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A graphical user interface to assess the neuroinflammatory response to intracortical microelectrodes

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Abstract

Background: Brain-implanted devices, including intracortical microelectrodes, are used in neuroscience applications ranging from research to rehabilitation and beyond. Significant efforts are focused on developing new device designs and insertion strategies that mitigate initial trauma and subsequent neuroinflammation that occurs as a result of implantation. A frequently published metric is the neuroinflammatory response quantified as a function of distance from the interface edge, using fluorescent immunohistochemical markers.

New method: Here, we sought to develop a graphical user interface software in Matlab to provide an objective, repeatable, and easy-to-use method for analyzing fluorescence immunohistochemistry images of neuroinflammation. The user interface allows for efficient batch-processing and review of images, and incorporates zoom and contrast features to improve the accuracy of identifying the ‘region of interest’ (ROI).

Results: The software was validated against previously published results and demonstrated equivalent scientific conclusions. Furthermore, a comparison between novice and expert users demonstrated consistency across levels of training and a rapid learning-curve.

Comparison with existing method(s): Existing methods published in the intracortical microelectrode literature include a wide variety of procedures within ImageJ and Matlab. However, specific procedural details are often lacking.

Conclusions: The distribution of the methodology may promote efficiency and reproducibility across the field seeking to characterize the tissue response to implanted neural interfaces. It may also serve as a template for researchers seeking to perform other types of histological quantification as a function of distance from an ROI.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jneumeth.2019.01.003>.

Conflicts of interest

The authors declare no conflict of interest.

Keywords

Neuroinflammation; Intracortical microelectrode; Neurodegeneration; Brain implant; Craniotomy; Matlab

1. Introduction

Brain-implanted devices have been utilized for a number of research and clinical purposes (Ajiboye et al., 2017; Hochberg et al., 2012; Pancrazio and Peckham, 2009). While we believe the following method has utility in multiple research areas, the current study focuses on intracortical microelectrodes. Intracortical recording microelectrodes are used for electrophysiology, cortical mapping, and studying neural circuitry. Within non-clinical research, microelectrodes are used ubiquitously and in large numbers, while within clinical research they are used selectively in smaller numbers of patients for exploratory rehabilitation applications. For example, they are often utilized in brain computer interface (BCI) systems that restore natural motion to patients with paralysis and limb loss. Certain entrepreneurs are simultaneously exploring their use in other, more futuristic, endeavors (Pancrazio and Peckham, 2009; Wu and Rao, 2017). While tremendous achievements have been made in the BCI field, there is a need for better neural interfaces that maintain viable levels of performance for longer duration (Jorfi et al., 2015). Typical implants begin degrading within the first weeks of implantation and many fail within just a few years (Jorfi et al., 2015).

For intracortical recording microelectrodes, it has become apparent that the neuroinflammatory response likely plays a role in their deterioration in recording performance over time (Barrese et al., 2016, 2013; Rennaker et al., 2005; Biran et al., 2005). Accordingly, the reduction of the neuroinflammatory response to implanted materials in the brain is an active area of research (Hermann et al., 2018; Bedell et al., 2018; Nguyen et al., 2016; Michelson et al., 2018; Cody et al., 2018). Efforts are focused both on understanding and mitigating initial iatrogenic trauma as well as the chronic neuroinflammatory foreign body response resulting from implantation of the devices (Goss-Varley et al., 2017).

Several aspects of the neuroinflammatory response due to implantation are potentially detrimental to the efficacy of the intracortical microelectrode (Shoffstall and Capadona, 2018). The release of cytotoxic soluble factors by localized activated microglia and recruited macrophages contributes to oxidative corrosion and degradation of the microelectrode interface (Jorfi et al., 2015). Hypertrophic astrocytes in the region of the injury aid in scar formation around the implant, which may ultimately reduce recording quality over time (McCreery et al., 2016). Neurodegeneration around the implant can also reduce the functionality of the microelectrode (Biran et al., 2005). While many potential biological factors have been identified, there is still the need for continued research to improve our understanding of the biological system and the prognostic role of the various biomarkers (Michelson et al., 2018; McCreery et al., 2016). In addition to electrophysiological and motor behavioral studies, the majority of groups researching in this area rely on histological

and immunohistochemistry methods to measure the biological response at the neural device-tissue interface (Jorfi et al., 2015; Hermann and Capadona, 2019).

Analysis of the neuroinflammatory response in post-implantation tissues can be accomplished using various histological techniques. There are dozens of groups actively working in this area and each has developed slightly different methods for their analyses (McCreery et al., 2016; Potter et al., 2012a, b; Sohal et al., 2016; Oakes et al., 2018; Lo et al., 2018). Histology can be used to categorize inflammatory markers based on their relative distance from the resulting hole after explanting the microelectrode implant in order to gain a greater understanding of the spatial reach of the neuroinflammatory response to implantation. For example, McCreery et al. refer to the measurement of neuron and glial fibrillary acidic protein (GFAP) density within ‘overlapping concentric annuli’ from the center of the microelectrode tip (McCreery et al., 2016). Some groups use Matlab to quantify fluorescence intensity via calculation of concentric ellipses or generation of rotational intensity sweeps around an implant region of interest; others use LabView to perform a pixel extraction algorithm (Sohal et al., 2016; Potter et al., 2012b; Sohal et al., 2016; Oakes et al., 2018; Lo et al., 2018). Similar measurements are reported with slight variations throughout the literature. The Capadona lab previously published a software called MINUTE (‘Microelectrode INTERface Universal Tool for Evaluation’), which semi-automates the process of generating intensity-based quantification as a function of distance from a selected region of interest. However, it has been cited relatively infrequently throughout the literature, potentially due to user-interface challenges (Potter et al., 2012a; Lee et al., 2017). Some of these challenges include long processing times for single-file sequential processing, inability to zoom or contrast images, an assumption that the implant hole geometry is purely elliptical, and difficulty in reviewing or updating analyses. Within the broader literature that provides openly available methods, various groups have demonstrated different strategies for quantifying histological outcomes. The efforts include pure intensity-based measurements and cellular counts using fully automated versus fully manual methods, but these methods often lack in simple user-interfaces and require a high level of training for proficiency [Shoffstall et al., 2018-26]. Additionally, variety among these histological quantification analysis methods contributes to a range in data error and reproducibility among specific users and groups.

There does not appear to be any obvious field-specific consensus regarding the best practices to study the neuroinflammatory response due to implanted intracortical microelectrodes. Therefore, we sought to develop an easy-to-use and open-source graphical user interface (GUI) in Matlab, coined ‘SECOND’ (alluding to both its improved speed compared to MINUTE, and its being the 2nd version of the program), to process and analyze immunohistochemistry fluorescence-based images. The methods of construction and use, as well as the code’s validation, are presented here. The goal of this work was to achieve a software that is openly available to the field to improve the efficiency, accuracy, and standardization of results across groups.

2. Materials and methods

2.1. Overview

SECOND is a Matlab (Mathworks, Inc., Natick, MA) program with a basic Graphical User Interface (GUI) for histological image configuration and fluorescence intensity analysis. The histological images can be efficiently viewed and analyzed in large sets. The user-driven configuration and automated processing method in SECOND allows for variations in image quality and implantation procedures. SECOND can be used across various fields in need of analyzing fluorescence intensity as a function of distance from a defined region of interest (ROI).

The GUI for the program contains multiple tools necessary in order to work from image setup to data analysis and collection. The flow of the program follows the GUI toolbar from left to right, the details of which are outlined in Supplementary Figs. 1 and 2. The user first opens the desired set of images for analysis under the File menu in the toolbar. In the next step under the Setup menu, the program requires the user to manually configure the image parameters, including contrast adjustments and area definitions applied to all channels within the image, and to save the results in a Matlab file. After a set of images is configured, the user selects “Batch Run” from the Analysis menu for a collection of images and the corresponding configuration files are automatically processed for fluorescence intensity quantification. Once results are compiled under the Review menu, the script produces a collection of analysis files containing the averaged fluorescence intensities in each of the predefined buckets of distances emanating from the ROI.

The results are compiled and output three ways: a Matlab file with raw analysis data, a raw intensity plot, and a final contrast-adjusted image with overlaid exclusion and distance masks. The final “Compile Results” step is valuable for integrating the data into another program for more efficient review and revision of the total set of images. According to specific study requirements, the user may combine results further and perform statistical analysis with the resulting data after using SECOND for measuring intensities.

A step-by-step video tutorial has been uploaded to YouTube to help orient first-time users of the program: <https://www.shofflab.com/research/videos>

2.2. Sample images

For the purpose of demonstration, the sample images throughout the manuscript were taken from a previous study of cortical rat brain (Shoffstall et al., 2018). Tissue was sliced, 20 μm in the transverse direction after removal of a planar silicon microelectrode array implanted in the primary motor cortex. The removal of the device after perfusion-fixation with formaldehyde leaves a characteristic hole which is clearly visible in the center of the image (Fig. 1). The slides were stained for GFAP (glial fibrillary acidic protein) following published protocols (Shoffstall et al., 2018).

In order to be compatible with SECOND, image files must be saved in the TIFF grayscale format with 16-bit depth. Bit depths of 8 do not provide sufficient resolution of intensity levels for most analysis applications. If multiple channel analysis is being performed, each

channel must be represented by a unique grayscale image file. All the files pertaining to a single multi-channel capture must be located in a single folder by themselves. It is recommended that each file be designated with a channel in its name with an underscore at the end. For example, a typical naming scheme we have adopted is “SpecimenID_# (SliceID)_Channel.tif”. There are no other defined limits as to the pixel resolution or other dimensions of the files. However, it is recommended that the images be of sufficient resolution to allow for all features to be resolved (i.e., not pixelated or blurry). Since the tissue response is normalized to a background level at a set distance from the region of interest, it is also important the images contain a sufficient distance away from the electrode such that the response plateaus to a baseline value. In our case, we perform image stitching on a 3-by-3 or 4-by-4 tiled region at 20x magnification to ensure a large enough region, roughly 1500 μm -by- 1500 μm microns has been captured. In our experience the inflammatory response for most of our markers (e.g. GFAP, CD68, IgG), return to baseline by 300–500 μm away from the implant edge. Pixel resolution is determined by the combination of the objective magnification and the resolution of the camera used to capture the images. The calibration factor must be determined for each experimental setup and input during the configuration process in SECOND.

2.3. Pre-analysis image configuration

16-bit monochrome TIF image files are loaded into the program, one for each channel (e.g., each fluorophore: DAPI, GFP, Cy5, etc.). The user performs contrast adjustments on each channel separately to view the details within the images (Fig. 1). Contrast adjustments allow the user to more easily define the implantation hole as the ROI and to define any necessary image exclusions, such as bubbles on the slide and tears in the tissue. The contrast bounds, ROI mask, and exclusion masks as a result of the user’s adjustments are saved in a Matlab configuration file (`‘_sconfig.mat’`) for the image.

2.4. Data analysis

2.4.1. Load configuration and parameters—Further analysis requires the user to ‘batch run’ a set of microscope images and the configuration files. The code loads the configuration data, which includes image files for each channel, bounds for contrast adjustment, definitions for the ROI and any exclusions, and general settings. The settings include the grouping width for defining concentric distance buckets around the hole, micron-per-pixel conversion factor (determined by the microscope camera configuration), and configuration date. The code is optimized for use with 16-bit TIF monochrome images collected with 12–16 bit CCD cameras.

2.4.2. Generate exclusion mask—After loading all configuration data, the program creates an exclusion mask as a combination of the ROI (center) and exclusions (periphery) as defined by user input (Fig. 2). The exclusion mask initializes as an array of ‘ones’ that has the same dimensions as the image. The program inserts values of ‘NaN’ (‘not a number’) in the array for each pixel within the exclusion boundaries. The exclusion mask is applied to each channel within the image.

2.4.3. Generate distance map—Next, the program creates a map of pixel distances emanating linearly outward from the ROI using the Matlab function ‘bwdist’ (Supplementary Fig. 3). These pixel distances are converted to micron distances based on the predefined conversion factor for the image. The distance map is further bucketed based on the predefined micron grouping width so that fluorescence intensity can be measured and averaged within each bucket. For a sample micron grouping width of 50 μm , the intensities would be calculated in a bucket of 0–50 μm , 50–100 μm , etc. around the hole (Supplementary Fig. 4, Fig. 3).

2.4.4. Fluorescence intensity analysis—Analysis of the fluorescence intensity in the non-excluded areas of the image is automatically performed by the program (Fig. 4). The mean intensity for the fluorescent objects of interest in each channel is measured and categorized based on the predefined distance buckets. Accordingly, the total number of pixels within each distance grouping increases at larger distances away from the ROI. The fluorescence intensity data is formatted into a plot and output by the program. The final image files with contrast adjustments, area definitions, and distance bucket mapping are also output by the program during this step, generating individual ‘_analysisresults.mat’ MAT-files for each set of images.

2.5. Data compilation

After SECOND analyzes the fluorescence intensity data for each channel of a set of images, the user may compile the data for further analysis and review (Fig. 5). Compiling the results collates the data into the user’s program(s) of choice for data processing and statistics (e.g. Excel, R, etc.), including intensity profiles and final images. This allows the user to perform further graphical and statistical analyses for entire batches of images, resulting in a wider view of the measurements of interest. As referenced in previously published papers, the raw intensity curves are further normalized to a baseline level established far from the injury site and aggregated using discrete area-under-the-curve tabulations (Bedell et al., 2018).

Image processing and fluorescence quantification via the script keeps the user blind to the experimental group, but further analyses can be accomplished after accumulating the data for a set. Blinding the user from knowing the specific experimental group aims to minimize bias in image configuration and masking selections. To categorize data after using SECOND, the compiled results should be given unique identifiers based on analysis user, date, or lab-specific attributes. These can be added as an additional column which can be used as a grouping variable in most data analytics software (e.g. Excel, Minitab, SPSS, SAS, R, etc.).

3. Results & discussion

3.1. Scientific validation testing

In order to validate that the software would perform similarly to previously published results, and result in equivalent scientific conclusions, we analyzed the same set of data using both SECOND and MINUTE. Additional details about the methods and data of the study represented in Fig. 6 can be found in the referenced publication (Potter et al. (2012a)).

For the purposes of clarity in this manuscript, we have chosen to simply refer to the experimental groups as “1, 2, 3, etc.”.

The Capadona lab previously studied the accuracy of fluorescence intensity quantification at the tissue-electrode interface using various immunohistochemical methods, and we used the same experimental images to compare the quantification methods in SECOND versus MINUTE (Fig. 6). We determined that the two analysis methods provided very similar data output, and as a result the scientific conclusions from the study would have been the same. Statistics were run using a ANOVA with Tukey tests for pairwise inter-group comparisons (Minitab).

3.2. Novice vs expert analysis (training efficiency testing)

Two groups, “expert” and “novice”, were tested using SECOND to analyze three histological tissue images from two different animals for a total of six images. The six tissue samples were imaged with three fluorescent channels each, DAPI, ED1 (CD68), and GFAP. The findings from the GFAP analysis are shown in Fig. 7, while results from the other channels are shared in Supplementary Fig. 5. The “novice” testing group was comprised of nine students chosen from an undergraduate lab course. The novices were given no formal training to use SECOND, only told to follow the prompts from the program to analyze the intensities among the set of six images. The four experts were chosen from the Capadona lab, since they had regularly used SECOND to perform similar analyses. The two groups completed the program flow from manual contrast adjustments and area definitions to automated micron bucketing and analysis of fluorescence intensity.

The resulting intensity values from their analyses were normalized and bucketed into groups of 50 μm outwards from the implant hole (Fig. 7A). There were no statistically significant differences between the experts and the novices at any of the distance intervals (Table 1). The majority of the error was contributed by image-to-image variability rather than user-to-user variability (Extended ANOVA results are shown in Supplementary Table 1). However, it can be noted in Fig. 7B that there was at least one novice user that yielded outlier results. Given additional training, it is anticipated that the inconsistency could be resolved. On average, even with no training, the novices were able to obtain very similar intensity profiles for the images, showing that SECOND continues to be efficient among users of various training levels.

3.3. Processing algorithm & efficiency

Substantial efficiency was gained using SECOND compared to MINUTE, providing the ability to generate image masks and a compiled dataset within several hours compared to several days of analysis. The efficiency was primarily gained as a result of the following:

- 1) Batch-processing compared to sequential-processing allowing for user flexibility, and
- 2) Fast processing algorithm taking only 30–60 seconds compared to 15–20 minutes to analyze each image. Most of the efficiency was gained by using the

‘bwdist’ function rather than an iterative calculated expansion of concentric ellipses mapped to pixel-space.

- 3) Built-in image contrast and zoom to make hole and exclusion definition easier and more precise
- 4) Ability to define non-ellipsoidal holes

Data analysis was also made more efficient through a number of other means. The manual contrast adjustment by the user takes only a few seconds and allows for more accurate detection of image details. The adjustment is applied only to the visualization while the underlying data remains unaltered. Defining the implant hole and exclusions is additionally made simpler by a program prompt to zoom in on a smaller area of the image containing the definition of interest. These user-driven annotations take a few minutes to complete per image, but the result is an image that can be automatically processed and analyzed in seconds with increased accuracy and ability for post-hoc review.

A distance map (‘bwdist’) is created in order to later measure the fluorescence intensity as a function of distance from the implant hole (Supplementary Fig. 3). The distance map for the image is generated more efficiently than other algorithms that rely on iterative methods, such as direct calculation of elliptical coordinates or image dilation (Potter et al., 2012a). Elliptical coordinates oversimplify the neural interface right at the edge where it matters most, and image dilation is a very processing-heavy task. Previous methods in MINUTE iteratively calculated ellipses expanding from the ROI before mapping discrete pixels to the area to determine distances. The built-in Matlab function ‘bwdist’ provides a very rapid and efficient method for tabulating the intensity as a function of distance, and does not force the geometry to be perfectly ellipsoidal.

The image (TIF format) is loaded into Matlab as an unsigned 16-bit integer array (Fig. 1A). Without any contrast and level adjustment, the image is dark with indistinguishable details. This would make any user interaction to define the hole edges very challenging. Therefore, a contrast procedure is performed (Fig. 1B). The contrast adjusted image is easier to see. While the display shown to the user is adjusted, the underlying pixel values are kept constant. This is accomplished by adjusting the image display bounds (‘CLim’) within Matlab image properties.

While the goal during sectioning and cover-slipping tissue slides is to minimize tears and bubbles, these issues are sometimes unavoidable. Artifact removal is a necessary part of research, so the methods provided give a simple means to annotate and review exclusions. An exclusion mask is created as a combination of the defined ROI and selected exclusions (Fig. 2). The exclusion mask serves to eliminate regions from the image that should not contribute to fluorescence intensity measurements. The inside area of the implant hole is excluded, because its perimeter is set as the origin for categorizing distances emanating outwards from the hole. Bubbles on the slide, major tissue tears, and any abnormalities to the image are excluded by the user to reduce error in the fluorescent intensity measurements. The user interface provides a simple method for removing artifacts from the analysis.

Due to the changes above, we estimate the average user-input time decreased by 2.5-fold, from 5 to 2 min, and computation time decreased by 20-fold, from 20 to 60 s, per image.

3.4. Special considerations

While SECOND attempts to generalize the analysis of neuroinflammation in histological images, there are a few factors to consider for each experiment-specific goal.

3.4.1. Pixel-to-Micron conversion factor—The conversion factor is a vital component to accurately reporting the image analysis and will vary based on lab-specific instrumentation. Pixel distances are converted to micron distances based on a predefined conversion factor (Supplementary Fig. 3). In order to perform analysis of the fluorescence intensity surrounding the hole, we further group micron distances into buckets based on a predefined group width. Therefore, it is highly recommended that users verify that the image processing techniques are accurately reporting distances. One possible method for checking accuracy would include collecting an image with a calibrated slide containing a ruler (Supplementary Fig. 6).

3.4.2. Intensity normalization—Specific selection of the normalization band should be done with care and justification. Near the implant hole, fluorescent intensity is typically highest due to the inflammatory response, and exponentially decreases to a dimmer background level. The separately included 'CompileResults' code normalizes the intensity data for each channel according to a selected band deemed far enough away from the hole that the inflammatory response has subsided to baseline. In our experience, depending on the stain, this can vary between 200–500 μm away (Bedell et al., 2018).

3.4.3. Image review for quality control—Especially for analysis being performed by multiple users, it is important to have a system for reviewing images for quality control. For this reason, the final image output displays the contrast adjustments, ROI definition, and perimeter lines for each micron bucket emanating from the ROI (Fig. 3). The review image is valuable for the user to manually check the details of the area definitions and boundaries of the measurements. Shown in Fig. 5 is a montage of the review JPG images for a single subject. While code for generating the panel montage is provided, it will require customization according to the user-specific naming scheme.

3.4.4. Appropriateness of intensity metric—Typical immunofluorescent labeling procedures used by the Capadona lab and analyzed with SECOND include GFAP, IgG, and CD68 stains, among others (Hermann et al., 2018; Bedell et al., 2018; Nguyen et al., 2016; Goss-Varley et al., 2017; Potter et al., 2012a, b). These stains correspond to protein biomarkers expressed at different amounts near the tissue-electrode interface, with quantities depending on the level of inflammation or injury to the tissue. While it is appropriate to analyze some IHC markers using an intensity-based approach, we have found that for some stains, including those that are sparsely populated and/or discretely stained (e.g., neuronal nuclei), the more scientifically relevant metric may rather be a cell count or density. In that case, an alternative analysis method would be required.

3.4.5. Considerations for multi-tined electrodes—The analysis of a matrix-style array type electrode would also be possible using the SECOND code. In this case, each tine would have to be analyzed one-at-a-time. While this is feasible, it would be highly cumbersome. In that case, it would be best to make a slight alteration to the code that would enable multiple ROIs to be defined (1 for each tine of the electrode). The remainder of the code would not require alteration.

4. Conclusion

Intracortical microelectrodes represent one of the principle types of brain-implanted devices currently under investigation. Beyond intracortical microelectrodes, there is interest in developing other novel neural interfaces for the central and peripheral nervous systems. A common goal is to develop interfaces that reduce the inflammatory response, so as to reduce tissue impedance and the distance between the device and neural tissue. Since many disparate research groups are each using slightly varied methods to achieve similar measurements of the neuroinflammatory response to implanted materials, reporting in the literature is equally varied and is potentially leading to inefficient duplication of efforts.

Using SECOND, the intensity of various fluorescent molecules on histological images can be quantified as a function of distance from a user-specified ROI. The code is openly available to the field, and if adopted, may improve the efficiency, accuracy, and standardization of results across groups. The ultimate goal of providing the analysis method here is to improve implanted neural device efficacy and longevity. While the code is currently optimized for use with microelectrodes implanted in cortical tissue, it may be adapted for broader study applications involving intensity based-measurements radiating from a specifically defined region of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Contrast adjustment within SECOND. A) Unadjusted monochrome GFAP-stained image showing a void in the center of the image created from explanting a single-shank silicon microelectrode device. B) Contrast-adjusted image according to user-selected bounds. Bubbles are obviously visible at the top and bottom edge of the images – the bubbles are excluded from the analysis in later steps. Scale bars = 400 μm .

fx1

Code Snippet 1: The built-in Matlab function 'cellfun' is used to call a custom function 'applyContrast' to adjust each of the channels loaded into the cell array image storage variable 'm1'.

Fig. 2.

Binary exclusion mask, showing the tissue void at the center from the explanted device as well as 3 other regions to remove bubbles as image artifact. The regions are all user-defined and saved in the image configuration file.

fx2

Code Snippet 2: An exclusion mask is generated from user-defined input of the 'hole' ROI and any other artifacts such as bubbles and tears. Again the functions are serialized using the built-in Matlab function 'cellfun' for efficiency and modularity.

Fig. 3.

Contrast-adjusted image with overlaid 'Bucket' grouping lines. Red denotes the hole. Yellow denotes exclusions applied to the image. Both are removed from the analysis. Scale bar = 400 μm .

fx3

Code Snippet 3: Sub-indices are generated from the distance map to aid in bucket grouping.

Fig. 4.

Intensity profile as a function of distance from the neural interface region of interest. The intensity decays to a constant background level, typically within the first 500 μm from the hole edge. This example reflects a single trace of raw intensity data.

fx4

Code Snippet 4: Intensity profiles as a function of distance from the hole edge are calculated by leveraging the function 'accumarray' which allows for transformation of data along sub-indices.

Fig. 5.

Image Review. The panel shows the entire array of slices and stains performed for a given subject. The review makes it apparent that there are two panels that require re-masking. Panels “EGFP/106” and “EGFP/114”, where there are two apparent areas void of cellular staining toward the bottom-right edge. Results from a minimum of 3–4 image replicates for a given tissue sample are suggested to be averaged together in order to achieve statistical power. Red denotes exclusion of imaging artifacts (bubbles, tears, etc.). Yellow denotes the hole definition. Scale bars = 400 μ m.

Fig. 6. SECOND versus MINUTE. A comparison between the data results found using the two different algorithms. There were not statistically significant differences between MINUTE and SECOND for any of the datasets tested.

Fig. 7.

Experts versus novices analysis. A) Expert users are compared to novice users in their analysis of the same set of 6 images stained with GFAP. Normalized intensities resulting from the two user groups and based on distance buckets are shown. The error bars represent the standard deviation of the average normalized intensities for each user group. B) The same data is shown with individual normalized intensity (0–50 μm bucket) measurements for each user (black-diamonds) overlaid on each group's average for each of the 6 images analyzed.

Table 1

Analysis of Variance (P-Value) Table. The image contributes most significantly to the source of variability. There were no significant differences between the expert and novice users in either of the image sets tested (stained with GFAP and ED-1).

P-Values from ANOVA-Tukey Tests	Distance Bucket (µm)			
Marker 1 (GFAP) Source	0–50	50–100	100–150	150–200
<i>Expert/Novice</i>	0.158	0.548	0.595	0.202
<i>Image</i>	< 0.001	< 0.001	< 0.001	< 0.001
Marker 2 (ED-1) Source				
<i>Expert/Novice</i>	0.753	0.838	0.869	0.947
<i>Image</i>	< 0.001	< 0.001	< 0.001	< 0.001