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Immunofluorescence Cell Counting and Analysis in Fiji

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We use this protocol and it's working

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Abstract

Objective: To quantify specific starter populations of AAV infected dopamine (DA) neurons within the Substantia Nigra pars compacta (SNc) or Ventral Tegmental Area (VTA) in multi-channel immunofluorescence images of brain sections.

Experimental Model System:

This study utilizes a dual recombinase-based strategy to genetically target and label distinct subpopulations of midbrain dopamine (DA) neurons. To label a specific DA neuron subtype, we employed a transgenic mouse model expressing Cre recombinase under a subtype-specific promoter and FlpO recombinase under the dopamine transporter (DAT) promoter. These animals were stereotactically injected with a CONFON (Cre-ON/FlpO-ON) virus driving the expression of eYFP, which labels the targeted subtype in green. Concurrently, a COFFON (Cre-OFF/FlpO-ON) virus driving mCherry expression was co-injected. This virus labels the broader population of DAT-expressing DA neurons that are not part of the specific Cre-expressing subtype in red. This approach results in the following labeling:

- **Green (eYFP):** DA neurons of the specific genetic subtype.
- **Red (mCherry):** Other DA neurons within the injection site vicinity.

To confirm dopaminergic identity, dopamine neurons are also immunostained with Tyrosine Hydroxylase (TH) with a Far-red labelled secondary antibody such as Alexa fluor 647. Nuclei are labelled with DAPI. Quantification of these starter populations within the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) is critical for interpreting subsequent axonal staining patterns in downstream target regions. The protocol below details the method for quantifying these DA neuron populations through image adjustment, application of standardized regions of interest (ROIs), and manual cell counting based on strict criteria.

Video and image document provided

Attachments



[Cell Counting Protoc...](#) [video1082986225 \(1\)....](#)

2.2MB



68.5MB



Materials

Materials:

- Fiji (ImageJ) software
- Multi-channel TIFF image files (Channels: C0001, C0002, C0003, C0004)
- Pre-defined ROI files (.roi or .zip)

Image Loading and Channel Alignment

- 1 Launch the Fiji application.
- 2 Load the TIFF image stack using one of the following methods:
 - 2.1 **Method A:** Drag and drop the image file directly into the Fiji window.
 - 2.2 **Method B:** Navigate to File > Open... and select the target image file.
- 3 Separate and align the channels:
 - 3.1 Navigate to Color > Merge Channels....
 - 3.2 Assign the images to their corresponding color channels as follows:
Red: C0002
Green: C0001
Blue: C0003
Gray: C0004 (This channel will be displayed as Teal)
 - 3.3 Ensure the following boxes are checked:
☒ Create composite
☒ Keep source images (Recommended)
☒ Ignore source LUTs
Click OK

Image Cropping

- 4 Using the Rectangle tool, select a region of interest (ROI) that encompasses the entire stained brain section
Note: Ensure the cropped area includes a margin of space around the section to accommodate subsequent ROI placement.
- 5 Crop the image by navigating to Image > Crop.

Rationale: Cropping reduces file size and RAM usage, improving software performance.

Channel-Specific Signal Adjustment

- 6 Open the Brightness/Contrast tool by navigating to Image > Adjust > Brightness/Contrast.
- 7 For each channel, perform the following:
 - Select the target channel using the channel slider at the bottom of the image window. The active channel is indicated by the color of the image title.
 - In the Brightness/Contrast window, click Reset followed by Auto
 - Manually adjust the Minimum and Maximum sliders to optimize the signal-to-noise ratio based on the criteria below

Blue Channel (C0003, Cell Bodies):

 - Adjust to maximize the number of visible, distinct circular cell bodies.
 - Reduce background haze without causing faint cell bodies to disappear.
 - Avoid settings where individual cells converge into a single blob.

- Red/Green Channels (C0002/C0001, Axonal Markers):

 - Adjust to visualize punctate circular regions, which represent cell bodies, amidst the axonal staining.
 - Precise cell body delineation is not required as axons are not the counting target.

- Teal Channel (C0004):

 - Adjust to preserve distinct ring-like structures while minimizing background signal.
- 8 Apply the settings by clicking Apply.
Repeat for all channels.

Saving the Adjusted Composite Image

9 Save the adjusted and cropped composite image.

9.1 Navigate to File > Save As > Tiff....

9.2 Give the file a descriptive name and save it in the appropriate directory.

- **Note:** All subsequent counting and analysis must be performed on this saved image. Failure to save will result in loss of adjustments and any counting data.

Managing Regions of Interest (ROIs)

10 Open the ROI Manager by navigating to Analyze > Tools > ROI Manager

11 Load the pre-defined ROIs by clicking More >> and selecting Open.... Select the appropriate ROI file.

12 Assign and modify ROIs for each cell type:

12.1 Four ROIs are required, corresponding to the following cell types:

1. GFP+ in SNcMid (Bregma 3300)

2. mCherry+ in SNcMid (Bregma 3300)

3. GFP+ in SNL

4. mCherry+ in SNL

13 Ensure each ROI is assigned to the correct channel (green for GFP, red for mCherry) by selecting the ROI and then the corresponding channel with the slider.

14 To create a new ROI or modify an existing one, use the selection tools (e.g. wand tool)

15 Click Add [t] in the ROI Manager to save a new selection. Click Update to save modifications to an existing ROI.



- 16 Use the Show All function to visualize all ROIs simultaneously and ensure proper alignment across channels. Example Image A

Manual Cell Counting

- 17 Initialize the Cell Counter plugin by navigating to Plugins > Analyze > Cell Counter.
- 18 In the Cell Counter window, initialize a new counter. **Ensure the adjusted composite image is saved.**
- 19 Assign counter types to the four cell types (Type 1-4). Systematically count cells within each ROI based on the following hierarchical criteria:
Quality Check: After counting, review each ROI to ensure no cell has been erroneously assigned to both red and green channels. Use the Delete Mode in the Cell Counter to remove any duplicate marks.
- 20 **Counting rules:**
- **Step 1: Blue nucleus presence (required).**
 - Count if nucleus is circular, dot-like, or fragmented but enclosed by a circular boundary.
 - If nucleus appears as a blob, estimate the number of individual cells.
 - **Step 2: Red/Green overlap.**
 - Count if the nucleus is entirely covered or surrounded by Red or Green.
 - Do not count if only partially covered or surrounded by axonal fibers.
 - **Step 3: Teal overlap.**
 - Count if nucleus is surrounded by a teal ring or teal marks are visible on the nucleus.

Data Saving and ROI Measurement

- 21 Save the cell count data:
- In the Cell Counter window, click Save Markers. Use the default naming convention to ensure file accessibility.



22 Measure ROI areas:

- In the ROI Manager, select one ROI.
- Ensure measurement settings are correct (Analyze > Set Measurements...; ensure Area is selected).
- Click Measure. A results window will appear.

23 Export the data:

- Copy the measurements from the Results window into a spreadsheet (e.g., Excel, Google Sheets), ensuring each ROI area is recorded alongside its corresponding cell count. Image B