publication: Costes, S. V., Daelemans, D., Cho, E. H., Dobbin, Z., Pavlakis, G., and Lockett, S. (2004). Automatic and Quantitative Measurement of Protein-Protein Colocalization in Live Cells. Biophys J 86, 3993-4003.

# Step 2.2: Automatic Thresholding

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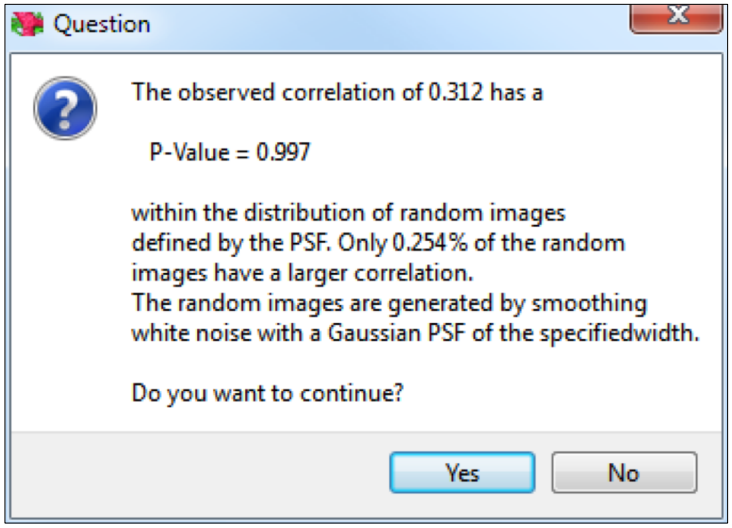
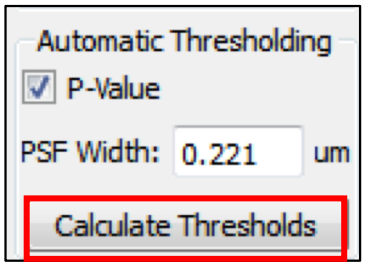
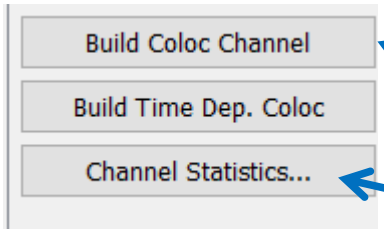
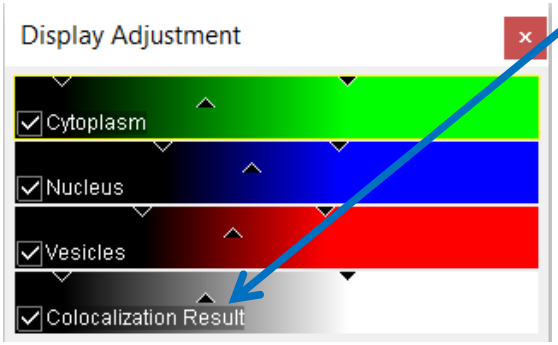
- P-value depends on **PSF Width**.
  - Default PSF width = longest diagonal of 1 voxel.
  - This calculation does NOT affect the automatic thresholding values, and is only used to check if colocalization is significant.
- Specific, non-random colocalization depends on resolution (both PSF size and voxel size), and signal to noise ratio.
- Deconvolution may help.

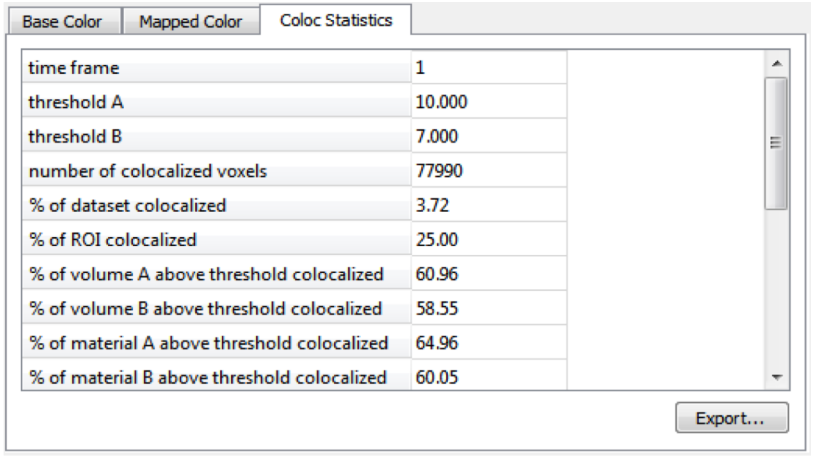


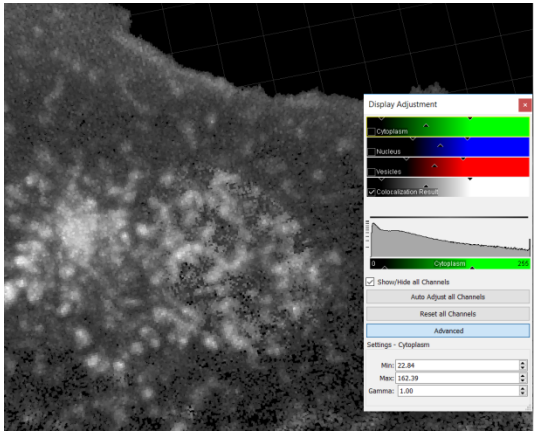
# Step 3: Quantification and Visualization




- Colocalization result is saved as a channel
- Click ‘Channel Statistics’ or the coloc channel to access final statistical values.
- Don’t use the preview statistics in Coloc viewer.
- Coloc channel can be visualized and measured just like any other data channel.



# Special Case: Time Dependent Auto-Threshold

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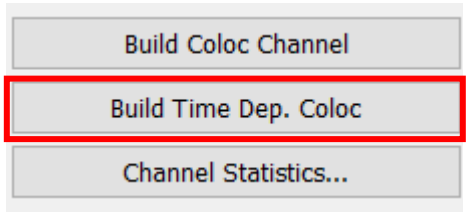
- Build time-dependent Coloc channel.
- The relationship between correlation and intensity could change over time.
- For automatic thresholding, scatterplot histogram is analyzed separately for each time-point.
- Different threshold result is possible at each time-point, for both image and statistics output.




Base Color | Mapped Color | Coloc Estimated Statistics

| time frame | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| threshold A | 1.000 | 3.000 | 2.000 | 2.000 | 2.000 |
| threshold B | 3.000 | 2.000 | 3.000 | 3.000 | 2.000 |
| number of colocalized voxels | 321267 | 336929 | 323047 | 338339 | 334260 |
| % of dataset colocalized | 11.02 | 11.55 | 11.08 | 11.60 | 11.46 |
| % of ROI colocalized | 11.02 | 11.55 | 11.08 | 11.60 | 11.46 |
| % of volume A above threshold colocalized | 66.25 | 39.15 | 55.68 | 48.54 | 53.72 |
| % of volume B above threshold colocalized | 78.85 | 95.54 | 87.34 | 90.06 | 90.41 |

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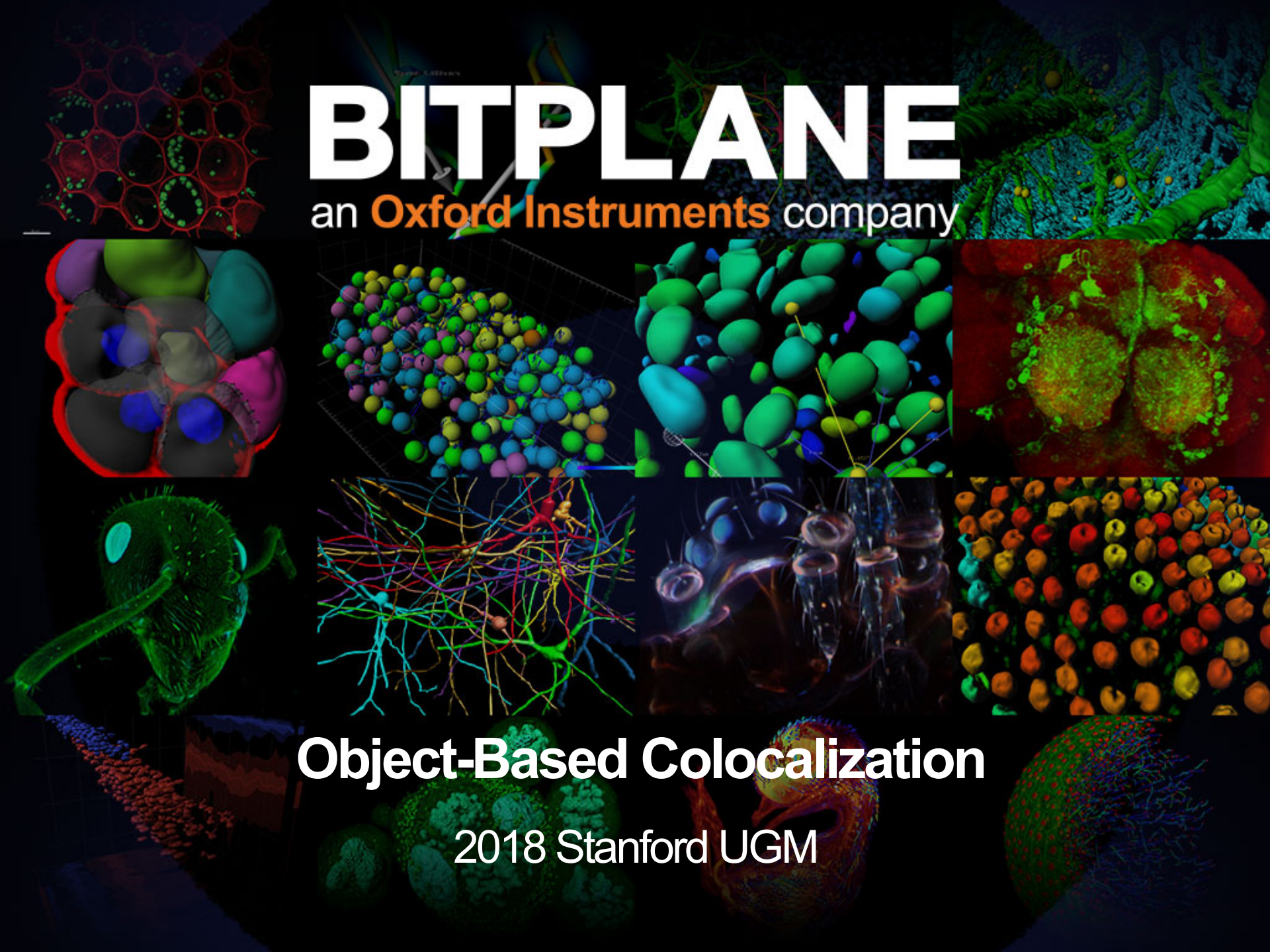
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Object-Based Colocalization
2018 Stanford UGM

# Object-Object Relationships

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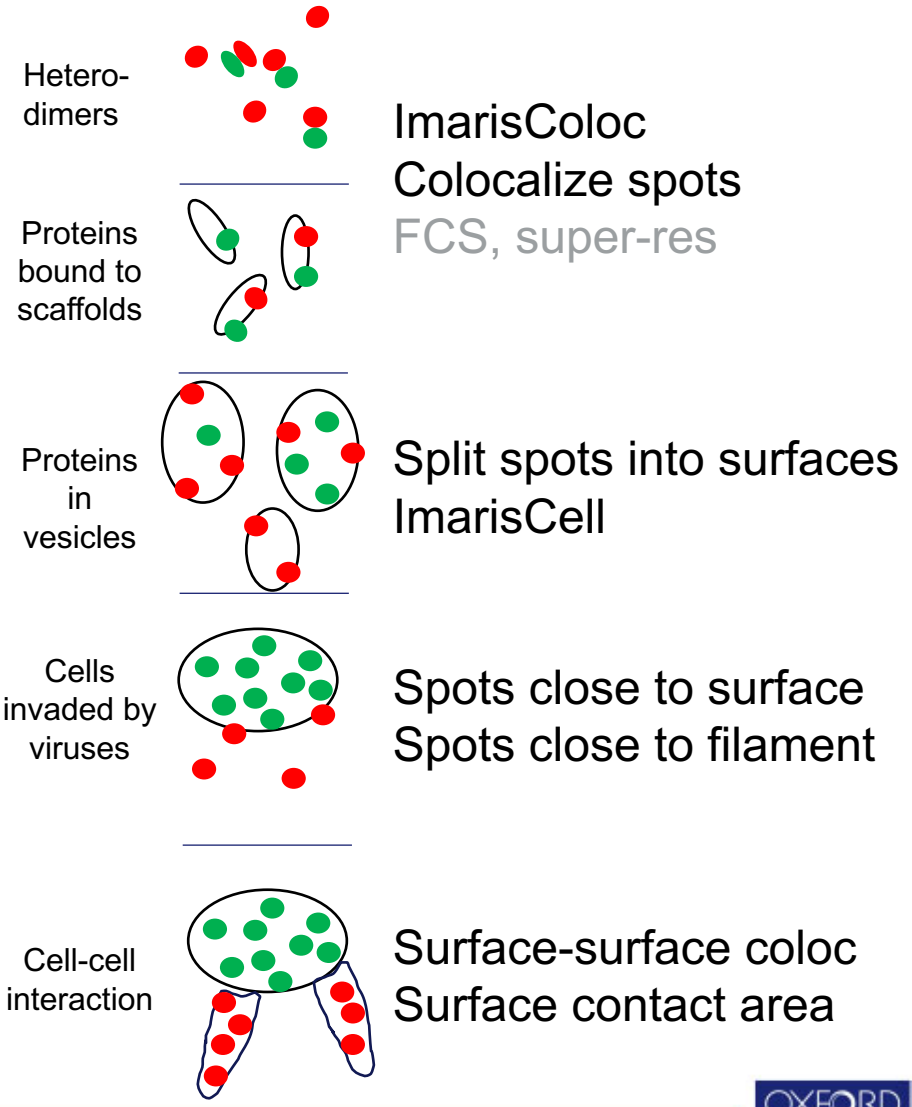
- XTensions (Built-in):
  - Distance transform
  - Colocalize spots
  - Split spots into surfaces
  - Spots close to surface
  - Spots close to filament
- Imaris Open (not officially supported):
  - Surface-surface coloc
  - Surface contact area
  - Triple spots coloc
  - Kiss and run (tracking)
- Other Less Conventional Methods:
  - ImarisCell module
  - Intensity filtering

**Possible methods**

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# XTension: Colocalize Spots

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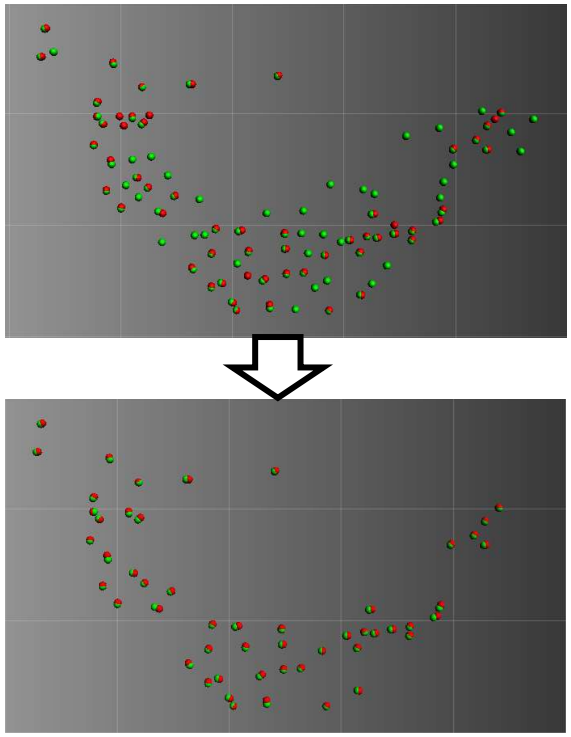
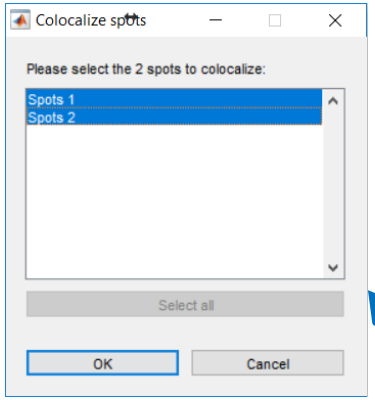
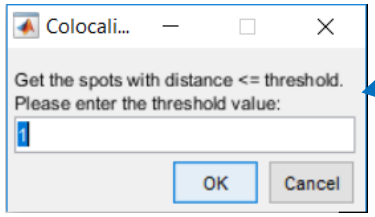
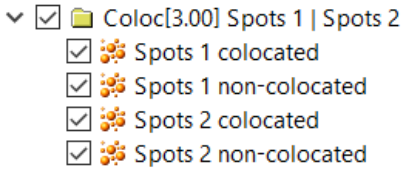
All red and green spots



Colocalized spots



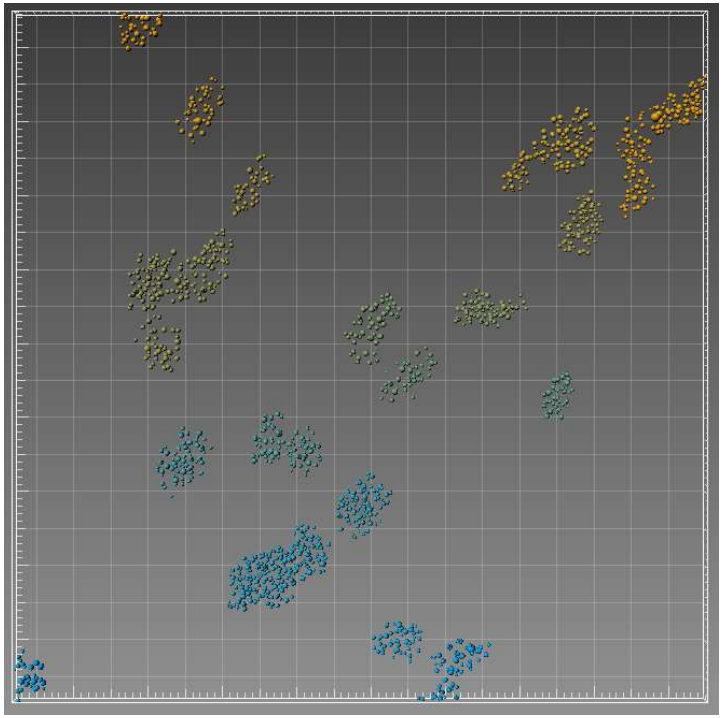




1) Create Spots using channel 1.
2) Create Spots using channel 2.
3) Run Colocalize Spots XT, select the two Spots objects.
4) Define distance threshold.
5) New Spots objects are generated based on colocalization for further analysis.

# XTension: Spilt Spots into Surface Objects





- This tool is able to segment spots that lie within a surface volume.
  - It generates a new spots object for each surface.
  - Quantification of #spots/cell.
- If a Spot object lies outside one of the isosurface volumes, it will not be counted.
- Cell module (Surfaces = Cells, Spots = Vesicles) would provide a better solution.
  - More statistical options.
  - Automatic and batchable.
  - All information within one Cell object.

# XTension:
# Find Spots Close to Filaments/Surface

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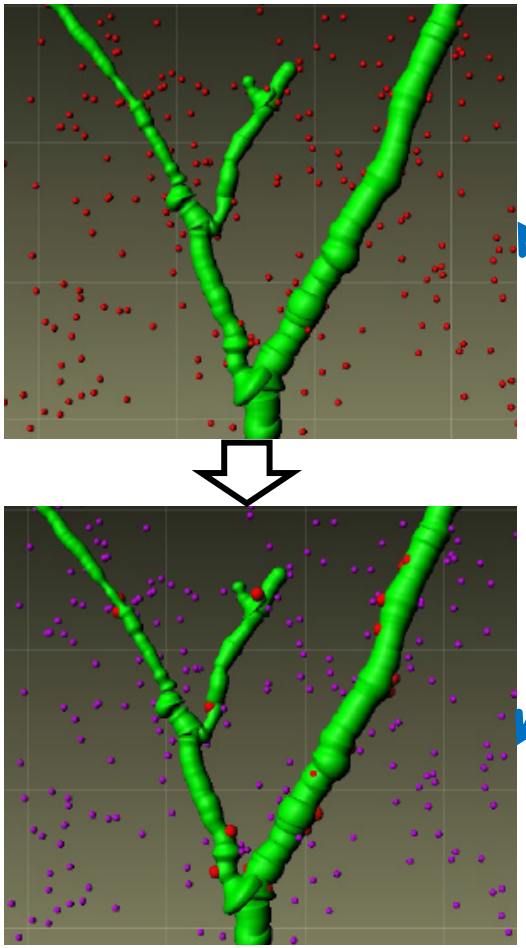
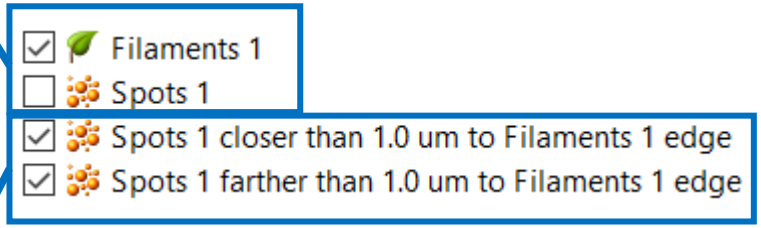
Total Spots & Filament



Spots close and far from the Filament

1) Generate Filaments and Spots.
2) Run Find Spots Close to Filaments XT.
3) Spots closer and further than X µm of Filament are separated into 2 new groups.




- Similar workflow for Find Spots Close to Surface

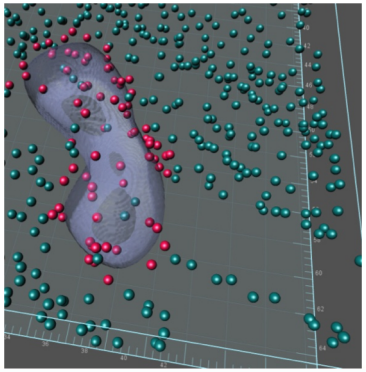


Image courtesy of Dr. Marc Landry, Bordeaux

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# Exercise 1: Synthetic Spots Coloc

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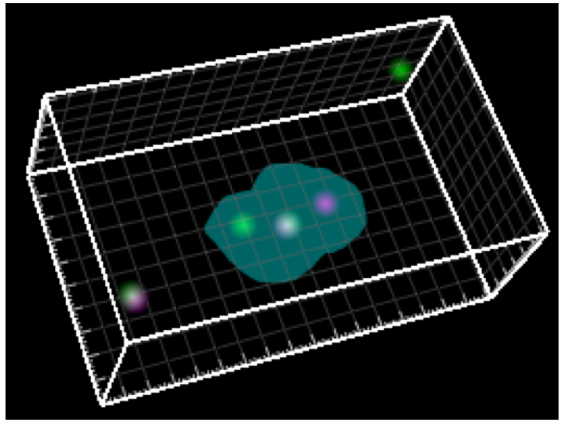
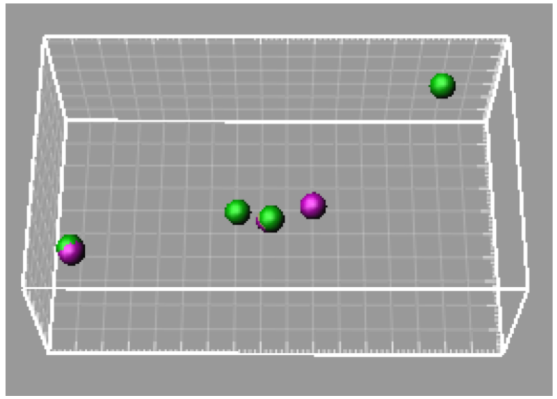
- Load SyntheticColoc_new.ims.
  - Analyze as if you are looking for overlap between Ch1 and Ch2 within the Ch3 region.
  - Set the threshold in an appropriate manner for this dataset.
    - Hint: Are these better described as well-defined “objects” or diffuse “patterns”?
  - Export Coloc Statistics.



- Next, Create Spots objects from Ch1, Ch2, and Colocalization Result Channel.
  - Color each Spots object differently.
  - Save the Surpass Scene file.



- BONUS: Ch4 is a noisy version of Ch1 with background offset = 1. Use Ch4 instead of Ch1 and repeat the analysis. Which statistical value is the most/least robust?

# Exercise 2: Intensity-Based Coloc Exercise

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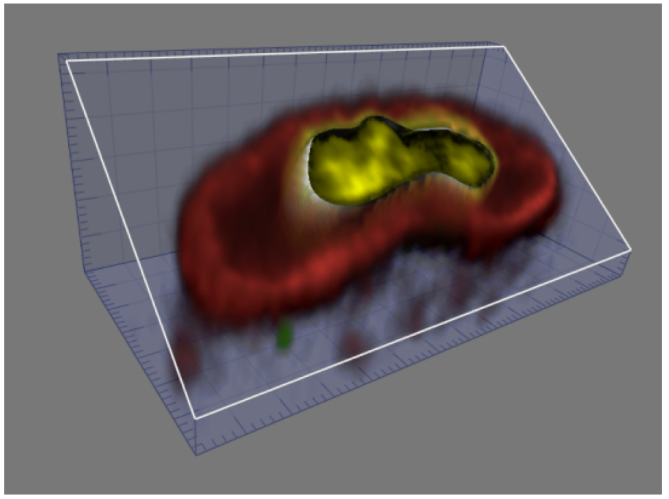
- Load EX1_Costes-Crop-12T.ims.
  - Create Surface based on the green channel. Use that surface to make a Coloc ROI (be careful to exclude any bright green signal outside that central object by filtering in Surfaces by number of voxel).
  - Mask new surface (edit tab of Surfaces) setting voxels outside Surface to zero & inside Surface to 255.
  - Set the thresholds A & B in an appropriate manner for this image, using the surface mask as ROI.
  - Create 3D Coloc Surface, highlighting the new results.
    - Modify Surfaces, transparency, clipping plane.



# Exercise 3: Object-based Coloc Exercise

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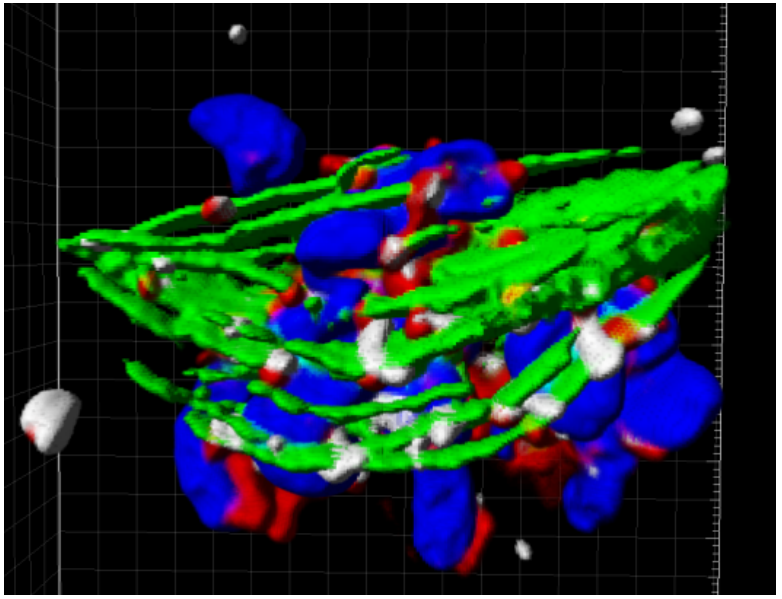
- Load PtK2Cell.ims in Arena (Imaris demo images).
  - Build Spots group on Kinetochores (Ch1) and a 2nd Spots group on dynactin (Ch4).
  - Click on cogwheel icon of one of the Spots groups and choose ‘Colocalize Spots’.
  - Enter a distance threshold in microns (0.2um = 200nm, etc).
  - BONUS: Create Surface of DNA channel (Ch3) & run ‘Split Spots into Surfaces on both the ‘Colocalized Kinetochore Spots’ and ‘Colocalized Dynactin Spots’.
  - BONUS: Also create Surface of Microtubules channel (Ch2), and use ‘Surface-Surface coloc’ to estimate the overlapping volume of DNA and Microtubules.



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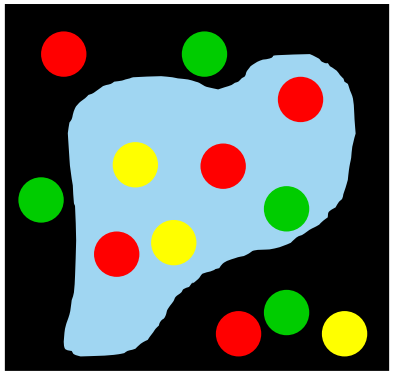
# Colocalized Volume/Material






- Overall image = *200* voxels, ROI = 100 voxels
- Each circle = **6** voxels
- Intensity inside red circle = 3
- Intensity inside green circle = 4
- Intensity outside circles = 1 for both A and B

- Set threshold A = 2, threshold B = 2

- Volume = # of voxels
- Material = Sum of intensity value

- Number of colocalized voxels = 2*__6__ = 12
- % of dataset colocalized = (2*__6__)/*200* = 6%
- % of ROI colocalized = (2*__6__)/100 = 12%
- % of Volume A above threshold colocalized = (2*__6__)/(5*__6__) = 40%
- % of Volume B above threshold colocalized = (2*__6__)/(3*__6__) = 67%
- % of Material A above threshold colocalized = (2*__6__*3)/(5*__6__*3) = 40%
- % of Material B above threshold colocalized = (2*__6__*4)/(3*__6__*4) = 67%
- % of ROI material A colocalized = (2*__6__*3)/((100-5*__6__)*1+5*__6__*3) = 23%
- % of ROI material B colocalized = (2*__6__*4)/((100-3*__6__)*1+3*__6__*4) = 31%

# Pearson's coefficient



$$r = \frac{\sum_i (A_i - \bar{A})(B_i - \bar{B})}{\sqrt{\sum_i (A_i - \bar{A})^2 \sum_i (B_i - \bar{B})^2}}$$

- Measures the pixel-by-pixel covariance in the signal levels of two images; assume linear relationship
- r ~ 0 → no correlation; r~1 → high correlation
- Advantages:
  - Simplicity, widely available
  - Independent of signal levels and signal offset
- Disadvantages:
  - Hard to interpret intermediate values
  - Meaningful only if A and B have comparable quantity, or only analyze in colocalized volume
  - Unlabeled region can artificially inflate r as both A and B are simultaneously low

# Mander's coefficient

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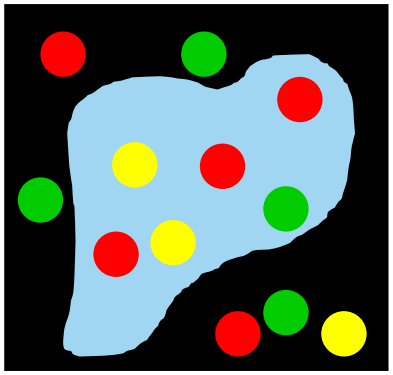
$$M_A = \frac{\sum_i (A_i * Coloc)}{\sum_i A_i} \qquad M_B = \frac{\sum_i (B_i * Coloc)}{\sum_i B_i}$$

For $M_A$:
- Original Mander's:
  - Coloc = 1 if Bi > 0
  - Otherwise Coloc = 0
- Thresholded Mander's:
  - Coloc = 1 if Bi > threshold B
  - Otherwise Coloc = 0




- ROI = 100 voxels
- Each circle = **6** voxels
- Intensity inside red circle = 3
- Intensity inside green circle = 4
- Intensity outside circles = 1 for both A and B

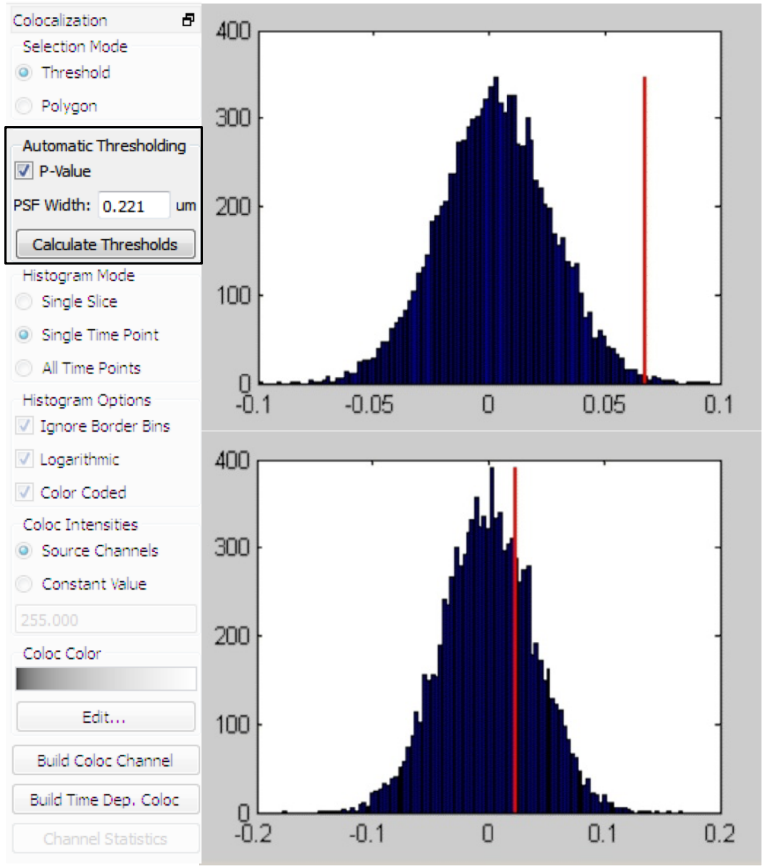
- Set threshold A = 2, threshold B = 2

- Original $M_A$= $M_B$= 100%, *due to all pixel intensity > 0!*
- Thresholded $M_A$= (2*__6__*3)/((100-5*__6__)*1+5*__6__*3) = 23%
- Thresholded $M_B$= (2*__6__*4)/((100-3*__6__)*1+3*__6__*4) = 31%

- Strictly measures co-occurrence independent of signal proportionality
- How to define threshold?
  - Manual threshold may introduce biases.
  - Costes et al.'s automatic thresholding method is available.

# Automatic Thresholding Detail (1)






- Before the thresholds for the channels are computed, ImarisColoc performs a separate analysis which determines the probability of having non-random colocalization.
- The Pearson correlation coefficient (PCC) is computed for the acquired (masked) channels (red line) and compared to the PCC obtained with randomized images (blue histogram) which are smoothed with a PSF similar to the acquired data.
- Each blue line shows the PCC for one randomized version. The collection of blue lines shows the distribution of PCC for all randomizations.
- If the PCC of the acquired (masked) channels is not larger than the PCC for 95% of the randomized images, then it can be concluded that too much of the overlap is random, and it is recommended not to proceed (lower image).

# Automatic Thresholding Detail (2)

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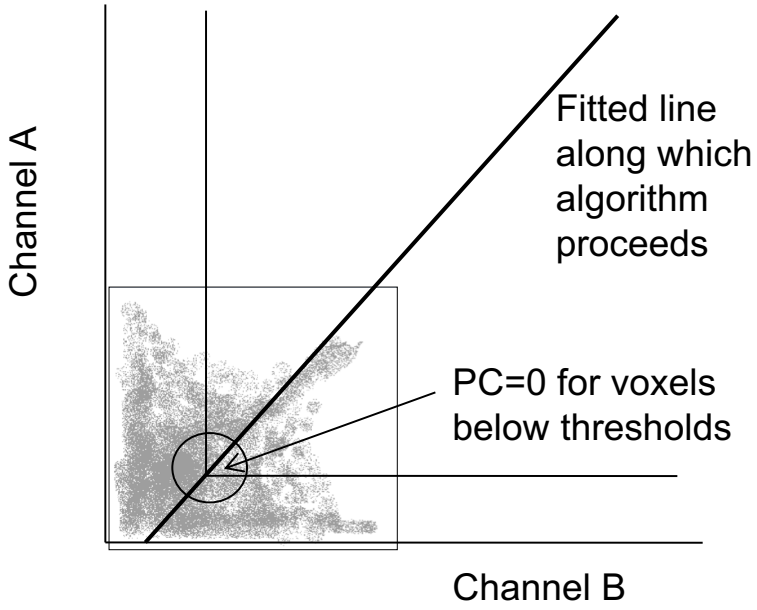
- The algorithm developed by Costes et al. is based on the exclusion of intensity pairs that exhibit no correlation (Pearson's correlation below zero).
- Starting with the highest intensity value, the algorithm reduces the threshold value step by step along a line shown below and computes the correlation coefficient of the image using only voxels with intensities above the threshold.
- The algorithm continues reducing the thresholds until the correlation reaches 0, thus defining the automatic threshold.



# XTension: Surface-Surface Coloc

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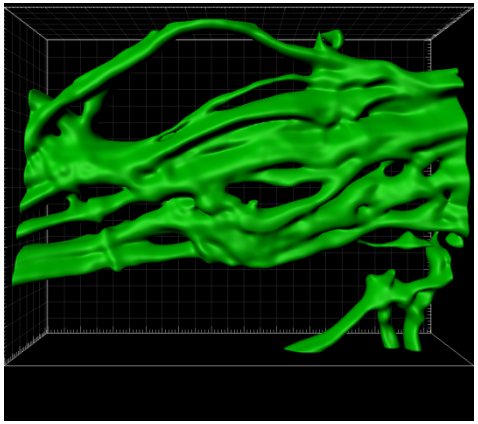
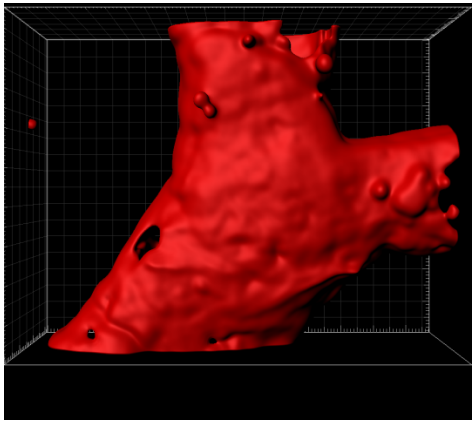
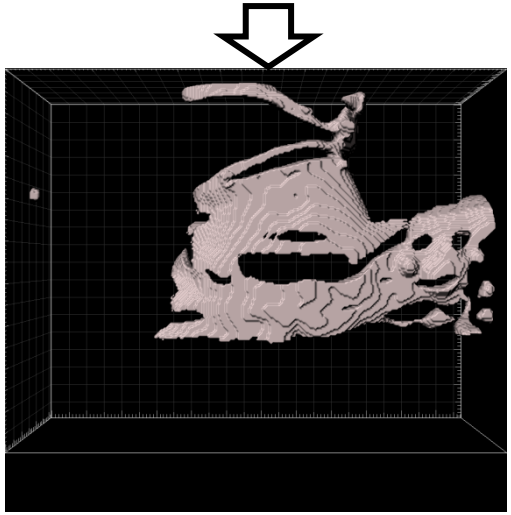
Surface 1



Surface 2





New Surface representing Surface 1& 2 colocalization