

Gonad Analysis Pipeline
Step-by-Step Protocol
Libuda Lab
Date: 11/16/2020

Contents:

[Section I: Stitching in FIJI](#)

[Section II: Creating Surface objects to identify nuclei](#)

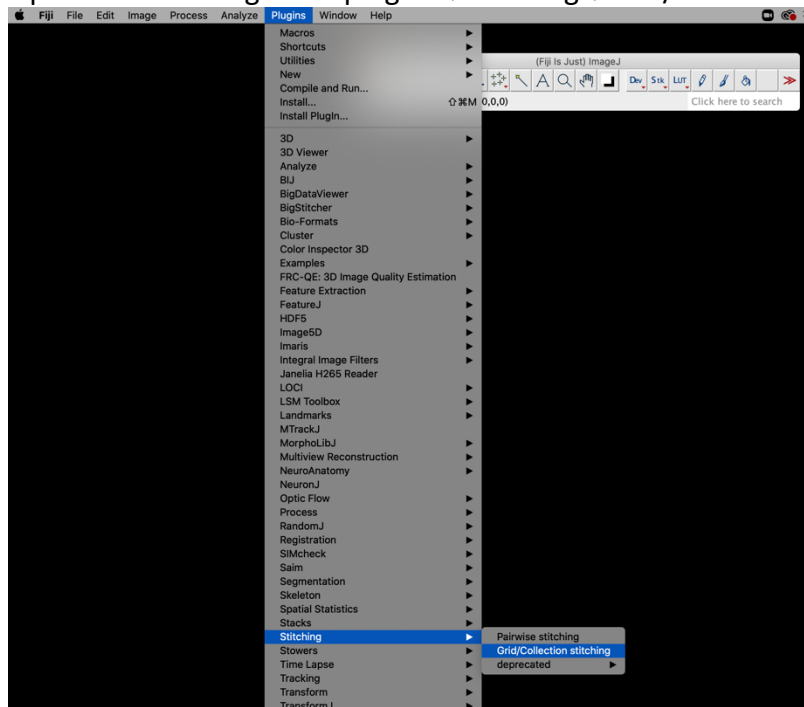
[Section III: Creating Spots objects to identify foci](#)

[Section IV: Creating colocalized surfaces](#)

[Section V: Linearization of nuclei](#)

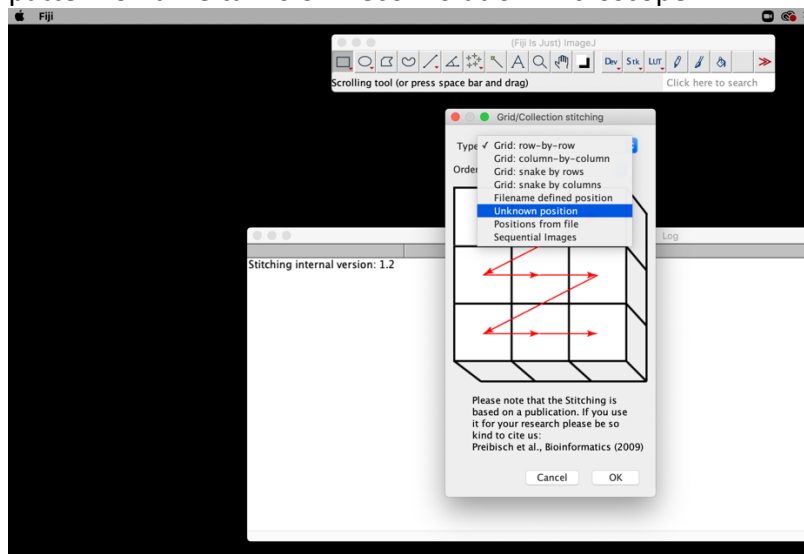
Section 1: Stitching in FIJI

1. Open FIJI and navigate to plugins→Stitching→Grid/Collection stitching

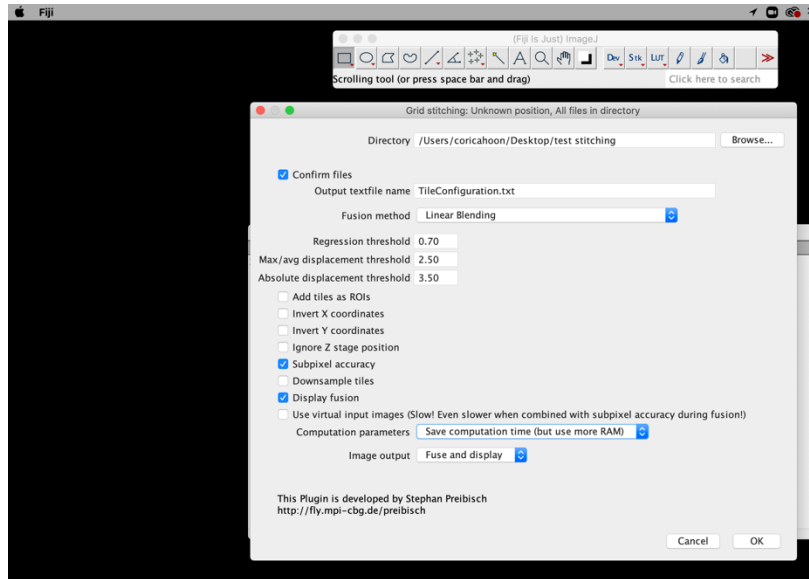


2. Click on the drop-down menu for “Type” to change to “Unknown positions” and click “ok”.

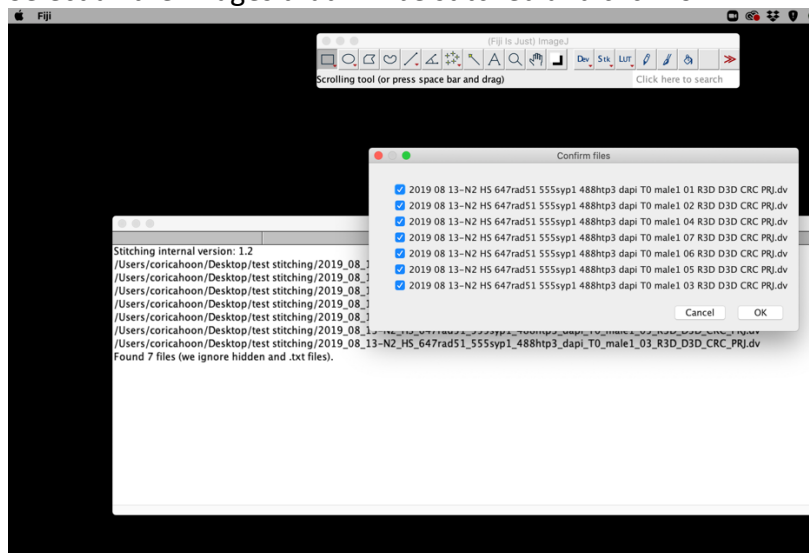
*Note: These options refer how the images were collected and depending on the acquisition and microscope other options in this dropdown menu may work better. We collect images in a non-grid pattern on a Delta Vision Deconvolution Microscope.



3. Enter Directory path to the file containing the images. The “Browse...” button can be used to navigate to the image file. In the screenshot below are the settings we use for stitching. A wiki page with extensive information about each of these settings can be found here https://imagej.net/Image_Stitching.

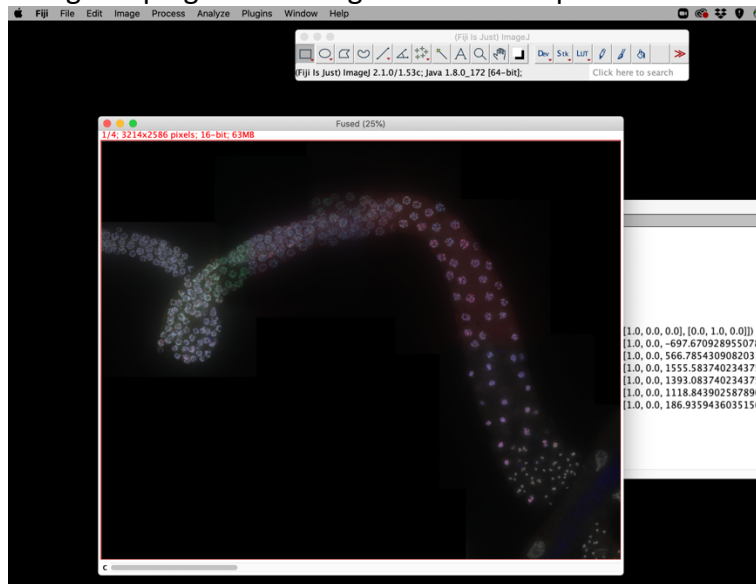


4. Select all the images that will be stitched and click “ok”.



5. Stitched image will be displayed and can be saved to import into imaris as a TIFF or using the imaris plugin “image to imaris” (can be found under plugin→Imaris→Image to Imaris)
 *Note: if the image does not stitch properly after adjusting settings in step 3, using the pairwise plugin instead of the grid/collection stitching option in step 1 works well.

Using this plugin each image is stitched in pairs of two instead of a large group of images.



Suggested FIJI modules to look into for 3D image thresholding, nucleus segmentation, spot maxima identification and generating the spots per nucleus: 3D ImageJ plugins library and MorphoLibJ suite in the IJPB-plugins library (note: we have not thoroughly tested out the functionality of these modules for the purpose of *C. elegans* germline nuclei quantification).

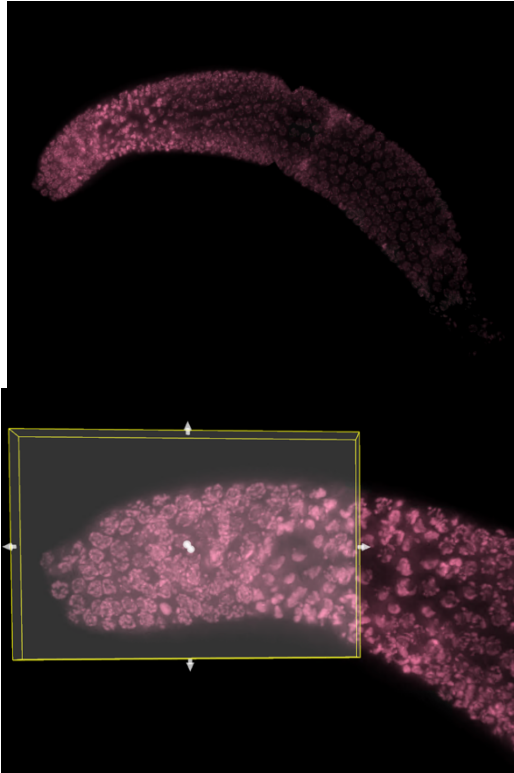
Section II: Creating Surface objects to identify nuclei

Create surface objects:

1. Open an image in Imaris using the 3D view window



Immunofluorescence image of an extruded *C. elegans* gonad, oriented with the pre-meiotic tip on the far left



2. Select Surface object creation tool



Creation wizard step 1/5

- Select “Segment only a Region of Interest”

-Nuclei in each region of the gonad have different volumes, which makes creating a single set of surfaces for all nuclei across a whole gonad impossible.

- Individual regions of interest allow for surface creation parameters to be tailored for each section and accurately model nuclei morphology.

-we begin by creating objects pre-meiotic tip and name each section sequentially (e.g. this section will be named Section 1).

Creation wizard step 2/5

- Select the source channel off which you will build the surface objects
- Smoothing should be selected, the default value will be 0.137
- Choose a thresholding method (Absolute intensity or Background subtraction based on local contrast)
 - this choice is based on image quality and personal preference. We use Background subtraction by default.

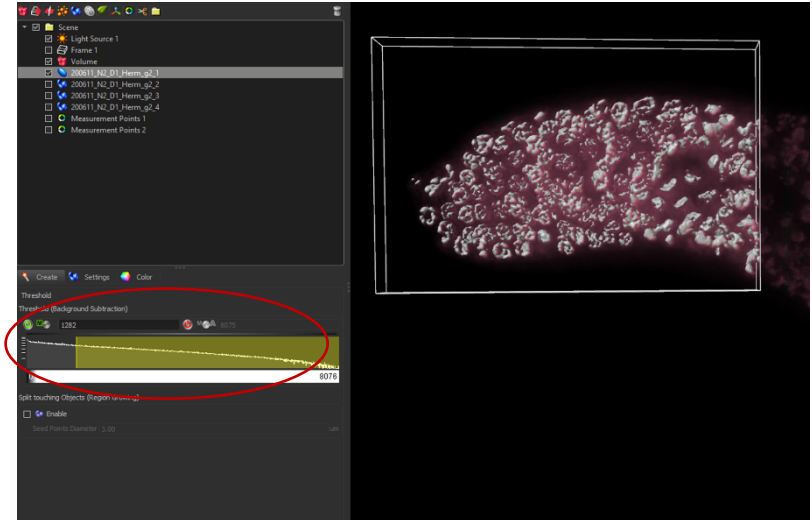


- Estimate the XY-diameter of your nuclei using the “Slice” window



Creation wizard step 3/5

- **Thresholding:** Here you assess whether you are appropriately capturing spots surfaces based on your channel of choice.
-comparing the channel of choice alone to the surface objects being created is a good way to assess this
- **Manual thresholding:** If the automatic thresholding setting is not appropriately capturing spots then you can shift to manual thresholding by dragging the edges of the highlighted box on the histogram to incorporate more or fewer surface objects. When this is done the lighted “A” button (for automatic) will switch over to a lighted “M” (for manual). To toggle back to the automatic setting simply click the button between “M” and “A” again.
- **Split Touching Objects (Region Growing):** This setting can be used to automatically separate objects which are touching and might otherwise be considered a single object. Imaris will try and parse apart touching objects, using the “seed point diameter” submitted as the cutoff object size.
-selecting this option will add an additional thresholding step to the wizard



Creation wizard step 4/5

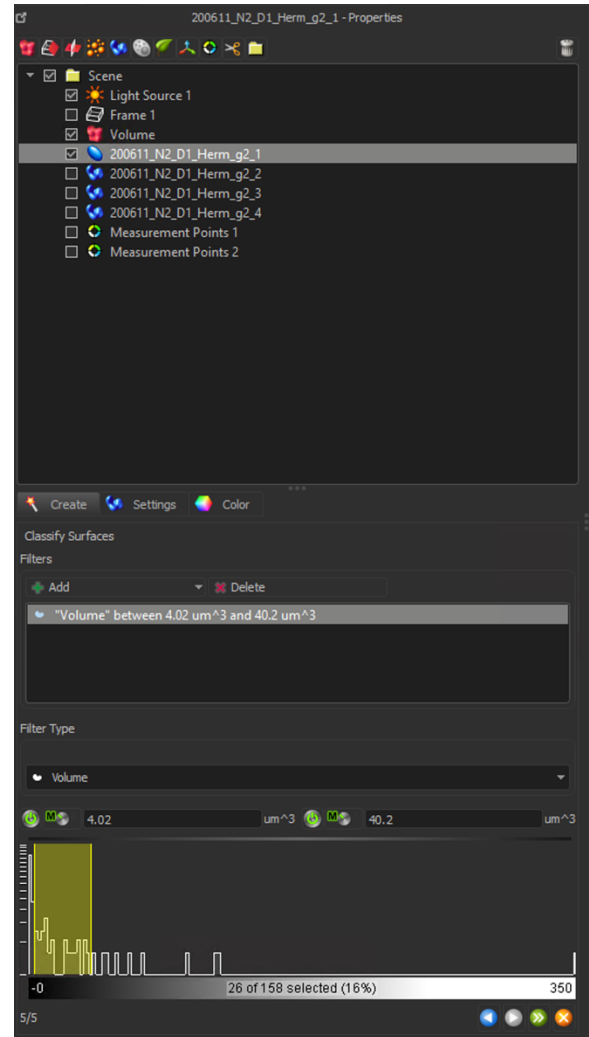
- Surfaces are then filtered for quality.
-we commonly use a Volume filter to exclude partial nuclei and surfaces made of combined nuclei.
-after object creation objects can also be manually combined (in the case of disconnected components of a single nuclei) and can also be split (in the case of combined nuclei)

To build surfaces based on nuclei in various regions of the gonad we used the parameters included in this table. Experiment with parameter settings so that they best fit your needs.

Parameter	Early Pachytene (small nuclei)	Late Pachytene (large nuclei)
Smooth	0.15	0.15
Background	3.5	4
Thresholding	Manual adjustment of bar on histogram	
Seed Point Diameter	2-3	3-4
Volume Filter*	8-55 (adjust as needed)	10-50 (adjust as needed)

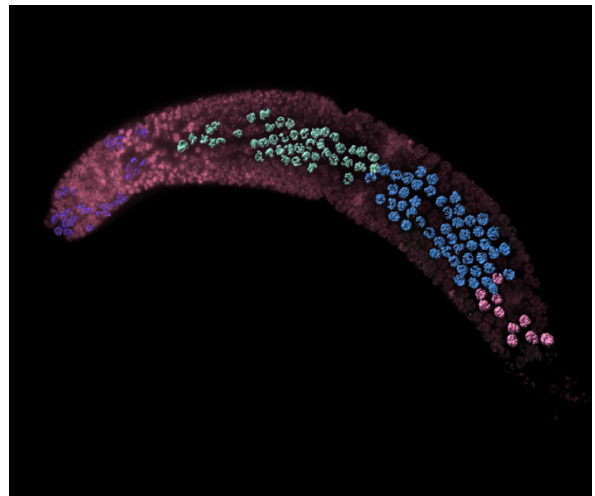
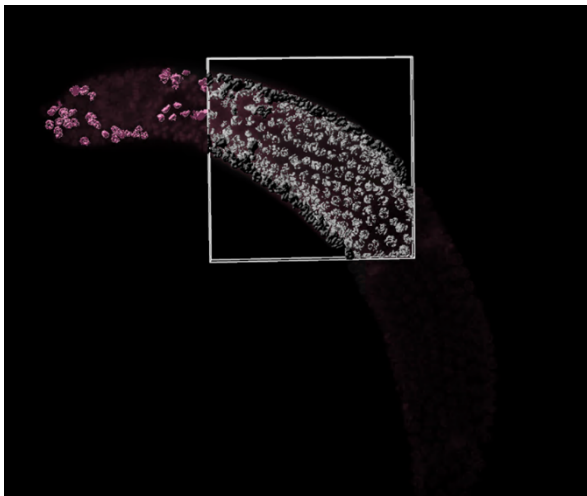
Creation wizard step 5/5

- Surfaces can then be sorted or classified by various input, including Diameter, signal Intensity (mean, med or sum), Position, Area or Volume.
 - Spots will be color coordinated to indicate classification groups
 - We do not commonly use this
3. Naming surface objects:
 - Using a standard naming scheme is recommended in order to more effectively organize and access resulting data
 - Our standard naming convention is yrmoday_strain_sex_treatment_gonad#_stage_# of nuclei included in section (e.g. 18918_N2_male_hs_g07_1)
 4. After the first section of surface objects is finished, continue creating sections until you have reached the end. It is important that when starting the next section **do not overlap** with the previous section (as overlap creates duplicate surfaces for individual nuclei)



Cropping individual surface object sections:

1. Depending on the computational capacity of your computer it is best to crop these sections individually and SAVE AS NEW IMAGE as to not overload the software.



- The objects when cropped will maintain their xy coordinates but the original image will have to be referenced for measurement statistics.
* If only a few surfaces are being analyzed (~20-50) you can skip cropping it all depends on your computer.

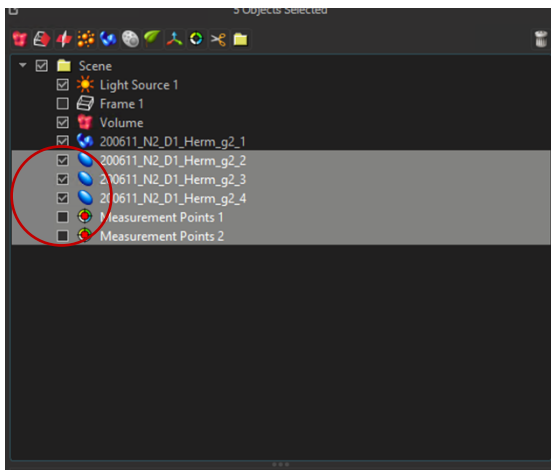
- Naming convention: same image name + name of section you are analyzing

Ex. If the original image was named

200611_n2_d1_herm_rad-51_488_dsb2_555_g2_r3d_d3d_crc_STITCHED

and the section you are now cropping down was section 1 save the new image file as

200611_n2_d1_herm_rad-51_488_dsb2_555_g2_r3d_d3d_crc_STITCHED_1.



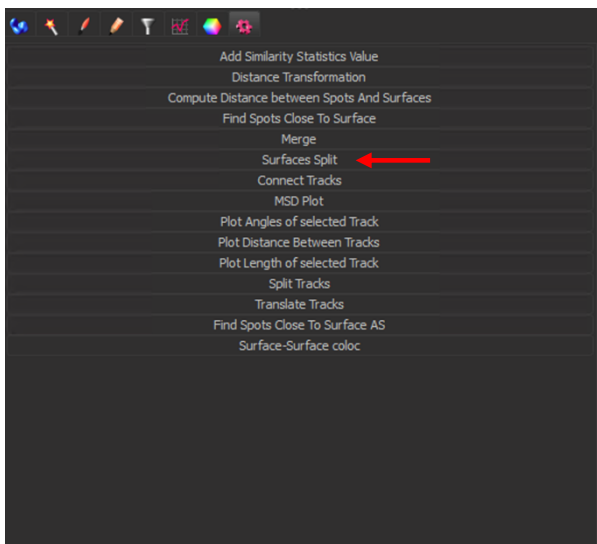
- Once the new image file is saved, highlight and delete all objects/sections you are not working on. This increases the processing speed.

- To crop section you will be analyzing press CTL + W and select the surrounding area of Surface Object

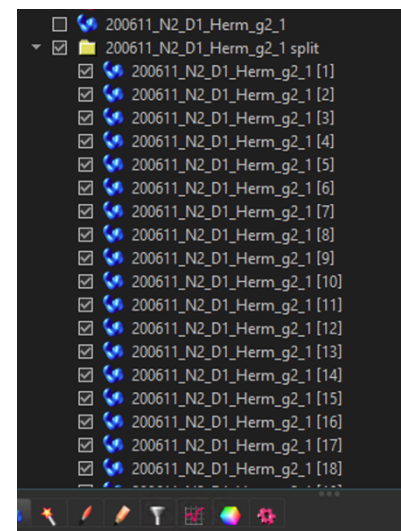


Splitting surface objects into individual nuclei surfaces and associate with spots objects (see Section III for Spots objects creation):

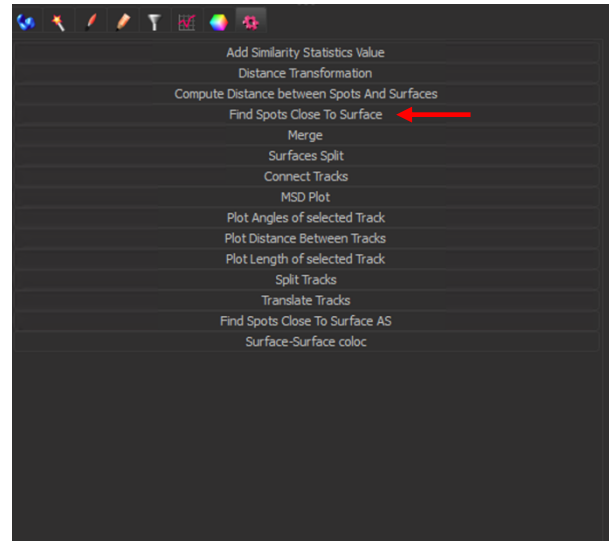
- To split the overall object into individual nuclei surfaces, select the object and click Surfaces Split.



- A matlab window and script will pop up and process.
- Now each nuclei surface has an individual ID to reference and it's own statistics.
- repeat these steps with the remaining sections generated in the gonad.

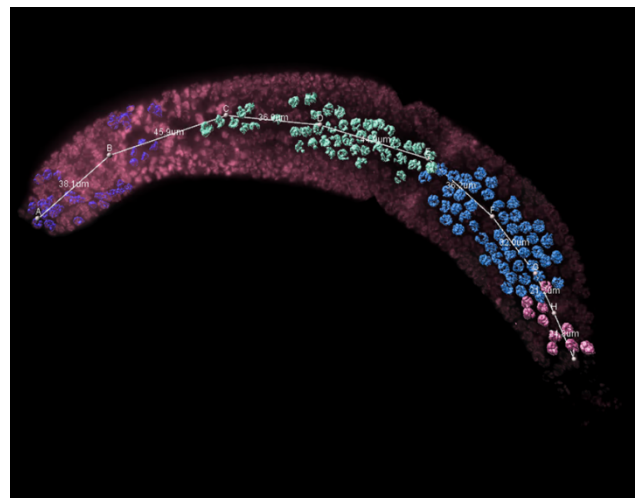
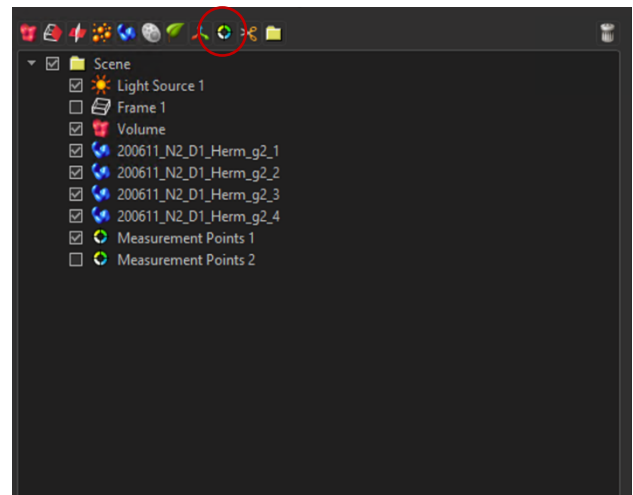


2. Associate individual nuclei surfaces with spots
 - Use “Find Spots Close to Surface” tool
 - A matlab window will pop-up
 - Select the surfaces you would like associated with spots (we select all of the split/numbered nuclei surfaces)
 - Select the spots object you would like associated with nuclei surfaces
 - Input a threshold number when prompted (the max distance (um) that the objects can be apart while still being considered “close to” or associated with each other)
-we use 0.1



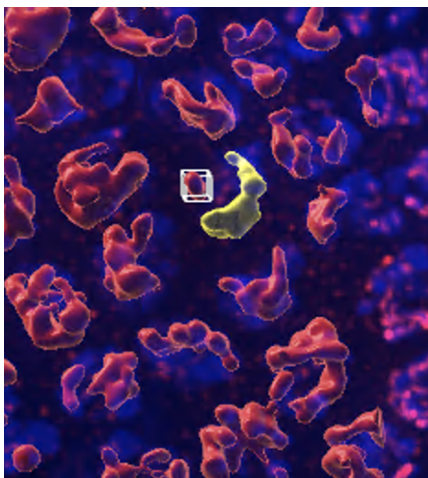
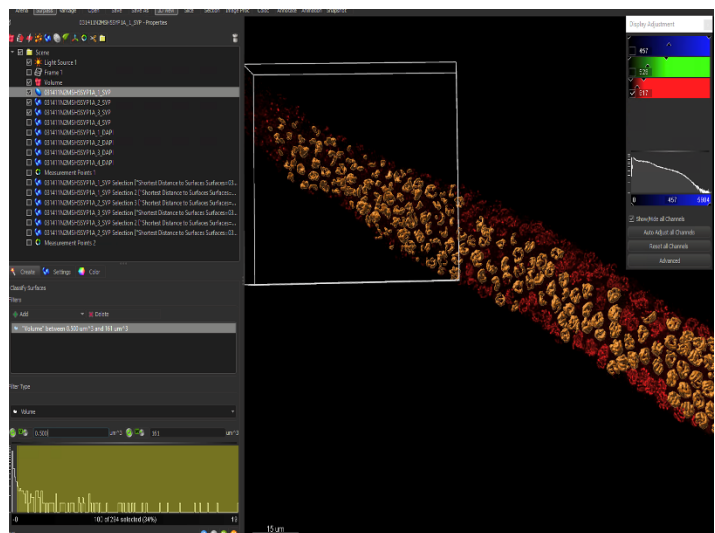
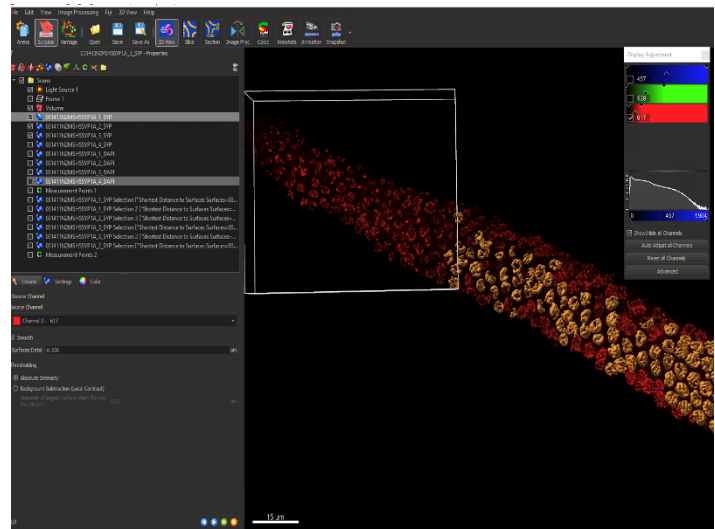
Measurement points throughout gonad/image:

- In order to use the whole gonad linearization algorithm (see Section V) it is important to draw a measurement points through the center of the gonad.
 - Open the original image with all sections included.
1. Select the Measurement Points tool and highlight. Press the Shift Key and left click your mouse to put down the first point, continue to hold down the Shift Key and put down as many additional points that go through the center of your image and conform to the shape as accurately as possible.



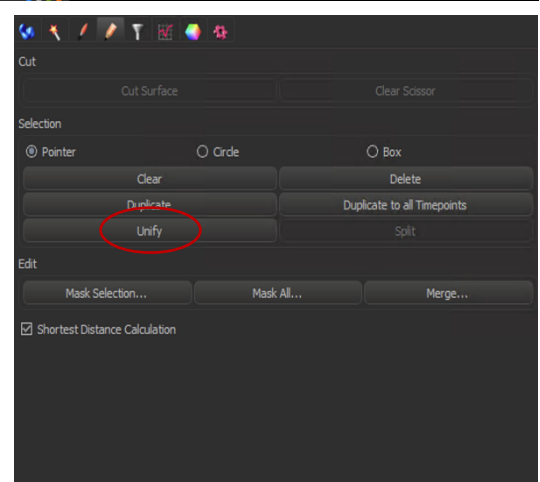
Section II b. Creating surfaces with more detailed structures (e.g. surfaces built from synaptonemal complex components like SYP-1)

1. Create a new surface object
 - Select “Segment only a Region of Interest”
 - In this example we start at the onset of SYP-1 staining and use parameters that most accurately fit the signal within that region (we use Smooth 0.22 um + Absolute Intensity).
 - Threshold accordingly to include most surfaces generated (e.g. 0.5um and above).
2. After the first section is finished move on to the subsequent sections and verify that there is no overlap between sections.
3. Return to each section to check the quality of objects generated.
 - Scan the surface objects for incomplete/fragment surfaces and fused surfaces



- Multiple small fragments of a surface object that were not originally captured as one single object can be selected and unified as one.

- After we unify objects as needed, we then delete objects that are partial.



Filtering touching/nearby objects

If surfaces have been generated in two separate channels, surface objects that are touching can be filtered and duplicated into a new surface object for further analysis.

- Example: If the aim is to only analyze surfaces where two different signals/channels are present (e.g. only DAPI-stained nuclei where the synaptonemal complex component SYP-1 is also present) then this protocol can be used to create a subset of DAPI-based surfaces that also have SYP-1

1. Select a surface object already generated

Click on the Edit icon  and check the box marked “Shortest Distance Calculation”

2. Select the Filter icon 

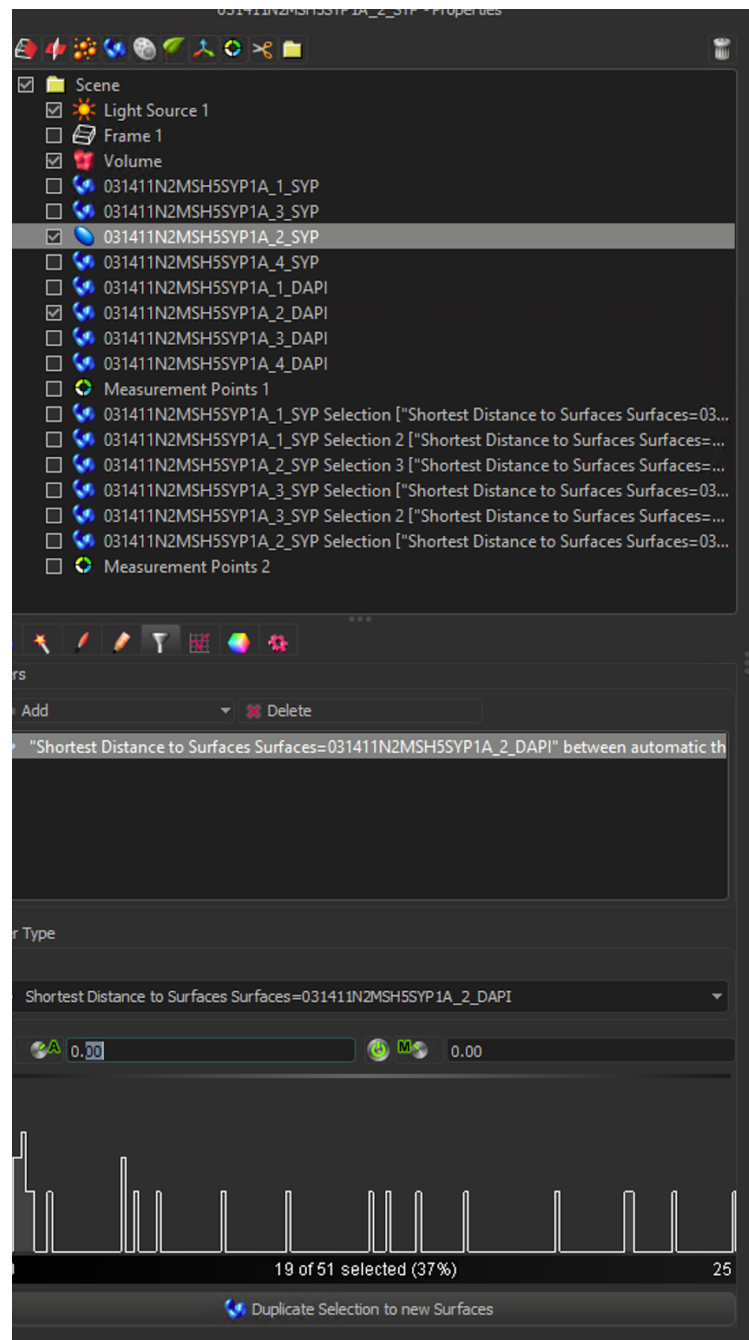
-Add a filter

-Choose filter type “Shortest Distance to Surfaces=name of the other surface that overlaps with the surface you are working with.

3. For objects that are touching use the criteria 0.00um. This will highlight only those objects that are touching.

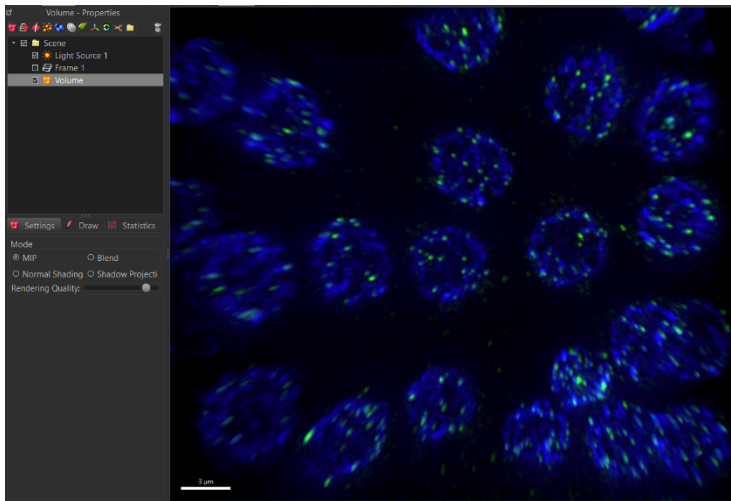
4. Select “Duplicate Selection to new Surfaces” and name the surface accordingly

5. Further analysis such as spot association can be done on the duplicated objects.



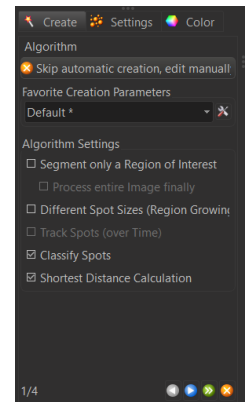
Section III: Creating Spots objects to identify foci

1. Open an image in the 3D view window



Immunofluorescence image of *C. elegans* oocytes. Chromosomes are stained with DAPI (blue) and sites of DNA damage are identified by localization of the recombinase RAD-51 (green foci).

2. Select the Spots object creator icon A creation wizard panel will open. →

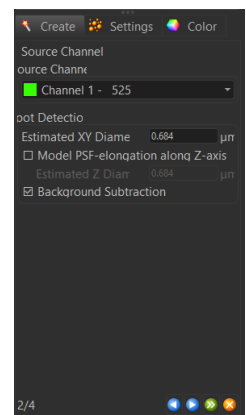


Creation wizard step 1/4

- Spots can be created across the entire image or the “Segment only a Region of Interest” box can be checked
- If spot volume needs to be considered check the “Different Spot Sizes (Region Growing)” box. Otherwise all spots will be created to have the same volume

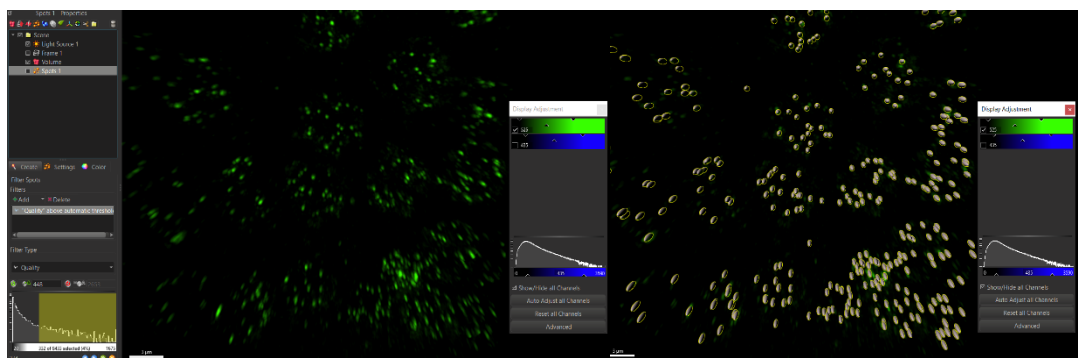
Creation wizard step 2/4

- Choose the appropriate channel from which your spots objects will be built
- Estimate the XY-diameter of your foci using the “Slice” window
- Z-axis elongation can be accounted for by checking the “Model PSF-elongation along Z-axis” box

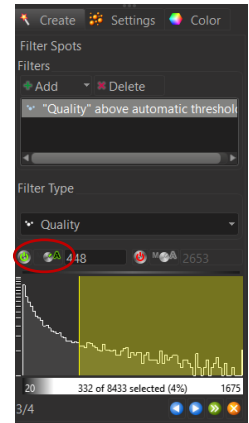


Creation wizard step 3/4

- Thresholding: Here you assess whether you are appropriately capturing spots surfaces based on your channel of choice.
-comparing the channel of choice alone to the spots objects being created is a good way to assess this:

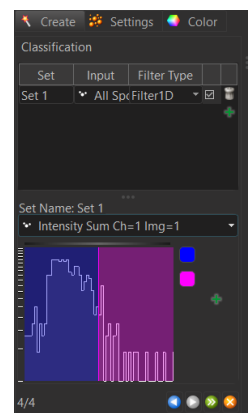


- We have found “quality” to be an effective filter type
-unless the signal has a high background/noise ratio the automatic thresholding setting works well
- Manual thresholding: If the automatic thresholding setting is not appropriately capturing spots then you can shift to manual thresholding by dragging the edges of the highlighted box on the histogram to incorporate more or fewer spots objects. When this is done the lighted “A” button (for automatic) will switch over to a lighted “M” (for manual). To toggle back to the automatic setting simply click the button between “M” and “A” again.



Creation wizard step 4/4

- Spots can be subsequently sorted or classified by various input, including Average Distance to 3 nearest neighbors, Diameter, signal Intensity (mean, med or sum), Position, or Volume.
- Spots will be color coordinated to indicate classification groups



3. Once Spots objects have been created there are various Xtensions available to assess them.
 - To assess spots associated with specific surfaces (e.g. to count the number of RAD-51 foci associated with individual nuclei) we use the “Spots Close to Surfaces” Xtension. (See Section II) <https://imaris.oxinst.com/learning/view/article/spots-close-to-surface-xtension>
 - To log onto the Imaris Open page, please use your existing Bitplane customer portal account (<http://portal.bitplane.com/>), username and password. To see a list of all XTensions already uploaded, approved and available for download, select the Imaris Open - File Exchange page. In order to refine and limit the list of the XTension to a certain set of Modules/ Applications, you can use either the Browse by Module or Browse by Application option (<https://imaris.oxinst.com/learning/view/article/integrate-a-xtension-downloaded-from-the-imaris-open-page-within-imaris>)

Section IV: Creating colocalized surfaces

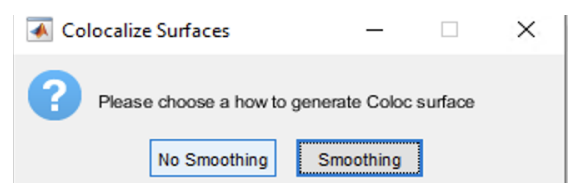
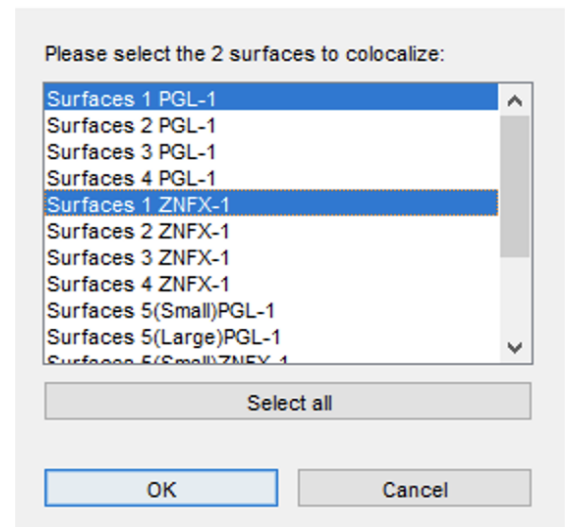
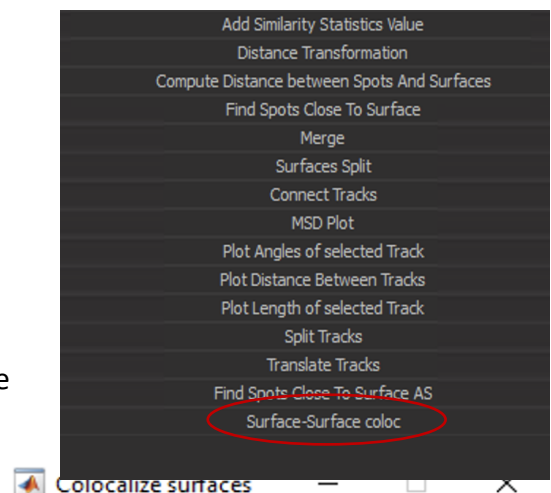
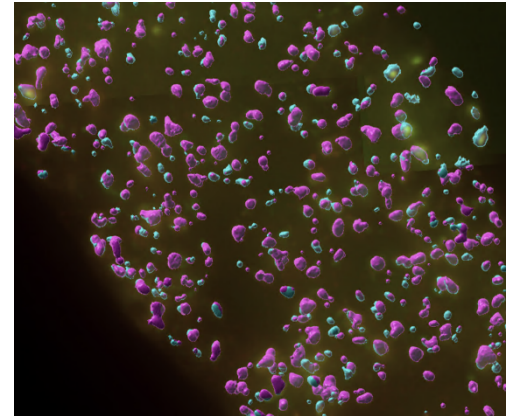
This protocol allows for the identification of individual colocalized surfaces, as well as statistics relating to the colocalized regions.

*requires Surface-Surface Colocalization Xtension

1. Select two surface objects for which you want to analyze overall colocalization.

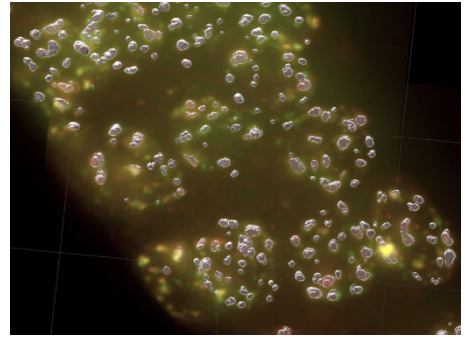
Example: the surfaces shown were generated in separate channels (e.g. determining colocalization of two different proteins) however, other permutations such as surfaces generated in the same channel but with different parameters (e.g. determining colocalization of same protein that has large and small morphology) can also be analyzed.

- Select the extension Surface-Surface coloc and then select two surfaces of interest and click ok.
- Choose whether or not you want to smooth the new colocalized surfaces generated.
- A new colocalization surface will be generated and the volumes of these new surfaces represent the amount of overlap between the two selected surface sets. Name this surface based on the initial two surfaces used to create it so you can backtrack. (e.g. PGL-1+ZNFX-1)

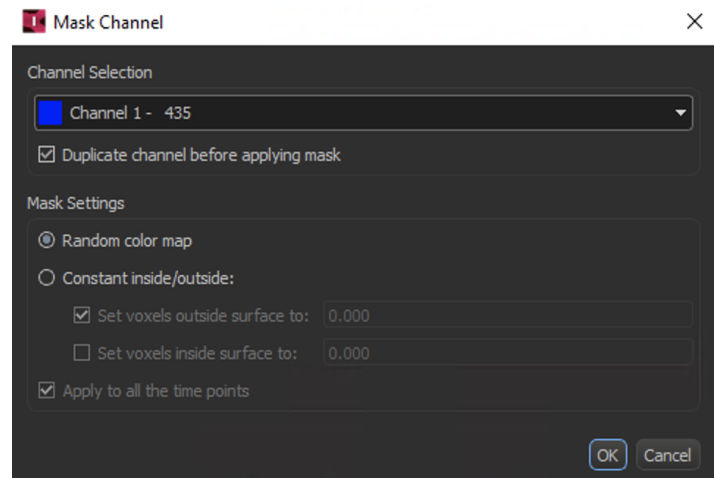
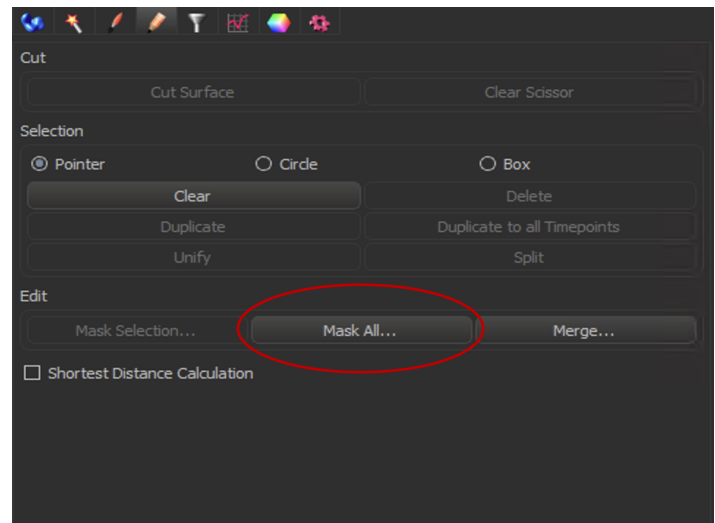


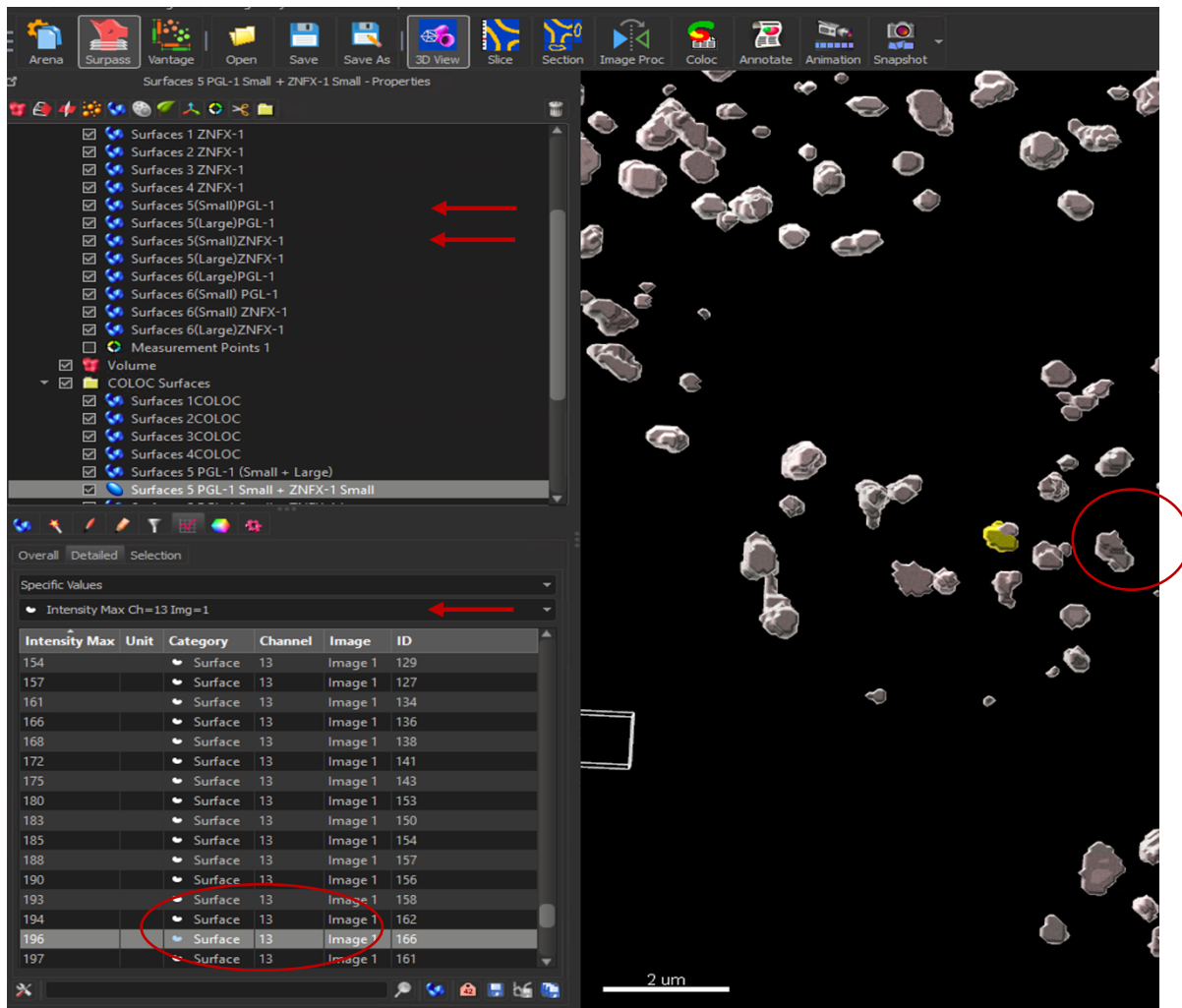
Determining which two specific surfaces are colocalized out of a large surface data set.

If you want to identify and gather information on a subset of surfaces that are colocalized (e.g. large with small) this covers how to identify specific surfaces that are touching based on the statistics that are exported.



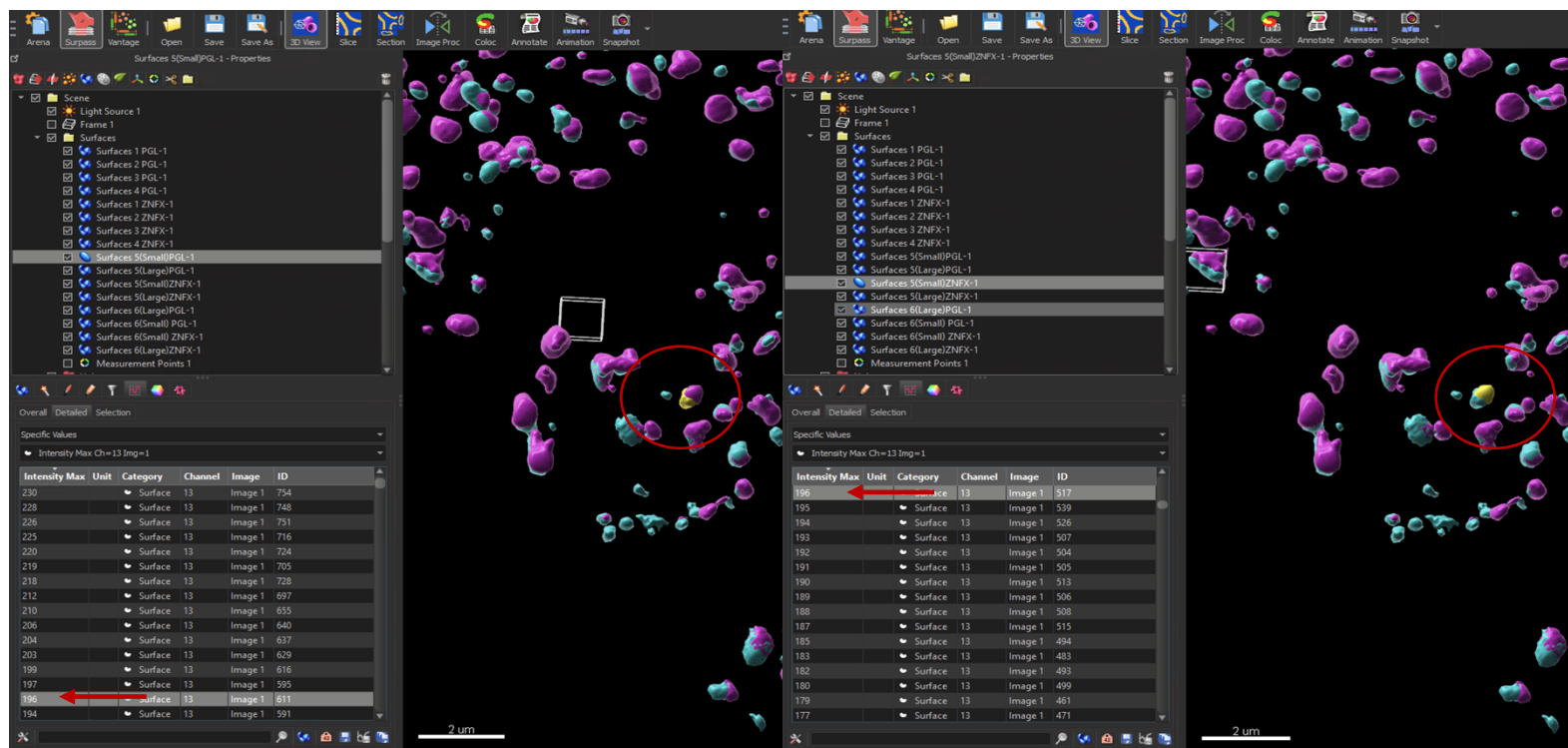
1. Select a colocalized surface
2. Click on the Edit icon 
3. Select "Mask All"
 - this will prompt a screen that creates a new channel and the assigned number of that channel will be the reference for the two colocalized surfaces.
4. Select one of the two channels you are comparing and keep it consistent for all future comparisons. If you are comparing surfaces within one channel, simply select that channel.
5. Select "Random color map" and click "OK"
 - As you create more color maps uncheck them as you proceed to prevent the graphics card from being overloaded.





6. The newly made channels are specific to the two surfaces used to generate the colocalized surface.

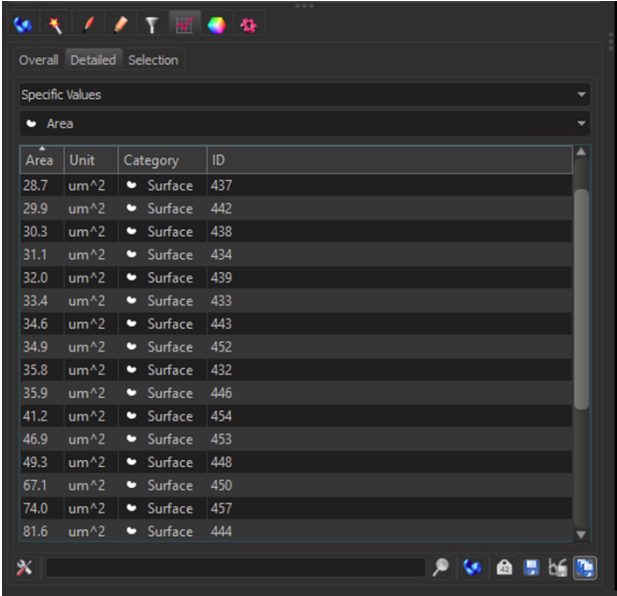
- Example: Above the selected object is Surfaces 5 PGL-1 Small + ZNFX-1 Small which describes the two surfaces I used to make that new colocalization surface.
- Select the statistics icon  and a the “Detailed tab”
- Intensity Max for Channel Number associated with the colocalized surface of interest should be selected from the drop-down menu. In this example shown the associated channel is Channel 13.
- The values given in intensity max are unique IDs that are shared between all three surfaces. There should not be any values that are zeros present in the colocalized surface statistics.



- For reference, the colocalization data for intensity max value 196 will be associated with the individual surface object present in Surfaces 5(Small)PGL-1 and the Surfaces 5(Small)ZNFX-1. There will be zeros present in these data sets due to the fact that of the surfaces in PGL-1 surface are not touching another surface in the ZNFX-1 surface and can be ignored

Export statistics of surface objects/measurements/spots objects:

- Select the statistics button and click on the bottom far folder to export all statistics.
- If there are multiple objects or spots within a folder click on the folder to export statistics of everything within that folder.



The screenshot shows a software interface with a dark theme. At the top, there are icons for various tools. Below them are tabs labeled 'Overall', 'Detailed', and 'Selection'. A dropdown menu labeled 'Specific Values' is open, showing a list of 'Area' values. Below this is a table with four columns: 'Area', 'Unit', 'Category', and 'ID'. The table contains 15 rows of data, all with 'um^2' as the unit and 'Surface' as the category. The 'Area' values range from 28.7 to 81.6, and the 'ID' values range from 437 to 444. The interface also has a search bar and several icons at the bottom.

Area	Unit	Category	ID
28.7	um^2	Surface	437
29.9	um^2	Surface	442
30.3	um^2	Surface	438
31.1	um^2	Surface	434
32.0	um^2	Surface	439
33.4	um^2	Surface	433
34.6	um^2	Surface	443
34.9	um^2	Surface	452
35.8	um^2	Surface	432
35.9	um^2	Surface	446
41.2	um^2	Surface	454
46.9	um^2	Surface	453
49.3	um^2	Surface	448
67.1	um^2	Surface	450
74.0	um^2	Surface	457
81.6	um^2	Surface	444

Section V: Linearization

Organization of imaris statistics files

1. Create a folder on the computer to store stats files for analysis. This filepath will serve as your home directory when using the R-code to linearize nuclei across the gonad.
2. Stats should be exported from Imaris using the “export all stats” function into the home directory
 - The stats files need to be organized into folders. The script will sort through these folders and retrieve the correct files for analysis.

-For example, our whole gonad images are generally broken up into sections for analysis; premeiotic tip (PM) early pachytene (EP), mid pachytene (MP), late pachytene (LP), and occasionally diplotene/diakinesis (DD). Individual folders should be created for each gonad analyzed. Each folder should be named as Gonad info_Section (e.g. Gonadinfo_EP)

-In addition, a folder should also be created to contain positional stats for the measurement line defining the center of the gonad. This folder should be named: Gonadinfo_Z

-Make sure that the Measurement folder’s name comes last alphabetically. The order of the non-Measurement folders doesn’t matter, just as long as the Measurement folder comes last; if it doesn’t, whichever folders fall after it will be missing from the dataset.

Example:

Gonadinfo_s###_EP

Gonadinfo_###_MP

Gonadinfo_###_LP

Gonadinfo_###_Z ←(here the Measurement folder comes last)

Link to gonad linearization algorithm

Implementation of the gonad linearization algorithm can be found at <github.com/libudalab/Gonad-Analysis-Pipeline>. The current implementation is sufficient for use in associating spots (eg. RAD-51 foci) with nuclei. Use of this method for other nuclear features will require modification by the user.