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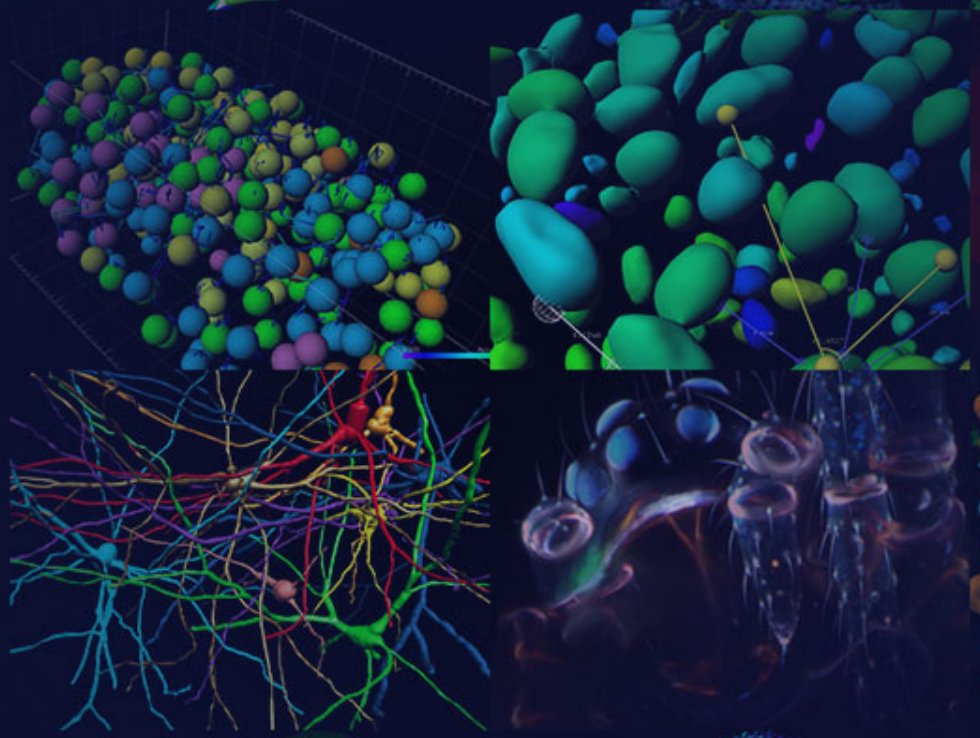

Colocalization Tools

Colocalization as a tool for multichannel analysis

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- Imaris has several tools to quantify and visualize colocalization
  - Each addressing specific hypotheses
- Which method is right for you?
  - Do you want to visualize the colocalization in 3D space?
  - Ultimately what data do you want to export?
    - Colocalization statistics (threshold method)
    - Counts (association/overlap of different puncta)
    - Surface to surface colocalization
    - Surface/Filament to spot relationships

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Key Features of ImarisColoc

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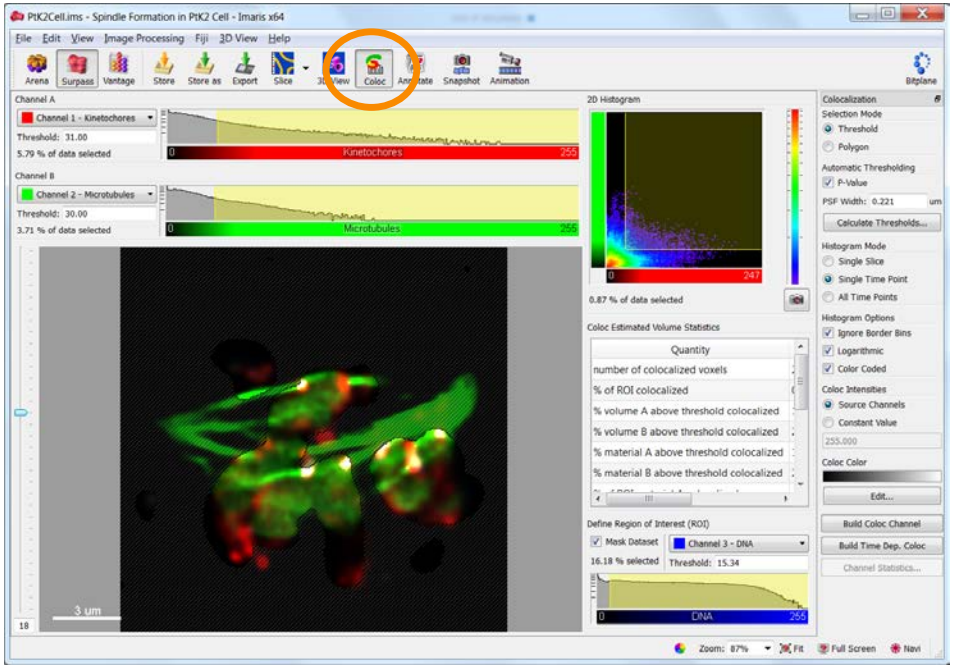

- ✓ Automated, standardized determination of colocalized voxels.
- ✓ Processes 2D, 3D, and 4D images.
- ✓ Real-time feedback on changes in selection.
- ✓ Display of colocalized voxels with original channels as defined in channel visibility editor.
- ✓ Output of colocalized voxels into new channel allows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

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Acquisition parameters

- Adequate signal/noise
- Consistent microscope settings (laser power, PMT gain, exposure time, etc.)
- Avoid saturated pixels
- 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.

Two primary kinds of colocalization

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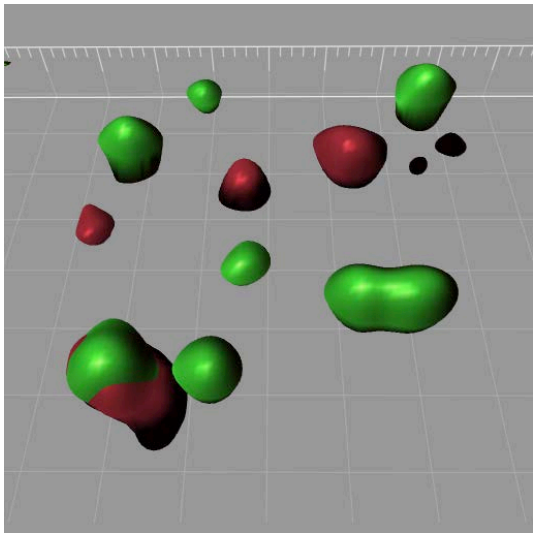
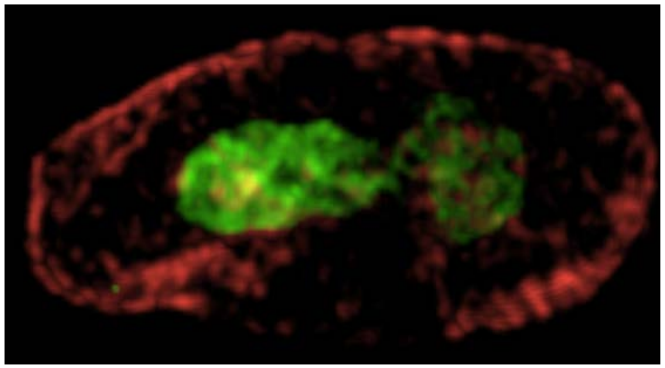
- Object-Object
  - Two distinctly labeled structures or puncta



- Protein-Protein spatial colocalization
  - No specific labeled structure
  - Two fluorescent markers are diffusely distributed



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APPROACH I: Protein-protein association

Based on Costes et al., Biophys J 86(6): 3993-4003

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- In case of quantifying the amount of co-localization between two diffuse protein patterns, an Automatic Thresholding technique is ideal:
  - No clear object delimitation – morphology is not a useful reference for threshold evaluation
  - Thresholding based on independent foreground/ background or shape evaluation of each channel doesn't work anymore.

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Step 1: Region of Interest

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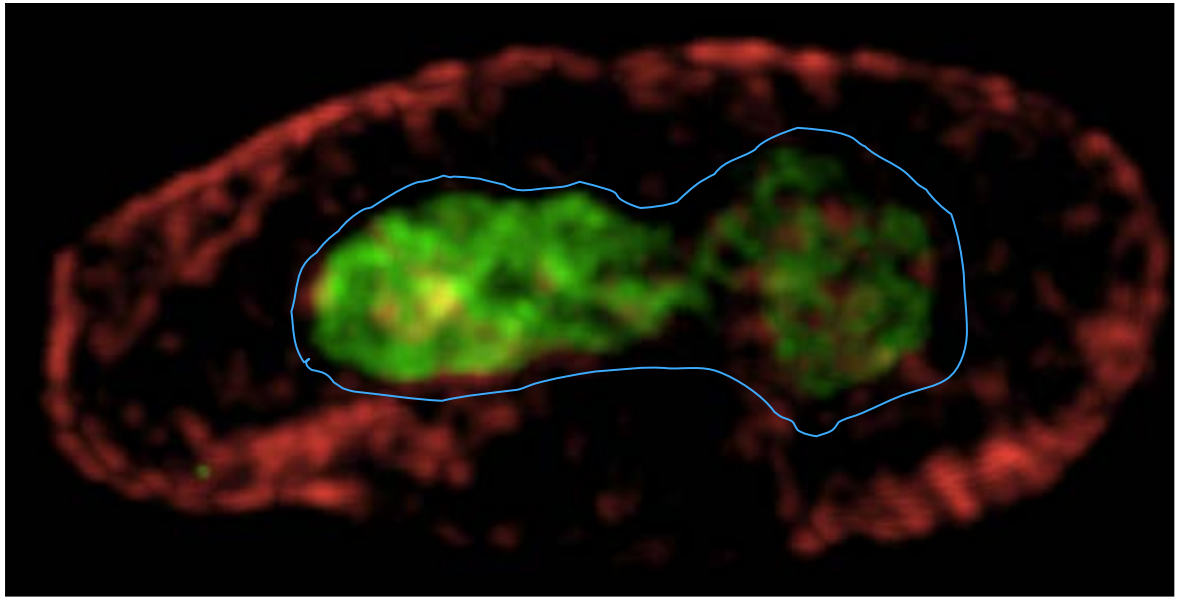
The automated colocalization analysis takes into account the whole image. For accurate results you should define a region of interest in order to ignore background



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Step 1: Region of Interest

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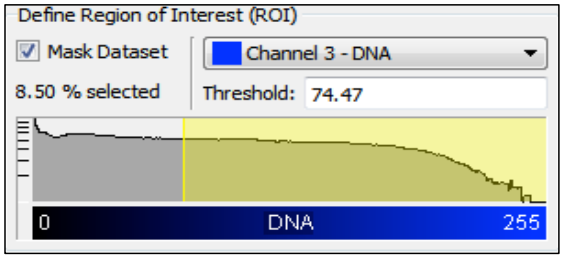
- Options:
  - Acquire a 3rd channel in which the labelling represents the Region of Interest
    - If the entire channel isn't specific enough, create a surface (intensity based or new manual methods) so you can use only part of the channel by masking with the surface
  - Draw a manual Contour Surface and use it as a mask to create an ROI channel
    - Duplicate (any) channel before applying mask
    - Set outside to 0, inside to any mid-range value
  - Use ImarisXT (Channel Arithmetics) to merge the two channels into one mask channel

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



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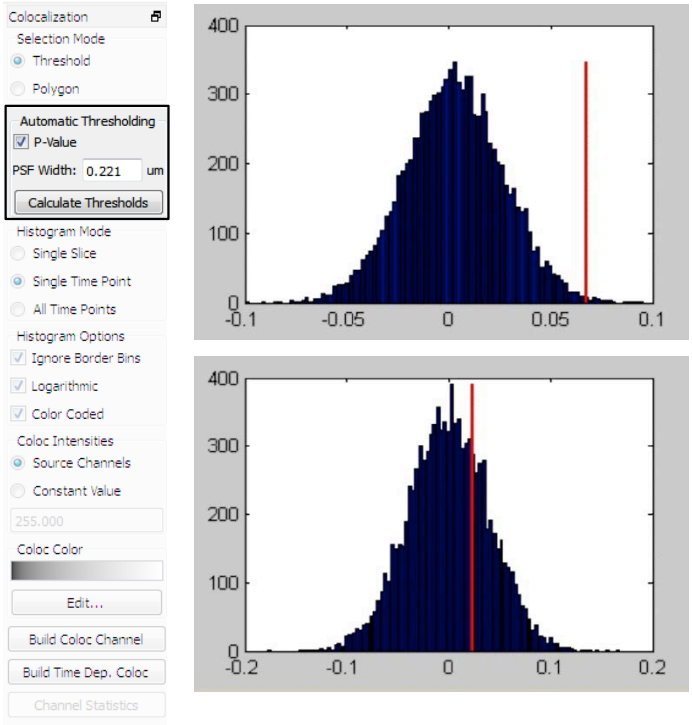
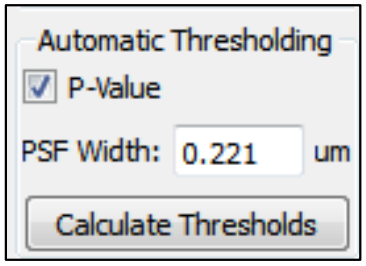
Step 2: Pretest for automatic thresholding

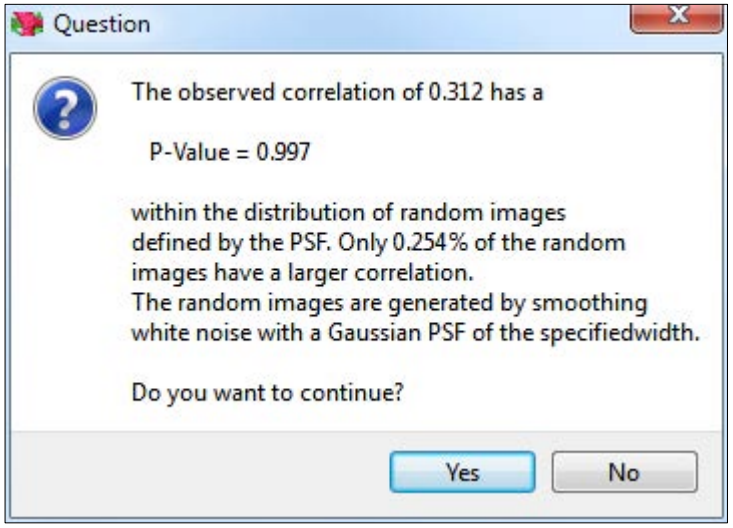


Automatic and Quantitative Measurement of Protein-Protein Colocalization in Live Cells
Sylvain V. Costes, Dirk Daelemans, Edward H. Cho, Zachary Dobbin, George Pavlakis, and Stephen Lockett
Biophys. J. 2004 86: 3993-4003

- 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 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).

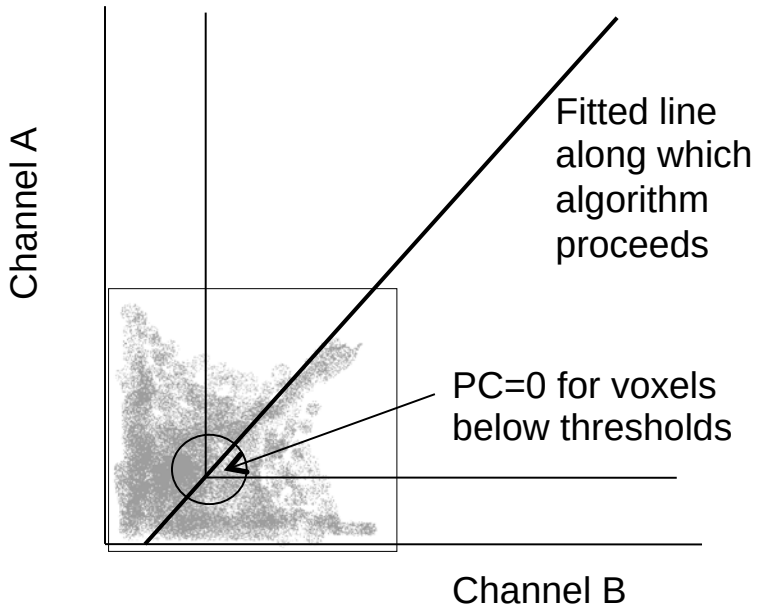
Step 2: Pretest for automatic thresholding





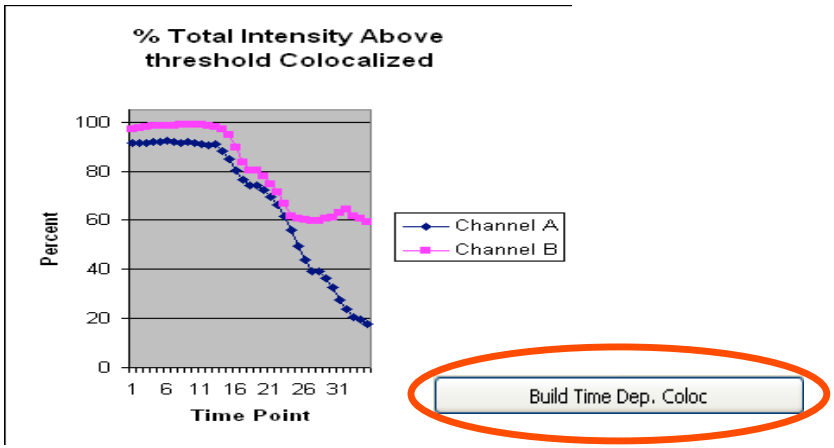
- P-value depends on PSF Width (default is just a “guess” based on voxel size only (longest diagonal of 1 voxel)
- Specific, non-random colocalization depends on resolution (both PSF size and voxel size), and signal to noise
- deconvolution may help

Step 3: Automatic threshold



- The algorithm developed by Costes and Lockett (NCI/NIH) 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 to the left and computes the correlation coefficient of the image using only voxels with intensities below the threshold. The algorithm continues reducing the thresholds until the correlation reaches 0, thus defining the automatic threshold

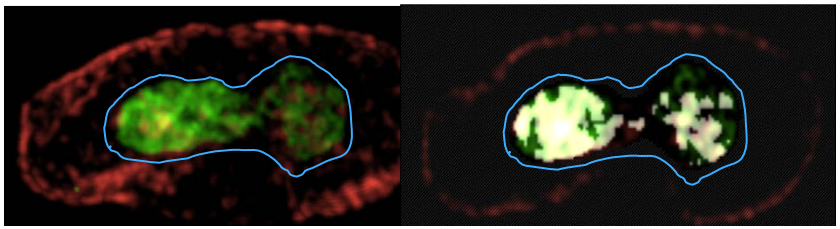
Special case: Time dependent Auto-Threshold

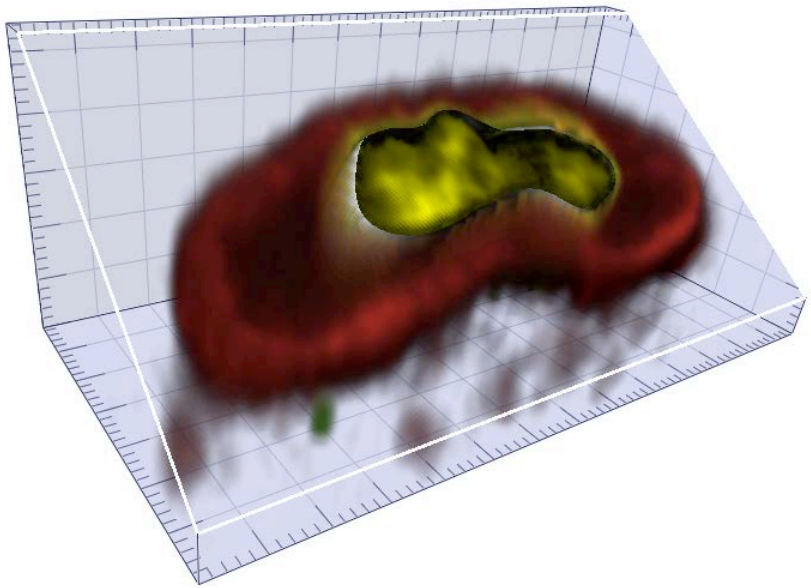


	A	B	C	D	E
1	time frame	1	2	3	4
2	threshold A	50	50	49	50
3	threshold B	25	22	18	16

- 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

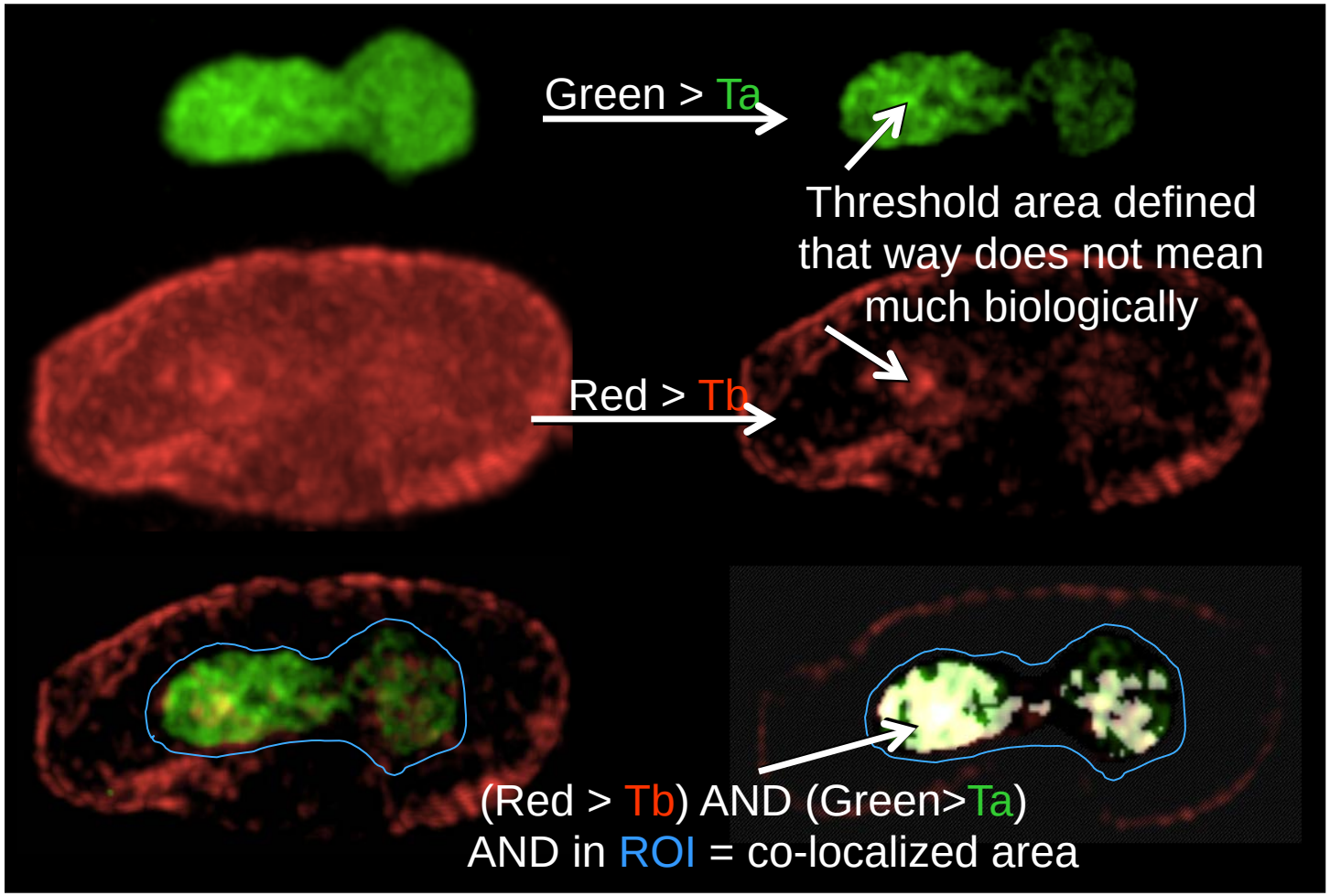
ImarisColoc: Scientific Cooperation





- ImarisColoc – especially the implementation of the automated thresholding – was developed together with Dr. Sylvain Costes, today working at the Lawrence Berkeley National Laboratory
- Dr. Costes accepted to share yet unpublished results and methods with Bitplane and we entered a very fruitful co-operation implementing some of his algorithms for ImarisColoc in 2003.
- 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.

Illustration



Blue shows ROI

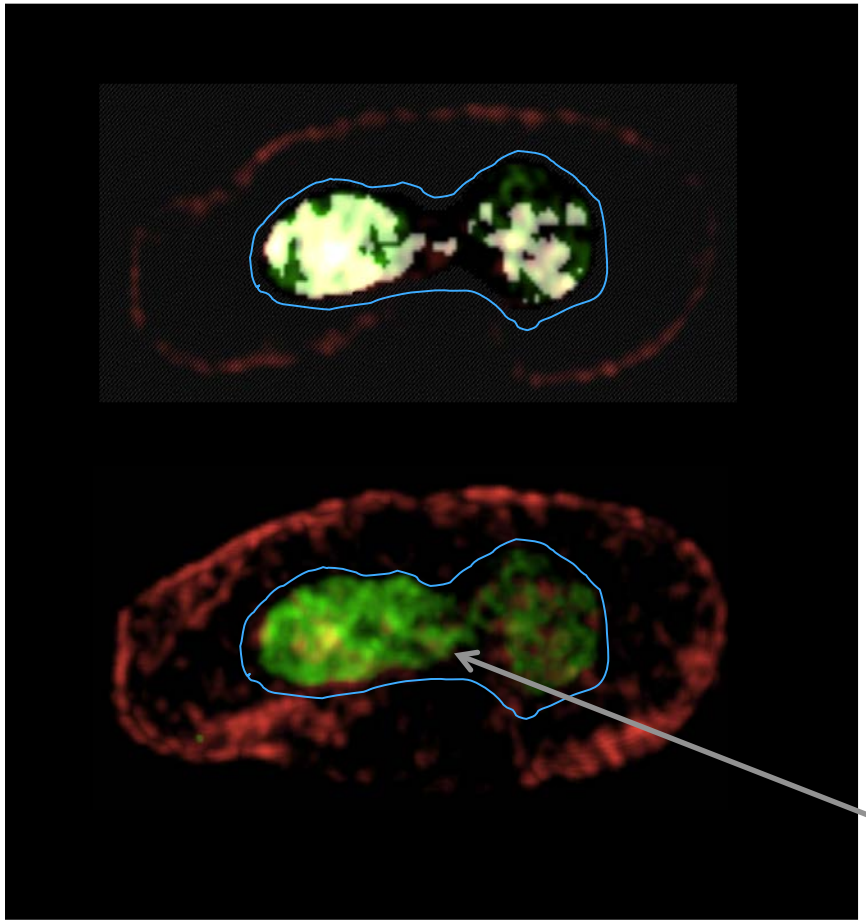
Top row: Green pixels before and after Ta

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

Percent ROI Material



- % of ROI Material A colocalized = Sum of green intensity in colocalized (white) area/ Sum of all green intensity inside ROI
- % of ROI Material B = Sum of red intensity in colocalized (white) area/ Sum of all red intensity inside ROI
- Above-mentioned values include intensity below Ta and Tb from the ROI (in the denominator). To exclude voxels of the ROI that are below Ta and Tb from the denominator, use “% material above threshold” instead (although title doesn’t mention ROI, it’s being applied).

Even voxels below threshold are in the denominator, provided they’re contained by the ROI

APPROACH II : distinct objects

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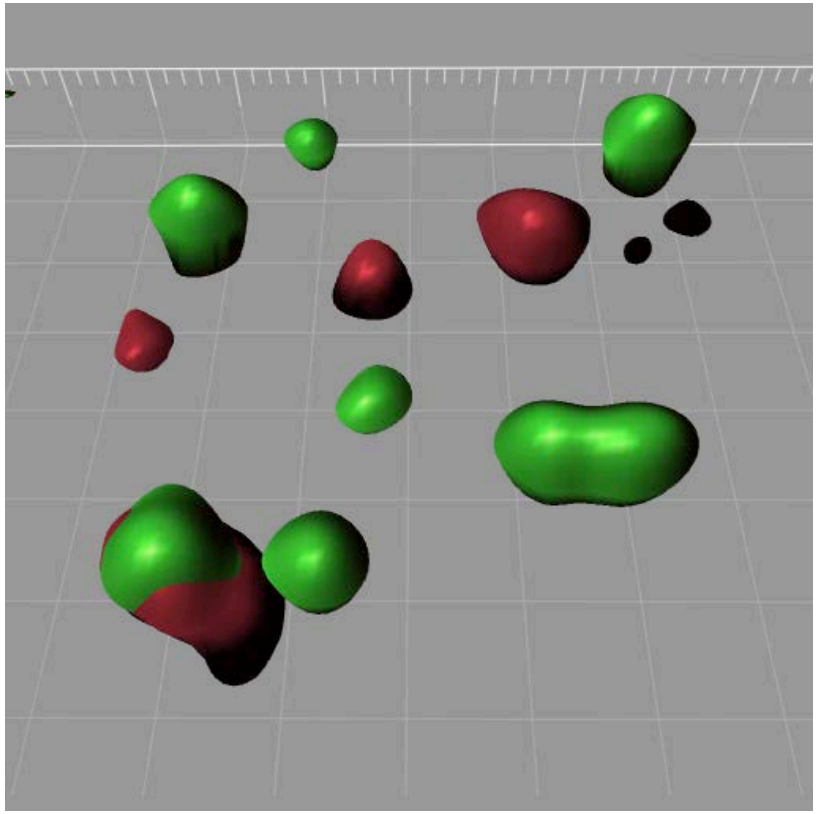
- Assume Channel A and Channel B are green and red objects, respectively
- Define threshold values Ta and Tb that segments each object
  - Intensity above threshold can then be interpreted as the object of interest
  - Automatic Thresholding based on Costes et al. is generally not used – signals A and B do not correlate well, because the differing structures are resolved
- Overlap volumes contained by ROI are then considered colocalized volume



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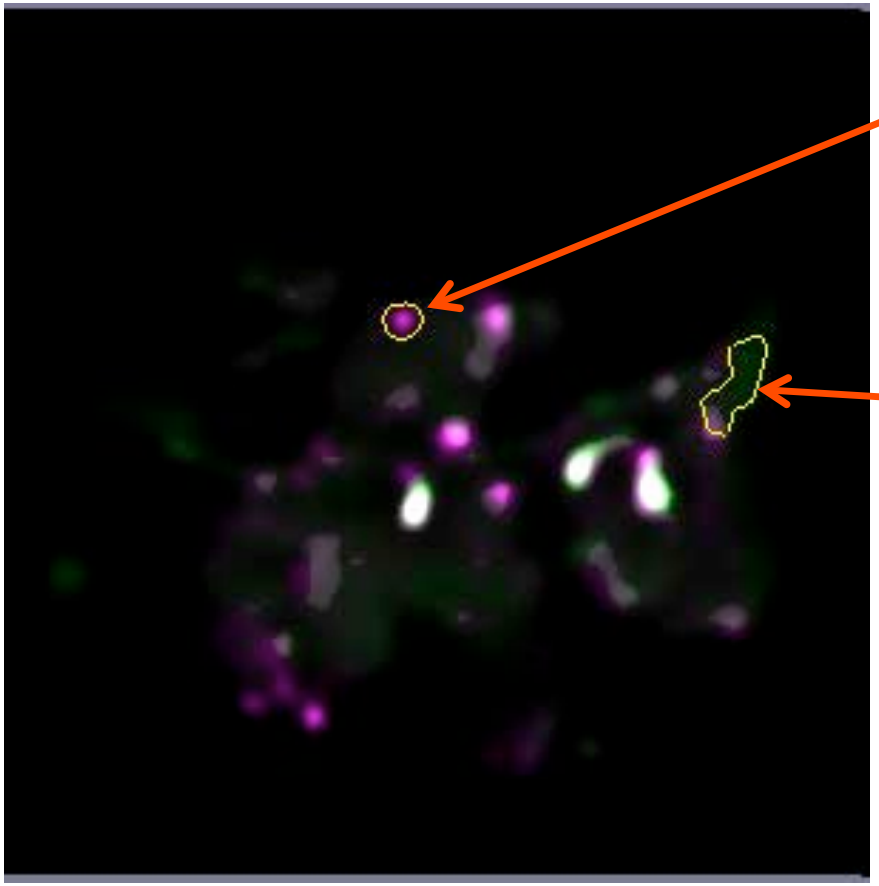
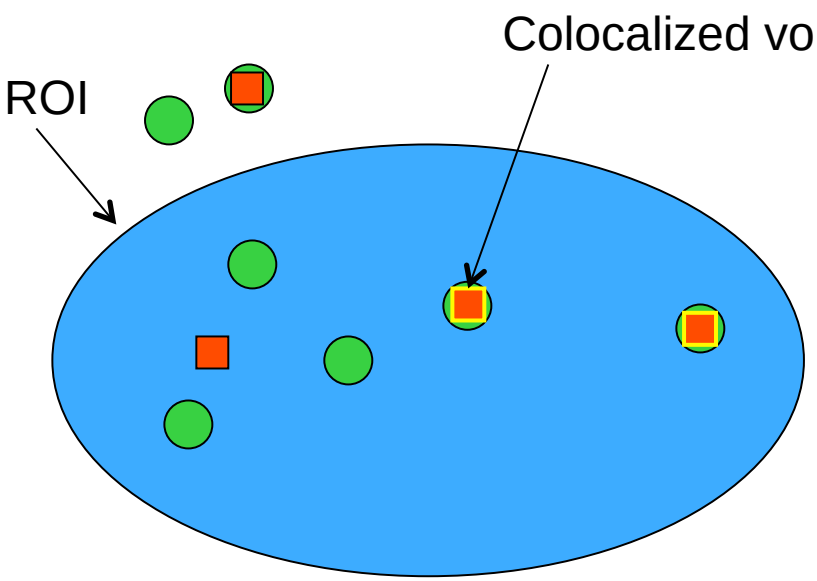
Manual Thresholding



- An IsoLine tool is used to visualize the area above the threshold next to the mouse
- Click on bright and drag toward dark to set Channel A threshold
- Hold Shift Key and do same to set Channel B threshold

Percent Volume above Threshold

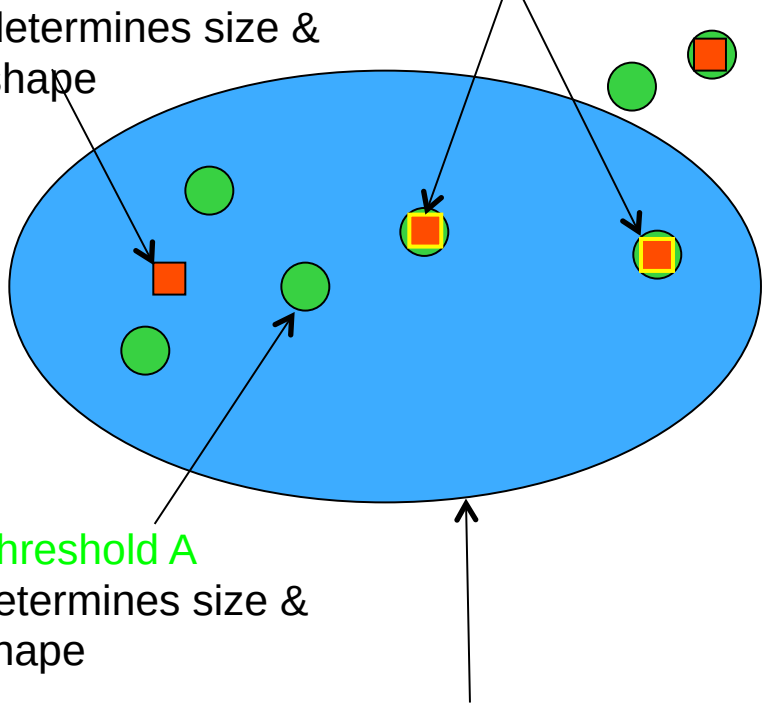


% ROI Colocalized =
colocalized volume / Full ROI volume
= very small number in this case

% volume A colocalized =
colocalized volume / volume of green objects *inside ROI* ~ 2/5

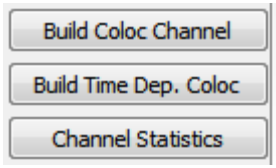
% volume B colocalized =
colocalized volume / volume of red objects *inside ROI* ~ 2/3

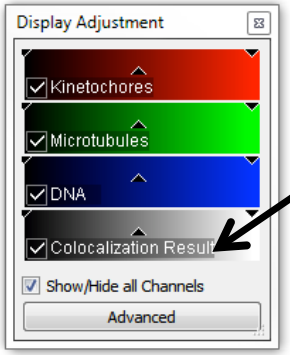
Percent Material above Threshold



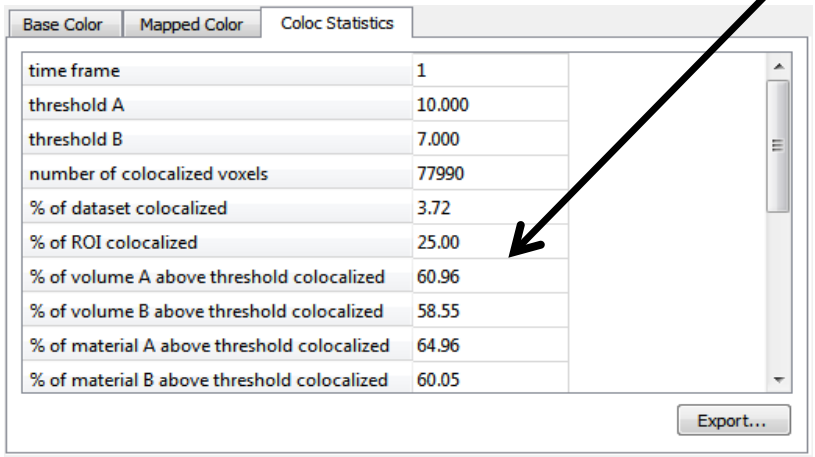
- % Material A above threshold colocalized = Sum of A intensity in colocalized volume / Sum of A intensity in all the circles contained by ROI
- % Material B above threshold colocalized = Sum of B intensity in colocalized volume / Sum of B intensity in all the squares contained by ROI

Colocalization Result





Colocalization result can be visualized and measured just like any other data channel



Don't use the preview statistics in Coloc viewer, click "Channel Statistics" for the final values, and for export

Imaris Xtensions

- Colocalize spots
- Spilt spots into surfaces
- Distance Transform
- Spots close to surface
- Spots to spots closest distance (nearest neighbor)

XTension: Colocalize Spots

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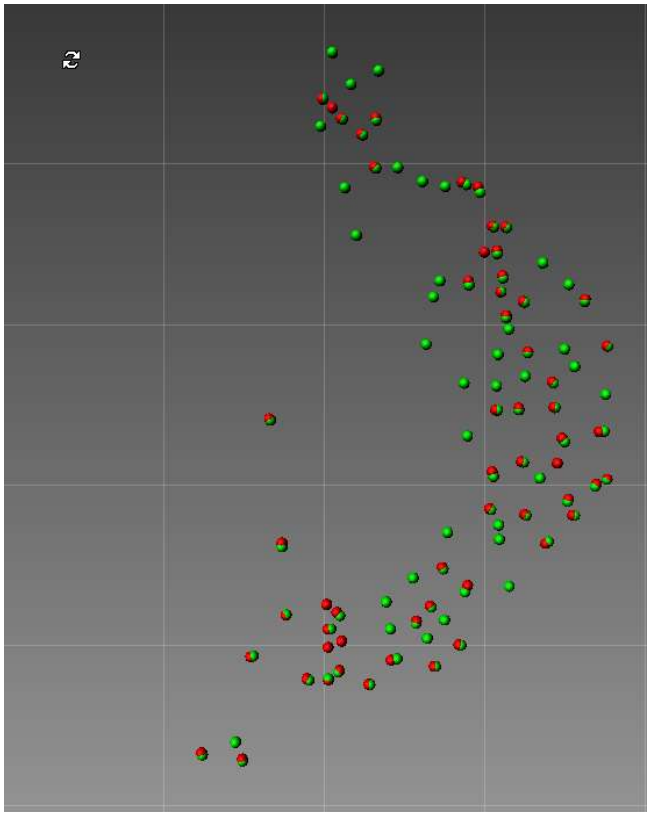
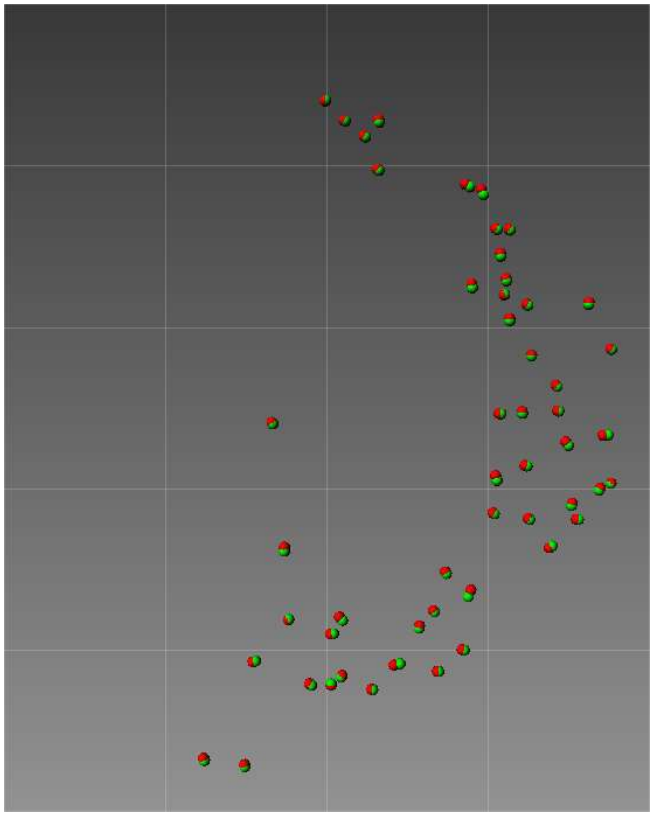
All red and green spots



Colocalized spots (within 0.5 um)



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XTension: Spilt Spots into Surface Objects

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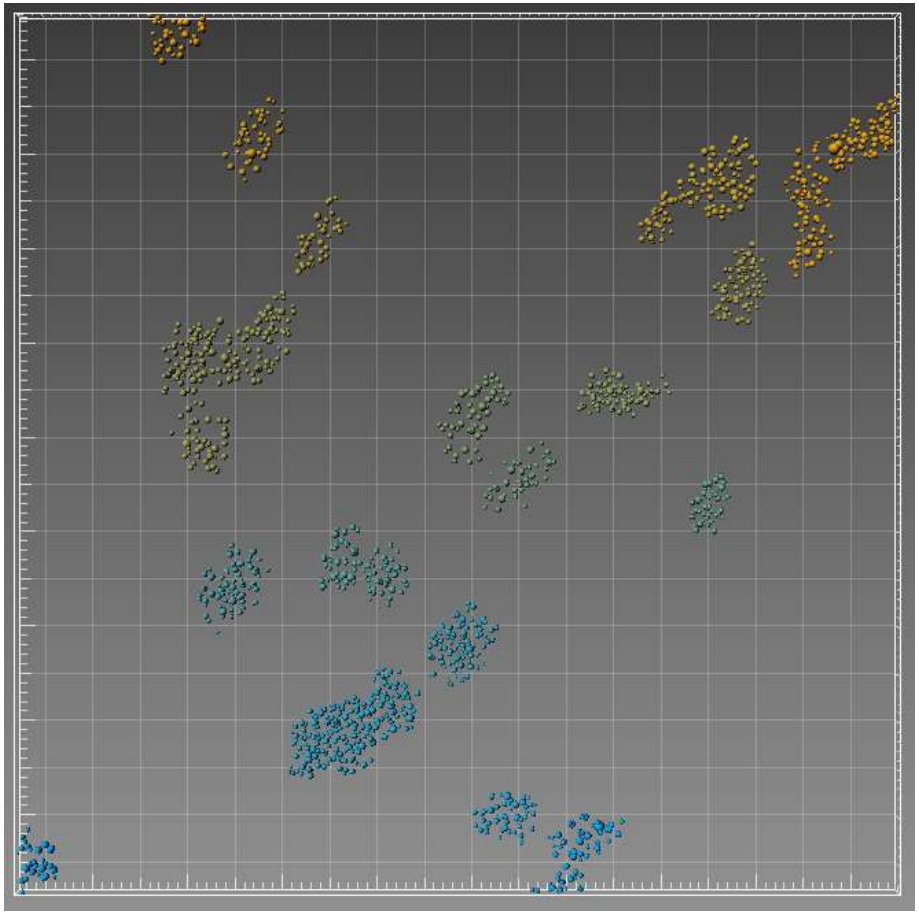

- This tool is able to segment spots that lie within a surface volume
  - It generates a new spots object for each surface
  - Quantification of #spots/cell
- If a Spot object lies outside one of the isosurface volumes, it will not be counted.

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XTension: Spots Close To Surface

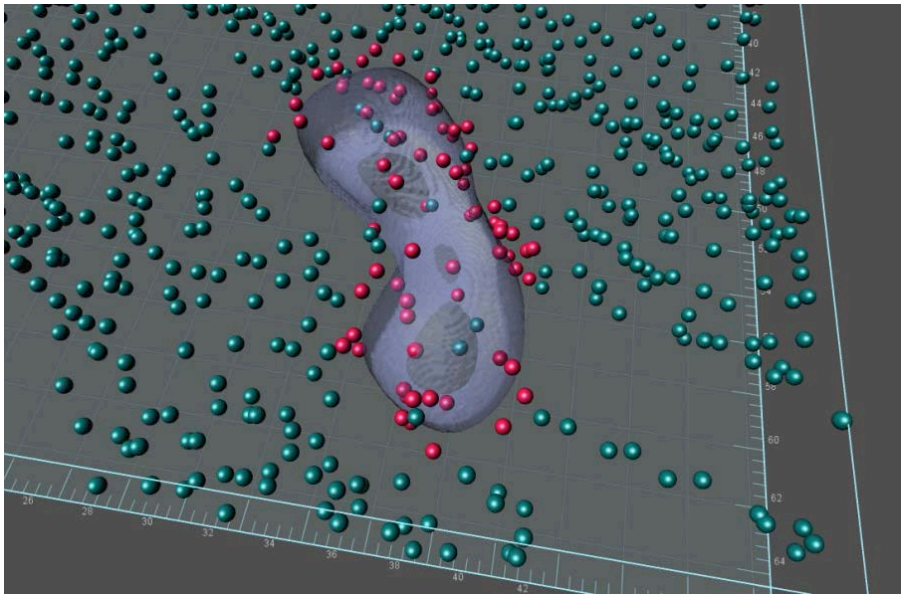

Image courtesy of Dr. Marc Landry, Bordeaux

- Separates those spots in an Imaris Spot component that are close to the specified surface
  - Usage:
    - Neuron plus postsynaptic markers (such as PSD, or pre/ post synaptic receptors)
  - Result:
    - Spots are segmented and recolored that are within the specified distance of the surface object

Manders Coefficients

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- "Original Mander's A" =

  (Total A intensity in all voxels where B > 0) / (Total A intensity)

  - vice versa for "Original Mander's B"
  - In literature often named M1, M2

- "Thresholded Mander's A" =

  (Total A intensity in all voxels where B > threshold) / (Total A intensity)

  - same as above, but only within the region defined by the coloc threshold
  - vice versa for "Thresholded Mander's B"
  - 0 no colocalization, 1 perfect colocalization

Manders, E., Stap, J., Brakenhoff, G., van Driel, R., and Aten, J. (1992). Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. J Cell Sci 103, 857-862.

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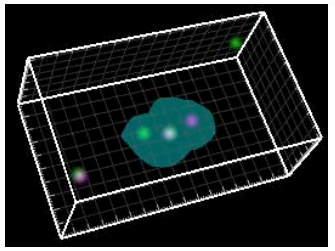
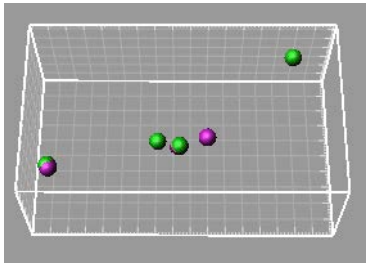
Exercise 1



- Load ‘desktop’/data/Exercise Coloc/SyntheticColoc.ims
  - Analyze as if you’re looking for overlap between Ch 1 and Ch 2 within the Ch 3 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 3 Spots objects from Ch A, Ch B, and Colocalization Result Channel
  - Color each Spots object differently
  - Save the Surpass Scene file



Exercise 2

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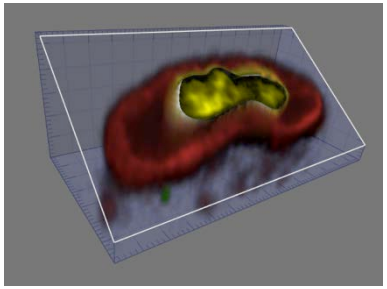
- Load ‘desktop’/data/Exercise Coloc/Costes-Crop-12T.ims
  - Create an ROI that is only from the largest green object (be careful to exclude any bright green signal outside that central object)
  - Set the thresholds A & B in an appropriate manner for this image
  - Export the Coloc Statistics to a .csv file
  - Create a 3D scene in Surpass to visualize the results. Be creative, there are many possibilities for using different objects here (Surfaces, Volume, Clipping Plane, etc.).



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