

Image quantification of whole *C. elegans* gonads

Updated 1/12/21

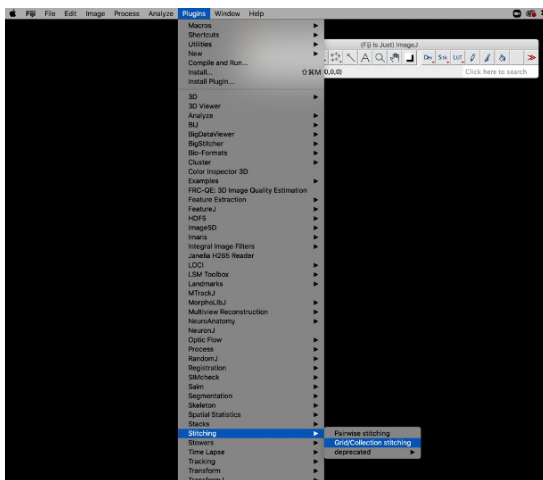
For the latest up-to-date version of this protocol, please go to the "Publications" section of www.libudalab.org

Protocol

Section 1: Stitching in FIJI

Step 1

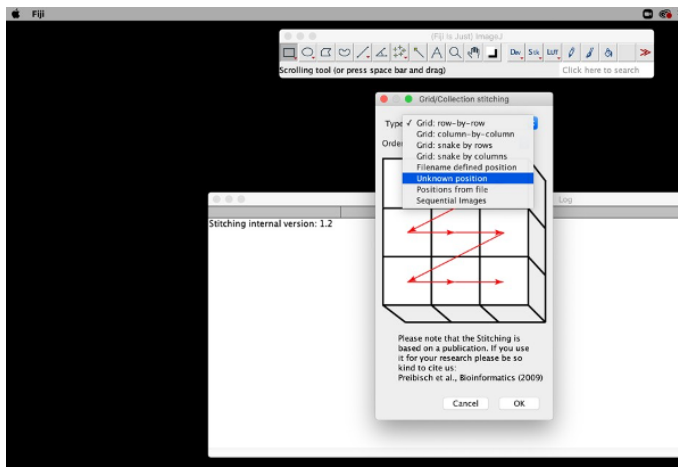
Open FIJI and navigate to plugins→Stitching→Grid/Collection stitching



Step 2

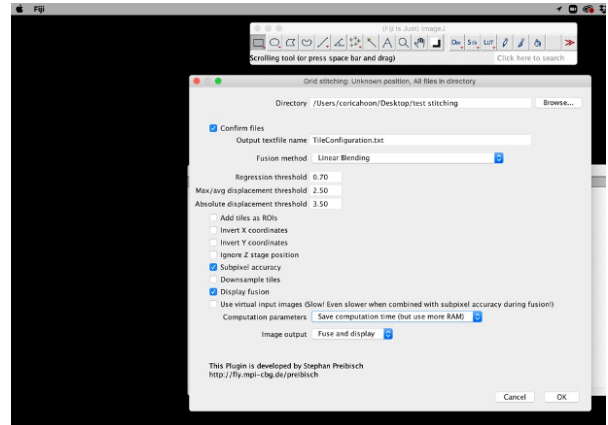
Click on the drop-down menu for "Type" to change to "Unknown positions" and click "ok".

- 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 DeltaVision Deconvolution Microscope.



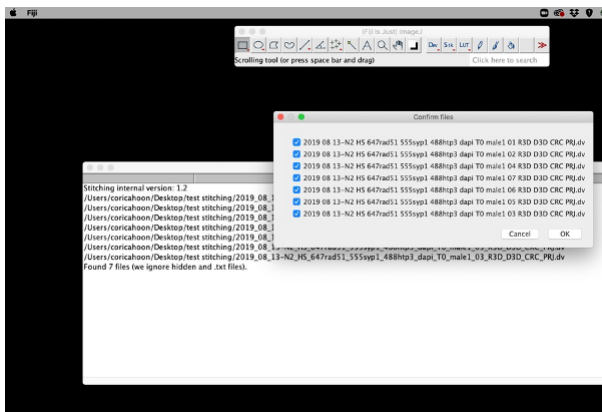
Step 3

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.



Step 4

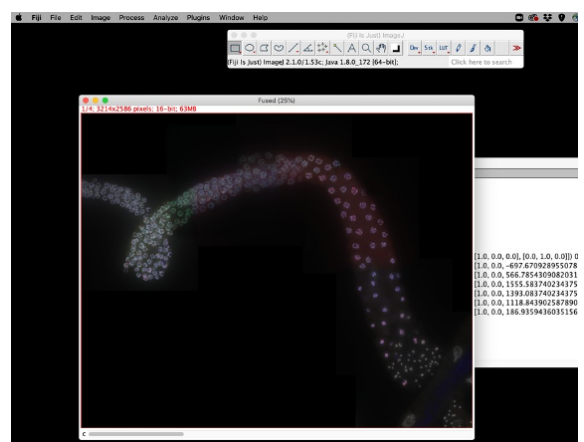
Select all the images that will be stitched and click "ok".



Step 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. This plugin will stitch each image in pairs of two rather than a group of images.



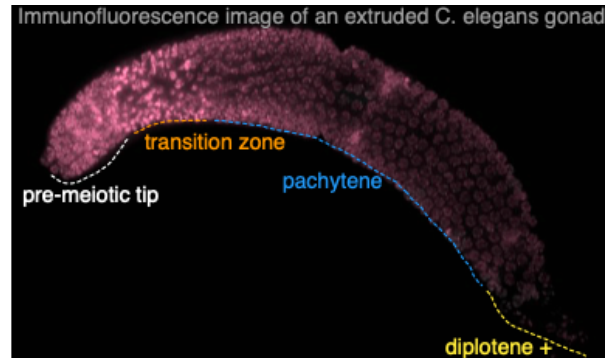
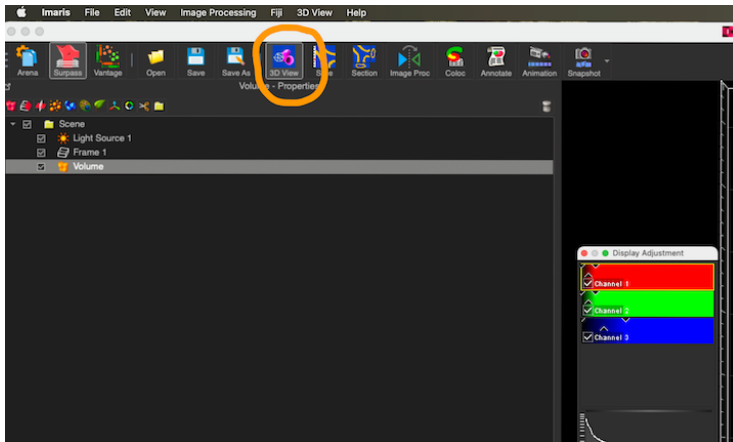
Suggested FIJI modules to look into for 3D image thresholding, nucleus segmentation, spot maxima identification and generating the spots per nucleus: 3D ImageJ plugins library and MorphoLibJ suite in the JPB-plugins library (Note: we have not thoroughly tested out the functionality of these modules for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

Section II: Creating surface objects

Creating surface objects for nuclei

Step 6

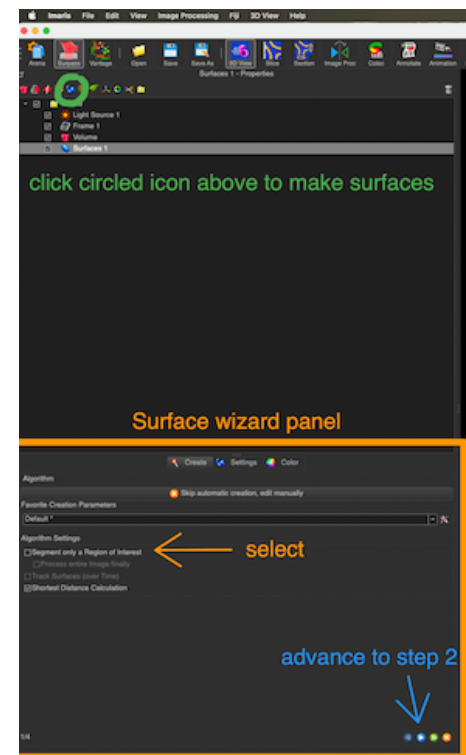
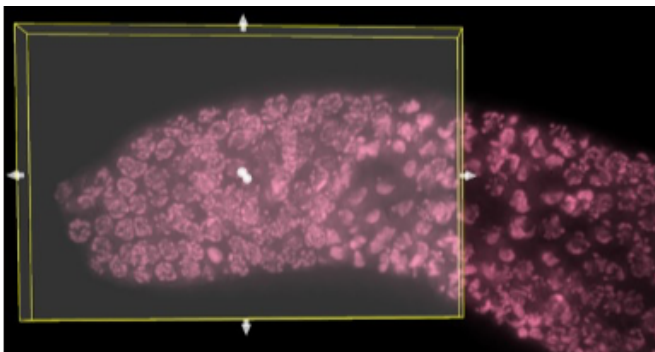
Open an image in Imaris using the 3D view window



Step 7

Select Surface object to reveal the creation tool Creation wizard step 1/5

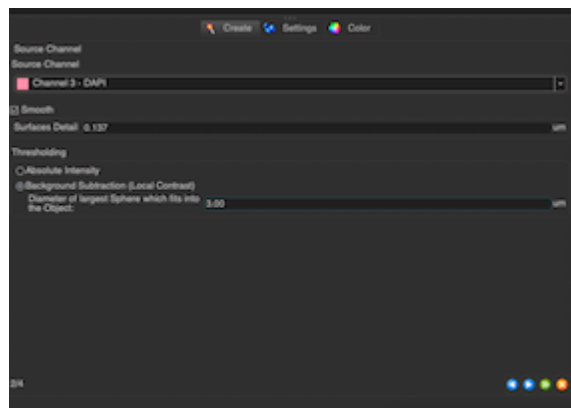
- Select "Segment only a Region of Interest"
 - Nuclei in each region of the gonad have different volumes, which makes creating a single set of surfaces for all nuclei across a whole gonad impossible.
 - Individual regions of interest allow for surface creation parameters to be tailored for each section and accurately model nuclei morphology.
 - We start creating objects at the pre-meiotic tip and name each section sequentially (e.g. this section will be named Section 1).



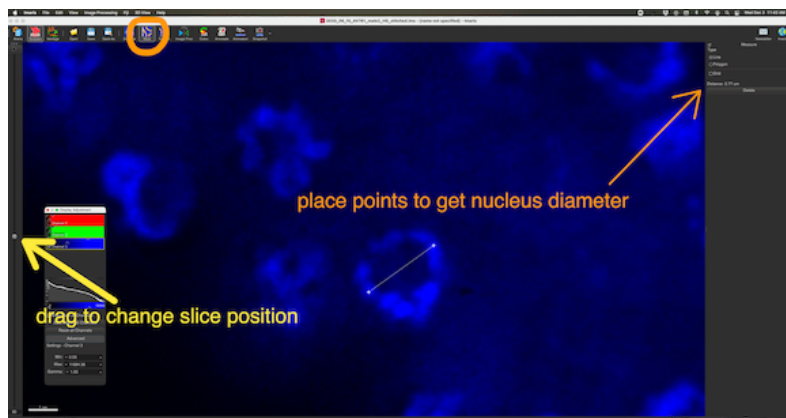
Step 8

Click blue single arrow at the bottom of the wizard to advance to step 2/5

- Select the source channel off which you will build the surface objects
- Select smoothing and use 0.15
 - this can be adjusted depending on the image quality
- 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.



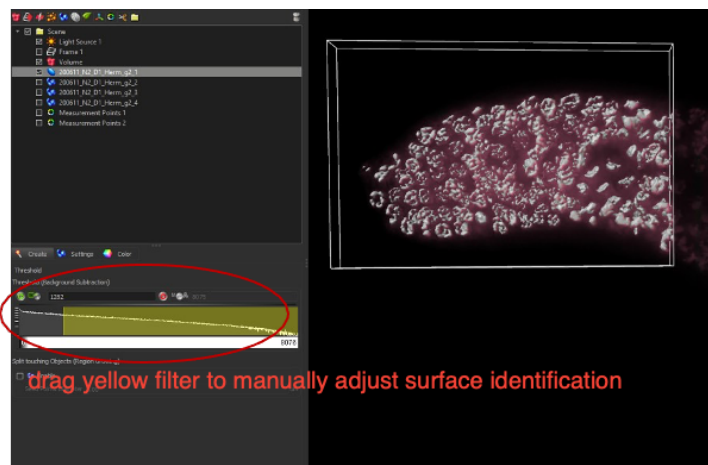
- Note: The xy diameter of your nuclei can be estimated using the "Slice" window (indicated by the orange circle in the screenshot below)



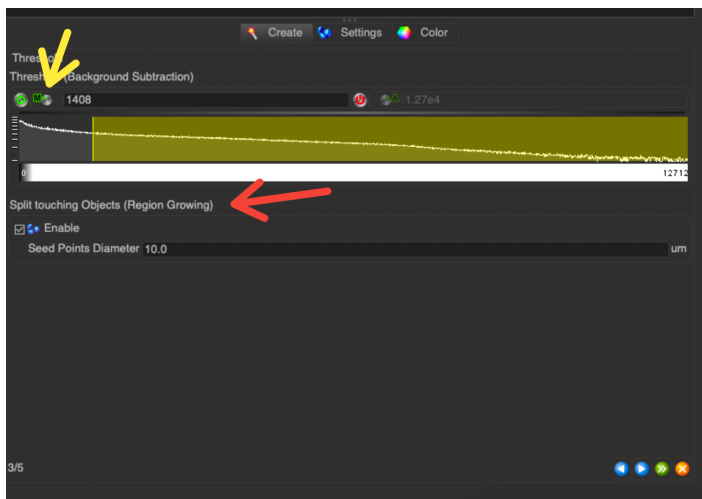
Step 9

Advance to creation wizard step 3/5

- Thresholding: Here you assess whether you are appropriately capturing spots surfaces
 - Turn off other channels in the image not being surfaced to more easily view each surface imaris generated.



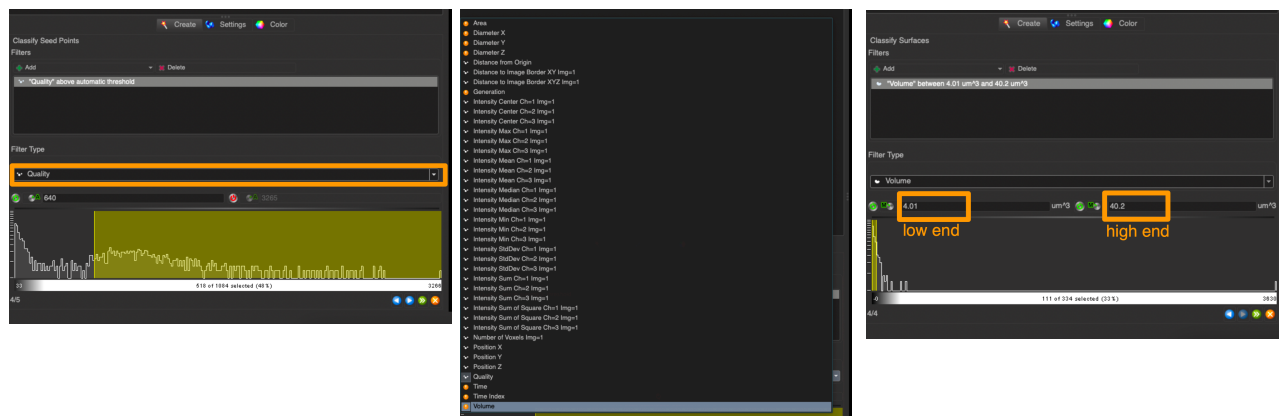
- Manual thresholding: use if the automatic thresholding setting is not appropriately surfacing the nuclei in the image
 - Drag the edges of the highlighted box on the histogram to incorporate more or fewer surface objects.
 - When this is done the lighted "A" button (for automatic) will switch over to a lighted "M" (for manual). To toggle back to the automatic setting simply click the button between "M" and "A" again (see yellow arrow in screenshot below).
- 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 (see red arrow in screenshot below). 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 and we use this with different values depending on the region of the germline we are trying to surface (see table below).



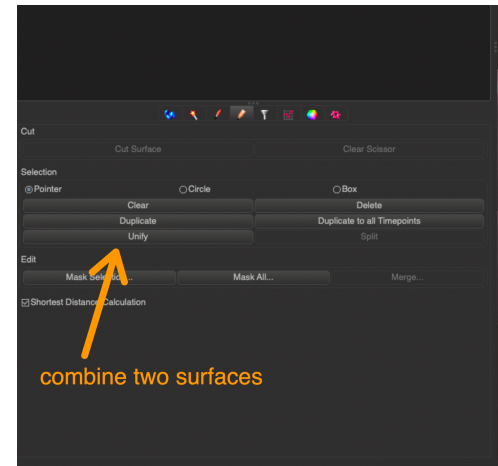
Step 10

Advance with the blue arrow to creation wizard step 4/5

- Surfaces are then filtered for quality either automatically or manually moving the yellow filter on the histogram like with background subtraction threshold in step 9.
- Clicking on the orange boxed region in the left image below, will reveal the list of filtering options in the image to the right. We commonly use a Volume filter to exclude surfaces with partial nuclei and combined nuclei.



- Keep in mind that surfaces can be manually combined (in the case of disconnected components of a single nuclei) and split (in the case of combined nuclei) following the initial creation using the selection pointer and the pencil panel



- The parameters we use to generate surfaces of nuclei in gonad is in the table below.

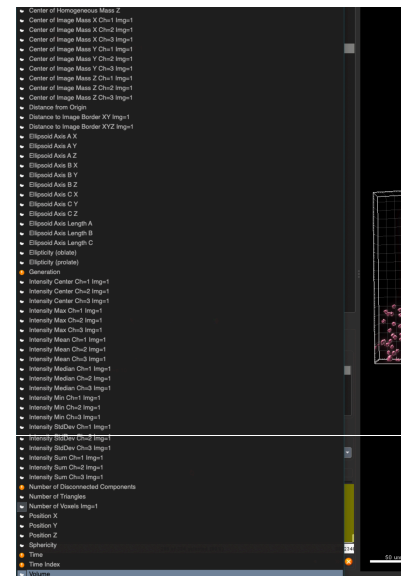
Nucleus surface thresholding settings

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)

Step 11

Advance with the blue arrow to creation wizard step 5/5 - we do not usually do this step

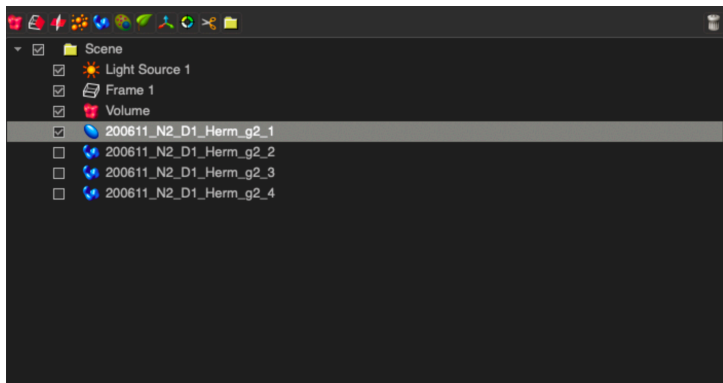
- Surfaces can then be sorted or classified by various input: Diameter, signal Intensity (mean, med or sum), Position, Area, Volume, etc (see screenshot of filter list).
- Spots will be color coordinated to indicate classification groups



Step 12

To finish creation wizard click the green double arrow button and rename the surface group to keep the data organized for the downstream processes in R.

- Our standard naming convention is yrmoday_strain_sex_treatment_gonad#_stage_# of nuclei included in section (e.g. 18918_N2_male_hs_g07_1)



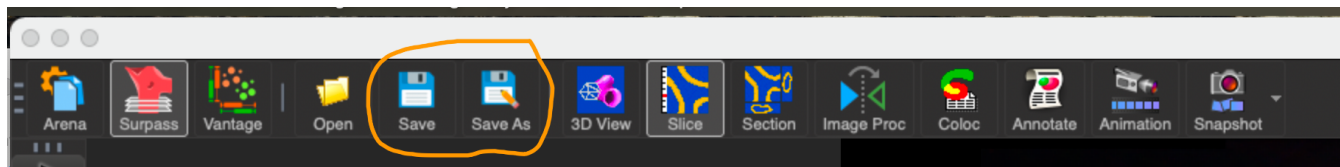
Step 13

After the first section of surface objects is finished, continue creating sections along the gonad.

It is important that when starting the next section **do not overlap** with the previous section (as overlap will generate duplicate surfaces for individual nuclei).

Step 14

RECOMMEND saving often using the SAVE (local save within Imaris) or SAVE AS (writes image to computer).



Creating surfaces with more detailed structures

The following are instructions on how to make surfaces of the synaptonemal complex protein SYP-1

Step 15

Create a new surface object and follow steps outlined above using the following settings

- Select "Segment only a Region of Interest"
- Smooth = 0.22 μm and select absolute intensity
- Threshold to include the most surfaces generated = $>0.5\mu\text{m}$

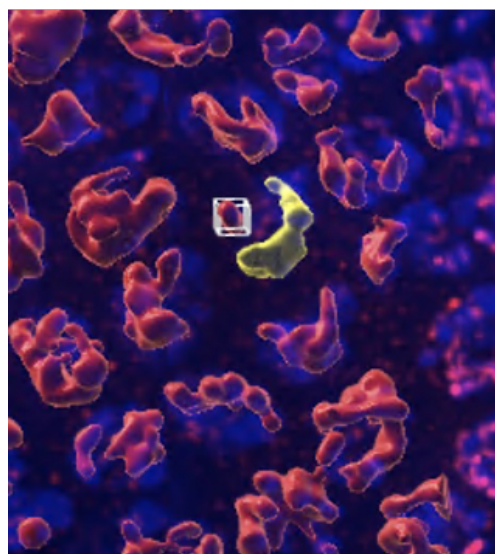
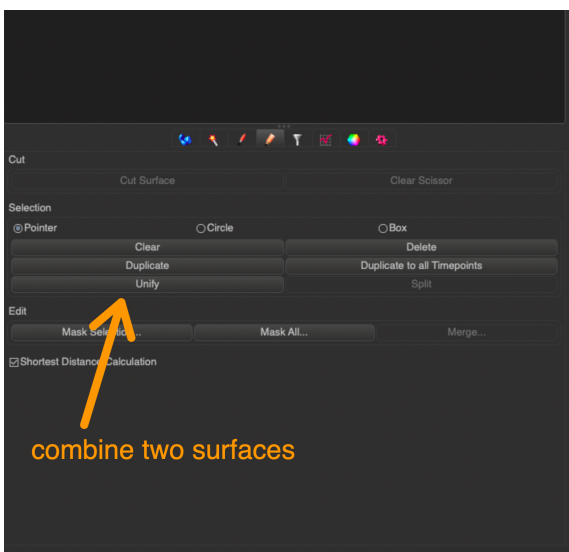
Step 16

After the first section is finished move on to the subsequent sections and verify that there is no overlap between sections.

Step 17

Return to each section to check the quality of objects generated.

- Scan the surface objects for incomplete/fragment surfaces and fused surfaces
- Using the pencil icon, manually unify small fragments of a surfaces that were not originally captured as one single object. It can be helpful to select surfaces and have them light up in yellow to identify those there are only partials or are unconnected segments. Additionally, under the checkmark graph setting you can view the surfaces by size which is also helpful in identifying the surfaces that are very small and likely partials or unconnected parts of surfaces.
- Partial surface that only encompasses part of the object are deleted



Filtering touching/nearby objects

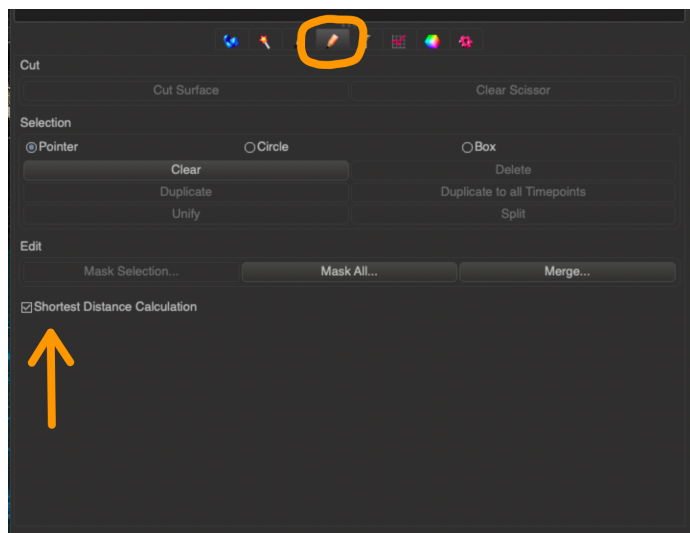
If surfaces have been generated in two separate channels, surface objects that are touching can be filtered and duplicated into a new surface object for further analysis. For example, if the aim is to only analyze surfaces where two different channels are present such as you only want the nuclei that have SYP-1 staining. Then, this protocol can be used to create a subset of DAPI-based surfaces that also have SYP-1. This is super useful and can be used in many different contexts.

Step 18

Select a surface object already generated

Step 19

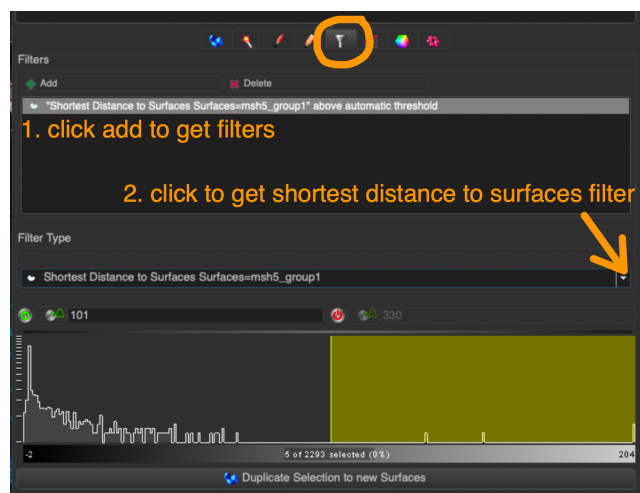
In the surface menu at the bottom, click on the Edit icon and check the box marked "Shortest Distance Calculation"



Step 20

In the surface menu, select the Filter icon and add a filter type "Shortest Distance to Surfaces=name of the other surface".

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



Step 21

Select "Duplicate Selection to new Surfaces" at the bottom and name the newly generated surface to get a surface that has only nuclei associated with the "= name of the other surface"

Step 22

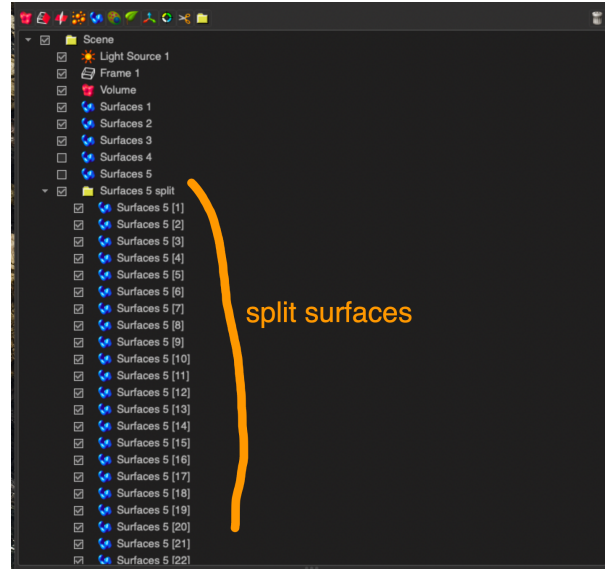
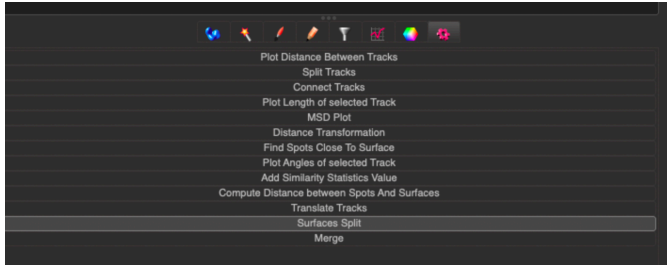
Further analysis such as spot association (see section III below) can be done on the duplicated objects.

Splitting surface objects into individual surfaces:

Step 23

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. This script is separating out each surface, so now each nuclei surface has an individual ID to reference and it's own statistics.
- Repeat the process for each of the surfaced regions.



Cropping individual surface object sections:

We have found that processing all of the surfaces at once from an entire gonad is too much for our computer to handle, so we crop the gonad into sections and only process the surfaces within each cropped region.

Step 24

Before crop click SAVE AS to rename to image to the cropped image, this prevents you from accidentally saving over the uncropped image with all the surfaces.

- 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

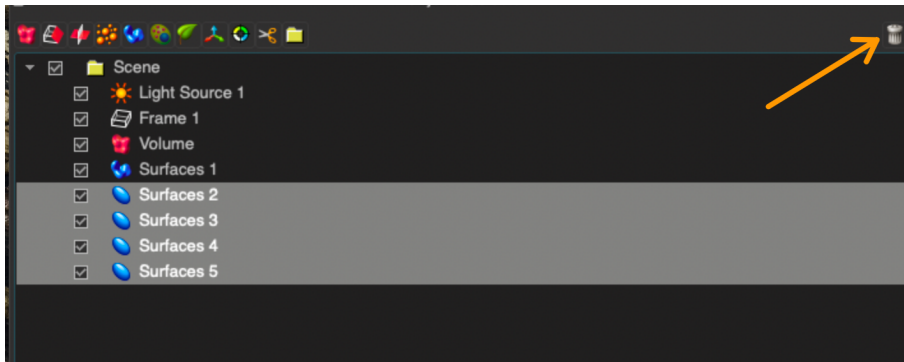
- Example:

original image was named: 200611_n2_d1_herm_rad-51_488_dsb2_555_g2_r3d_d3d_crc_STITCHED

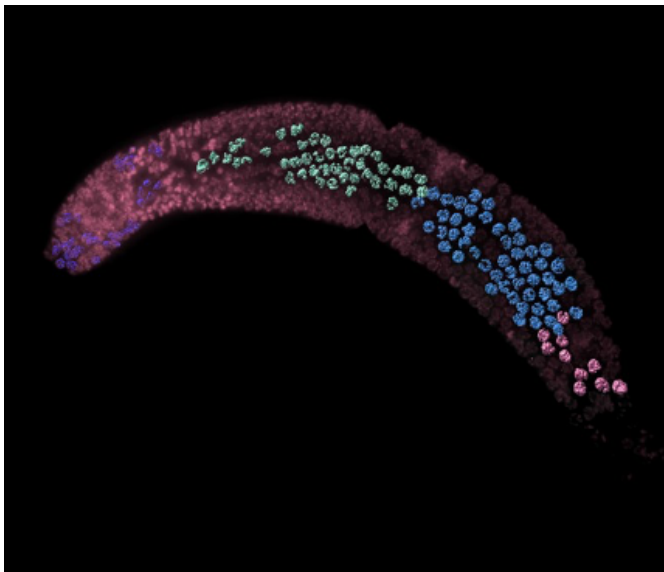
cropped section save image name as: 200611_n2_d1_herm_rad-51_488_dsb2_555_g2_r3d_d3d_crc_STITCHED_1

Step 25

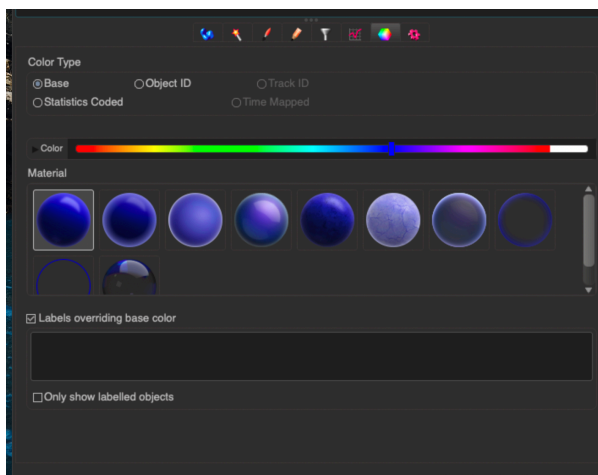
Delete all the surfaces you are not working with by selecting the surfaces and pressing the trash can.



- For example, if you are working on the green surfaces, delete the blue and red ones in the image below.

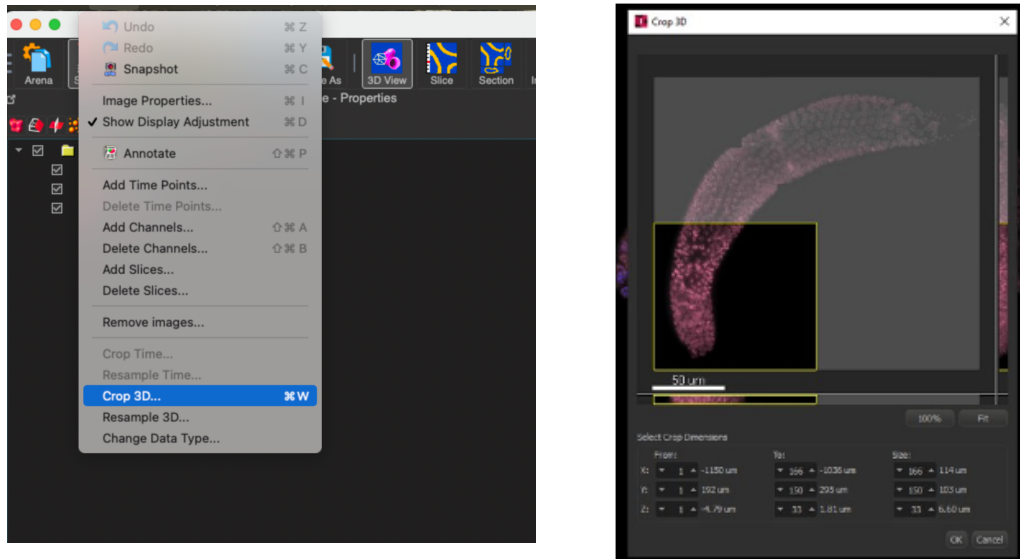


- Note: you can change the color of the surfaces in the surface menu in the rainbow cube icon



Step 26

To crop the image with only the surfaces press CTL + W (or COMMAND + W for those on a Mac) or navigate to the 3D crop (edit>crop 3D). Then, move the yellow box around in the crop window to crop the gonad.



- This is a best estimate, there is no exact way to do this. So if you end up cropping too large or too small a region use CTL + Z (or COMMAND + Z for those on a Mac) to undo and try the crop again.
- Note: The cropping does not have to be exact, but all surfaces in this region need to be within the cropped area. Any extra nuclei from the image that have not been surfaced will be ignored and so will the spots/other features associated with those nuclei since we threshold these elements away using distance from surface.

Step 27

Save the cropped image using SAVE AS

Step 28

Close the cropped image and open the original uncropped image with the all the surfaces to repeat this process for the other surface regions.

Measurement points throughout gonad/image:

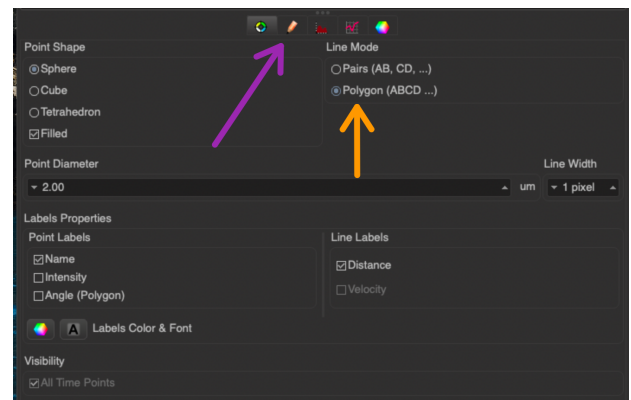
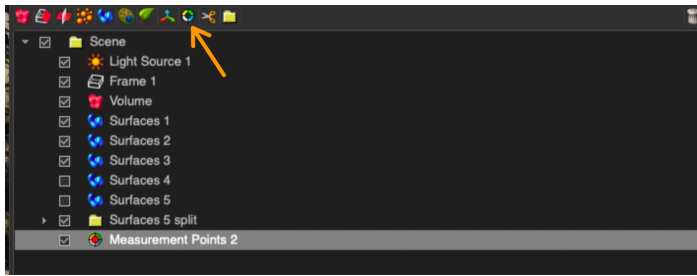
In order to use the whole gonad linearization algorithm (see steps 49-50) it is important to draw a measurement points through the center of the gonad.

Step 29

Open the original image with all the surfaced regions.

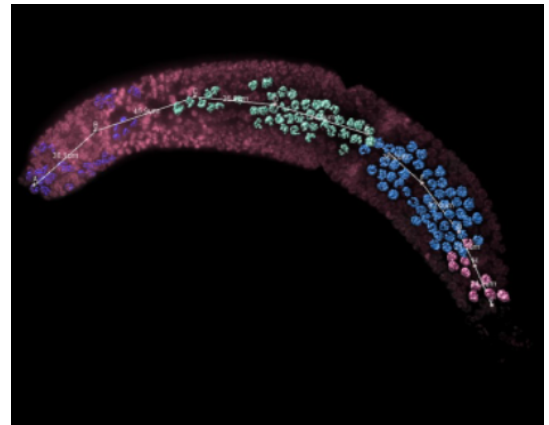
Step 30

Select the Measurement Points tool and make sure the polygon is selected.



Step 31

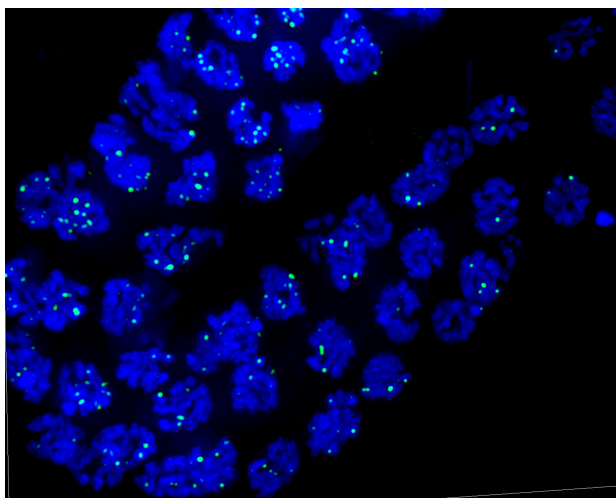
Click on the pencil icon (see purple arrow in screenshot above) and using the Select pointer (to change pointer look for panel in right corner of Imaris window) press SHIFT + mouse LEFT click to put down the first point. Continue holding down SHIFT draw a line through the center of the gonad by LEFT clicking and placing points. Place as many points as is necessary to conform to the shape of the gonad.



Section III: Creating Spots objects to identify foci

Step 32

Open an image in the 3D view window (see step 6 for details)

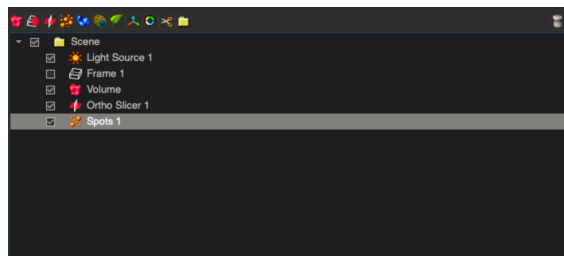


Immunofluorescence image of *C. elegans* spermatocytes. Chromosomes are stained with DAPI (blue) and sites of DNA damage are identified by localization of the recombinase RAD-51 (green foci).

Step 33

Select the Spots object creator icon

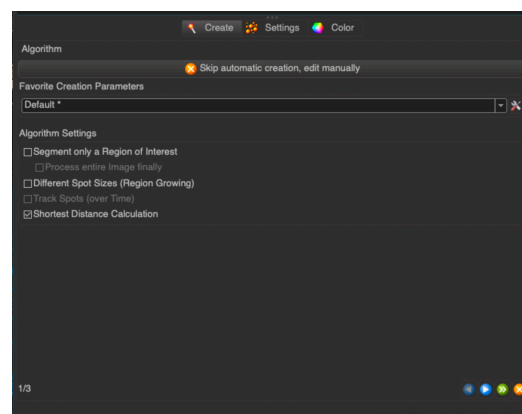
- a creation wizard will open at in the spots panel in the bottom left corner.



Step 34

Creation wizard step 1/3

- Spots can be created across the entire image or a region of interest
 - (select "segment only a region of interest")
If spot volume needs to be considered select the "Different Spot Size
 - (Region Growing)" option. Otherwise all spots will be created to have the same volume
- Use the blue arrow at the bottom right corner to advance to the next step in the creation wizard

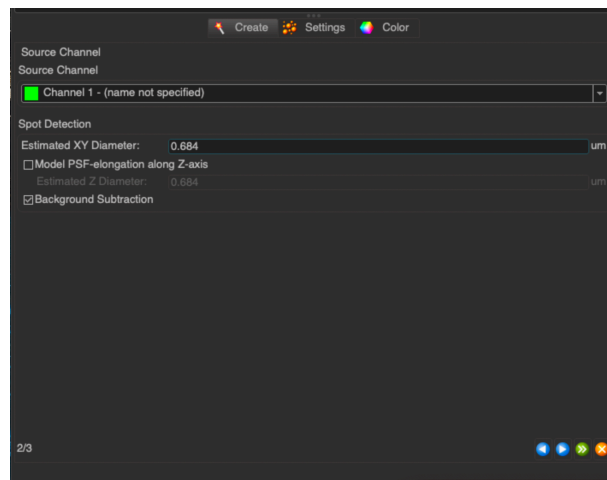


Step 35

Creation wizard step 2/3

- Choose the channel that you want spots objects to be generated from
- Estimate the XY-diameter of your foci using the "slice" window (see step 8)

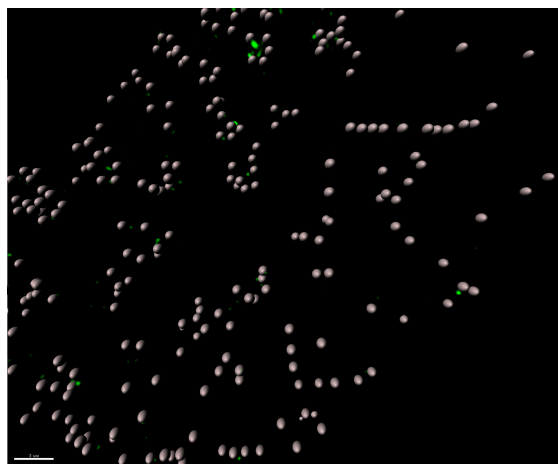
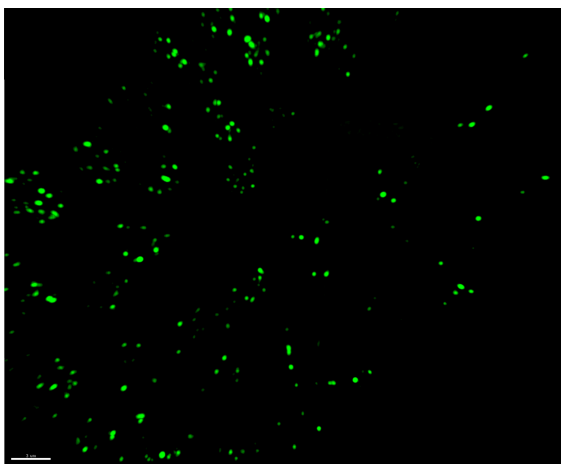
Z-axis elongation can be accounted for by selecting "Model PSF-elongation along Z-axis" - we usually select this to have the spots fit better to the observed foci in the image (see the elongated ovals in the screenshot of the spots below, whereas without this selected the spots are spheres).



Step 36

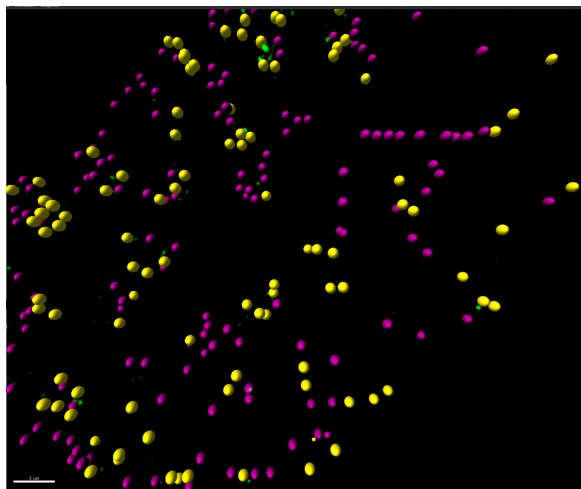
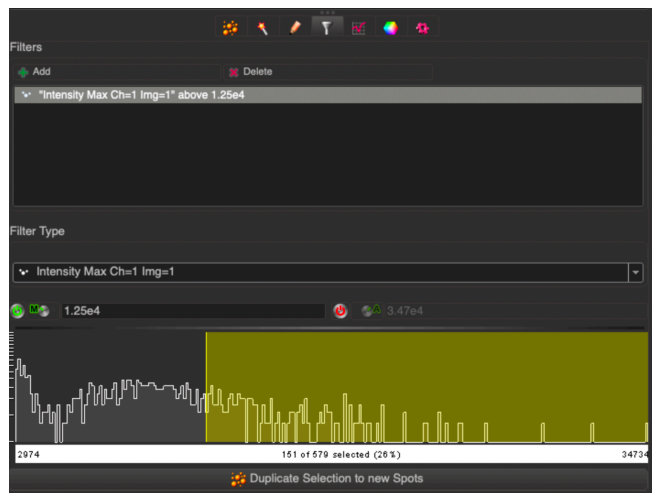
Creation wizard step 3/3:

- Thresholding: Here you assess whether you are appropriately capturing spots surfaces based on the channel you selected
 - Recommend turning off all other channels in the image for this step to visualize the foci and easily compare the image foci to the spots surfaces generated by Imaris
 - The "quality" filter and automatic thresholding appears to work well for our foci so long as signal-to-noise ratio is high
 - Similar to the thresholding surfaces above you can use the automatic threshold function or drag the yellow bar left and right to manually threshold (see step 9 for surface object thresholding)



Step 37

After spots are created they can be sorted by many different filter options (such as volume, intensity, position, etc) under the filter icon and each filter can be used to color code the filtered spots in the rainbow cube icon



Step 38

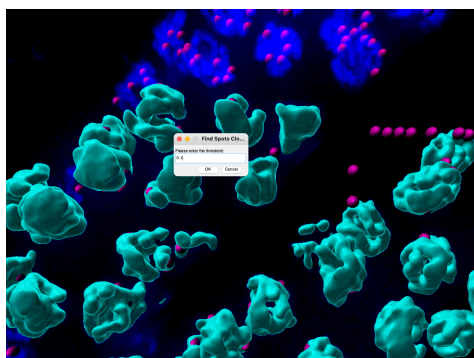
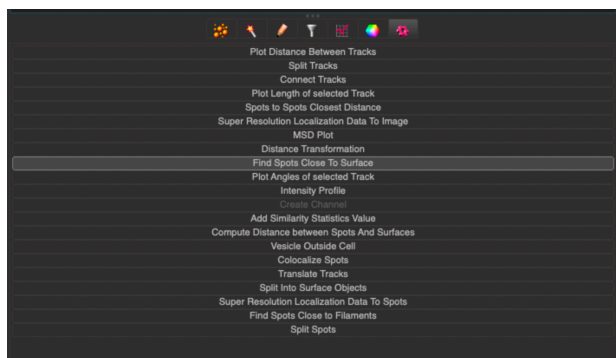
Once Spots objects have been created there are various Xtensions available assess them.

- To access the Xtensions, log onto the [Bitplane customer portal](#). 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. Imaris has a nice help page on all of this too, found [here](#).

Step 39

To assess spots associated with specific surfaces we use the ["Spots Close to Surfaces" Xtension](#) (e.g. to count the number of RAD-51 foci associated with individual nuclei).

- Under the gear icon in either the spots or the surfaces, select the "Spots Close to Surfaces".
- This will launch a MatLab code that will allow you to select the surfaces and spots you want to associate and a threshold value (ie: the max distance in μm that the spots and surfaces can be apart to be considered associated).
- We select all split surfaces and threshold at 0.1 μm for the RAD-51 spots to be considered associated with the nuclei surfaces.



Section IV: Creating colocalized surfaces

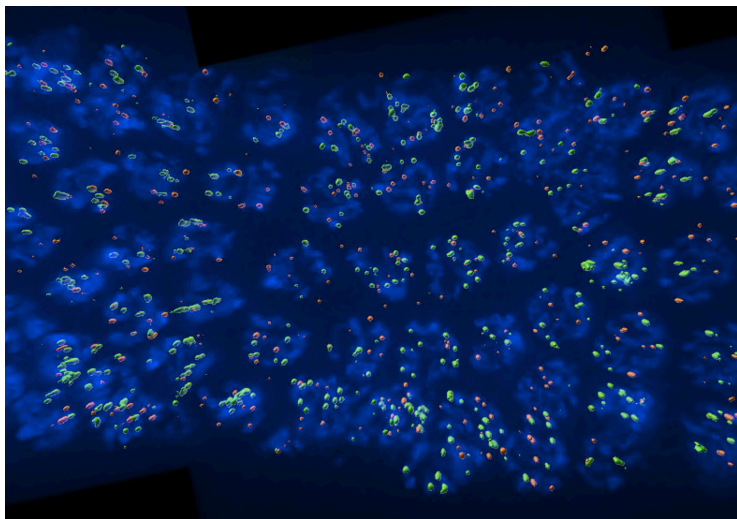
This protocol allows for the identification of individual colocalized surfaces, as well as statistics relating to the colocalized regions.

***requires Surface-Surface Colocalization Xtension**

Step 40

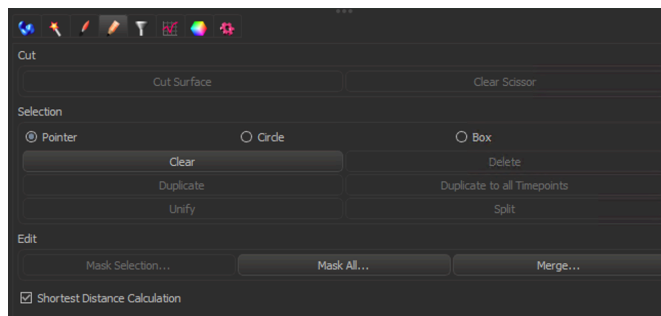
Select two surface objects for which you want to analyze overall colocalization (e.g. Surfaces RAD-51 & MSH-5.)

- Example: the surfaces shown were generated in separate channels to determine the colocalization of two different proteins



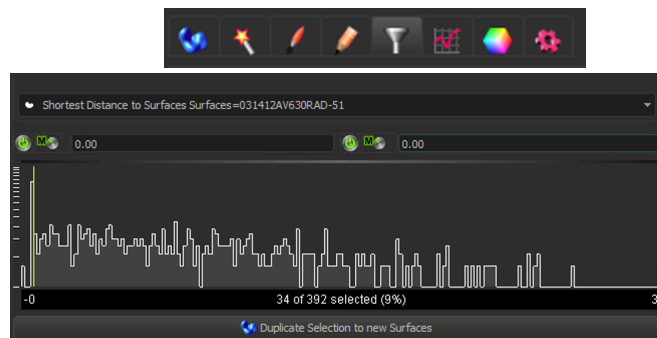
Step 41

- Pick one of those surfaces (e.g. MSH-5) and click the little pencil to check the Shortest Distance Calculation function.



Step 42

- Click the funnel icon and select the other surface you are
- trying to gather colocalization information from (e.g. RAD-51). Click on the range values and set them both to 0.0 then click duplicate selection to new surfaces. This duplicates all surfaces from the MSH-5 surfaces that are touching RAD-51 surfaces.

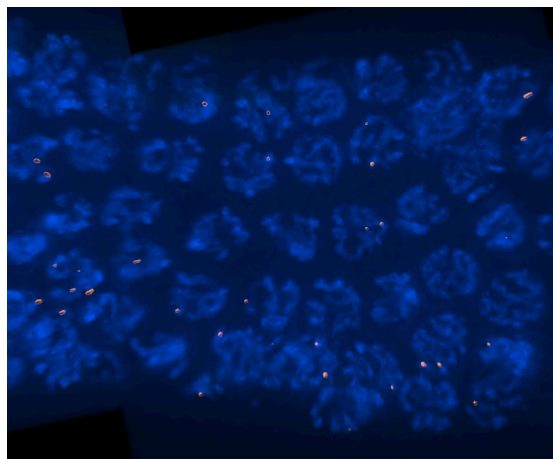


Step 43

A new duplicated surface will be generated containing only the surfaces that are colocalized with the other surface.

- Recommend naming this surface based on the initial two surfaces you used to create it (eg: RAD-51 & MSH-5) by double clicking on the surface name.

☒ MSH-5 Selection ["Shortest Distance to Surfaces Surfaces=RAD-51" between 0.00 and 0.00]

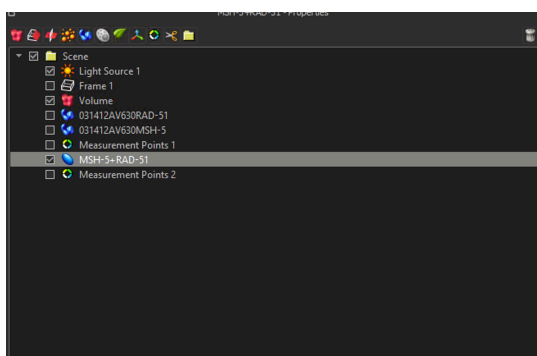


Determining which two surfaces are colocalized from a large surface data set.

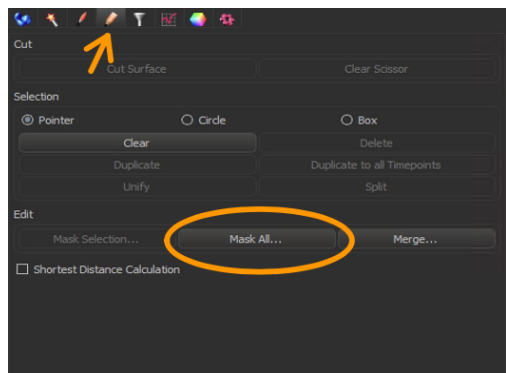
If you want to identify and gather information on a subset of surfaces that are colocalized this covers how to identify specific surfaces that are touching based on the statistics that are exported.

Step 44

Select the duplicated surface



In the surface menus at the bottom, select the edit icon (pencil) and click on "Mask All..."



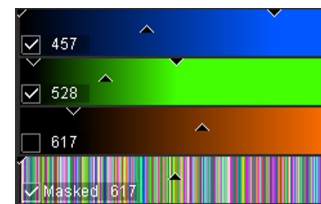
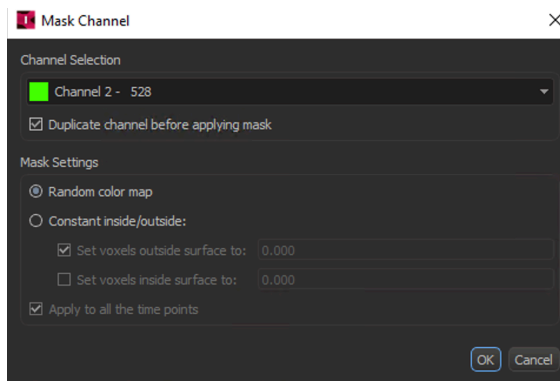
- This will prompt a screen that creates a new channel and the assigned number of that channel will be the reference for the two colocalized surfaces in this example Channel 4 has been generated.

Step 45

Select one of the two channels you are comparing and random color map in mask settings.

It's important that you keep the channel selection option consistent for all future comparisons. If you are comparing surfaces within a channel, simply select that channel.

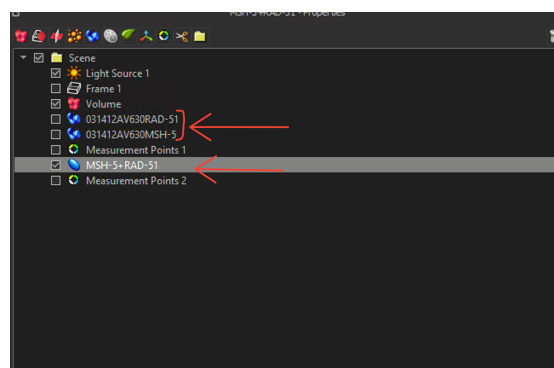
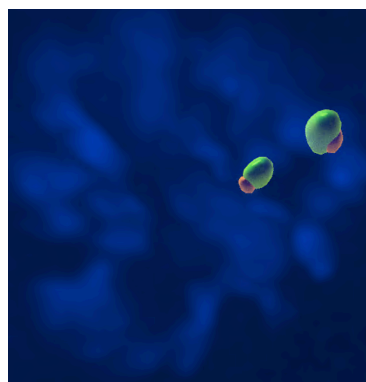
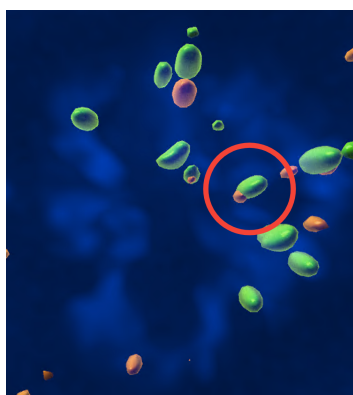
As you create more color maps uncheck them as you go to not overload the graphics card.



Step 46

To determine which two specific surfaces are touching out of a large data set they will have a shared ID located under the Intensity Max Statistics.

- Example: The selected object is RAD-51 + MSH-5 (the duplicated surface), Intensity Max value 15 in the duplicated channel is associated with Intensity Max value 15 in both the individual RAD-51 and MSH-5 surfaces because they are touching.



Overall Detailed Selection

Specific Values

Intensity Max Ch=4 Img=1

Intensity Max	Unit	Category	Channel	Image	ID
15.0		Surface	4	Image 1	11
16.0		Surface	4	Image 1	12
17.0		Surface	4	Image 1	13
19.0		Surface	4	Image 1	15
20.0		Surface	4	Image 1	16
21.0		Surface	4	Image 1	17
22.0		Surface	4	Image 1	18
23.0		Surface	4	Image 1	19
24.0		Surface	4	Image 1	20
26.0		Surface	4	Image 1	21
27.0		Surface	4	Image 1	22
29.0		Surface	4	Image 1	23
30.0		Surface	4	Image 1	24
31.0		Surface	4	Image 1	27
32.0		Surface	4	Image 1	30
33.0		Surface	4	Image 1	28

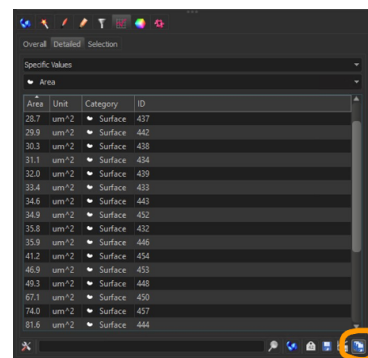
- Select the statistics icon and under the "detailed tab"
- Intensity Max for Channel Number associated with the colocalized surface of interest should be selected from the drop down menu. In this example shown the associated channel is Channel 4 generated from the previous Mask All function.
- The values given in intensity max are unique IDs that are shared between all three surfaces. There should not be any values that are zeros in the duplicated surface statistics.
- For reference, the colocalization data for intensity max value 15 will be associated with the individual surface object present in Surfaces RAD-51 and the Surfaces MSH-5. There will be zeros present in these data sets due to the fact that the some RAD-51 surfaces are not touching another surface in the MSH-5 surface and can be ignored.

Section V: Export statistics of all surface objects/measurements/spots objects

Step 48

Select the statistics button and click on the bottom farfolder to export all statistics.

- Note: If there are multiple objects or spots within a folder click on the folder to export statistics of everything within that folder.



Section VI: Linearization

Organization of imaris statistics files

Step 49

Create a folder on the computer to store stats files for analysis. This filepath will serve as your homedirectory when using the R-code to linearize nuclei across the gonad.

Step 50

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.
 - 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. image-name_EP)
- In addition, a folder should also be created to contain positional stats for the measurement linedefining the center of the gonad. This folder should be named: image-name_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:
 - image-name_s###_EP
 - image-name_###_MP
 - image-name_###_LP
 - image-name_###_Z (this last files needs to be the Measurement folder)

Link to gonad linearization algorithm

Implementation of the gonad linearization algorithm can be found [here](#) .

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.