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http://www.ncbi.nlm.nih.gov/pubmed/22474665; http://cshprotocols.cshlp.org/content/2012/4/pdb.prot068601.long
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Quantitative analysis of NMJ morphology v1.02c

Introduction

Image analysis and quantification are best performed in 3D, e.g. with the software Imaris by Bitplane, but can also be performed by a simpler method for 2D analysis using the free software ImageJ that is presented in the following protocol. My intention is to propose ideas of how such an analysis could be performed with ImageJ, rather than providing a stiff protocol, where the steps have to be followed without modifications.
Although execution of most of the tools will be described via the ImageJ menu, most are also readily accessible through icons in ImageJ MBF (see below) toolsets. In addition, these toolsets can very easily be adapted for higher efficiency.

ImageJ can be downloaded for free at http://rsb.info.nih.gov/ij
It is, however, highly recommended not to use the standard version of ImageJ but a modified bundle, already containing a collection of useful plugins, such as *ImageJ MBF* or *Fiji*.
ImageJ MBF includes a very useful collection of plugins and toolsets for microscopy and can be downloaded at http://www.macbiophotonics.ca/imagej/
Fiji is a modified version of ImageJ, aiming at a complete integration of plugins, especially for image analysis in the context of (Drosophila) neurobiology.
It can be found at http://pacific.mpi-cbg.de/wiki/index.php/Fiji
Please note that ImageJ (and especially Fiji) constantly gets updated. The function of plugins can change with an update. The method as described here works for sure with versions **1.42q**-**1.44c**.

Adjusting the Settings and Background Correction

1. Make sure to acquire your data according to the Nyquist-Shannon theorem (i.e. with twofold oversampling). Use the full intensity range for image acquisition, i.e. saturate the signal but avoid oversaturation. Use a pseudo-colored lookup table with range indicators when adjusting laser intensities.

2. Open your images, preferably from the original file generated by the microscope software (e.g., *.lif*, *.lsm* or *.oib*) and not from exported TIFFs (this preserves scale settings). ImageJ MBF supports opening these file types. Check the box *Split channels*.
   If you open Olympus *.oib* files check *Autoscale*.

3. Set measurements (*Analyze/Set Measurements…*). Check *Area*, *Min & Max Gray Value*, *Mean Gray Value*, and *Limit to Threshold*. Choose *Redirect To: None*, *Decimal Places: 2*.

4. Check whether the scale settings are correct (*Analyze/Set Scale…*). These settings will be preserved even during image resize operations and in TIFF files saved by ImageJ.

5. If you have acquired Z-stacks, generate maximum intensity projections of all channels (*Image/Stacks/Z Project…*, *Projection Type*: *Max Intensity*).

6. Change the lookup table (LUT) to *Fire* (*Image/Lookup Tables/Fire*).

7. Optionally: Remove irrelevant (non-synaptic) structures from the image, e.g. tracheae, by using the *Freehand selections* (standard toolbar) and *Fill* tools (*Edit/Fill* or *f*). For this you have to set the *Color Picker* tool to 0,0,0 (black) first (*Image/Color/Color Picker…*).

8. Remove Background: Mark part of the image background using *Freehand selections* and copy it to the *ROI manager* (ROI = region of interest) by pressing *t*. Repeat this twice to obtain three representative regions. If you didn't remove tracheae, make sure not to include them in your selections. Measure the ROIs in all three channels (*Analyze/Measure* or *m*). Subtract the *Mean Gray Value* (*Process/Math/Subtract…*) of one of your ROIs (i.e., of the image background) from the whole image (i.e., deselect any selection in your image first).

9. Shift the intensity distribution back to its original scale (i.e., the range prior to background correction). If you have used the full intensity range for image acquisition, multiply the whole image by the factor 255/(255 – *n*) (*Process/Math/Multiply…*), where *n* is the value used for background correction in the previous step. If the brightest pixel in your stack is lower than 255, use the intensity value of this brightest pixel instead of 255. If you compare several genotypes, you must use the same subtraction and multiplication factors for all genotypes. Repeat Steps 8 and 9 for all channels; use the same ROIs.

10. Save the images.

Creating a Mask of the Synaptic Area

Perform steps 11-20 for each of the channels.

11. Apply a threshold to remove irrelevant lower intensity pixels: Set the lower threshold to a fixed gray value *n* that cuts off unwanted structures but preserves your actual signal (*Image/Adjust/Threshold…, Lower Threshold Level: n, Upper Threshold Level: 255*, press *apply*). It is best to zoom in while choosing the lower threshold. The result will be a black and white image, save it. Write down the value used for the lower threshold.

12. To facilitate segmentation, generate minimum overlays of the binary masks generated in the previous step (Step 11) with the images saved in Step 10 (*Process/Image Calculator*). *Image1*: Mask from Step 11, *Image2*: Saved image from Step 10. *Operation: Min*, check *Create New Window*. In the image produced in this step, the white areas of the mask created in Step 11 will again show the signal from your original image.

13. Change the LUT to *Fire* (*Image/Lookup Tables/Fire*).

14. Segment the remaining fused synapses in the overlay images. It is recommended to perform this step semiautomatically via the command *Find Maxima*. Unfortunately, there have recently been some changes to this function. Thus, here the protocol branches off, depending on which version of ImageJ you use.

    - ImageJ v1.43j and older:
      Process/Binary/Find Maxima…, *Output type: Segmented Particles.*
      Choose an appropriate value for *Noise Tolerance*.

    - ImageJ v1.43k and newer:
      Set the lower threshold of each segmented image to a gray value of 1, the upper threshold to 255, and do not press apply. (*Image/Adjust/Threshold…, Lower Threshold Level: 1, Upper Threshold Level: 255*, do not press *apply*!).

      The location of the function has also been changed some time ago:

      - ImageJ v1.43r and older: *Process/Binary/Find Maxima…*
      - ImageJ v1.43s and newer: *Process/Find Maxima…*

    - *Output type: Segmented Particles*, check *Above lower threshold*.
      Choose an appropriate value for *Noise Tolerance*.

15. Repeat Steps 12 and 13: Create overlays of the masks you created during the last steps with the images you saved in Step 10.

16. Most probably you will not be able to separate all synapses by using *Find Maxima*. In addition, you might be worried that *Find Maxima* doesn't do the job as well as your eye. Therefore, you have to consider continuing the segmentation manually.
    Draw lines between fused synapses using *Freehand line selections* with a line width of 1 pixel (*Image/Adjust/Line Width…*). The *Color Picker* tool has to be set to 0,0,0 (black) (*Image/Color/Color Picker…*).
    Freehand line selections can be accessed by right-clicking on the *Straight line selections* tool. The selections are filled with color using the *Fill* tool (*Edit/Fill* or *f*).

17. Save the segmented masks.

18. Remove high-frequency noise from the masks by applying a gaussian blur filter (*Process/Filters/Gaussian Blur…*). Not that here you do not apply this filter to your original data but only to the mask. Do not choose a Sigma (Radius) that merges neighboring signals (AZs or PSDs). Zoom in to observe this. Usually 0.6 is a good value.

19. Apply another threshold to create black and white masks again, set the lower threshold to the same level *n* as in step 11, the upper threshold to 255 (*Image/Adjust/Threshold…, Lower Threshold Level: n, Upper Threshold Level: 255*, press *apply*). Now you have created masks that can be used for analysis of single AZs or PSDs.

20. Repeat Steps 12 and 13: Create overlays of the masks you created during the last steps with the images you saved in Step 10.

Analysis

21. For the next step, *Analyze Particles*, to work correctly, set the lower threshold of each segmented image to a gray value of 1, the upper threshold to 255, and do not press apply. (*Image/Adjust/Threshold…, Lower Threshold Level: 1, Upper Threshold Level: 255*, do not press *apply*!).

22. Analyze the dimension and intensity of all remaining individual structures (AZs or PSDs) (*Analyze/Analyze Particles…*). Choose a reasonable (minimum) *Size* (e.g., 4 pixels [2*2]). *Show Outlines*; check *Display Results*, *Clear Results*, *Exclude on Edges*.

23. Save the outline drawing and compare the outlines of the areas used for the analysis with your original image and, if necessary, remove additional structures and segment further (repeat Steps 14-23 on the basis of the mask saved in Step 17).

24. Copy the results to a spreadsheet or statistics application and continue the analysis with these applications. If the scale settings were correct in Step 4, these settings have been preserved throughout the segmentation and normalization steps. Thus, the results (in micrometers) should need no further conversion. To be sure, double-check the scale settings (*Analyze/Set Scale…*) of the images you performed the particle analysis on.

25. Measure the total synaptic area and synaptic density in a similar manner. Instead of analyzing particles, measure the total area highlighted in the mask from Step 19 (Analyze/Measure). In case of immunostainings, pre- and postsynaptic area can be related to a generic neuronal marker such as Hrp to assess synaptic density. The normalization to an Hrp signal will cancel out variations in animal size to some degree. Optimally, these values should be normalized to the size of the respective muscle or the segment length to minimize errors due to variations in animal size. Because this is often difficult to achieve under in vivo imaging conditions, using NMJ sizes for normalization is another option.