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DIO-SPOTlight Image Processing and Analysis v.250227

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We use this protocol and it's working

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Abstract

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This protocol contains the image processing and analysis steps that follow the image acquisition protocol.

Partner protocols: *DIO-SPOTlight Immunohistochemistry v.250227* & *DIO-SPOTlight Confocal Imaging Acquistion v.250227*

1 **Image Analysis**

2 Open Fiji (ImageJ) perform batch conversion from IMS to TIFF format

a. **Process** > **Batch**

i. Batch convert window opens. Select input and output files. Output format: TIFF; Interpolation: none; Scale factor: 1.00. Press convert.

3 Background subtraction and ROI cellular masking.

a. Open image and duplicate

b. Select duplicated image and perform a 15-pixel Gaussian blur.

i. **Process** > **Filters** > **Gaussian Blur**

1. Gaussian Blur window opens. Type 15 in the “Sigma (Radius)” box. Select OK.

c. Subtract blurred image from the original.

i. **Process** > **Image Calculator**

1. Image Calculator window opens. For Image 1, select the original. For Image 2, select the blurred duplicate. For Operation, select subtract. Check box “Create in new window.” Select OK.

d. Select the new background subtracted image and perform a 2-pixel Gaussian blur.

i. **Process** > **Filters** > **Gaussian Blur**

1. Gaussian Blur window opens. Type 2 in the “Sigma (Radius)” box. Select OK and save the image for mask making.

e. Locate the desired plane containing a high number of the cells of interest and duplicate that plane for the original and background subtracted image.

f. Select the background subtracted image and create binary masks of the anti-ChAT and nuclear stained channels (channels 1 and 4):

i. **Image** > **Adjust** > **Threshold**

4 ROI selection of binary images

a. Select the wand tool and select a binary cholinergic neuron and add to the ROI manager. For the same cell switch to the binary nuclear stained channel and add the nucleus of the cell to the ROI manager.

b. Segregate the cytoplasmic and nuclear signal using the XOR function in the ROI manager.

i. **ROI manager** > **More** > **XOR**

c. Repeat for the other cells of interest

d. Save the coordinates.

5 Select the original raw image corresponding to the masked cells. Measure the fluorescent signal of the selected ROI and save or directly transfer to a spread sheet. Label as ChAT.

6 Create a binary mask of neighboring non-cholinergic neurons.

a. Select the RFP and GFP channels (channels 2 and 3) from the background subtracted image.

b. Create an additive image of both RFP and GFP channels to limit biasing ROI selection toward cells expressing low levels of either fluorescent protein.

i. **Process** > **Image Calculator**

1. Image Calculator window opens. For Image 1, select RFP channel. For Image 2, select GFP channel. For Operation, select Add. Check box “Create in new window.” Select OK.

ii. Create a threshold binary mask for the new additive image the same as above for the ChAT and nuclear stained channels.

7 ROI selection of binary images for neighboring neuron selection.

a. Add ROI to ROI manager and exclude nucleus the same as described above.

b. Include background measurements to ROI manager from neurons where Cre recombination failed.

c. Save neighboring neuron ROI coordinates.

8 If cells did not include a nucleus in the analyzed plan no XOR function was performed. In some instances, manual ROIs were drawn.

9 Select the original raw image corresponding to the masked cells. Measure the fluorescent signal of the selected ROI and save or directly transfer to a spread sheet. Label as neighboring neurons

10 Cytoplasmic measurements only were used for analysis.