

Published in:

Braude JP, Vijayakumar S, Baumgarner K, Laurine R, Jones TA, Jones SM, **Pyott SJ**. (2015) Deletion of *Shank1* has minimal effects on the molecular composition and function of glutamatergic afferent postsynapses in the mouse inner ear. *Hear Res.* 321:52-64. [auditory epithelium]

Sadeghi SG, **Pyott SJ**, Zhou Y, Glowatzki E. (2014) Glutamatergic signaling at the vestibular hair cell calyx synapse. *J. Neurosci.* 34:14536-50. [vestibular epithelia]

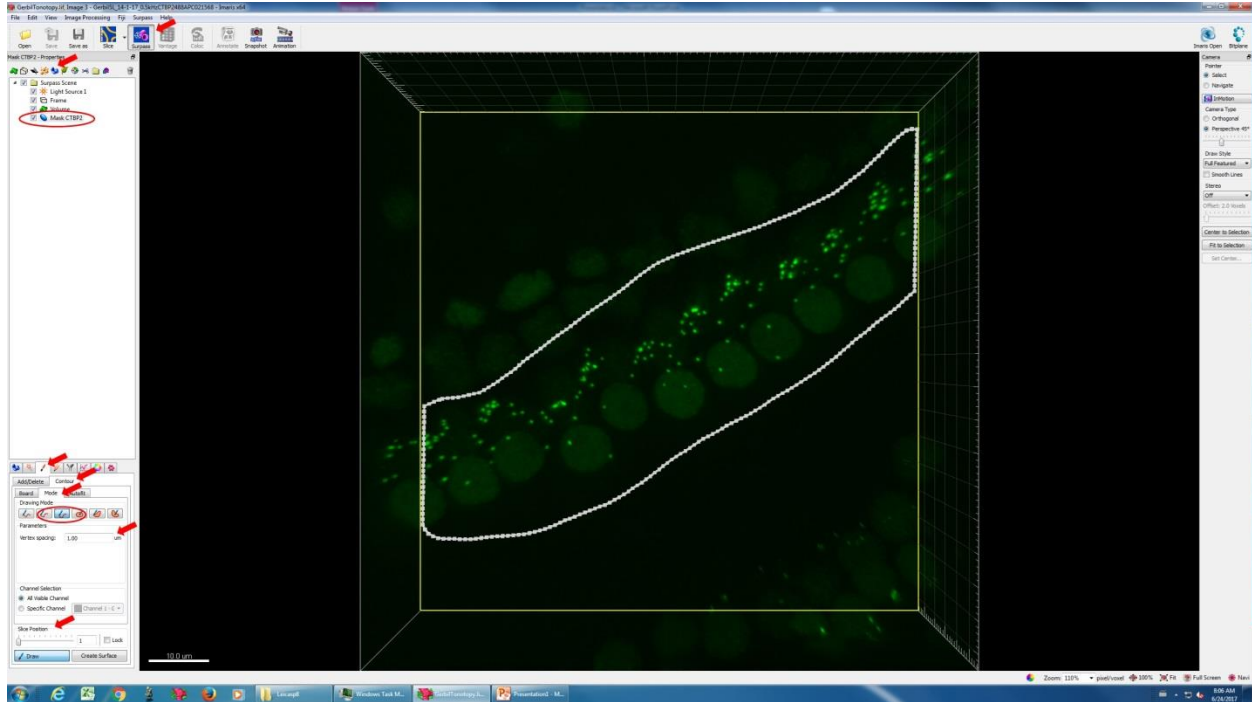
Tips for opening and examining files in Imaris

- Adjust intensity (lower arrow) and contrast (upper arrow) in the “display adjustment” screen. Go to Edit > Show Display Adjustment (or press Ctrl+D) to show the “display adjustment” screen. Channels can be turned on or off from viewing by selecting or deselecting from this window.
- Press the escape key to switch between the selection (pointer) and the rotation (double arrows) mode.
- To zoom, scroll in or out.
- To rotate, left click and hold.
- To drag, right click and hold.
- Save files in .ims format (preferably “[original filename]_Edit”). Save files often!

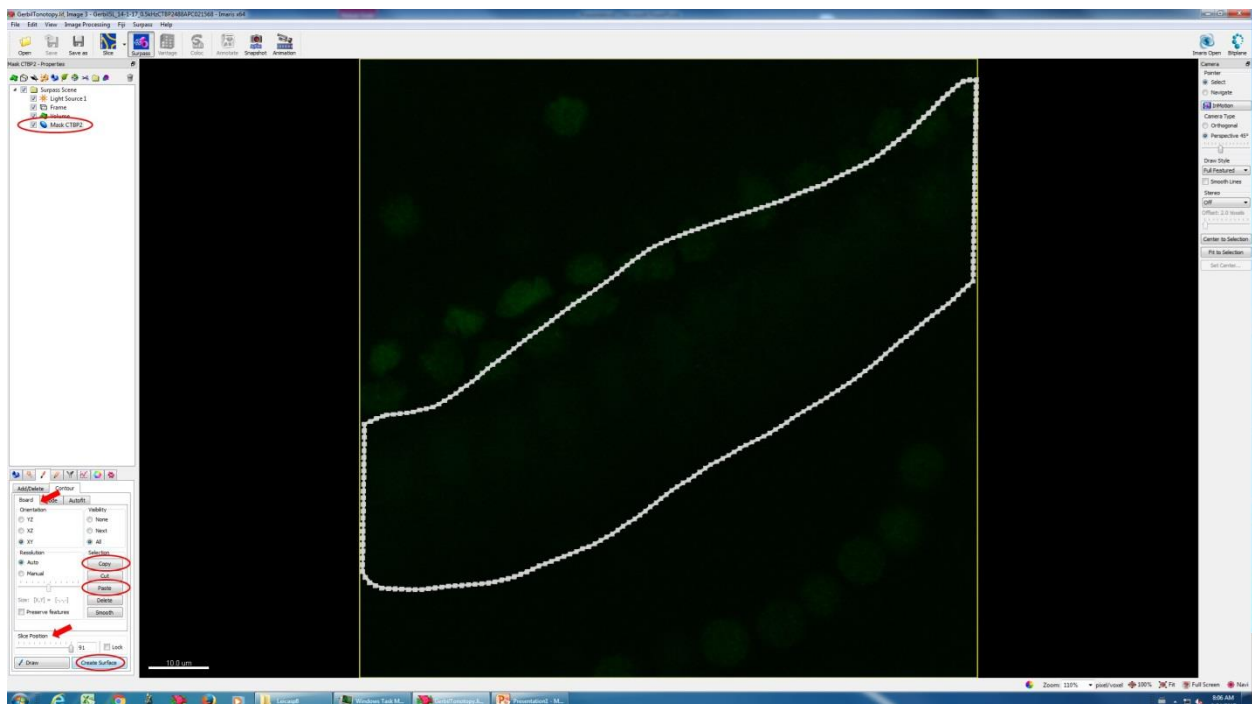
Selecting ROIs using a “free form” crop

Sometimes it is necessary to analyze only a ROI (for example, inner hair cells but not outer hair cells or even a subset of hair cells from the entire field of view). While Imaris has a cropping feature, it allows only square ROIs to be cropped. To perform a square crop go to Edit > Crop 3D. Follow the directions below to crop a free form ROI. In this protocol, cropping is actually done by “masking” everything outside a selected ROI that is defined by a surface.

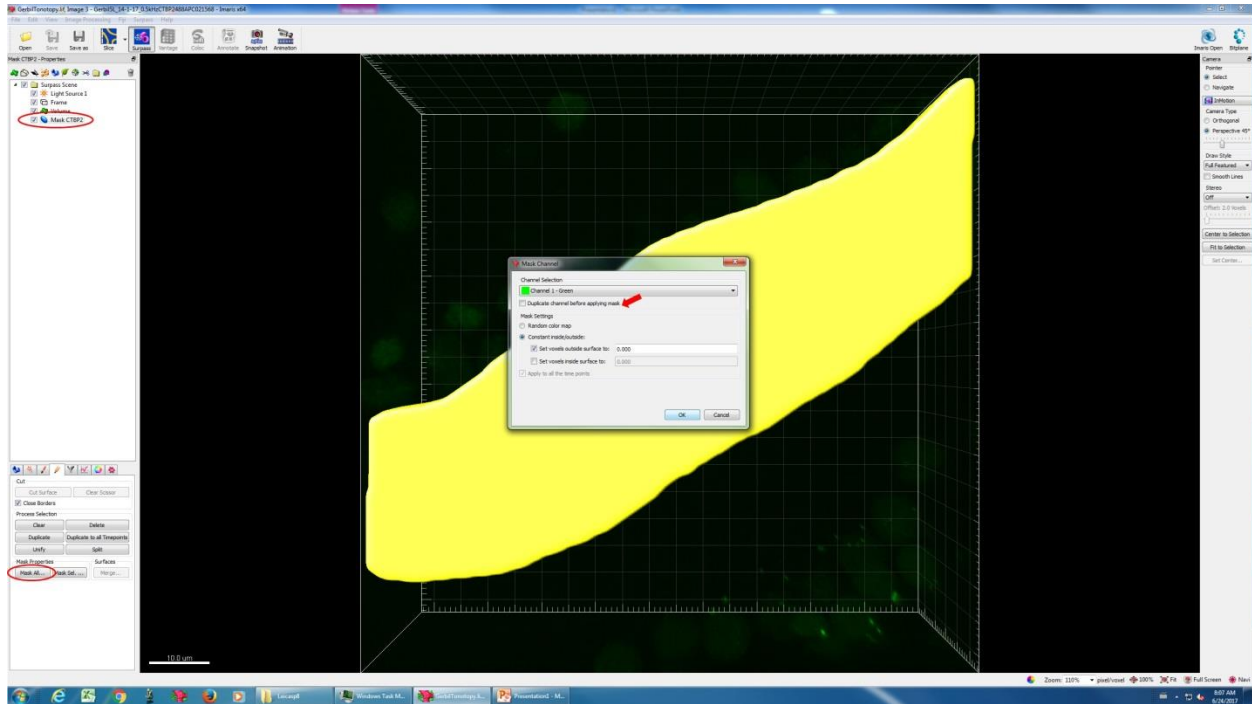
1. Add a surface by clicking the “surface” icon (blue blobs) in the properties window. You can rename the surface to be created. (In this case it has been named “Mask CTBP2”.)
2. “Skip” automatic creation (wizard).
3. Go to the paint brush > contour > mode window and select drawing mode to insert vertex every 1 μm . This distance can be adjusted as appropriate. Adjust the slice position slider to view the entire stack.
4. To demarcate the ROI, toggle to the select mouse pointer (single arrow) by pressing esc. Then press ctrl+space to get a crosshair pointer. Now outline the ROI while holding the left click. Be sure to press ctrl+space (to go from the cross hair to single arrow pointer) immediately after selecting the ROI.



5. Go to the paint brush > contour > board window and hit “copy”, scroll through to the opposite end of the stack with the slice position slider, hit “copy,” and then hit “Create Surface.” This will create a surface of the outline through the entire stack.



- Now you can “mask” (not see or analyze) data outside of this ROI (surface). Go to the Pencil window and enter Mask all.... Select the channel to be masked. Be sure to deselect the “duplicate channel before masking” option. Repeat for all channels.



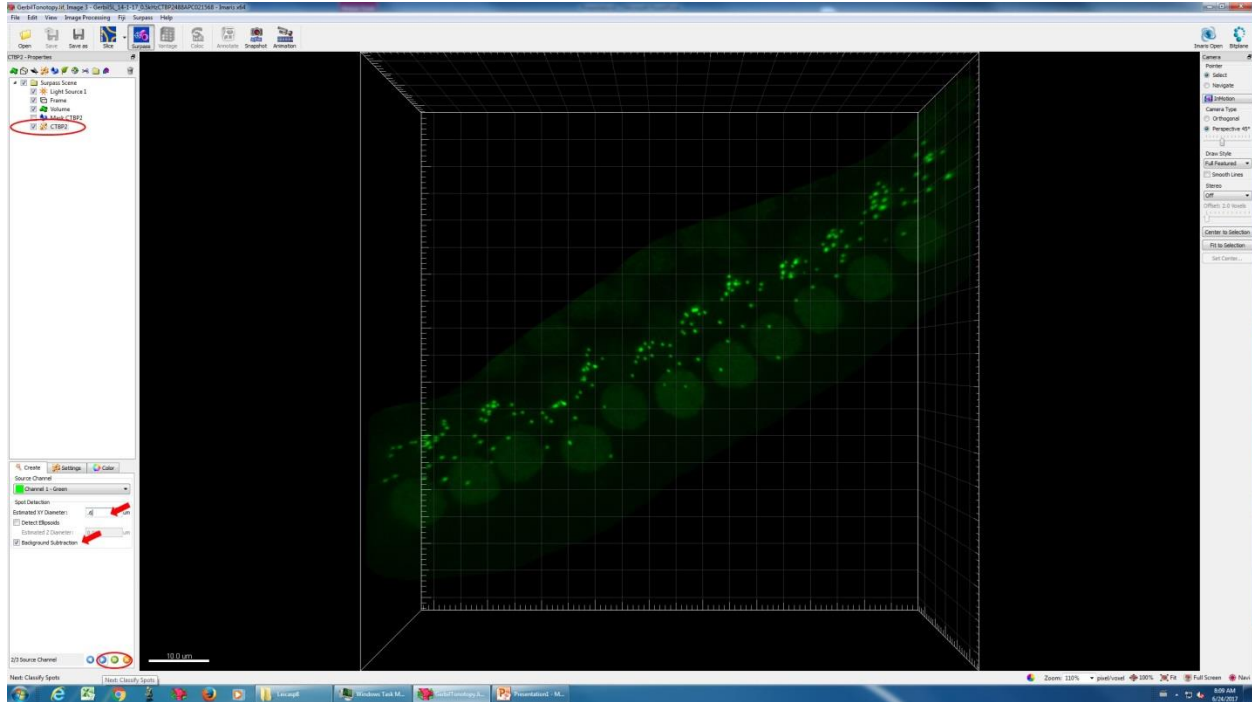
- Deselect the surface to view the ROI.

Detecting immunopuncta of interest with the “spots” function

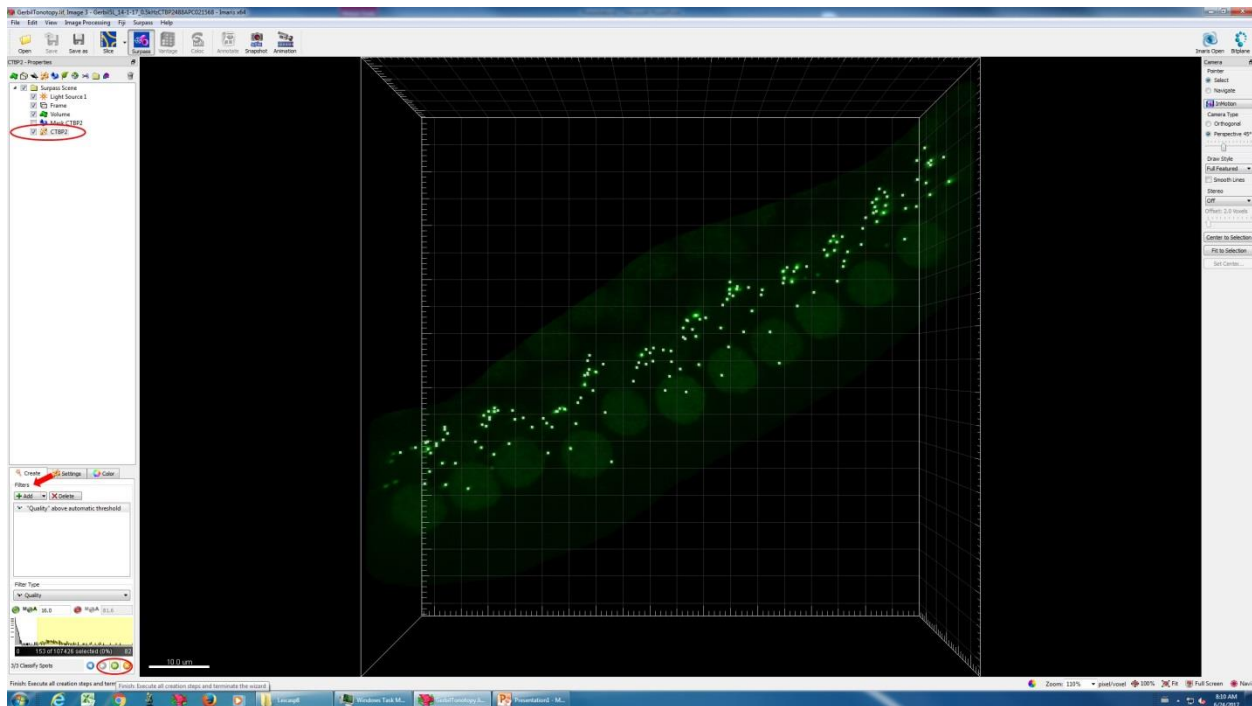
Points of interest can be tagged for counting and determination of x, y, z coordinates using the “spots” function. Spots can be created both manually and automatically. In our experience, automatically detecting immunopuncta of interest saves time but should be checked and corrected manually.

Automatic detection:

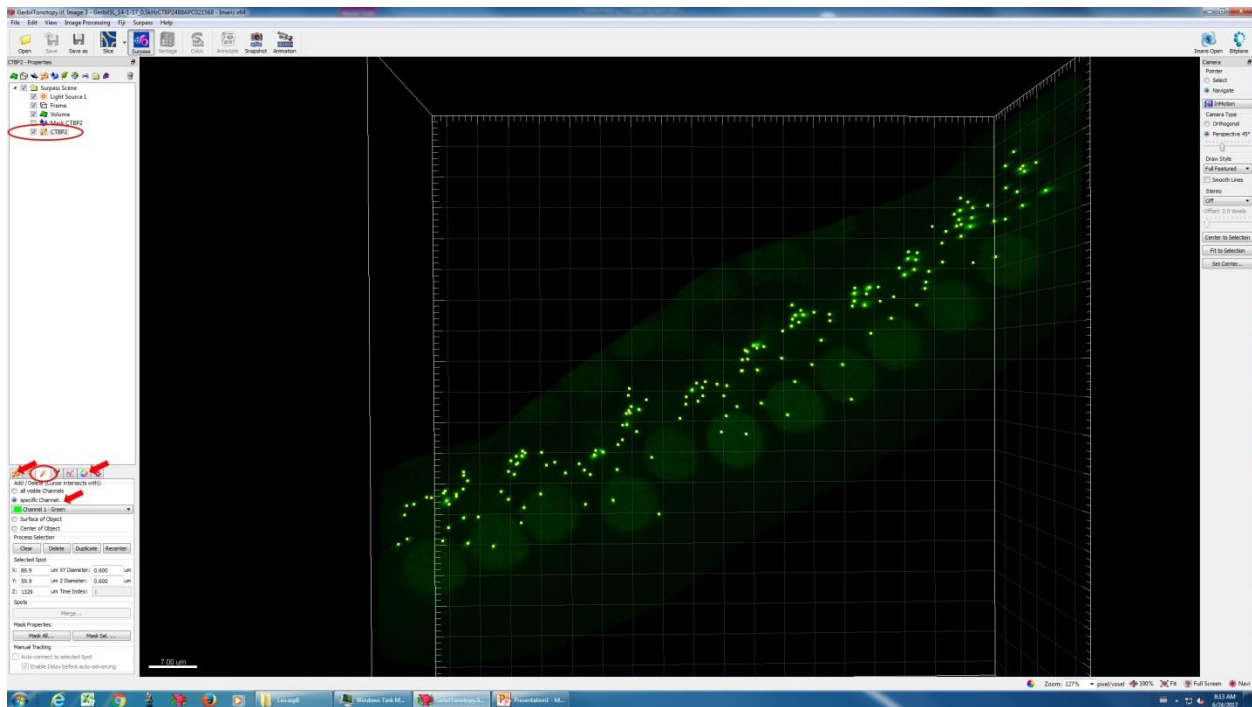
- Select the spots icon. You can rename the spots to be created. (In this case it has been named “CTBP2”.)
- Begin automatic creation on by clicking on the blue continue arrow on bottom of the wizard menu.
- Select the appropriate channel.
- Set the “estimated xy diameter” appropriately. (For example, 7 μm for detection of hair cell nuclei or 0.6 μm for detection of CTBP2 immunopuncta. Exact diameters for best detection will need to be adjusted for the specific points of interest.)
- Leave background subtraction on (checked).



6. After continuing, a pre-selection of spots will be displayed. Various criteria (“filters”) can be used to include/exclude spots by adjusting the slider. Spots can also be included/excluded later manually.
7. Press the double green arrow to complete the spots detection.



8. Additional spots can be included or excluded as described above. (Briefly, go to pencil > specific channel and select appropriate channel. Toggle to the select mouse pointer (single arrow) by pressing esc. Hold the shift key while left clicking the immunopunctum of interest to create a spot. Remove a spot by right clicking on that spot. The size, shape, and color of the spot can be adjusted from the spots and color wheel panels.)



9. Be sure to rotate the space and zoom in and out when manually verifying all spots.

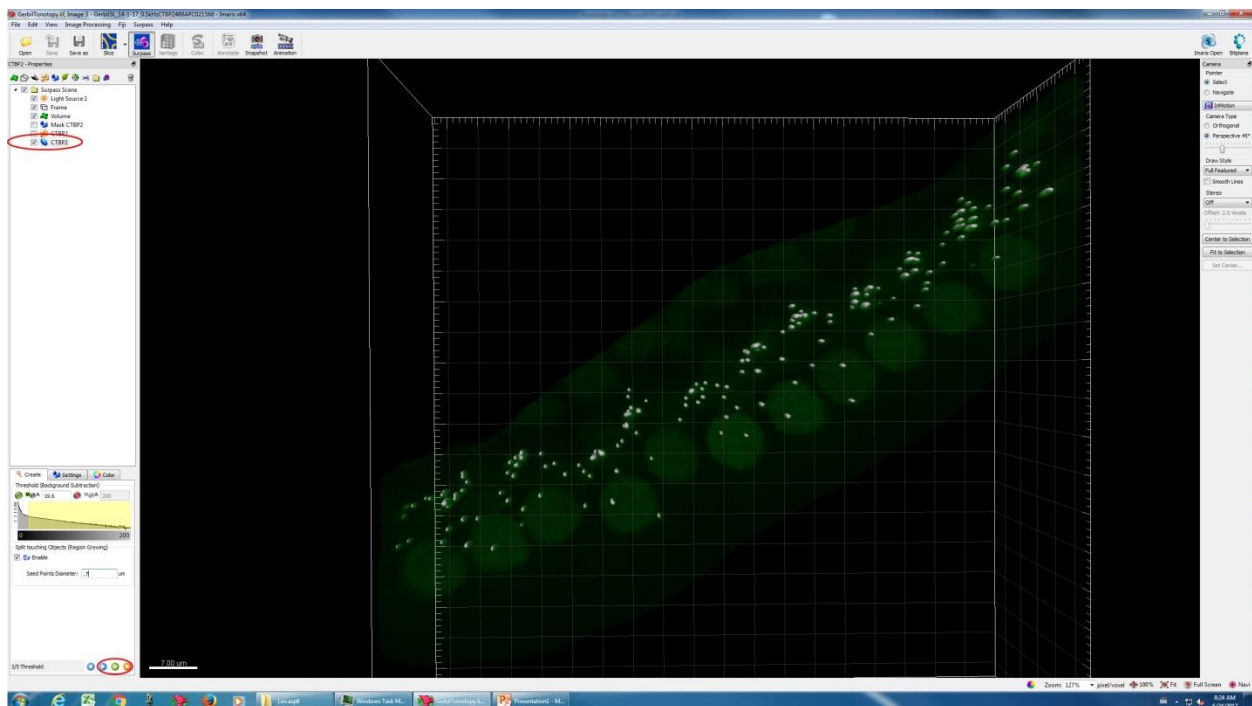
Tip: If the immunopuncta are not round (but, for example, oblong) the xy diameter should be adjusted for the smaller diameter. To measure the diameter (length) of the immunopuncta of interest, go to slice mode and click on one end of the object and then the next end. The length of the line created will appear on the far right corner of the screen.

Manual detection:

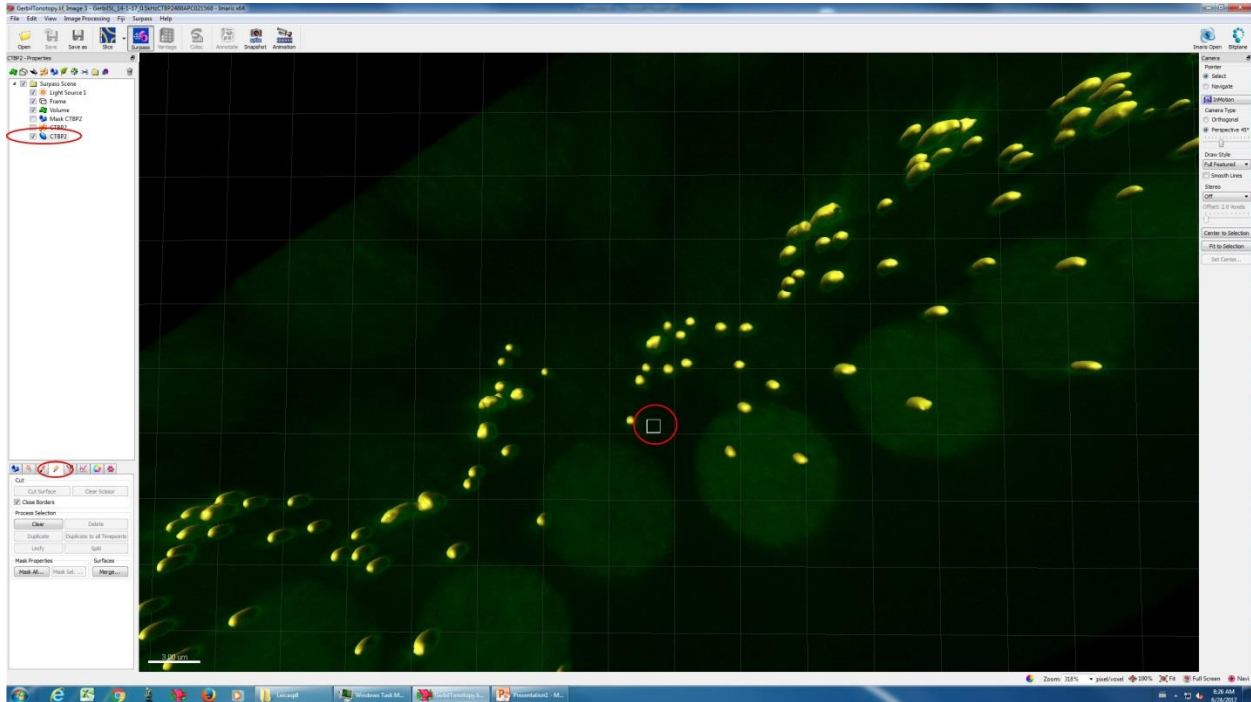
1. Select the spots icon (yellow-orange bubbles)
2. Skip the automatic creation.
3. Go to pencil > specific channel and select appropriate channel.
4. Toggle to the select mouse pointer (single arrow) by pressing esc. Then hold the shift key while left clicking the location of the immunopunctum of interest. Spots can be removed by left clicking for a second time on that spot. The size, shape, and color of the spot can be adjusted from the spots and color wheel panels.

Creating surfaces for immunopuncta of interest with the “surfaces” function

1. Select the surfaces icon (blue blobs). You can rename the surfaces to be created. (In this case it has been named “CTBP2”.)
2. Begin automatic creation on by clicking on the blue continue arrow on bottom of the wizard menu.
3. Select the appropriate channel.
4. Select appropriate surface level detail (generally one-tenth the approximate diameter of the immunopunctum of interest)
5. Under threshold, use either absolute intensity or background subtraction. (When fitting surfaces, test these parameters to get the best fit.)
6. Continue creation wizard by clicking the blue arrow forward. At any point before completing the surface, you can step back and make adjustments.
7. Adjust the threshold slider to achieve the best fit of the surface to the immunopuncta of interest. Various criteria (filters) can be used. “Quality” is generally the most helpful. Consistency between samples is most critical. Surfaces that are not well fit can be excluded later. Touching objects can be split by enabling “split touching” during the creation wizard. The seed point diameter for splitting touching objects can also be adjusted.
8. Continue creation wizard by clicking the blue arrow forward. A pre-selection of surfaces will appear. Again, various filters can be used to include/exclude surfaces, including the number of voxels, volume, and area.
9. Press the double green arrow to complete the surfaces detection.



10. Surfaces can be excluded. Briefly, go to pencil. Toggle to the select mouse pointer (single arrow) by pressing esc. Remove a surface by left clicking on that surface and then selecting delete. The size, shape, and color of the surface can be adjusted from the surface and color wheel panels.



11. Be sure to rotate the space, zoom in and out, and turn on and off the display channel when manually verifying the fit of all surfaces.

Exporting data for subsequent analysis

1. Select the spots or surfaces of interest.
2. Go to statistics (graph icon)
3. Select information to export by clicking the tools icon (left corner). Typically the spot ID, spot x, y, z coordinates, total number of spots and/or volumes of surfaces are selected.
4. Select the multiple floppy disk icon to export data to an Excel file.
5. Be sure to verify the correctness of the information exported and save the Excel file with other identifying information (specimen ID) that might otherwise not be exported.

