Automated Imaging and BrainJ Analysis Pipeline

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This manual provides a step-by-step guideline to installing and running Brain Analysis in ImageJ (BrainJ).

BrainJ combines a series of software tools, with a focus on automation and ease of use, to create a complete open source pipeline for reconstructing brain slice images to whole brains and performing analysis within a CCF, such as the ABA CCFv3. While many tools exist for the reconstruction and analysis of whole brain data, they are often focused on one part of the process, require more intervention, or require further understanding of a programming language to implement. BrainJ was created as a seamless way to deal with reconstructing and analyzing histological sections, without requiring any additional programming experience, and reliably deployable through the powerful and commonly used ImageJ/FIJI platform.

BrainJ allows brain slices acquired on confocal, wide-field, and high-throughput instruments to be recombined in 3D and aligned to the ABA. The approach enables analysis of large brain volumes in a manner similar to serial sectioning tomography approaches, with the caveat that it is highly sensitive to tissue integrity and requires consecutive intact tissue sections for successful analysis. Following reconstruction, brains can be analyzed in other existing tools (e.g. ClearMap) or within BrainJ, where appropriate. Image processing and machine learning are incorporated into the pipeline to provide an option for automated cell detection and mapping of axons and dendrites. Cells, either manually selected or via automatic detection, along with automatically detected axons/dendrites can then be analyzed in the context of the ABA. Furthermore, raw data can be transformed into the Common Coordinate Framework (CCF) to allow measuring intensity of brain regions, and visualization of raw image data in this space.

- Automated cell analysis for sparse populations of cells can be achieved with a pixel resolution of 2µm/pixel.
- For best results in quantifying cell numbers it is best to use a probe with nuclear localization, such as GFP fused to histone 2B (H2B-GFP).
- Be aware that oversaturation of axons/dendrites may result in false positive cell body detections when using automated analysis. If analyzing both cells and neuronal projections it is important to ensure your images are free from oversaturation.

I. BrainJ Overview and Features

BrainJ has been designed to provide a readout of cell locations and intensities, axon/dendrite densities, and brain region intensities, within the context of the Allen Brain Atlas (ABA). It does this while minimizing the burden of manual processing and annotation of tissue sections into datasets suitable for this analysis. Emphasis has been placed making the process as automated as possible whilst taking into account common challenges of sectioning, labelling, and scanning of tissue sections. BrainJ processing generates a significant amount of data per dataset (2-3 fold original data), to allow validation of results, and reprocessing when required, this intermediate data can deleted following successful processing.

Overview

BrainJ is split into 5 steps, this allows batch processing of multiple brains, with minimal user intervention, even when they are from different experiments or imaging conditions.

1. Set Experiment Parameters
    - This is a quick step required to create a parameters file that defines how the brain was scanned, which channel you are using for a counterstain (such as DAPI, NeuroTrace, or NeuN), what resolution you prefer to perform the rest of the analysis with, and any rotation or flipping that may be required to ensure all sections are in the same orientation. Run this step for each brain before proceeding.

2. Reformat Sections
    - This step processes multiple brains at once and cleans up the data for further analysis. During this process each section is centered and made horizontal in the image frame, while any surrounding tissue or debris are removed. It also creates a montage and stack preview of the dataset used to check section integrity, ordering, and orientation.
        Duration: ~ 10-20 sec/section. ~15-30 minutes per brain.

3. Section Flipper / Replacer
    - If sections have been floating during immunolabelling then some may require flipping horizontally for consistency. This process takes a list of sections requiring flipping, then updates the dataset and slice preview.
        Duration: ~3 sec / section. ~1-3 minutes.

4. Set Analysis Settings
    - Another quick step required to create a parameters file defining the type of analysis you wish to perform. It includes options for machine learning processing, and cell/projection threshold settings.

5. Registration and Atlas Analysis
    - This final step performs a slice by slice registration and creates a 3D volume of the brain that is subsequently used for 3D registration to a template brain. It maps cells into the CCF, and uses reverse mapping to measure region volumes and perform projection density analysis as well as transforming the raw data into the CCF. Spreadsheets as well as optional image representations and heatmaps are generated during this process.
        Duration ~1 – 3 hours per brain.

II. Installing BrainJ

* BrainJ has been developed for PCs.

1. Install **Fiji/ImageJ**. Fiji can be downloaded at: http://fiji.sc/Fiji
2. Install BrainJ
    - Download BrainJ and copy the **BrainJ.jar** file into your ImageJ plugins folder
    - Store the **Atlas Files** folder somewhere on your computer. The pipeline will use the files in this folder to perform analysis and generate visualizations
3. Install **MultiStackReg**
    - Download and copy into your ImageJ plugins folder. MultiStackReg can be downloaded at : http://bradbusse.net/sciencedownloads.html
4. Install **TurboReg**
    - TurboReg can be downloaded at : http://bigwww.epfl.ch/thevenaz/turboreg/
5. Install **Elastix 5.0.0**
    - Elastix can be downloaded at: http://elastix.isi.uu.nl/download_links.php
6. Install **Ilastik 1.3.3post3**
    - *Only required for machine learning detection of cells and projections.
    - Ilastik can be downloaded at: https://www.ilastik.org/download.html

III. Step-by-step use of BrainJ

Preparing files for BrainJ

1. Place all the section files for a single brain into one folder. These files can be TIF format, or a proprietary image format supported Fiji's Bioformats importer. Each file should contain only a single tissue section. The dataset does not need to be an entire brain but should be complete for the range it is covering, and span at least 2mm in the A-P axis.
2. In some cases, BrainJ can use coordinate information in the image metadata to order sections according to their arrangement on the slide. Alternatively, sections must be named alphanumerically in an anterior to posterior order (e.g. Section001, Section002... Section009, Section010...). Be careful not to use numbering system that may place sections out of order (e.g. Section1, Section2... Section9, Section10...)

Setting Parameters for BrainJ

BrainJ is designed to batch process multiple brains. To process each brain some parameters are required in order to reorient, rescale, and clean up the tissue sections for subsequent alignment and analysis. The parameters for processing each brain should be set for each brain individually following these instructions.

3. Run **Set Experiment Parameters** (In Fiji, Plugins>BrainJ>1 Set Experiment Parameters).
4. In the GUI that appears, select the folder containing the section images, and then complete the other fields. Refer to Box 1 for further information.

Box 1 | Experiment Parameters

This Box describes the parameters needed in the Experiment Parameters GUI, as well as some advice and additional considerations.

- **Select folder:** the location of the folder containing the tissue section images.
- **Sample type:** the type of sections being processed.
- **Section rotation/flipping:** used to specify any rotation required to correct for how the samples were imaged or placed on the slide (e.g. if sections were mounted at a 90° or upside down)
- **File order:** used to specify how the sections will be processed. Using stage coordinates or relying on the existing order of how the files are named (alphanumerically).
- **Order of sections on slide:** the arrangement of sections on the slide (if using stage coordinates for file ordering). These are based on Nikon Elements stage coordinates, further modification may be required to support this feature with other datasets.
- **Final resolution:** The resolution used for analysis, 2um/pixel provides a good performance
- **Section cut thickness:** section thickness, if using a sub-series multiply accordingly (e.g. every second section, cut at 50µm = 100 µm).
- **Counterstain channel:** the channel containing DAPI, NeuroTrace, NeuN, or other global cell markers suitable for registration and alignment.
- **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 suitable for this setting, open a section image in Fiji and hover the cursor over the tissue and background. If set too low, the sections will not be isolated properly, too high and the sections will be over-segmented.

Reformatting and pre-processing tissue sections

5. Run **Reformat Sections** (In Fiji, Plugins>BrainJ>2 Reformat Sections (Batch Process)).
6. In the GUI that appears, select the folder/s containing the section images that you wish to process. GPU acceleration should be enabled to ensure faster processing of images.
7. This step will automatically process each folder and generate two subfolders:
    - **\1_Reformated_Sections**, which contains the cleaned and processed raw data ordered anterior to posterior at the resolution selected in the Experiment Parameters step
    - **\2_Section_Preview**, which contains a down sampled montage and slice preview of the entire brain useful for screening the dataset.

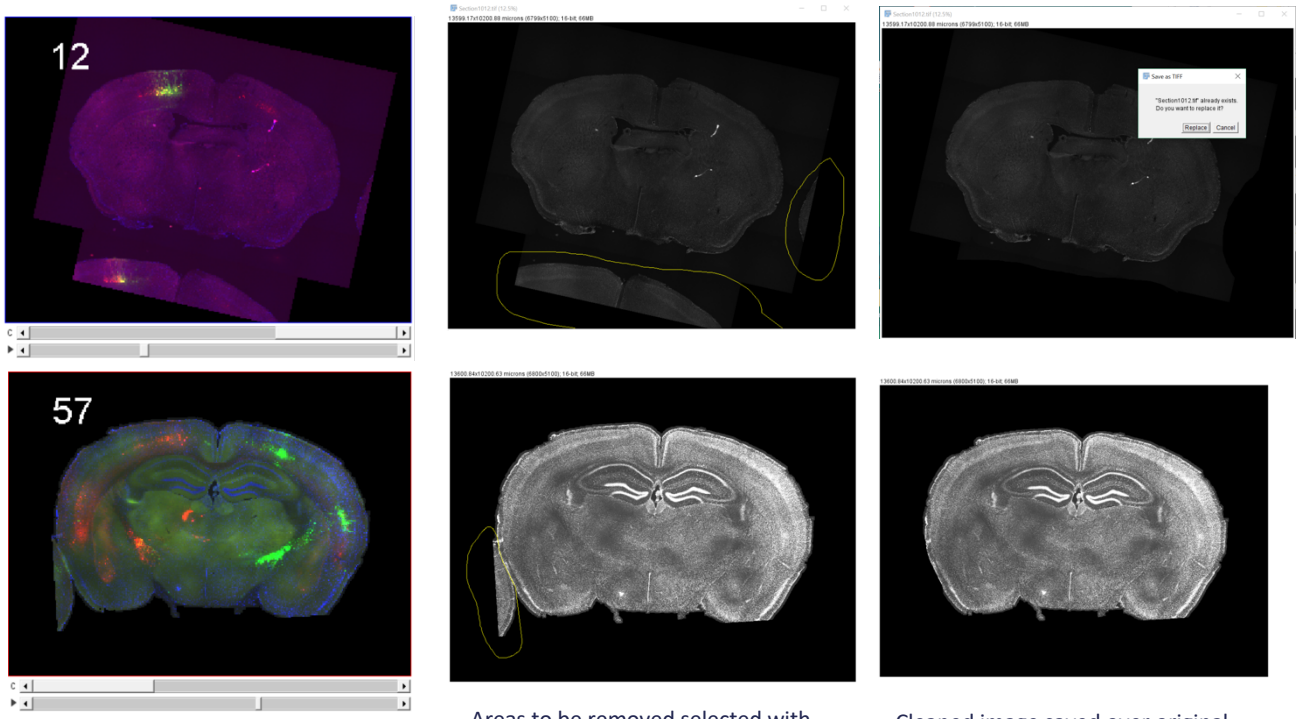
Review and correct processed tissue sections

8. Open **\2_Section_Preview\Section_Preview_Stack.tif** in Fiji, adjusting the brightness and contrast (Ctrl-Shift-C) as necessary to see the signal in each channel. Review the dataset, making a note of any problematic sections, and sections that require flipping or replacing. See Troubleshooting table step 8, for more information how to address badly damaged sections, or sections containing overlapping tissue or debris (Figure 2).
9. Depending on how the sections were mounted, it may be necessary to flip some sections to ensure they are all in the same orientation. For a section/s too badly damaged to be used for analysis, they can be replaced with neighbouring sections. To do this, run **3 Section Flipper/Replacer** (In Fiji, Plugins>BrainJ>3 Section Flipper/Replacer) and provide a list of the sections that need to be flipped and/or replaced.
   Warning: If multiple consecutive sections are badly damaged only a partial analysis may be possible or the dataset may not be suitable for analysis and should be excluded from the study. After correcting the full resolution section images this step will update the section previews images in the **2_Section_Preview** folder to allow final inspection before running the analysis.
10. *It's important to clean up any sections as described above and deal with any damaged sections before proceeding to the next step. If you encounter any issues using the pipeline, especially if you experience poor alignment to the ABA, it's very likely due to missing corrections at this stage or having a number of badly damaged tissue sections.*

Setting analysis settings

From the previous steps the dataset should be cleaned, ordered anterior to posterior, with the necessary sections flipped and any overhanging or problematic sections corrected.

11. Run **Set Analysis Settings** (In Fiji, Plugins>BrainJ>4 Set Analysis Settings).
12. In the GUI that appears, select the folder containing the section images, and then complete the other fields. Refer to Box 2 for further information.
13. If processing multiple brains/datasets, repeat steps 11-13 for each dataset.



*Checking sections using **Slice Preview Stack.tif***

Areas to be removed selected with freehand ROI

Cleaned image saved over original

*Figure 2: Examples of viewing **Slice_Preview_Stack.tif** file in the **2_Section_Preview** folder for two different brains. Example 1: section 12 has unusually high background and some neighboring sections visible. Example 2: section 57 has an overlapping section that need to be removed.*

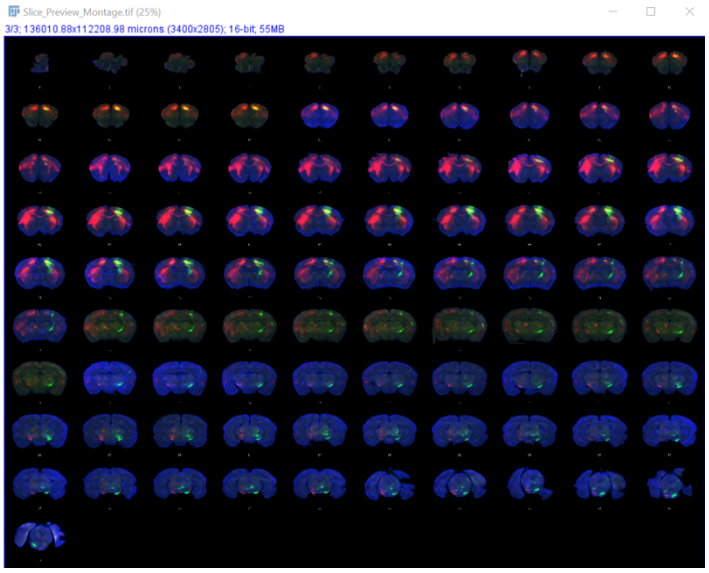


Figure 3. Montage preview

Box 2 I Analysis Settings

This Box describes the parameters needed in the Analysis Settings GUI, as well as some advice and additional considerations.

- **Select folder:** the location of the folder containing the tissue section images.
- **Reference section:** the type of the starting section used for initial section-to-section registration. This should be roughly in the middle of the dataset.
- **Background removal:** the radius of a rolling ball filter used to clean up images prior to cell detection and projection analysis. A value of **7** works well for the recommend lateral resolution of 2µm/px.
- **Generate full resolution registered image of reference channel:** Leave this off unless you are attempting to perform cell analysis on the reference channel, or otherwise need it at full resolution.
- **Perform a second pass section registration** – Leave this off. This feature can be used to register badly damaged sections with the help of fiducial markers, but is not required for normal use.

Cell analysis settings:

- **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 labelled 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 using Ilastik with BrainJ
- **Channel selection:** select which channel/s you wish to perform cell analysis on. Up to three channels can be analyzed. 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, signal intensity (probability value when using Ilastik typically 30-50).
- **Minimum cell area:** cell area used for cell detection (when using Ilastik for segmentation) For 2 micron/pixel a value of 20 for soma and 15 for nuclei.

Projection Analysis Settings:

- **Method for projection detection**:
  - Binary Threshold fluorescence above this threshold will be included in the mesoscale mapping analysis. This is fast 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 using Ilastik with BrainJ
- **Channel selection:** select which channel/s you wish to perform projection analysis on. Up to three channels can be analyzed. 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 determining thresholds below.

Program Location Settings:

- **Ilastik location:** the directory where Ilastik is installed.
- **Elastix location:** the directory where Elastix is installed.

Training Ilastik for machine learning based mesoscale mapping / cell detection

We recommend using Ilastik for best results, especially when analyzing densely labelled neurons.

14. Prepare representative images for training the Ilastik project.
    - Training Ilastik requires several representative images. These should come from multiple animals in the experiment, If only one animal then a minimum of 3 sections is recommended.
    - These images can come from the \1_Reformated_Sections or \3_Registered_Sections folder. During the BrainJ pipeline a 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 subtract applied. You can do this by running **Create Ilastik training images** (In Fiji, Plugins>BrainJ>Other Tools>Create Ilastik training images). Provide a list of representative sections, and it will create images suitable for training and save them in a folder \Ilastik_Training_Images

15. 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 BrainJ.
16. 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.
17. Copy in the images prepared for training from step 14.
    - It is not necessary to save these images into the project file but we recommend doing so 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.
18. Train the project file.
    - For further advice on training an Ilastik project see: https://www.ilastik.org/documentation/pixelclassification/pixelclassification
    - The Ilastik Project comes preconfigured with three labels: Cells (for soma/cell bodies), Projections (for axons/dendrites), and Background.
    - It 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.
19. Save the project file as : "Ilastik_Project_Channel_#" (# = channel number, e.g."Ilastik_Project_Channel_1" for channel 1) In the \Ilastik_Projects folder.
20. Repeat steps 14-19, to create an Ilastik project for each channel to be analyzed. These projects can be copied to other animals/datasets to be analyzed.

Performing the final registration and atlas based analysis

21. Run **Registration and Atlas Analysis** (In Fiji, Plugins>BrainJ>5 Registration and Atlas Analysis (Batch Process)).
22. In the GUI that appears, select the folder/s that you wish to process.
23. Select which registration, analysis, and visualization steps you wish to perform. Refer to **box 3** for further information.
24. This step will automatically process each folder and generate three subfolders:
    - **\3_Registered_Sections –** this folder contains the registered raw data at the resolution set for analysis. This folder can be deleted once you have confirmed the pipeline has run successfully.
    - **\4_Processed_Sections –** this folder contains processed images used for analysis, such as the output from Ilastik, and validation images for confirming the accuracy of cell segmentation. This folder can be deleted once you have confirmed the pipeline has run successfully.
    - **\5_Analysis_Output –** this folder contains all the final analysis tables and images. See Anticipated Results.

Box 3 | Registration and analysis GUI

This Box describes the options available in the registration and analysis GUI

- **Perform section registration?** - An initial rigid body registration of all tissue sections.
- **Perform atlas registration?** – Affine and the elastic registration to map the ABA on to the experimental brain.
- **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 run this step again with this and the following options enabled.
- **Generate cell analysis images? –** Generates images showing cell locations in the ABA and colored according to the ABA region colors
- **Perform projection density analysis? –** Analyzes the density of projections in the ABA brain regions and also the relatively density of the projections in the areas based on the total amount of projection labelling.
- **Generate projection density analysis images? –** Generates images showing the projections mapped to the ABA and heatmaps with ABA regions colored by density (0- 100).
- **Transform original channel images to ABA space? –** Transforms raw image data into the ABA, allowing you to visualize the raw data in the ABA and perform brain region specific intensity measurements.
- **Measure Intensities in ABA brain regions?** Perform brain region specific intensity measurements.
- **Generate mean intensity based atlas images?** Generates an atlas image with brain regions colored with their measured mean intensity.

Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
8	Sections are not cropped or centred, and/or background surrounding tissue sections is still visible	Background intensity of counterstain channel is set too low	Increase background intensity and re-run **Reformat Sections**
8	Sections have pieces missing or holes in them	Background intensity of counterstain channel is set too high	Decrease background intensity and run Reformat sections again
8	Some sections are flipped horizontally	Sections were free floating during immunohistochemistry labelling	Make a note of these sections. These can be quickly corrected using the **Section Flipper/Replacer**
8	Section is severely damaged or folded	Damage during sectioning or mounting	Make a note of this section number. These can be quickly corrected using the **Section Flipper/Replacer**
8	Section/s with debris or other tissue section overlapping the section edges	Tissue or fluorescent object overlapping tissue section during imaging	Make a note of this section number and open the corresponding counterstain image. E.g. if my DAPI channel is channel 3 and section 34 requires correction, open **\1_Reformat_Sections\3\Section1034.tif** Draw an ROI around the area requiring removal, cut (Ctrl-X), then re-save (Ctrl- S). No editing is required on the other channels
Ilastik	Trouble opening ilastik project files, or missing images used for training.	Training images were linked to project file but then moved	Newer releases of Ilastik allow using project files even if the training images have been moved, but it creates a problem for auditing or modifying projects. After copying images to the Ilastik project file, select all the images, right-click, select “Edit properties…” and update XX to “Copy to project file”. This copies the training images into the project file.
Ilastik	Segmentation using Ilastik is giving mixed or inaccurate results	Training of Ilastik project needs to be refined	The **\4_Processed_Sections\Probability_Masks** folder keeps the probability images output from Ilastik. In these images, cells will appear as red, projections as green, and background as blue. Review these and compare to the corresponding raw data image to confirm the data is being accurately represented. Refine the training in Ilastik as necessary.

IV. Additional Notes on Cell Detection and Projection Analysis

1. Manual Cell Detection

1. Run through the first 4 steps of BrainJ
2. Run step 5 of BrainJ, 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 ImageJ (e.g. Brain1/3_Registered_Sections/2)
    a. An "Open Folder" window will appear. Click "Yes".
5. Under **Edit > Options – input/output**
    a. Turn off Rows and Columns headers
6. Under **Analyze>Set Measurements**.
    a. Deselect everything except **Centroid.**
    b. Set Decimal places to 0.
7. Under **Image > Properties…**
    a. Set Unit of length to Pixel.
    b. Pixel Width, Pixel Height, Voxel depth = 1.
8. Select the Multipoint Tool.
9. Go through slice by slice and click on every cell.
    a. If you miss a cell it is ok to go back through the stack and add new cells.
    b. **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 a "**Cell_Points_Ch1.csv**" into the subfolder "**4_Processed_Sections\Detected_Cells"**
    a. Note: If you are counting cells on channel 2 this file would be called **Cell_Points_Ch2.csv**
13. Run step 5 of BrainJ
    a. Uncheck step 1 and 2 - already performed.
    b. Select 3) perform cell detection, and any other desired analysis steps enabled.

2. Determining Thresholds for Find Maxima Cell Counts and Binary Thresholds for Projection Analysis

1. Run through the first 3 steps of BrainJ.
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 BrainJ then:
    a. Confirm the typical intensities of the cells you wish to detect using Find Maxima, and use this value in the analysis settings.
    b. Use the Threshold tool (Ctrl-Shift-T) to determine the minimum threshold value used for binary thresholding of axons/dendrites.

3. Training Ilastik for cell and projection detection

1. Train Ilastik to detect the cell bodies of neurons, and their axons/dendrites.
2. 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 BrainJ. The supplied project file contains three masks – background (for tissue and slide background), cells (for cell bodies), projections (for axons/dendrites).
3. Ensure that you train this project on images that have already had a rolling ball filter applied (the same filter that will be automatically applied during processing in BrainJ). It is not necessary to save these images to the project file if using Ilastik-1.3.2rc2
4. For further advice on training an Ilastik project see - https://www.ilastik.org/documentation/pixelclassification/pixelclassification
5. Please ensure you name the project file according to the channel it will be applied to. E.g. the project file for channel 2 should be called "**Ilastik_Project_Channel_2.ilp**" (case sensitive)

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