SpineRacks and SpinalJ user guide **Version 5/2021**

Materials

Biological material

This protocol was established on fixed spinal cord tissue of adult C57BL/6J mice.

Reagents/Consumables

- Ultimaker Natural PVA filament (Ulimaker, product 9732)
- Paraformaldehyde 16% aqueous solution, EM grade (e.g. Electron Microscopy Sciences, product 15700)
- PBS 10x (e.g. Corning, product 46-013-CM)
- Conical tubes 15 ml (Fisher Scientific, product 14-959-49B)
- Sucrose (e.g. Fisher Scientific, product AC177140050)
- Polystyrene petri dish, 4 inch square (e.g. Carolina Biological Supply, product 741470)
- Tissue-Tek OCT Compound (Sakura Finetek, product 4583)
- Parafilm M (Fisher Scientific, product S37440)
- Truncated embedding mold, Peel-A-Way(R) T12 (Polysciences, product 189861)
- Absolute Ethanol (e.g. EMD Milipore, product EX0276-4)
- Dry ice
- Microscope slides, Fisherbrand Superfrost Plus (Fisher Scientific, product 12-550-15)
- Disposable microtome blades (Sakura Finetec, product 4685)
- NeuroTrace (Invitrogen, product N21480, N21483) or DAPI (Invitrogen, product D1306)
- Primary and secondary antibodies
- Mounting media, Fluoro-Gel (Electron Microscopy Sciences, product 17985)
- Cover glass 60mm (Fisher Scientific, product 12460S)

Equipment

- 3D-printer (Ultimaker3 or equivalent)
- 4 °C, -20 °C and -80 °C storage units
- Perfusion setup and tools for spinal cord dissection
- Chemical hood
- Orbital shaker
- Dissecting scope
- Spring scissors, 8mm (e.g. Fine Science Tools, product 15009-08)
- Forceps, blunted (e.g. Fine Science Tools, product 11253-20)
- Cryostat (Leica Biosystems CM3050S or equivalent)
- Paintbrushes for handling sections
- Wheaton staining dishes (optional, to wash multiple slides)
- Slide scanner microscope (Nikon Instruments AZ100 Multizoom with automated slide feeder; 4x 0.4 NA AZ Plan Apo objective; Andor Zyla sCMOS camera or equivalent system)
- A computer workstation running Windows with at least 32 GB of RAM. For benchmarking, the pipeline was deployed on a workstation running Window 10 Enterprise 64 bit, equipped with a 16 core Intel i9 7960x 2.8GHz CPU, 128 GB DDR4 memory, a 1TB Samsung 860 SSD and a 12 GB nVidia Titan X video card.

Before you start

- Prepare 1x PBS, store at RT
- Prepare 30% sucrose in ddH_2O, store at 4°C
- Prepare fresh 4% PFA in PBS
    - Download SpineRack.stl print files (https://github.com/felixfiederling/SpinalJ/blob/main/SpineRack.stl) and print using PVA filament. For the Ultimaker3 printer, we used the following slicing settings in Cura v4.8: Material: natural PVA; print core: BB 0.4; layer height: 0.15mm; print temp: 220°C, bed: 60°C; infill: 20%; build plate adhesion: brim 3mm.

  The print time for 50 SpineRacks on this device is about ~3h. Clean printed structures from brims and print artifacts that protrude into the wells if necessary by peeling off the first print layer. Store SpineRacks in a sealed container at room temperature in the dark with a desiccant bag to prevent absorption of ambient moisture.
- Download and install Fiji (Schindelin et al., 2012) software (http://fiji.sc/Fiji)
    - Download SpinalJ for Fiji (https://github.com/felixfiederling/SpinalJ)
    - Copy the SpinalJ.jar file into your Fiji plugins folder and install the plugin (*Plugins* > *Install*).
    - Store the segmentation masks Spli_mask.roi and Split_mask_scaled.roi somewhere on your computer.
    - Download the SpinalJ atlas files folder (https://data.mendeley.com) and store on your computer.
    - Install MultiStackReg. Download and copy into your Fiji plugins folder. MultiStackReg can be downloaded at: http://bradbusse.net/sciencedownloads.html
    - Install TurboReg (Thévenaz et al., 1998). TurboReg can be downloaded at: http://bigwww.epfl.ch/thevenaz/turboreg/
    - Install Elastix 5.0.0 (Klein et al., 2010). Elastix can be downloaded at: https://elastix.lumc.nl/download.php
    - Install Ilastik 1.3.3post3 (Berg et al., 2019). Only required for machine learning detection of cells and projections. Ilastik can be downloaded at: https://www.ilastik.org/download.html

Procedure

Tissue preparation (timing: ~1h + ~2d unattended)
1: Perfuse the deeply anesthetized animal with 4% PFA in PBS and expose the spinal cord (SC) by laminectomy or hydraulic extrusion as described elsewhere (Kennedy et al., 2013). Postfix spinal tissue in 4% PFA in PBS on an orbital shaker overnight at 4°C.

2: Wash SC 3x in PBS on ice, remove dura and meninges, then transfer into a 15ml tube filled with 30% sucrose solution and incubate at 4°C for 1-2 days until the tissue has sunk for cryo-protection.

Tissue embedding (timing: 10-15min + 20-30min unattended)
3: Prepare a ~5cm x 5cm piece of Parafilm, and pour some OCT on it. This pool of OCT will be used to wash and orient tissue pieces prior to embedding.

4: Transfer the SC together with the sucrose solution to a square petri dish (Figure 1a). Cut the SC into three equal parts (Figure 1b, c) using sharp spring scissors under a dissecting scope. Make sure to cut perpendicular to the long axis of the SC (angled cuts produce incomplete cross sections that cannot be used for registration). Keep track of the identity and rostrocaudal (r-c) orientation of the tissue pieces.

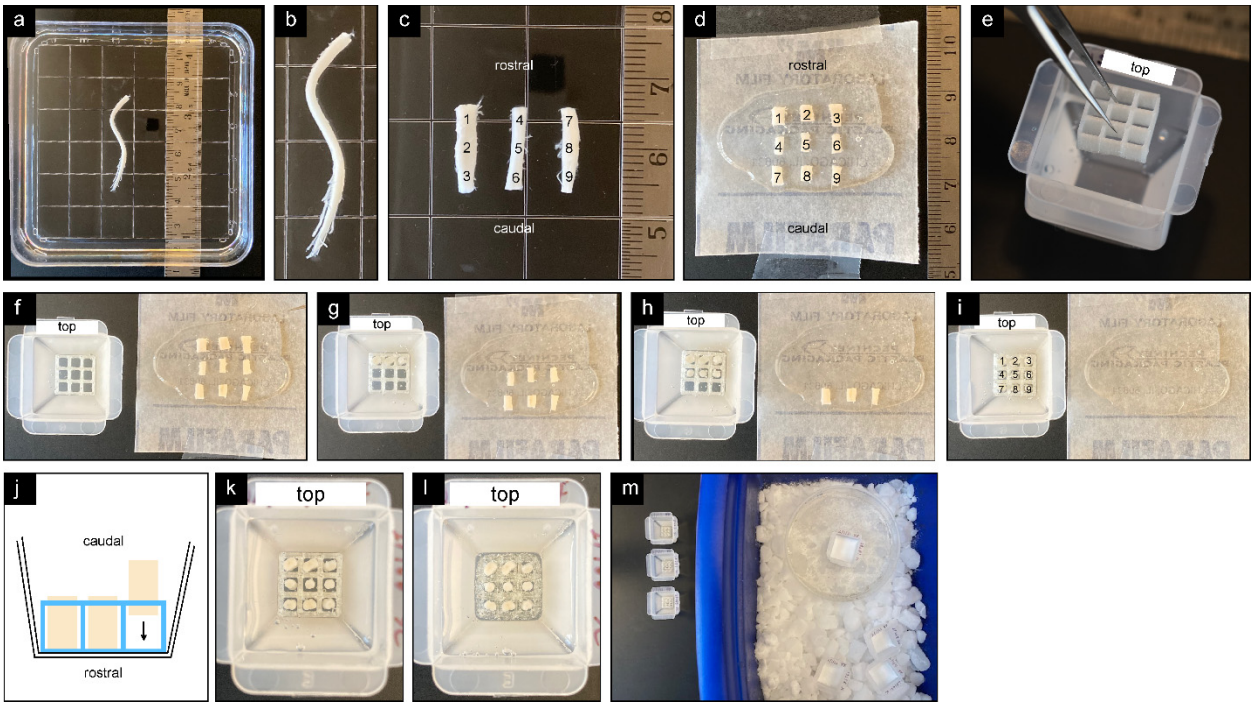

Figure 1: Embedding of SC tissue using SpineRacks
a, b) Isolated SC in sucrose solution. c) SC cut into 3 equal tissue pieces. Numbers 1-9 indicate r-c orientation and placement of next cuts. d) SC cut into 9 equal pieces arranged according to position in SpineRack in OCT. e) Submerging SpineRack in embedding mold filled with OCT. f-i) Placement of tissue pieces 1-9 into SpineRack. j) Vertical orientation of tissue pieces in the mold. k, l) Incubation of SpineRack in OCT expands and softens the material for sectioning (k: before, l: after 20 min incubation). m) Freezing the block.

5: Cut each piece into three equal sized pieces (Figure 1c, d). Starting with the most rostral segment, after each cut gently transfer the resulting tissue pieces 1-3 into the OCT puddle with its rostral end facing the top side of the Parafilm square using forceps. Repeat with the other pieces and collect resulting tissue pieces

4-6 and 7-9 in OCT. Keeping track of the identity and orientation of each tissue piece is critical. For maximal cryo-sectioning efficiency, all tissue pieces should be of equal length.

6: Fill a truncated embedding mold with OCT and mark one side for orientation. Submerge a SpineRack into OCT and push all the way to the bottom of the mold using forceps (Figure 1e). Release any trapped air bubbles.
Critical: After immersing the SpineRack into OCT, the PVA material will start to dissolve and will soften and expand in the process, and this may prevent positioning or placement of tissue pieces. Thus, tissue must be embedded immediately, within the first 15 minutes after immersing the SpineRack in OCT. To extend embedding time, cooling of OCT on ice can slow down the dissolving of the rack.

7: Transfer one tissue piece at a time into the mold, wash off any remaining sucrose solution, and load into the SpineRack from top-left to bottom-right in r-c order (Figure 1f-i). Using blunt forceps, carefully push each tissue piece within a well to the bottom of the mold with its rostral end facing down and the dorsal side facing the marked side of the mold (Figure 1j). Wide cervical segments 1-3 are rotated to fit the well diagonally.

8: After embedding all tissue pieces, incubate SpineRack and tissue for 20-30 minutes to soften the PVA material for sectioning (Figure 1k, l).

9: Freeze the block on dry ice in a slush of ethanol and crushed dry ice until solid (Figure 1m).
- *Optional stop point: Store blocks at -80°C until use.*

Sectioning and Staining (timing: ~2h + ~24h unattended)
10: Place the block into the cryostat chamber to equilibrate temperature. For sectioning, mount the block on the block holder with the marked side up and set sectioning thickness to 25µm (Figure 2a).

11: Section the block and collect all sections continuously on numbered slides. For automated imaging, collect eight sections in two rows of four sections with equal spacing on each slide (Figure 2b). Keep note of the order and orientation of sections on each slide, as this is crucial to maintain r-c order of images. Make notes of lost sections (slide # and position on slide; lost sections can be replaced by neighboring sections during image processing).
- *Optional stop point: Store slides at -80°C.*

12: Wash slides in a Wheaton dish filled with PBS for 5-10min (shaking at RT) to re-hydrate tissue and wash away SpineRack material and OCT (Figure 2c). Proceed with immunohistochemistry or other staining methods following standard protocols and mount slides under coverslips (Figure 2d). For subsequent image registration, staining with NeuroTrace (preferred) or DAPI is required.
- *Optional stop point: Store mounted slides at 4°C.*

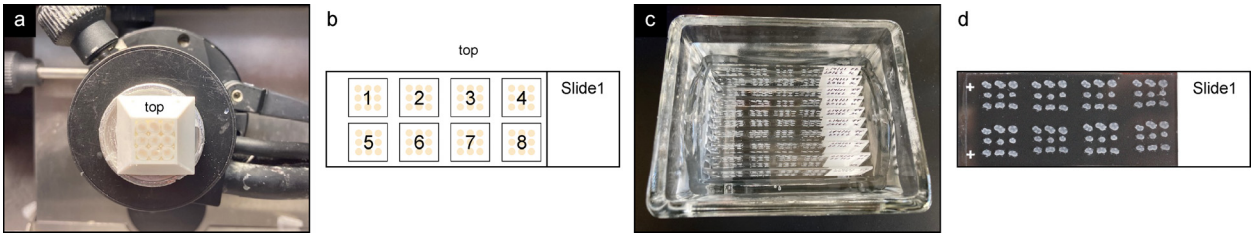

Figure 2: Sectioning, collecting, and staining of block sections
a) Tissue block mounted in cryostat. b) Schematic arrangement of 8 block sections on a slide in a right-and-down order. c) Slides washing in PBS. d) Slide after staining and mounting.

Imaging (timing: 10-15min + 3-5h unattended)
13: Image slides on a slide scanning microscope. Scan capabilities and acquisition software depend on the system used and, therefore, optimal scanning settings have to be determined by the user. For further processing in SpinalJ, images of individual tissue sections with alphanumeric file names that reflect r-c order, or images of block sections (array of 9 tissue sections) are required.

Image pre-processing in SpinalJ
14: Setting Pre-Processing Settings (timing: 5min)
SpinalJ provides a series of tools to pre-process SC section images for registration and analysis. These include segmentation of block section images, compensation for lost sections, r-c ordering of sections, cleaning out damaged sections, as well as re-orientation and horizontal alignment of sections. If pre-processing is already performed outside SpinalJ, skip to step 19.

To prepare image pre-processing, create a parameter file that contains information on how the images of a sample are processed. Run *Plugins > SpinalJ > 1 Set Preprocessing Settings*. In the GUI that appears, complete the fields below:

- **Image Data** - Location of the folder containing the raw sections. Can't handle files that are larger than 2GB.
- **Reference Channel** - Select channel containing NeuroTrace, DAPI, NeuN, or other global cell marker used for section registration.
- **Transform all Block Section Images -** Select transformation option to flip images as necessary so that sections are oriented with their dorsal side up and array order as follows: Top row, left to right: 1, 2, 3. Middle row: 4, 5, 6. Bottom row: 7, 8, 9 (cf. Figure 4).
- **Determine r-c File Order** - Method used to bring image files into r-c order (using stage coordinates or alphanumerical file names).
- **Order of Sections on Slide -** Only required if choosing stage coordinate sorting. For orientation of slide, see Fig.2b.
- **Segmentation Masks -** The location of the folder containing the segmentation masks .roi files.
- **Automatic Segmentation:**
    - **Min. Object Size** – Lower area cutoff for automatic detection of tissue sections.
    - **Max. Object Size –** Upper area cutoff for automatic detection of tissue sections.
    - **Min. Circularity** – Lower circularity cutoff for automatic detection of tissue sections.
    - **Max. Circularity** – Upper circularity cutoff for automatic detection of tissue sections.
- **Replace Lost Sections -** Check 'yes' to compensate for lost sections during cryo-sectioning by duplicating neighboring sections. Requires a .csv list of lost sections (see below).
- **Lost Sections -** Only required if choosing to replace lost section. The location of the folder containing a .csv list of lost sections. To create the list, enter pairs of values that specify the slide (column A) and the position (column B) on which the section was lost. E.g. if the fifth section (first in bottom row) on the third slide was lost, enter 3, 5. If several consecutive sections are lost, repeat the list entry. The first row contains the titles 'Slide' and 'Section', respectively. Save list as '_Lost_Sections.csv'.
- **Horizontal Alignment Sampling Interval** - Total number of sections / n sections randomly selected to determine the horizontal orientation for each tissue piece. Default: 10.
- **Minimal Test Angle -** Negative test angle extreme. Must be > -90°. Default: -50°.
- **Maximal Test Angle -** Positive test angle extreme. Must be < 90°. Default: 50°.
- **Test Angle Increment** - Interval between test angles. Default: 10.
- **Masking Channel -** Channel for section segmentation.
- **Thresholding Option for Masking -** Automatic thresholding option. 'Percentile dark' works best for NT, 'Min error' works best for DAPI.

Data generated during image pre-processing will be saved to subfolder _*Temp*\ and can be deleted after reformatted images have been created (step 18).

15: Segmentation of block section images (timing: 10-15min + 0.5-1h unattended)
Run *Plugins > SpinalJ > 2 Block Section Segmentation*. Confirm path to the folder containing raw image data. Input files cannot exceed 2GB in size and need to contain slide information as first part of the filename, e.g. “Slide4_Section3.nd2”. Block section images will be renamed to reflect r-c order and automatically segmented. Images that contain less than 9 tissue sections cannot be automatically segmented and are saved to folder *_Temp_0_Scaled* as a down-scaled version and displayed at the end of the script for manual segmentation. Position the segmentation mask displayed on top of each image so that each tissue section falls within the appropriate outline of the section array mask, reflecting segment identity (Figure 4a). Click *OK* to proceed to the next section. If identification of segment identity is impossible because of missing sections, hold *SHIFT* + click *OK* to skip segmentation of the current image. Output files are saved to folder *_Temp_I_Split* with names indicating tissue piece (segment) identity, e.g. “Segment05_Slide12_Image02.tif”.

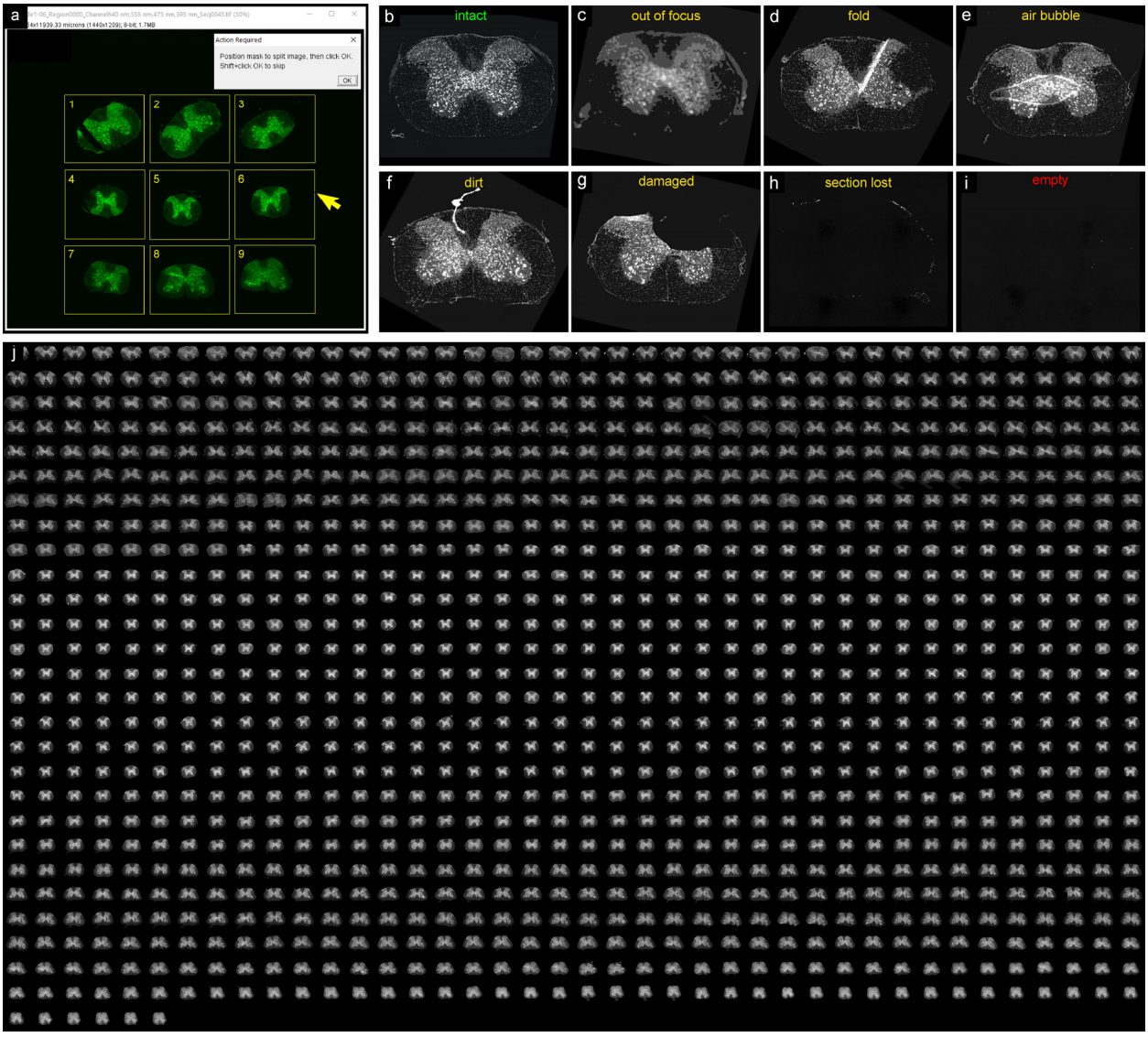

Figure 4: Image pre-processing
a) For manual segmentation of block section images, position the segmentation mask (yellow) to include all tissue sections 1-9. b-i) Cleaning out of compromised and empty section images. Example NT images of sections to keep (green), replace (orange), or delete (red). j) Montage of all horizontally aligned sections from a sample after pre-processing.

16. Segment Rotation (timing: 5min + 10min unattended)
Run *Plugins > SpinalJ > 3 Batch Rotation* to re-orient section images from tissue pieces embedded upside-down or rotated by 90°. Confirm path to the folder containing raw image data. A preview montage of all sections will show the reference channel of all section images ordered in rows representing tissue pieces 1-9. Identify rows in which all sections are oriented at an angle ≥ 90° or ≤ -90° and select those rows for rotation. Set rotation value and click *OK*. Confirm proper orientation in the updated preview montage saved at *_Temp_II_Preview_Split*.

17. Section Clean up (timing: 15-30min + 10min unattended)
Clean dataset of compromised and empty sections to obtain a continuous stack of intact sections. Run *Plugins > SpinalJ > 4 Clean Stack*. Confirm path to the folder containing raw image data. A reference channel preview of the image stack of individual tissue sections will be displayed. Navigate through the stack and decide for each section whether to 'keep', 'replace', or 'delete' the current image by pressing the *SPACE*, *CTRL,* or *ALT* key, respectively (Figure 4b-i). To the left and right of the stack window, additional windows show the previous (left) and next (right) image in the stack. Use 'replace' only after at least one previous image has been selected for 'keep'. Processed files are saved to folder *_Temp_III_Clean* and a preview montage of the reference channel is saved to *_Temp_IV_Preview_Clean* for validation.

18. Horizontal alignment of section images (timing: 20min unattended)
Horizontal alignment of sections improves image registration performance. Run *Plugins > SpinalJ > 5 Horizontal Alignment.* Confirm path to the folder containing raw image data. After automatic alignment is completed, a preview montage of the cleaned and aligned sections is created for validation (Figure 4j). In addition, pre-processed images are scaled to match the resolution of the atlas and saved to a new folder */_Output/ 1_Reformatted_Sections/* with subfolders for each image channel.

Image registration, atlas mapping and analysis in SpinalJ
19. Setting Registration and Analysis Parameters for SpinalJ (timing: 5min)
This step and all subsequent steps assume a single folder containing intact, consecutive single section images of a sample with an alphanumerical name format that reflects r-c order.

SpinalJ is designed to batch process multiple samples, even when the experimental conditions or analysis settings are different. This step creates a parameter file containing analysis information for individual samples. Run *Plugins > SpinalJ > 6 Set Analysis Settings*. In the GUI that appears, complete the fields below:

- **Select folder** - The location of the folder containing the sections.
- **Final resolution of image output (µm/px)** - The resolution used by the rest of the analysis, **2µm/pixel,** typically performs well.
- **Counterstain channel (DAPI or NeuroTrace)** - The channel containing NeuroTrace, DAPI, NeuN or another global cell marker.
- **Background intensity of counterstain channel** - this value should be slightly higher than the intensity of the background surrounding the tissue. *To determine the background intensity: open a section image in Fiji and hover the cursor over the tissue and background to find a suitable intensity level for this setting.* If set too low, the sections will not be adequately isolated, and if set too high, the sections will be patchy and over-cropped.
- **Background removal prior to segmentation (rolling ball radius in px, 0 if none) -** The radius of a rolling ball filter used to clean up images before cell detection and projection analysis. A value of **7** works well for the recommend image pixel size of 2µm/px.
- **Section cut thickness** - Section cut thickness. If using a sub-series, multiply accordingly. E.g. every second section, cut at 20µm = 40µm.

- **Spinal cord range (start segment, end segment)** - The range of segments included in the dataset (e.g. C2-L5). *To determine range, open preview stack of sections and determine the segment identity of the first and last section based on morphological landmarks and by comparing to neighboring sections.*
- **Reference section** - The starting section used for initial section-to-section registration. This should be roughly in the middle of the dataset. E.g. section 500 in a 1000 section dataset.
- **Generate full resolution registered image of reference channel –** Check only if attempting to perform cell or projection analysis on the reference channel.
- **Perform a second pass section registration** - This feature can be used to register badly damaged sections with the help of fiducial markers but is not required for normal use.
- **Method for cell detection**:
    - Manual Cell Count takes a list of cell locations created by manual cell counting. – see *manual cell counting*.
    - Find Maxima will locate the center of cells and is fast and effective for cells with clearly labeled soma.
    - Machine learning segmentation with Ilastik can yield better results, especially when cells are difficult to discern from brightly labelled axons and dendrites. This will require training of an Ilastik Project for each channel – *see Additional Notes.*
- **Channel selection -** Select up to three channel/s for cell analysis. Set the first selector to the channel to analyze. If no remaining channels will be analyzed, set the remaining selectors to 0.
- **Minimum intensity threshold:** The minimum threshold used for detecting cells when using Find Maxima – *see Additional Notes*.
- **Minimum cell area:** Area used for cell detection (when using Ilastik for segmentation).
- **Method for projection detection**:
    - Binary Threshold fluorescence above this threshold will be included in the density analysis. This is a fast and effective method, useful when it's not necessary to remove cell bodies from the analysis.
    - Machine learning segmentation with Ilastik can yield better results, especially when it's necessary to differentiate between cells and projections. This will require training of an Ilastik Project for each channel – *see Additional Notes.*
- **Channel selection:** Select up to three channel/s for projection analysis. Set the first selector to the channel to analyze. If no remaining channels will be analyzed, set the remaining selectors to 0.
- **Minimum intensity threshold:** this is the minimum threshold used for detecting projections when using Binary Threshold – *see Additional Notes*.
- **Ilastik location:** The directory where Ilastik is installed.
- **Elastix location:** The directory where Elastix is installed.

20. Atlas registration analysis (timing: 1-8h unattended)

This step will automatically process each folder and generate three subfolders that contain 1) the registered sections at the resolution set for analysis (*\3_Registered_Sections*), 2) the processed images used for analysis, such as the output from Ilastik, and validation images for confirming the accuracy of cell segmentation (*\4_Processed_Sections*), as well as 3) the final analysis tables and images (*\5_Analysis_Output*). Run *Plugins > SpinalJ > 7 Registration and Atlas Analysis*. In the GUI that appears, complete the fields below:

- **Select experiment/spinal cord folder(s)** - The location(s) of the folder(s) for analysis.
- **Select spinal cord atlas to be used** - The directory where the atlas files are located.
- **1. Perform section registration?** - Perform initial rigid body registration of all tissue sections.
- **2. Perform atlas registration?** – Perform affine and elastic registration for atlas mapping to experimental data.
- **3. Perform cell detection and analysis?** - Perform cell detection using the method selected in the previous step. If performing manual cell counting, run both registration steps first, manually count cells, then rerun this step with this and the following options enabled. The cell analysis generates two tables for each channel used for cell detection. The Detected Cells table provides information on the coordinates of each cell, the region and segment it is contained in, and the measured mean intensity in each channel. The Detected Cells Summary table provides a total cell count for each region and segment.
- **Generate cell analysis images and heatmaps? -** Generates images showing cell locations in the atlas colored according to annotation ID.

4. Perform mesoscale mapping projection analysis? –The mesoscale mapping analysis uses reverse mapping to generate output tables for each channel containing information on the density of axons/dendrites present in each atlas region, and on the relative density according to the volume of the entire labeled volume. Importantly, all density measurements are calculated using the region volume as represented in the dataset, allowing for accurate measurement even when a region is only partially represented in the experimental data.

- **Generate mesoscale mapping visualization images and heatmaps? -** Generates images showing the projections mapped to the atlas and density heatmaps.
- **5. Measure mean intensities of annotated regions?** - Perform region specific intensity measurements. Mean intensities for each channel, atlas region and segment are saved to output tables.
- **Generate intensity based atlas images?** Generates an atlas image with regions colored with their measured mean intensity.
- **6. Extract specific regions at full resolution (requires >128GB RAM)**
- **Provide annotation IDs for extraction (e.g. 4,10)** – List (comma separated) annotation regions for extraction.

After the analysis is complete, all output tables and graphs can be found in folder *\5_Analysis_Output*.

Additional notes

Setting up Nikon slide scanner for scanning block sections with NIS-Elements JOBS:

1. Load all slides in order into a slide magazine (from bottom to top) and initialize the slide scanner using 4x objective, 2.1x zoom.
2. Open JOBS scan file for SC sections and pre-scan 1-2 slides using the autofocusing channel (DAPI or NT). For this, edit settings before scanning as follows (Fig. A1a):
    - Under PreScanSlide set Save Image → always.
    - Disable Region Loop.
3. After pre-scanning, open the pre-scan image.
4. Open JOBS scan file and open scan parameters (Fig. A1b).
    - Set lower and upper intensity threshold to filter out background and unspecific signals (Fig. A1c).
    - Set size cutoff to exclude objects that are not tissue sections (Fig. A1d).
    - Set dilation factor to merge all sections of a block section into a single object (Fig. A1e). Avoid merging multiple block sections.
5. Save settings, disable save pre-scan, enable Region Loop and scan all slides.

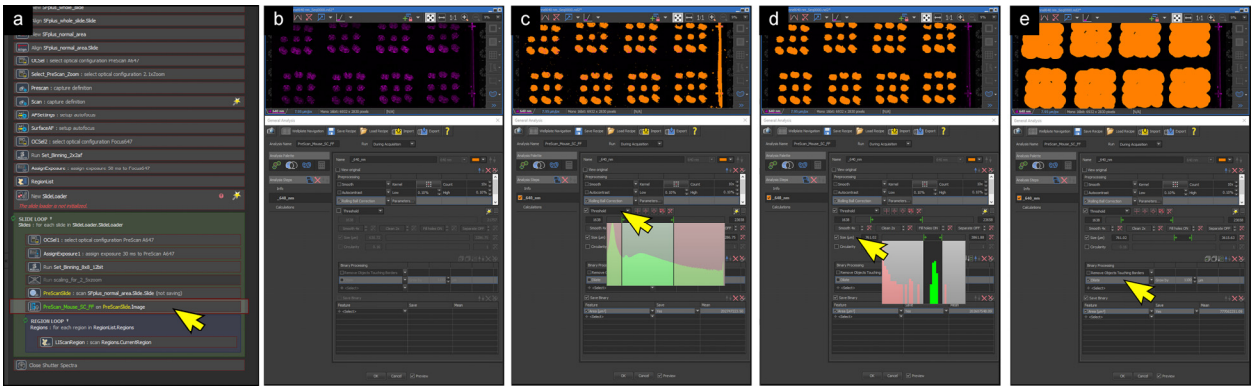

Figure A1: Scan settings for imaging block sections using NIS-Elements JOBS
a) Load a pre-scan image of a slide and open pre-scan settings in JOBS file. b) Check 'rolling ball correction'. c) Set lower and upper threshold so that all SC sections are marked orange. d) Apply size cutoff to filter out objects smaller and larger than SC sections. e) Apply dilation factor so that all sections of a block section are connected, but sections from different block sections do not touch. Save settings.

Manual Cell Detection:

1. Run through steps 1-19 of this guide. In step 19, select 'Manual Cell Count'.
2. Run step 20, but only perform 1. Section registration and 2. Atlas registration.
3. The experiment folder will now contain a sub-folder *\3_Registered_Sections*. Inside this folder, you will find subfolders for each channel. E.g. Folder *\2* corresponds to channel 2.
4. Drag the entire channel folder on Fiji. An 'Open Folder' window will appear. Click 'Yes'.
5. Under *Edit > Options > Input/Output…* turn off rows and columns headers.
6. Under *Analyze > Set Measurements* deselect everything except centroid. Set decimal places to 0.
7. Under *Image > Properties…* set unit of length to pixel. Pixel width, pixel height, voxel depth = 1.
8. Select the Multipoint Tool.
9. Go through slice by slice and click on every cell. If you miss a cell, it is ok to go back through the stack and add new cells. *CTRL+* click removes markers.
10. After counting, you can save this image if you wish to keep a record of how the cells were counted.
11. Press *CTRL+M* to get the centroid and slice position of each cell.
12. Save this list as 'Cell_Points_Ch#.csv' into the subfolder *\4_Processed_Sections\Detected_Cells* (if you are counting cells on channel 2 this file would be called Cell_Points_Ch2.csv).
13. Run step 20. Uncheck step 1 and 2 - already performed. Select 3. Perform cell detection and analysis, and any other desired analysis step.

Determining thresholds for Find Maxima cell detection and binary thresholds for projection analysis:

1. Run through steps 1-19 of this guide. Run step 20 and perform steps 1-3.
2. The experiment folder will now contain a sub-folder *\3_Registered_Sections*. Inside this folder, you will find subfolders for each channel. E.g. Folder *\2* corresponds to channel 2
3. Apply a rolling ball filter with the same diameter used within SpinalJ, then confirm the typical intensities of the cells you wish to detect using Find Maxima, and use this value in the analysis settings. For binary thresholding of axons/dendrites use the threshold tool (*CTRL+SHIFT+T*) to determine the minimum threshold value to be used.

Training Ilastik for machine learning based mesoscale mapping / cell detection:

We recommend using Ilastik for best results, especially when analyzing densely labeled neurons, although other cell detection methods are provided. Be aware that oversaturation of axons/dendrites may result in false positive cell detections when using automated analysis. If analyzing both cells and neuronal projections, it is important to ensure your images are free from oversaturated pixels. For best results in quantifying cell numbers, use a probe with nuclear localization, such as GFP fused to histone 2B.

1. Prepare representative images for training the Ilastik project.
   - Training Ilastik's pixel classifier requires several representative images. A minimum of 5 (better 10-20) sections from different r-c positions of the SC is recommended. If training a project on multiple animals or if the label is highly variable between sections, additional training images may be required.
   - Training images can come from the *\1_Reformated_Sections* or *\3_Registered_Sections* folders. During the SpinalJ pipeline, background subtraction is applied to all sections prior to running Ilastik. When adding images to the Ilastik project for training, ensure they also have this same background subtraction applied. You can do this by running *Plugins > SpinalJ > Other Tools > Create Ilastik training images* in Fiji. After providing a list of sections, images suitable for training will be created and saved to folder *\Ilastik_Training_Images*.
2. Open the provided Ilastik project file

- An example project is provided. Please use this project to build the projects for each of your channels as it contains settings required to export the necessary probability images used by SpinalJ.

3. Select the existing images in the project file. Right-click and choose 'Reset' - this clears out the existing training data, but keeps project file settings, allowing you to add new images for training.
4. Copy in the images selected for training.
   - We recommend saving these images into the project file for auditing and to avoid problems if further training is required. To do so, Right-click and select 'Dataset Properties…', in the window that appears, change 'Storage' to 'Copy into project file'. Then save the project.
5. Train the project file.
   - The Ilastik Project comes preconfigured with three labels: Cells (for soma/cell bodies), Projections (for axons/dendrites), and Background.
   - If you are not detecting cells, you do not have to train the cells label (likewise for projections).
   - For best results when training on 16-bit images, it can be helpful to adjust how the image is displayed in Ilastik. You can do this by right-clicking on 'Raw Input' in Group Visibility list (lower left-hand corner), then selecting 'Adjust thresholds'. Uncheck the 'automatic range' option and reduce the 'maximum value' so that you can clearly visualize low intensity structures.
   - For further advice on training an Ilastik project: https://www.ilastik.org/documentation/pixelclassification/pixelclassification
6. Save the project file as: 'Ilastik_Project_Channel_#' (e.g.'Ilastik_Project_Channel_1' for channel 1) in the *\Ilastik_Projects* folder.
7. Repeat steps 1-6, to create an Ilastik project for each channel to be analyzed. These projects can be copied to other animals/datasets to be analyzed.

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