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Quantification of Axonal Projections from Whole-Brain Slide Images

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Protocol status: Working
We use this protocol and it's working

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Abstract

Purpose
To provide a standardized method for processing and analyzing immunofluorescence images of mouse brain sections to quantify axonal projections. The pipeline consists of four main stages: Cropping individual brain sections from whole-slide scans, generating multi-channel TIFFs, warping images to a standard atlas for anatomical alignment, and applying a tubeness algorithm to identify and isolate axons for quantification.
Scope
This protocol applies to .VSI files generated from scanned immunofluorescence slides. It is designed for use with CellSense software for initial cropping and Fiji (ImageJ) for all subsequent image processing and analysis.

Please find an image PDF attached to help follow the protocol

Attachments

PDF

images.pdf
3.8MB

Materials

Materials

- CellSense Software
- Fiji (ImageJ) with the following plugins:
- BigDataViewer
- BigWarp
- Tubeness
- Reference
  Allen Brain Atlas images (corresponding to the Bregma levels of your sections)
- Pre-generated
  landmark files for the atlas images (if available)

Cropping Individual Brain Sections from Whole-Slide Images- To isolate a single brain section from a multi-section slide scan and remove non-brain background.

1 **File Identification:** Locate the .VSI file for the target slide, identified by Mouse ID, Genotype, Staining, and Slide Number

2 **Open File:** Open the .VSI file in CellSense Software

3 **Identify Target Section:** Using a reference brain atlas, identify the specific brain section (by micrometer level) to be cropped.

4 **Initial Crop:**
- From the upper toolbar, select **'Crop to new image'**
- Drag a rectangle around the entire target brain section and release.
- Wait for the process to complete (a green progress bar will be shown)

5 **Image Orientation:**
- **Flip** the image horizontally/vertically to match the desired hemisphere orientation.
- **Rotate** the image using the rotation slider until the brain section is symmetrical along the medial-lateral axis.
- *Note:Moving the slider to the right rotates the image clockwise.*

6 **Final Crop:** Use the **'Crop to new image'** tool again to tightly crop the image, removing all non-brain background area. Only the brain section should remain.

7 **Save:** Save the final cropped image as a new .VSI file.

Generating Multi-Channel TIFF Files-To export the cropped .VSI image as a standardized multi-channel TIFF file for analysis in Fiji.

8 Open Cropped VSI: Ensure the cropped .VSI file from Section 4.1 is open in CellSense.

9 Check Channels: In the right-hand panel, review the overview and staining sections. Delete any extraneous or blank (white pixelated) image channels so only the relevant fluorescence channels remain.

10 Export as TIFF

10.1 Go to File > Export as > Tiff.

10.2 Name the file according to the convention: [MouseID]_[MicrometerLevel].tif.

10.3 Save the file to the appropriate directory.

11

Atlas Warping for Anatomical Standardization- To align each brain section to a reference atlas, enabling consistent anatomical comparison across samples.

12 Open Images in Fiji

12.1 Open the TIFF file (e.g. Channel C004, typically a background stain like DAPI) for the brain section

12.2 Open the corresponding Allen Brain Atlas image for the matching micrometer level

13 **Optimize Contrast:** For both the brain image and the atlas image, navigate to Image > Adjust > Brightness/Contrast. Adjust the **maximum** sliderto enhance the visibility of anatomical landmarks (e.g., striatum, corpus callosum, ventricles). *Do not adjust the minimum slider.* Click **Apply**. (see image A)

14 Launch BigWarp Go to Plugins > BigDataViewer > Big Warp

14.1 In the dialog box: Set your brain image as the Moving Image. Set the atlas image as the Target Image

14.2 Under Landmarks File, click Browse and select the pre-defined landmark file for this atlas level.

14.3 **Uncheck** the box for *"Apply transform from landmarks"*.

14.4 Click OK

15 Align Images

15.1 Three images will open: your brain image (with pink landmarks), the atlas image (with green landmarks), and a landmarks list. See image B

15.2 Arrange the brain and atlas windows side-by-side

15.3 Press the space bar to toggle landmark node on/off

15.4 Mode ON (space bar down): Click and drag landmarks on your brain image to align them with the corresponding anatomical structures on the atlas image

15.5 Mode OFF (space bar up): Use the scroll wheel to zoom in/out for precision

15.6 **Important: Only move the pink points on your image. Do not move the green points on the atlas

16 Apply Transformation and Export

16.1 Once all landmarks are aligned, press 'T' to transform your image preview. Check for any unnatural distortion

16.2 If satisfied- File > Export Landmarks in the moving image window

16.3 Save the landmarks as [MouseID]_[MicrometerLevel]_[#Points]_Landmarks.txt

16.4 Go to File > Export Moving Image. In dialog: set resolution to 'moving'. Set Field of View to 'Target'. Click OK

16.5 Save the warped image as a TIFF with _WARPED appended to the filename (e.g., [MouseID]_[MicrometerLevel]_WARPED.tif). see image C

16.6 **Repeat for Other Channels:** Repeat this warping process for all other fluorescence channels (e.g., C002, C003) using the **same saved landmark file** to ensure perfect alignment

17 **Axon Identification using Tubeness Algorithm-**To isolate and create a binary mask of axonal projections based on fluorescence signal.

17.1 Open Image: Open the warped TIFF for the target channel (e.g., C003 for green fluorescence) in Fiji.

17.2 Adjust Contrast: Open Image > Adjust > Brightness/Contrast. Adjust only the maximum slider until the axonal signals are clear and the background is faint. Click Apply.

17.3 Apply Tubeness Filter:

17.4 Navigate to Plugins > Analyze > Tubeness.

17.5 Set the Sigma value to 0.5. Click OK. This creates a new image highlighting tube-like structures (axons).

17.6 Synchronize Windows: Open Analyze > Tools > Synchronize Windows. Select Image Scaling and click Synchronize All. Position the original and tubeness images side-by-side.

17.7 Thresholding:

17.8 With the Tubeness image active, go to Image > Adjust > Threshold.

17.9 In the Threshold window:

17.10 Set the lower slider to 0.

17.11 Slowly adjust the upper slider to the left until background noise is minimized while true axonal signals (confirmed by comparison with the original image) are preserved.

18 Quality Check: Zoom into both high- and low-density axonal regions on both the original and tubeness images to ensure fidelity (no data loss, no addition of spurious signal). View Image D

19 Create and Save Mask:

20 Once optimal thresholding is achieved, click Apply in the Threshold window. Check the box for Convert to Mask and click OK.

21 Save the resulting binary mask as a TIFF file. View Image E

22 Record Threshold Value: Record the final threshold percentage value used in a lab notebook or spreadsheet. Note: This value can be retrieved later by opening the mask TIFF, opening the Threshold tool, and sliding the lower bar completely to the right; the upper bar will show the used percentage.

23 **Data Analysis**
The resulting binary mask TIFF files are ready for downstream quantification analysis (e.g., pixel density, projection area) within the standardized atlas space

Untitled section

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