A semi-automated brain atlas-based analysis pipeline for c-Fos immunohistochemical data

J.R. Bourgeois a, G. Kalyanasundaram a, b,1, C. Figueroa a, A. Srinivasan a, A.M. Kopec a,∗

a Department of Neuroscience and Experimental Therapeutics, Albany Medical College, Albany NY, United States
b Rensselaer Polytechnic Institute, Troy, NY, United States

ARTICLE INFO

Keywords:
Rat brain atlas
Immunohistochemistry
C-Fos
Image warping

ABSTRACT

Background: The use of immunohistochemistry to quantify neural markers in various brain regions is a staple of neuroscience research. Numerous programs exist to automate quantification, but manual assignment of regions of interest (ROIs) within individual brain sections remains time consuming and can introduce interobserver variability.

New method: We have developed a novel open source FIJI-based immunohistochemical data analysis pipeline, Atlas-Based Analysis (ABA). ABA uses landmark-based image warping to adjust the experimental image to closely align with a published rat brain atlas. c-Fos positive cells are then quantified within predetermined ROI coordinates derived from the brain atlas. Image warping adjusts for natural variation in brain sections to ensure reliable alignment of ROIs for data analysis. This pipeline can be adapted for new atlases, landmarks, ROIs, and quantification measurements.

Results: ABA permits rapid quantification of immunoreactivity in multiple ROIs and produces results with high levels of interobserver consistency.

Comparison with existing methods: Compared to manual ROI designation, ABA reduces total analysis time by ~70%. With correct use of landmarks for image warping, ABA produces similar results to manually drawn ROIs, results in no interobserver variability, and maintains c-Fos+ pixel dimensions.

Conclusions: ABA reduces time to obtain reliable results when performing automated immunoreactivity quantification and allows multiple users to analyze data without compromising the reliability of data obtained.

1. Introduction

The era of ‘big data’ in neuroscience has spurred the simultaneous analysis of multiple brain regions. Immunohistochemistry is one technique that can effectively scale to permit parallel analyses in multiple regions of interest (ROIs) throughout the brain. However, collecting these data presents several challenges. Intact brain analyses require tissue clearing and specialized imaging tools (Salinas et al., 2018; Renier et al., 2016; Kim et al., 2015), while manual analysis of multiple ROIs throughout the brain is prohibitively time consuming and dividing the task among multiple individuals introduces the potential for inter-observer variability. For example, both the delineation of the boundaries of ROIs and what constitutes a positive cell for common immunohistochemically markers are likely to introduce discrepancies between experimenters. While there are numerous programs available for the automated counting of cells and other immunohistochemical results (Shi et al., 2016; Schuffler et al., 2013; Choudhury et al., 2010; Morriss et al., 2020), the difficulty of reliably applying these features simultaneously to multiple brain regions remains problematic.

Expression of the immediate early gene and transcription factor c-Fos within nuclei is commonly used as a marker for neural activity in response to various stimuli, including pharmaceuticals, seizures and nociception (Chang et al., 1988; Dragunow and Faull, 1989; Harris, 1998; Chiasson et al., 1997; Lin et al., 2018). Currently, c-Fos analyses may be performed by manually designating ROIs within brain sections for automated cell counting (Brynildsen et al., 2020), but this process remains cumbersome when applied across the brain, due to the varied size and shape of ROIs between sections. Herein, we describe a novel open source analysis pipeline utilizing FIJI for analyzing c-Fos immunoreactivity in multiple ROIs throughout the rat brain (Fig. 1a)
following acute morphine administration. This program combines pre-defined ROI coordinates based on atlas figures in the 2007 edition of The Rat Brain in Stereotaxic Coordinates by Paxinos and Watson (Paxinos, 2007) with a warping feature to enhance ROI alignment between the experimental image and the atlas. Together, these features automate the boundary assignments of ROIs in which c-Fos+ cells are quantified. We will refer to this program as Atlas Based Analysis (ABA) for simplicity. We validated the ability of ABA to reduce the time required to quantify c-Fos immunoreactivity in multiple ROIs while minimizing interobserver variability and maintaining c-Fos+ pixel dimensions. Finally, we highlight its versatility and adaptability for other brain-wide immunohistochemical analyses.

2. Materials and methods

2.1. Animals

Sprague-Dawley rats were purchased from Harlan/Envigo (Dublin, VA). All experiments and animal care were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital and Albany Medical College.

2.2. Morphine administration and tissue collection

Animals were injected with saline or 3 mg/kg morphine sulfate (free base) prepared in sterile saline 90 min prior to being euthanized, a time point at which stimulus-dependent c-Fos protein expression peaks (Morgan et al., 1987; Kovacs, 1998; Chaudhuri et al., 2000). Animals were deeply anesthetized with CO2, transecrally perfused with cold 0.1 M phosphate buffer (PB; pH 7.4), followed by 4% paraformaldehyde (PFA) in PB. Brains were removed and post-fixed in 4% PFA for 24 h at 4 °C. Analyses in Fig. 3 were conducted on tissue from a morphine-injected male (age postnatal day (P)22) and a vehicle-injected female (age P54). Analyses in Fig. 4 were conducted on tissue from the same P22 morphine-injected male. Analyses in Supplemental Fig. 3 were conducted on tissue from a vehicle-injected female (age P54).

2.3. Immunohistochemistry (IHC)

PFA-fixed brains were cryoprotected in 30% sucrose in 0.1 M PB for a least 2 days. Cryoprotected brains were embedded in Tissue-Tek O.C.T. and frozen on dry ice prior to cryosectioning 30 μm coronal sections in a 1:8 series. Sections were stored in 0.1 M PB with 0.1% sodium azide. IHC was performed with previously described procedures (Kopec et al., 2018) and overnight 4 °C incubation of mouse anti-c-Fos (Novus Biologicals NBP2-50037SS, 1:5000) or mouse anti-dopamine D1 receptor (D1r) (Novus Biologicals #NB110-60017; 1:5000). Sections were rinsed, incubated for 2 h at room temperature in donkey anti-mouse biotin-SP (Jackson Immunoresearch Laboratories, Inc., 715-065-150, 1:500). Antibody localization was resolved with a DAB chemical reaction using a VECTASTAIN ABC kit and ImmPACT DAB Substrate Kit (Vector Laboratories, Burlingame, CA), per manufacturer’s instructions. Sections were mounted onto gelatin subbed slides and dried before baking at 50 °C for 2 h. Slides were dehydrated and defatted with ethanol and
Histochoice Clearing Agent (Sigma-Aldrich, St. Louis, MO), respectively. For cresyl violet staining, slides were submerged in 0.1% cresyl violet in 0.1 M PB for 5–10 min prior to dehydration and defatting. All slides were coverslipped with DPX Mountant (Sigma-Aldrich, St. Louis, MO) and imaged at 20X with a Hamamatsu Nano Zoomer 2.0-RS Slide Scanner.

2.4. Required software and plugins

ABA requires FIJI, a special distribution of ImageJ analysis software, two new FIJI plugins designed by the Kopec lab (Warp Image and Batch Counter), and two sets of coordinate files that the plugins read to warp images onto atlas figures and designate ROIs for analysis. An additional plugin called Landmark Analyzer can be downloaded and used to edit the coordinates files used by Warp Image to make warping more accurate. FIJI can be downloaded for free from the ImageJ website (https://imagej.net/Fiji/Downloads). Plugins and corresponding text files used herein can be downloaded from the Kopec Lab Github account, https://github.com/kopeclab/Atlas-Based-Analysis.

2.5. Naming scheme and folder setup

Both the Batch Counter and Warp Image plugins require a similar folder setup (Supplemental Fig. 1). The parent directory must include the following folders which can be downloaded from the Kopec Lab Github account: “Atlas Analysis,” “Atlas Landmarks,” and “File Landmarks.” “Atlas Analysis” contains text files with ROI coordinates, “Atlas Landmarks” contains text files with landmark coordinates, and “File Landmarks” contains section-specific landmarks. Additionally, folders titled “Atlas Left” and “Atlas Right” should be created to store their respective hemissections following the cropping of bilateral atlas figures. The existing files in “Atlas Analysis,” “Atlas Landmarks,” and “File Landmarks” were created based on Paxinos and Watson’s 2007 rat brain atlas (Paxinos, 2007), and can only be used accurately with hemisections derived from this atlas. Folders of cropped images ready for Batch Counter or Warp Image should be stored in this parent directory.

2.6. Cropping atlas sections

Symmetrical left and right atlas figures must be prepared from bilateral atlas figures (e.g. from (Paxinos, 2007)) for ABA analysis. This can be accomplished manually through ImageJ or other image processing programs. For copyright reasons, we have not included atlas hemissections. We have included a macro named Picture_Splitter that crops an open bilateral coronal atlas figure when given the coordinates of a rectangle that encloses the bilateral atlas. The coordinates we used to create hemissections based on the Paxinos and Watson 2007 atlas (Paxinos, 2007) are included in the file named “Picture_Splitter coordinates - Paxinos 2007.” Creating hemissections from this specific atlas permits use of all ABA analysis files included on our Github website. Use of a different atlas would require defining ROIs and landmarks specifically for the new atlas.

2.7. Atlas section assignment and image preparation

Some pre-processing of the brain images must occur before ABA analysis (Fig. 1a). Experimental images, much like atlas figures, must be cropped into left and right hemispheres and, if necessary, rotated to match the orientation of the atlas. Each hemisphere needs to be assigned to the atlas figure that best matches the structures found in the brain section by comparing the brain image side by side with the atlas. If the angle of sectioning deviates from the angle shown in the representative atlas section, the image should be assigned to the atlas section that most closely matches relevant ROIs. The file name of the brain image must be updated to reflect whether it is the right or left hemisphere and which atlas figure will be used to perform the analysis (Fig. 1b).

2.8. Landmark placement and image warping

The Warp Image plugin increases overlay accuracy between an imaged brain section and its corresponding atlas. In the BigWarp (Bogovic et al., 2016) interface which is automatically included with FIJI, corresponding landmarks on the moving image (image of experimental brain section) and fixed image (atlas figure) are aligned through the Thin-Plate Spline. The user must manually move landmarks on the moving image (Fig. 2a) to accurately correspond to landmarks placed on the fixed image. While the Warp Image plugin works with superficial landmarks alone (Fig. 2b), deeper landmarks based on clearly distinguishable brain structures (e.g. ventricles or white matter) can increase overlay accuracy (Fig. 2c, Supplemental Fig. 2). The Landmark Analyzer plugin can be used to add deep landmarks, or to add or change existing superficial landmarks saved in the “Atlas Landmarks” folder.

2.9. ROIs

ROIs were manually defined by tracing atlas figure ROI boundaries using the FIJI Polygon tool, and the coordinates for each ROI were stored in the “Atlas Analysis” folder according to atlas figure. Batch Counter determines the location on each experimental image to analyze based on these text files. ROIs in the coronal plane that are included in the ROI text files on the Kopec Lab Github website include prelimbic cortex (PrL), infralimbic cortex (IL), cingulate cortex 1 (Cg1), cingulate cortex 2 (Cg2), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), ventral tegmental area (VTA), hippocampus (Hippo), anterior paraventricular thalamic nucleus (PVA), substantia nigra (SNR), posterior paraventricular thalamic nucleus (PVP), caudate putamen (CPu), lateral orbital cortex (LO), ventral orbital cortex (VO), piriform cortex 1, 2, and 3 (Pir1, Pir2, and Pir3), periaqueductal gray (PAG), and the ventral posterolateral nucleus of the thalamus (VPL).

ROIs can be added or removed by editing the ROI text files in the “Atlas Analysis” folder. To add new ROIs, first determine which atlas figures contain the ROIs to be added. Then, obtain the appropriate set of coordinates for the outline of the ROI for each relevant atlas figure and add them to the corresponding ROI text files. Note that only the coordinates from the right side of the atlas figure are necessary, as the Warp Image plugin will calculate the left hemissection’s coordinates using the right hemissection’s coordinates. To remove an ROI, delete the ROI abbreviation as well as the corresponding set of coordinates from each ROI text file of each atlas figure in which the brain region appears.

2.10. c-Fos quantification

The user is prompted to input which brain hemissection is being analyzed (left or right), the minimum and maximum size of positive cells, the folder of processed brain section images to be analyzed, and the folder to save results. Our image acquisition parameters yielded c-Fos positive nuclei between 20–100 pixels. This gate can be customized based on the intracellular localization of various markers, staining technique and imaging technique. ABA converts the image to binary and performs watershed segmentation (Soolie and Vincent, 1990), after which the Batch Counter plugin counts c-Fos positive cells. Data are saved individually by brain image and in summary format in the results folder specified by the user. Each brain image will have an individual results folder which will contain the cell counts for each ROI present in that section, as well as an image showing the atlas overlaid onto the warped image. The summary folder created will contain two summary spreadsheets. The first, “all data,” aggregates the data from all ROIs present in a single image. The second, “summary,” aggregates the data from a single ROI across all images in which it is present. Saving the data in these two ways allows comparison between ROIs (former), as well as comparison between anterior and posterior areas within each ROI (latter). Data are saved by left or right hemisphere due to the program analyzing each hemisection individually.
2.11. Data collection

Comparisons of time required for image processing were performed by three separate users: Users 1 and 2 assisted in designing the ABA process and were very familiar with the methodology, while User 3 was familiar with ImageJ/Fiji functionality, but had not participated in designing ABA. Each user performed both manual and ABA automated quantification on the same tissue sections for three separate days. Preliminary steps required for both types of processing, such as assignment of atlas sections and cropping of images, were not included in this comparison. The timed portion of manual trials included binarizing, watershed processing, manual outlining of ROI boundaries and automated cell counting through ImageJ, whereas the timed portion of automated processing using ABA included the placement of landmarks, image warping, and automated cell counting.

Comparisons for cell count accuracy were obtained on identical tissue sections with three users each performing manual processing, ABA automated processing using only superficial warping landmarks, and ABA automated processing using both superficial and deep warping landmarks, on three separate days.

Analysis of the effect of warping on individual c-Fos+ nuclei was performed on five randomly chosen, but readily identifiable, nuclei. Images were then made binary and underwent watershed processing, after which the Analyze Particle feature was used to obtain raw pixel sizes for each nucleus.

2.12. Statistics

All statistical analyses were performed using GraphPad Prism 8 software. All experiments were completed in triplicate (one per day for three days) by each of three different experimenters. Time comparison data were analyzed with Student’s t-test. Accuracy comparison data were analyzed with two-way ANOVA or repeated measures one-way ANOVA, and, when appropriate (p < 0.05), Tukey’s HSD post hoc analysis.

3. Results

3.1. ABA reduces time to obtain c-Fos+ cell counts from multiple ROIs

Three separate users performed both manual and ABA automated processing to obtain cell counts on 10 ROIs on three separate days each. ABA quantification resulted in a dramatic reduction in analysis time. Time required for automated cell counting (including the opening of images, adjustment of pre-coded landmarks, image warping and processing time) was decreased by ~70% compared to the time required for manual preparation of files (including the opening of images, outlining of ROIs, image conversion, automated quantification with ImageJ software, and recording output in a spreadsheet) (Fig. 3).
3.2. ABA produces consistent data between users

Comparison of cell counts per area were performed to assess the reliability of data produced by ABA quantification compared to manual quantification, and to confirm that results were consistent between users. Analyses were performed with either (i) manual outlining of ROIs, (ii) ABA using only superficial landmarks, or (iii) ABA using both superficial and deep landmarks. For many ROIs, ABA warping using only superficial landmarks maintained interobserver consistency (Fig. 4a-b) or eliminated interobserver variability (Fig. 4c) when compared to manual outlining of ROIs. In ROIs in which ABA warping using only superficial landmarks resulted in interobserver variability (Fig. 4d) or results that differed from manual ROI outlining (Fig. 4c-d), the addition of deep landmarks to the ABA warping process corrected these discrepancies. Indeed, the use of both superficial and deep landmarks in the ABA warping process consistently produced results with interobserver consistency and did not differ statistically from manually obtained results. We conclude that ABA with superficial and deep landmarks can thus increase ROI alignment accuracy for at least the analyzed brain regions. Furthermore, all ROIs quantified using ABA with superficial and deep landmarks had high statistical similarity between observers (Supplemental Table 1) and overall correlation was high in all ROIs between manually processed images and those quantified with ABA using both deep and superficial landmarks (Supplemental Fig. 2).

3.3. Warping with superficial and deep landmark placement does not affect c-Fos+ pixel dimensions

To examine how warping affects pixel quality of the data, we further analyzed infralimbic cortex which our data indicate is subject to warping-induced changes in c-Fos quantification (Fig. 4d). Representative images of the placement of the infralimbic cortex ROI boundaries on tissue sections using either manual outlining (Fig. 5a), ABA-mediated outlining without warping (Fig. 5b), ABA-mediated outlining following warping using superficial landmarks only (Fig. 5c) or both superficial and deep landmarks (Fig. 5d) indicate that the warping process induces subtle changes in which portion of the tissue section is positioned with the atlas-defined ROI boundaries (Fig. 5a-d). This is expected, as we are warping the experimental image to better align with an atlas figure. To determine what impact the warping process has on the pixel size of individual c-Fos+ positive nuclei, five neurons within this ROI were selected for measurement prior to warping (Fig. 5f), after

Fig. 4. Comparison of c-Fos data via manual vs. ABA-mediated analysis. Automated c-Fos positive cell counts per ROI were calculated within the same section by three different observers using manual preparation, ABA with superficial landmark only, and ABA with superficial and deep landmarks. Cell counts in representative ROIs, the (a) prelimbic cortex and (b) cingulate cortex area 1 were not different between observers or methodology. (c) Piriform cortex showed intraobserver variability between manual processing and ABA processing using superficial landmarks only, as well as interobserver variability using manual processing. Both discrepancies were ameliorated by quantifying using ABA with both superficial and deep landmarks. (d) Infralimbic cortex showed intraobserver variability between manual processing and ABA processing using superficial landmarks only, as well as interobserver variability with ABA processing using superficial landmarks only. Both discrepancies were ameliorated by quantifying with ABA processing using both superficial and deep landmarks. *p < 0.05; two-way ANOVA with Tukey HSD post-hoc analysis; n = 3 trials per user on different days.
warping using superficial landmarks only (Fig. 5g), and after warping using superficial and deep landmarks (Fig. 5h). Warping of the infralimbic cortex with superficial landmarks only increased in the size of c-Fos positive nuclei. However, warping of the infralimbic cortex using both superficial and deep landmarks returned the size of c-Fos positive nuclei back to pre-warping levels (Fig. 5i). These data are in line with our total c-Fos + cell quantifications by ROI, in which discrepancies between manual processing and ABA-mediated processing following warping with superficial landmarks only are corrected by the addition of deep landmarks prior to warping (Fig. 4).

4. Discussion

Herein we have described a semi-automated method for quantifying c-Fos positive cells in multiple ROIs. ABA reduces analysis time compared to a fully manual procedure (Fig. 3), while maintaining high levels of interobserver consistency (Fig. 4) and maintaining c-Fos+ pixel dimensions (Fig. 5). While the reduction in processing time is based on 10 ROIs within a section, quantification of additional predetermined ROIs will have minimal impact on the time required for automated processing, whereas in a manual pipeline each ROI would need to be individually outlined. Thus, as additional ROIs are added to any analysis, time saved by using ABA should increase. Currently, 19 ROIs are included on the Kopek Lab Github website and additional ROIs may be added by the end user as needed. While our analysis uses coronal sections, ROIs can be added in horizontal or sagittal planes using the described methodology, with the understanding that sagittal sections do not contain the symmetry present in other orientations, and horizontal atlas sections would require a 90° rotation to allow for left-right symmetry. Following the addition of any ROIs, users should verify that landmarks are appropriately placed for accurate warping, as we have observed that a selection of ROIs are more accurately quantified by using a combination of superficial and deep landmarks (Fig. 4c,d). Additionally, end users should take into account any underlying effects of their rodent model that may affect how accurately tissue sections match anatomical boundaries of a typical atlas image. In such cases, preliminary experiments should be performed to ensure that obtained results are reliable.

Our sample analysis utilizes c-Fos as a marker of neural activity due to its common use in neuroscience publications and its ubiquitous distribution throughout the brain (Chang et al., 1988; Dragunow and Faull, 1989; Harris, 1998; Chiasson et al., 1997), making it an ideal target for whole brain analysis. However, ABA-mediated processing is adaptable (Table 1) and not restricted to this marker. As c-Fos at this time point is observed predominantly in nuclei (Perrin-Terrin et al., 2016), this program is already optimized for any protein with nuclear localization. Relatively simple adjustments can be made to change the parameters of what qualifies a cell as being positive for a specific marker depending on the subcellular localization of that marker. For example, for virally labeled cells or endogenous cytoplasmic markers, pixel size thresholds could be increased to include sizes typical for whole cell bodies. Similarly, quantification need not be restricted to cellular counting, as the code for many currently available FIJI analysis tools can be substituted for counting-based measurements in ABA, such as standard densitometry and colocalization analyses. To this end, we performed an additional analysis using IHC labeling of dopamine D1 receptor for quantification by standard densitometry measurements (Supplemental Fig. 3), as proof of principle that this application can be adapted for

Table 1

<table>
<thead>
<tr>
<th>Change to ABA</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add new landmarks</td>
<td>Increase warp accuracy</td>
</tr>
<tr>
<td>Alter analysis function</td>
<td>Analyze for different output</td>
</tr>
<tr>
<td>Change particle size</td>
<td>Analyze other chromogenic stains</td>
</tr>
<tr>
<td>Alter image thresholding</td>
<td>Analyze fluorescent staining</td>
</tr>
<tr>
<td>Add new ROI coordinates</td>
<td>Analyze new brain regions</td>
</tr>
<tr>
<td>Change atlas</td>
<td>Analyze cell count in other animal models or different orientations (i.e. transverse of sagittal sections)</td>
</tr>
</tbody>
</table>

Table highlighting various adaptation options with the expected effect on output.
alternative markers and quantification methods. We have included this secondary analysis code, named ABA Densitometry, on our github website.

The warping function in ABA permits standardized rat brain atlas images to be adapted for wider usage. While the Paxinos & Watson’s The Rat Brain in Stereotaxic Coordinates uses male Wister rats between 270 g and 290 g (Paxinos, 2007), brains of different sizes are frequently used in current neuroscience research. Indeed, as current NIH funding requirements stipulate the use of both male and female animals in protocols (National Institutes of Health, 2015), appropriately placed landmarks facilitate image size adjustment for concurrent, accurate analysis of the same ROIs in brains of both sexes. Furthermore, developmental studies assessing younger rats can also be adjusted for size with appropriate warping, permitting analyses over various time points.

5. Conclusions

In summary, ABA is a versatile and time-saving open source analysis pipeline for parallel IHC-based analyses in multiple brain regions and brain sizes.

Credit authorship contribution statement

All authors contributed to conceptualization. CF and GK developed methodology, and GK designed and developed the software. CF, GK, and AS performed data curation and validation. JRB and AMK conducted formal analysis and manuscript writing (original draft). All authors contributed to writing (review and editing).

Acknowledgements

We thank Helen Yang and Isha Doshi for assistance with immunohistochemistry, and the Pathology Clinical Research Core at Albany Medical Center for assistance with imaging. This work was supported by Albany Medical College start-up funds to AMK.

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.2020.108982.

References