HERBS: Histological E-data Registration in rodent Brain Spaces

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Abstract

Recording technologies for rodents have seen huge advances in the last decade, allowing users to sample thousands of neurons simultaneously from multiple brain regions. This has prompted the need for digital tool kits to aid in curating anatomical data, however, existing tools either provide limited functionality or require users to be proficient in coding to use them. To address this we created HERBS, a comprehensive new tool for rodent users that offers a broad range of functionalities through a user-friendly graphical user interface. Prior to experiments, HERBS can be used to plan coordinates for implanting electrodes, targeting viral injections or tracers. After experiments, users can register recording electrode locations (e.g. Neuropixels, tetrodes), viral expression or other anatomical features, and visualize the results in 2D or 3D. Additionally, HERBS can delineate expression from multiple injections across tissue sections and obtain individual cell counts. Regional delineations in HERBS are based either on annotated 3D volumes from the Waxholm Space Atlas of the Sprague Dawley Rat Brain or the Allen Mouse Brain Atlas. HERBS allows users to scroll through the digital brain atlases and provides custom-angle slice cuts through the volumes, and supports free-transformation of tissue sections to atlas slices. Furthermore, HERBS allows users to reconstruct a 3D brain mesh with tissue from individual animals. HERBS is a multi-platform open-source Python package that is available on PyPI and GitHub.

Introduction

Understanding the structure, function, or physiology of neural circuits requires the ability to consistently target brain regions prior to experiments and, afterward, to maintain an accessible, objective record of the areas studied (Simmons and Swanson 2009). Traditionally, post-hoc anatomical record-keeping has relied on histological approaches where users delineate anatomical features in tissue sections, then register them manually with a reference atlas. While these and other approaches are critical for grounding experiments anatomically, doing them has become increasingly challenging with the emergence of large-scale recordings spanning multiple brain regions.

Several software tools have been developed in recent years which use computer vision technology to accelerate and systematize the mapping of experimental results in various model species (e.g Shamash et al. 2018; Song et al. 2020; Claudi et al. 2021; Andy 2022). When combined with the appropriate reference atlases (e.g. George Paxinos 2008; Wang et al. 2020; Claudi et al. 2020), these tool kits give users the ability to reconstruct electrode tracks, viral expression, anatomical projections(Oh et al. 2014; Zingg et al. 2014; Winnubst et al. 2019), cell types (Fürth et al. 2018), or

to detect cells (Tyson et al. 2021), gene expression patterns or functional nodes (Lein et al. 2007; Ortiz et al. 2020) in the brain.

Different types of tool kits have been developed for different brain preparations, including volumetric whole-brain data or sliced tissue sections, with each approach bringing its own strengths and limitations. Whole-brain volume analyses have the advantage of readily conveying the 3D spatial relationships between neuronal pathways and neighboring structures. For instance, ITK-SNAP(Yushkevich et al. 2006) is a popular biomedical software used for automatic image segmentation and delineation of regions of interest in human brain imaging data. For animal models there is *Brainrender* (Claudi et al. 2021), which displays any of several atlases incorporated from the BrainGlobe Atlas API in 3D. It provides fast visualisation for user-defined data sets, such as reconstructed electrode tracks, viral expression or anatomical projections. These can be obtained when *Brainrender* is combined with sibling software, Brainreg (Tyson, Rousseau, and Margrie 2020), which maps whole-brain data sets to atlases using supervised automatic image registration. While whole-brain data sets provide a holistic quantitative dimension to analyses, generating them is time and resource intensive, requiring either a light sheet scanning microscope or magnetic resonance imaging.

Because of the comparatively lower cost and simplicity, anatomical mapping using histological tissue sectioning still dominates in most labs (e.g. Clancy, Orsolic, and Mrsic-Flogel 2019; Zutshi et al. 2022; Lagartos-Donate et al. 2022; Gardner et al. 2022). This, however, also has its own challenges. One common issue lies in matching a given tissue slice correctly to a reference atlas template. Previous studies have sought to solve this problem using multiple software platforms, like ImageJ (Schneider, Rasband, and Eliceiri 2012), Adobe Illustrator (Adobe Inc. 2019a) or Adobe Photoshop (Adobe Inc. 2019b), which can be time-consuming for manual curation and is not systematic. Software packages have been developed to address this issue, including the electrode reconstruction tool kit, SHARP-TRACK (Shamash et al. 2018). This software uses a global warping transformation method to fit histological images back to a mouse atlas. Afterward, it provides a read-out of the estimated number of channels in each brain region as well as 3D visualizations. As currently configured, the tool kit works only with mouse atlases and is MATLAB-based, which requires a paid license and some programming knowledge.

Despite the number of tool kits available, there currently is no single software that combines functionalities spanning the gamut of pre-surgical planning and post-experiment registration without requiring programming. To help fill this methodological gap we developed HERBS, an open-source Python package which brings together a range of functionalities in a user-friendly graphical front-end interface. HERBS can be used to generate pre-surgical coordinates, reconstruct electrode tracks after recordings, mark viral expression volumes or single cells; delineate regional boundaries in brain slices, and view post-hoc anatomical reconstructions in 2D or 3D. There is no upper limit for the number of electrodes which it can reconstruct, so it can be used with microelectrode arrays or multi-tetrode bundles. To serve both rat and mouse users, HERBS has the in-built function to download and run the Waxholm Space Atlas of the Sprague Dawley Rat Brain (Papp et al. 2014) or the Allen Mouse Brain Atlas (Wang et al. 2020). The software also contains an atlas explorer that can scroll freely through either rat or mouse atlases to display coronal, sagittal and horizontal planar surfaces in 2D, as well as the corresponding mesh or volume of the atlas in 3D.

For accurate and robust image registration, HERBS provides rigid registration as well as interactive, non-rigid registration using triangulation-based piece-wise affine transformation. It is compatible with most tissue staining methods and image formats, as long as the regions of interest are visible and can be delineated by the user. For users who wish to augment or add functionalities of their own in Python, we provide a standalone Python pipeline tool kit, TRACER (**T**oolkit for **R**econstructing **A**natomical **C**oorindat**E**s in **R**ats), in parallel with HERBS (see Materials and Methods for more information). To maximize general accessibility, HERBS requires minimal programming knowledge and is compatible with Windows, macOS and Linux systems. It is intended as an open community resource available for further expansion and refinement. We hope it offers a valuable addition to the anatomical methods available for the neuroscience community.

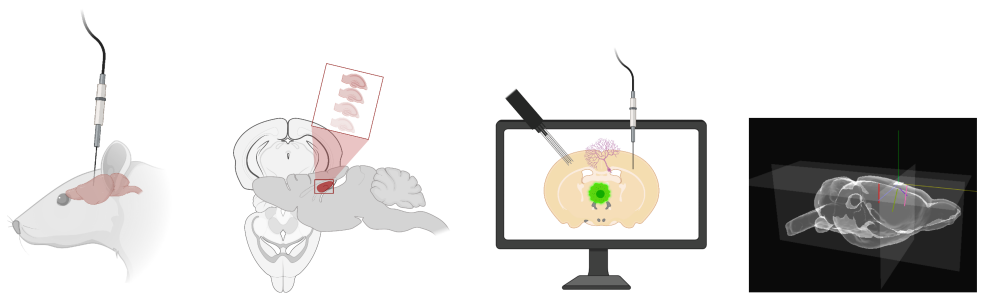
Results

General Outline

HERBS was designed as an intuitive, generic software tool to provide rodent users with four core functionalities, shown in Figure1A. These include (a) generating anatomical target coordinates when planning a surgery; (b) processing and editing raw histological data; (c) registering and reconstructing objects implanted in the brain; (d) visualizing annotated brain volumes in 3D. Users can carry out each functionality by following a series of steps described in the HERBS Cookbook using a keyboard and mouse. The layout of the user interface for HERBS, including color-coded panels used to execute its functionalities, are shown in Figure1B.

When installing HERBS, users can choose whether to use the Waxholm Space (WHS) Atlas of the Sprague Dawley Rat Brain (Papp et al. 2014) or the Allen Mouse Brain Atlas (Wang et al. 2020) as the anatomical reference (see Cookbook, chapter 6.1). Either atlas can be downloaded through HERBS, as they are both integrated as parts of the software package.

A. General functionalities



(a) Generate surgical coordinates (b) Process and edit histological images (c) Register and reconstruct objects or volumes (d) Visualize results in 3D

B. HERBS GUI

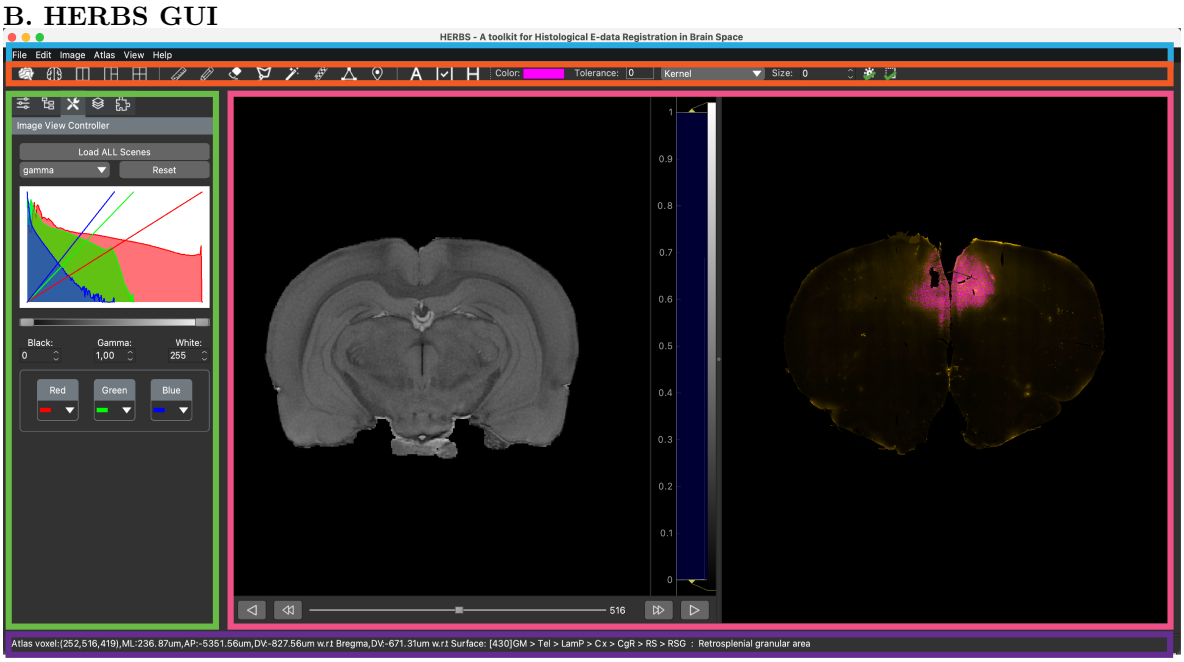


Figure 1. The core functionalities and user interface of HERBS. (A) HERBS can be used to (a) plan electrode insertions or viral injections before surgery,(b)process and edit histological images, (c) register objects such as probes, virus, cells and slice boundaries from histological images, (d) visualize the brain in 3D with defined functions. **(B)** The user interface of HERBS consists of the Menu Bar for navigating through the functions, the Tool Bar from which users select actions to perform in the Plot Window, such as registering or reconstruct objects. The Side Bar provides tools for visually editing objects or layers. The Status Bar (bottom) shows the brain regions and coordinates displayed in the Plot Window. The WHS or Allen Mouse Brain atlases can be downloaded and loaded directly through HERBS from the Menu Bar with a click of a button.

Generating pre-surgical coordinates

One of the essential steps in planning a surgery is defining the stereotaxic coordinates to reach specific brain areas of interest. HERBS facilitates this process by allowing users to determine the insertion parameters required to target one or several regions along a linear trajectory. This is done by simply clicking the start point and end point of the desired path on a 2D brain slice image, and there is no limit to the number of trajectories (e.g. recording probes) that can be planned. HERBS then automatically computes the insertion angle and length needed to reach the target brain region, as well as the location of the trajectory relative to Bregma defined in the atlas. The user can print and bring the read-out to the surgery room or save for record keeping. The planning functionality is equally applicable for targeting lenses for imaging, injection pipettes for tracer or virus injections, or making focal lesions.

The working steps for generating pre-surgical coordinates are illustrated in Figure 2, in which we plan the insertion of a multi-shank Neuropixels 2.0 probe through retrosplenial cortex, visual cortex and the intermediate hippocampus in the right hemisphere of a rat. The step-by-step protocol is described in the HERBS Cookbook, Chapter 6.4. Neuropixels users should note that estimates of probe length include the 175um channel-less shank tip, and that HERBS eliminates it when estimating the number of recording channels in the brain. In the first step, the user scrolls to the brain slice of interest (Figure 2A). This is aided by the color coding of each brain area in the atlas, which provides visual landmarks for defining boundaries between regions. Next, the probe trajectory is defined in the region(s) of interest with clicks of the mouse (Figure 2B). Once completed, the probe trajectory is saved as a layer in the Side Bar, after which it is saved to a 3D representation showing the trajectory of the probe in a WHS brain volume (Figure 2C). After the probe is merged on the Side Bar, a read-out is generated for a 4 shank probe (Figure 2D) that includes the location and regions traversed. A more detailed read-out table for each shank of the 4-shank probe can also be made (Figure 2E) which includes inclination, length of probe inserted, number of recording channels per region, and entry and exit coordinates of the probe. Either or both read-outs can be saved per the user's desire. As there is no limit to the number of probes that can be planned for insertion, the user simply repeats the steps above for each probe.

Reconstructing electrode tracks

Another core functionality of HERBS is reconstructing electrode paths after surgeries or *in vivo* recordings, and there is no upper limit on the number of electrodes that can be reconstructed. Prior to reconstructing probe trajectories, histological images can be pre-processed before they are overlaid on their corresponding atlas images. Pre-processing can include rotating the section to an optimal plane, changing brightness, gamma curves, etc., and can be done using the image view controller in the Side Bar, or the Edit option in the Menu Bar, as indicated in the interface panel in Figure1B (see HERBS Cookbook chapter 6.6).

After the image has been loaded and adjusted the user can begin the steps of the reconstruction pipeline shown in (Figure 3), which uses an example section with a track from a Dil-stained Neuropixels 1.0 probe (red) progressing laterally from visual to auditory cortex. The first step is

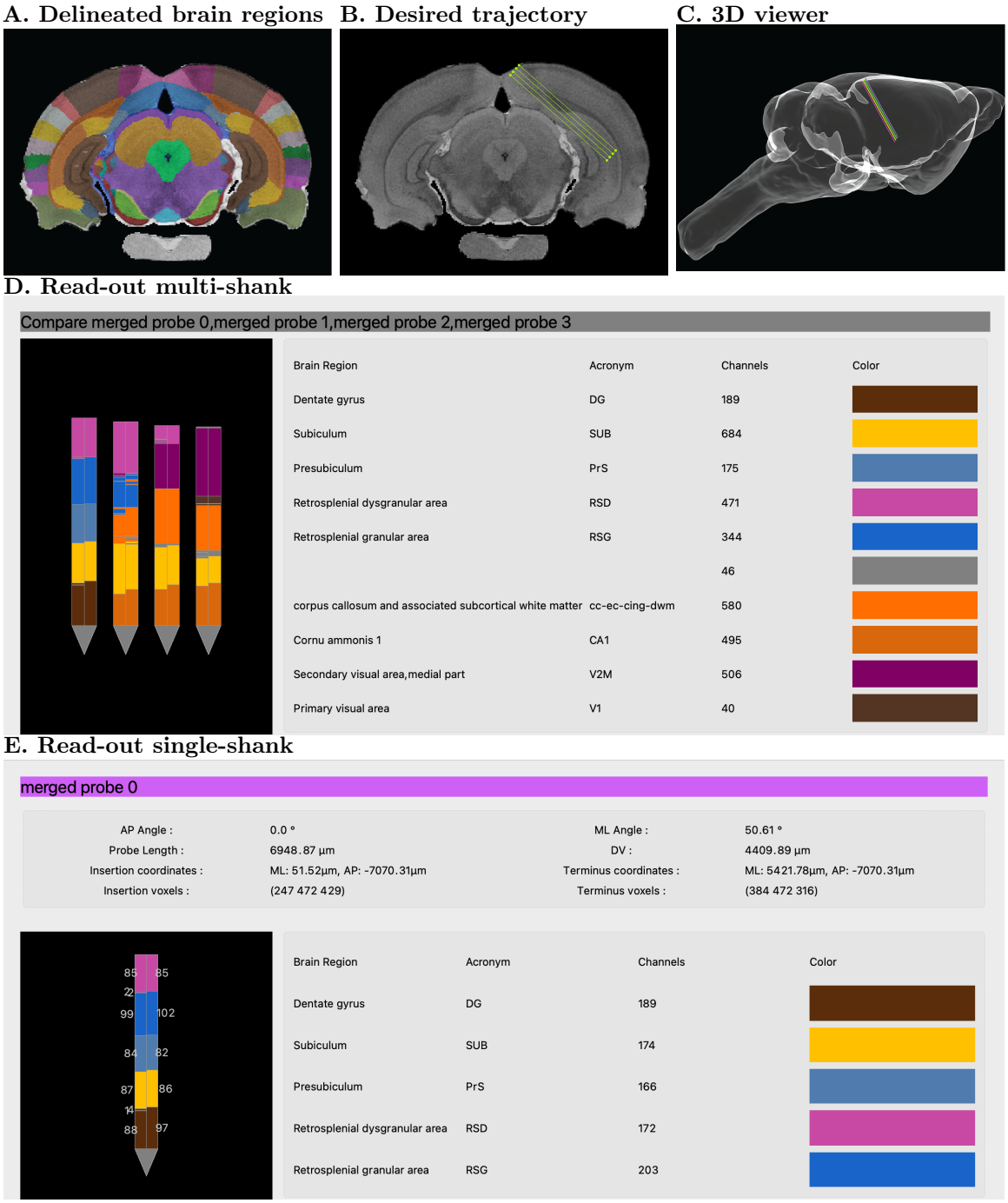


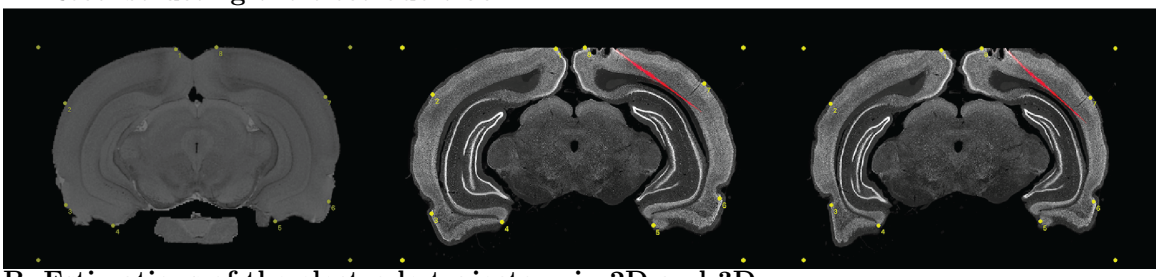
Figure 2. Using HERBS to plan a Neuropixels 2.0 probe implant before surgery. (A) Once the plane of choice is set (coronal in this example), 2D atlas brain sections with regional delineations appear and the user scrolls to the slice with the region(s) they wish to target. **(B)** The user clicks along the desired trajectory of the probe (the insertion angle here is 48 degrees lateral tilt, at Bremga -7.0mm). **(C)** A linear probe trajectory is projected into the Waxholm space in 3D. **(D)** A read-out table for all 4 probes shows the regions the probe has traversed and the number of channels in each region. **(E)** A detailed read-out for a single probe provides the entry position, probe angle and insertion length to reach the target regions, as well as an estimation of the number of recording channels that will be each region in the experiments.

choosing the plane of sectioning (coronal, sagittal, horizontal) and scrolling to the corresponding atlas brain slice. Once the corresponding slice is located, the user defines anchor points to warp the histological section onto the atlas section (Figure 3A) (see Cookbook, Chapter 6.4 and 6.5). Once the anchor points are defined and the matching step is complete, the user then clicks a minimum of 4 points along the probe track in the tissue, after which a 3D rendering is generated showing how the probe is situated in the volume of the brain (Figure 3B) (Cookbook Chapter 6.6.1). A detailed read-out table showing the inclination and length of the probe, regions traversed and number of recording channels per region is generated automatically (Figure 3C). In cases where histological sections are cut out of plane, HERBS allows the user to tilt the atlas brain up to 30 degrees to facilitate template matching. Note: Electrode track reconstructions can be performed for any type of electrodes, tetrodes or recording arrays, as well as with different sizes of sections.

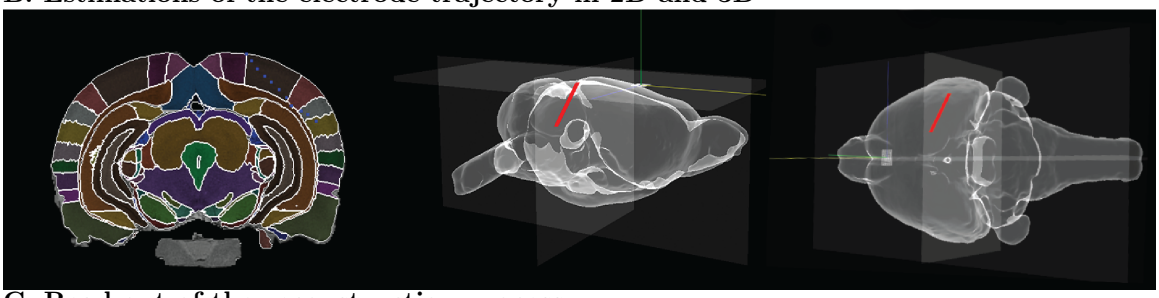
Reconstruction of viral expression

Beyond electrode reconstructions, HERBS can be used for anatomical applications including visualization of virus expression, anatomical tracer labeling, or marking lesions or neurodegeneration. Here we provide a brief overview of the procedure for reconstructing a spatial volume of adenovirus (AAV) expression (see Cookbook, Chapter 6.7 for this and other protocols). The procedure is similar to that for probe reconstruction, but the regions defined in each slice are 2-dimensional, and the volume to be delineated will likely extend over multiple slices (Figure 4). As with reconstructing a probe track, the first step in reconstructing a viral volume is matching the starting histology section with the corresponding slice in the atlas brain and overlaying the two. The initial slice from an example viral reconstruction is shown in Figure4A. The user then defines the perimeter of virus expression by selecting the magic wand tool from the Tool Bar and clicking on a fluorescent patch of tissue in the histological section (Figure 4B). Note that the granularity of the region selected will depend on the magic wand tolerance set by the user. These steps are repeated for each slice included in the visualization. Finally, the marked fluorescent regions from several 2D sections are combined and projected into a 3D brain volume (Figure 4C), and the list of brain regions in contact with the virus are shown in the read-out with color-coded labels (Figure4D). The same functions and steps are used for similar tasks such as labeling single cells, marking fibers of passage or visualizing lesions. Note also that the resolution of the 3D reconstruction will depend on how many sections the user includes in the reconstruction; the example in Figure 4C consisted of 11 tissue slices.

A. Reconstructing the electrode track



B. Estimations of the electrode trajectory in 2D and 3D



C. Read-out of the reconstruction process

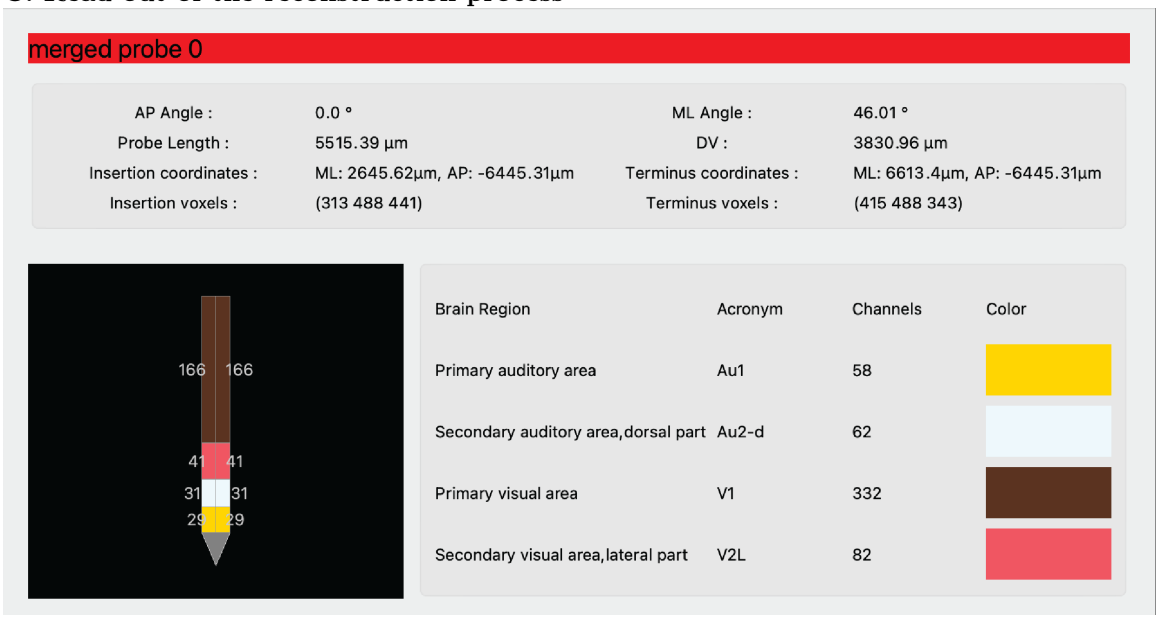


Figure 3. Reconstructing a recording probe trajectory from histological sections. (A) The initial steps include scrolling to the atlas brain slice (left) that best matches the user's histological section (middle). User-defined anchor points are then used to stretch and conform the histological section onto the atlas section; once they are merged a new image is generated with the histology warped to overlay the atlas slice (right panel). **(B)** The user then generates an estimate of probe placement and length by clicking at least four points spanning from the entry of the probe in the brain to the distal tip (left). The 3D reconstructed placement of the probe is shown in sagittal plane (middle panel) and viewed from the top (right panel) in the WHS volume. **(C)** A read-out from the merged probe shows details of the probe insertion, the coordinates, as well as brain regions the probe traversed in different colors.

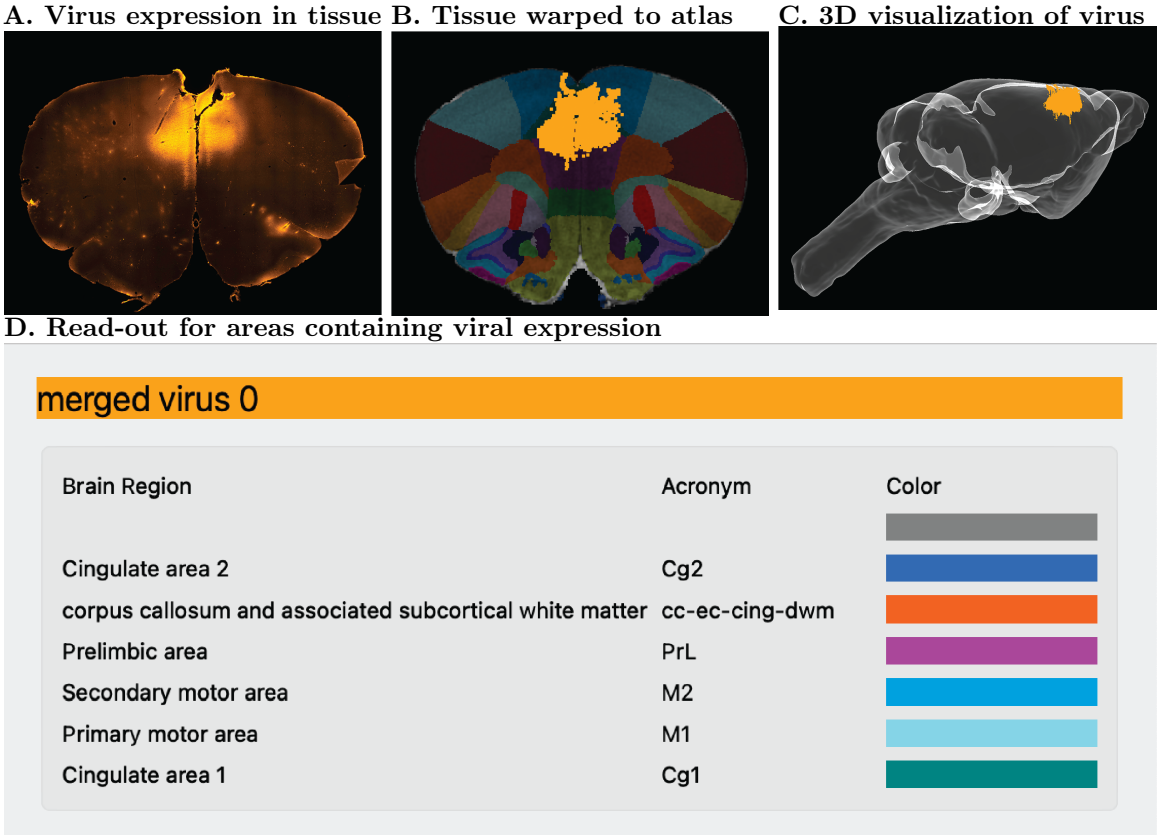


Figure 4. 3D visualization of virus expression across multiple tissue sections. (A) Shows the spatial extent of AAV-mediated mCherry expression in a coronal section from the frontal cortex of an adult rat. **(B)** The region of viral expression is defined by clicking the fluorescent region of tissue with the magic wand tool; areal boundaries are included at this stage. **(C)** A volumetric rendering of the brain shows the extent of viral expression in cingulate cortex and surrounding areas. **(D)** A read-out of the the regions in which the virus was expressed.

Discussion

With HERBS, we seek to furnish rodent users with a user-friendly, click-button GUI that simplifies the process of curating histological data . The functionalities include planning coordinates ahead of surgeries, as well as registering and visualizing anatomical data in 2D or 3D after experiments are completed. The GUI can be used to delineate a range of features such as recording locations, lesions or viral expression and is compatible with potentially any histological staining method. With HERBS, the user marks regions of interest on tissue images directly and each slice is mapped to a template, which should greatly reduce subjective judgement and variability across samples and brains. For rat users, HERBS references the newest version (v4) of the WHS rat brain atlas (Papp et al. 2014), which contains over 200 labeled cortical and sub-cortical regions at sub-millimeter scales, and for mouse users it references the widely-adopted Allen Mouse Brain Atlas (Wang et al. 2020).

Novelty and utility of HERBS in relation to other tool kits

Although several software packages with similar functionalities have been released in recent years (see Supplementary Table 1), there are a number of features which distinguish HERBS from them. Foremost is its simplicity, since it works via a visually intuitive, click-button interface designed to minimize user effort, and it requires no coding or added software. It is also unique in that it brings

together both pre and post-surgical registration and mapping functionalities in one program. The fact that it specifically includes rats also stands out among the many high-quality rendering and digital atlas resources for mice, such as Brain Mesh (Yaoyao-Hao 2020), SHARPTRACK (Shamash et al. 2018), cocoframer (Lein et al. 2007), and others (e.g. Jin et al. 2019, Chon et al. 2019). Another recently available anatomical tool kit is *Brainrender* (Claudi et al. 2021), which produces visually appealing anatomical renderings from a variety of source atlases through the BrainAtlasglobe API, but differs from HERBS in important ways. The most essential difference is that *Brainrender* was designed as a *visualization* tool. As such, it renders anatomical data in 3D volumes, but it cannot register anatomical to a reference atlas on its own. Registration must be done beforehand with separate software such as Brainreg (Tyson et al. 2022) or CellFinder(Tyson et al. 2021). HERBS, in contrast, was designed primarily as a *registration* tool, which comes configured to work with primary source atlases for rats or mice. The HERBS GUI can both download and load these reference atlases, whereas *Brainrender* relies on separate software and plugins which require programming knowledge in Python. The output from HERBS could be used as input for *Brainrender* if formatted appropriately, though HERBS offers some of its own visualization functionalities for simplicity. Finally, Brainreg and *Brainrender* use whole-brain data from light sheet imaging, whereas HERBS works with commonly used histological tissue sectioning methods. The Allen Institute recently released the Neuropixels Trajectory Explorer (Andy 2022) a MATLAB-based platform which can be used to plan coordinates for electrophysiological probes prior to surgery in mice and rats. Currently, the pre-surgical registration step is the only functionality available, as it does not yet have the option to load histology for post-hoc processing of probe tracks or viral expression. Unlike *Brainrender* and HERBS, it runs in MATLAB, which typically requires a paid software license. Thus, the main features that distinguish HERBS from other tools are that it (i) offers a combination of functionalities found separately in *Brainrender* and Neuropixels trajectory explorer, (ii) all functionalities in HERBS are accessible with button clicks and keystrokes, and (iii) it can be used immediately with the WHS Rat Brain Atlas or the Allen Mouse Brain Atlas without requiring additional plugins. Beyond these features, HERBS, similar to Brainreg (Tyson et al. 2022), can incorporate other atlases given the correctly formatted files, such as the Paxinos and Watson atlases for rats or mice, provided the user has the appropriate licensing permissions.

Sources of error and future improvements

While we hope that HERBS proves useful in its current form, there are areas where it can be improved and expanded in the future. For example, the template matching step can be time consuming if data sets contain a large number of histological slices, as with virus expression or tracer labeling. We aim to streamline these processes by implementing algorithms for pre-processing user images and matching reference slices in subsequent versions. Furthermore, although we designed HERBS to minimize the need for programming, installing it could be challenging for users who have not installed Python before.

In developing HERBS we also encountered sources of error which appear general to working with anatomical tissue that has been preserved with fixatives. For instance, we found that probe length estimates can be less consistent when larger numbers of histological slices are used for reconstructions. We suspect these errors could arise from averaging across clicked points defining the probe trajectory, and it is possible that misalignment of clicked points between slices can add to this. Another issue is that fixing tissue with paraformaldehyde causes non-uniform shrinking of the tissue. We found this could lead to small discrepancies when comparing Neuropixels channel counts from HERBS, which uses fixed tissue, against LFP measures taken while the animals were still alive. Although tissue shrinkage is inherent to histological processing and cannot be removed mathematically, HERBS offers a solution by fitting warped tissue to the atlas template using multiple anchor points per slice, which should substantially reduce inconsistencies due to shrinkage. Accurately registering raw neuroanatomical data in a reference atlas framework, though critically important, is time consuming, tedious and user variable. HERBS was developed to expedite, simplify and systematize this process by providing a single tool for generating anatomical

coordinates, as well as annotated 2D and 3D visualizations and data tables summarizing its outputs. We wish to emphasize that HERBS is a completely open source software with which we aim to help our fellow research community. We therefore welcome users to submit suggestions for improvements and to report bugs on our GitHub page, as this will help us continually improve the software and user experience.

Materials and Methods

Python packages and resources

HERBS is written entirely in Python version three using basic Python packages such as numpy, opencv, scipy as mentioned in the table below. Details on the specific dependencies used in HERBS can be found in HERBS Cookbook(chapter 3). Documentation and step-wise instructions for installing and implementing HERBS can be found in the HERBS Cookbook (Chapter 4). HERBS is an open-source software with hard-coded Python scripts. Should a user wish to customize Python-pipeline, one can use the code of TRACER - which is open-source and available at the Github repository. To use HERBS we recommend users to create a PyCharm project with Python installation(Version 3.8.10) and other supporting packages which are specified in the dependencies section in the HERBS Cookbook (Chapter 4).

Table 1. Key resources for HERBS

Resource	Type	Source and reference
Software, algorithm	Numpy	Harris et al. 2020
Software, algorithm	Scipy	Virtanen et al. 2020
Software, algorithm	OpenCV	Bradski 2000
Software, algorithm	PyOpenGL	Woo et al. 1999
Software, algorithm	PyQT5	PyQT 2012

Neuropixels and virus expression

All experiments were performed in accordance with the Norwegian Animal Welfare Act and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. All procedures were approved by the Norwegian Food Safety Authority (Mattilsynet; protocol IDs 27175 and 25094). All tissue for in-house testing came from adult (>15wk) Long-Evans hooded rats. Detailed steps of the surgical preparation and post-operative care are described in Mimica et al. 2018.

Neuropixels: the probes (version 1.0 Neuropixels, IMEC, Belgium) were coated with DiI (Vybrant DiI, catalog no.V22888, Thermo Fisher Scientific, USA) by repeatedly drawing a 2µL droplet of DiI solution at the tip of a micropipette up and down the length of the probe shank until all DiI had dried onto the shank, causing the shank to appear pink. The probes were angled in the arm of a stereotaxic frame and inserted at a rate of 100-300µm per minute. The chronically implanted animal was kept < 1 week following surgery, after which it was perfused with 4% paraformaldehyde and the brains were removed.

The brain was transferred to 2% dimethyl sulfoxide (DMSO; VWR, USA) solution for cryoprotection for 1-2 days, after which the Neuropixel shank was removed, the brain was frozen and sectioned in 50µm slices with a sliding microtome (Microm HM430, Thermo Fisher Scientific, USA). The tissue sections then underwent fluorescent immunostaining against NeuN (catalog no.ABN90P, Sigma-Alrich, USA), followed by secondary antibody-staining with Alexa 647-tagged goat anti-guinea pig antibody (catalog no.A21450, Thermo Fisher Scientific, USA), after which the

sections were rinsed, mounted, coverslipped and stored at 4^0C. The detailed immunostaining protocol is available per request. Next, the sections were digitized using an automated scanner for fluorescence and brightfield images at the appropriate illumination wavelengths (Zeiss Axio Scan.Z1, Jena, Germany), and were saved in .jpeg format for processing in TRACER.

Virus expression: pulled glass pipettes were used to inject 500nL of AAV5-mDIx-Chr2-mCherry-Fishell-3 (plasmid no.83898, Addgene, USA; AAV produced at Kavli Viral Vector Core Facility, NTNU) at a rate of 50nL per minute. Bilateral injections were targeted to the cingulate cortex (Cg1; +2.0AP, ±0.5ML, -2.0DV). 5 weeks after surgery the animal was sacrificed and perfused with 4% paraformaldehyde. The brain was removed, post-fixed overnight in 4% paraformaldehyde at 4^0C, then cryoprotected for 24hr in 2% DMSO. The brain was then frozen in dry ice and 40µm sections were collected as described above. Fluorescent signal from the virus was amplified by immunostaining against Red Fluorescent Protein (RFP) (catalog no.5F8, Chromotek GmbH, Germany), followed by secondary antibody-staining with Alexa 546-tagged Goat Anti-rat IgG (catalog no. A-11081, Thermo Fisher Scientific, USA). The detailed immunostaining protocol is available upon request.

Data availability, software and citation policy

The software described in this manuscript is an open-source software written completely in Python 3.8. HERBS is fully supported by Windows, macOS and Linux. Source code is available on https://github.com/Whitlock-Group/HERBS. HERBS Cookbook and documents are available on the Whitlock group Github page.

The Waxholm Space rat brain atlas files can be found here from the NITRC website (https://www.nitrc.org/projects/whs-sd-atlas). The required atlas files to run HERBS (downloaded upon installation of HERBS) and TRACER are cited below:

WHS rat brain atlas v4:

- WHS_SD_rat_atlas_v4.nii.gz (Kleven et al., in preparation)
- WHS_SD_rat_atlas_v4.label (Kleven et al., in preparation)

Previous version of the WHS rat brain atlas (v1.01):

- WHS_SD_rat_T2star_v1.01.nii.gz (Papp et al. 2014)
- WHS_SD_rat_brainmask_v1.01.nii.gz (Papp et al. 2014)

Citation Policy:

We kindly ask users to cite this paper when using HERBS or TRACER in their studies, and to cite the appropriate version of the Waxholm Space rat brain atlas.

Refer to the WHS atlas by its RRID: SCR_017124, and cite the first publication (Papp et al. 2014) along with the version of the atlas that is used. For example, cite Kjonigsen et al. 2015, v2 of the atlas if the user's work makes particular use of delineations of the hippocampal region. Or cite Osen et al. 2019, v3 of the atlas if the user's work makes particular use of delineations of the auditory system.

License:

The Waxholm Space atlas of the Sprague Dawley rat brain is licensed under the Creative Commons Attribution ShareAlike (CC BY-SA) 4.0 license:
https://creativecommons.org/licenses/by-sa/4.0/

The Allen Mouse Brain Atlas software and wiki are freely available at:
https://github.com/cortex-lab/allenCCF.

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References

[1] Adobe Inc. *Adobe Illustrator*. Version CC 2019 (23.0.3). Apr. 1, 2019. URL: https://adobe.com/products/illustrator.

[2] Adobe Inc. *Adobe Photoshop*. Version CC 2019. Mar. 6, 2019. URL: https://www.adobe.com/products/photoshop.html.

[3] P. Andy. *Neuropixels trajectory explorer*. https://github.com/petersaj/neuropixels_trajectory_explorer. 2022.

[4] G. Bradski. "The OpenCV Library". In: *Dr. Dobb's Journal of Software Tools* (2000).

[5] U. Chon et al. "Enhanced and unified anatomical labeling for a common mouse brain atlas". In: *Nature communications* 10.1 (2019), pp. 1–12.

[6] K. B. Clancy, I. Orsolic, and T. D. Mrsic-Flogel. "Locomotion-dependent remapping of distributed cortical networks". In: *Nature Neuroscience* 22.5 (2019), pp. 778–786.

[7] F. Claudi et al. "BrainGlobe Atlas API: a common interface for neuroanatomical atlases". In: *Journal of Open Source Software* 5.54 (2020), p. 2668. DOI: 10.21105/joss.02668. URL: https://doi.org/10.21105/joss.02668.

[8] F. Claudi et al. "Visualizing anatomically registered data with brainrender". In: *Elife* 10 (2021), e65751.

[9] D. Fürth et al. "An interactive framework for whole-brain maps at cellular resolution". In: *Nature neuroscience* 21.1 (2018), pp. 139–149.

[10] R. J. Gardner et al. "Toroidal topology of population activity in grid cells". In: *Nature* 602.7895 (2022), pp. 123–128.

[11] K. B. F. George Paxinos. *The Mouse Brain in Stereotaxic Coordinates,Compact 3rd Edition.* Elsevier, 2008.

[12] C. R. Harris et al. "Array programming with NumPy". In: *Nature* 585 (2020), 357–362. DOI: 10.1038/s41586-020-2649-2.

[13] M. Jin et al. "SMART: An open source extension of WholeBrain for iDISCO+ LSFM intact mouse brain registration and segmentation". In: (2019). DOI: 10.1101/727529.

[14] L. J. Kjonigsen et al. "Waxholm Space atlas of the rat brain hippocampal region: three-dimensional delineations based on magnetic resonance and diffusion tensor imaging". In: *Neuroimage* 108 (2015), pp. 441–449.

[15] M. J. Lagartos-Donate et al. "Postnatal development of projections of the postrhinal cortex to the entorhinal cortex in the rat". In: *Eneuro* 9.3 (2022).

[16] E. S. Lein et al. "Genome-wide atlas of gene expression in the adult mouse brain". In: *Nature* 445.7124 (2007), pp. 168–176.

[17] B. Mimica et al. "Efficient cortical coding of 3D posture in freely behaving rats". In: *Science* 362.6414 (2018), pp. 584–589. ISSN: 0036-8075. DOI: 10.1126/science.aau2013. eprint: https://science.sciencemag.org/content/362/6414/584.full.pdf. URL: https://science.sciencemag.org/content/362/6414/584.

[18] S. W. Oh et al. "A mesoscale connectome of the mouse brain". In: *Nature* 508.7495 (2014), pp. 207–214.

[19] C. Ortiz et al. "Molecular atlas of the adult mouse brain". In: *Science Advances* 6.26 (2020), eabb3446.

[20] K. K. Osen et al. "Waxholm Space atlas of the rat brain auditory system: Three-dimensional delineations based on structural and diffusion tensor magnetic resonance imaging". In: *Neuroimage* 199 (2019), pp. 38–56.

[21] E. A. Papp et al. "Waxholm Space atlas of the Sprague Dawley rat brain". In: *Neuroimage* 97 (2014), pp. 374–386.

[22] PyQT. "PyQt Reference Guide". In: (2012). URL: http://www.riverbankcomputing.com/static/Docs/PyQt4/html/index.html.

[23] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri. "NIH Image to ImageJ: 25 years of image analysis". In: *Nature methods* 9.7 (2012), pp. 671–675.

[24] P. Shamash et al. "A tool for analyzing electrode tracks from slice histology". In: *BioRxiv* (2018), p. 447995.

[25] D. M. Simmons and L. W. Swanson. "Comparing histological data from different brains: sources of error and strategies for minimizing them". In: *Brain research reviews* 60.2 (2009), pp. 349–367.

[26] J. H. Song et al. "Precise mapping of single neurons by calibrated 3D reconstruction of brain slices reveals topographic projection in mouse visual cortex". In: *Cell Reports* 31.8 (2020), p. 107682.

[27] A. L. Tyson, C. V. Rousseau, and T. W. Margrie. *brainreg: automated 3D brain registration with support for multiple species and atlases*. Version 0.1.5. Aug. 2020. DOI: 10.5281/zenodo.3991718. URL: https://doi.org/10.5281/zenodo.3991718.

[28] A. L. Tyson et al. "A deep learning algorithm for 3D cell detection in whole mouse brain image datasets". In: *PLoS Computational Biology* 17.5 (2021), e1009074.

[29] A. L. Tyson et al. "Accurate determination of marker location within whole-brain microscopy images". In: *Scientific reports* 12.1 (2022), pp. 1–8.

[30] P. Virtanen et al. "SciPy 1.0: Fundamental Algorithms for Scientific Computing in Python". In: *Nature Methods* 17 (2020), pp. 261–272. DOI: 10.1038/s41592-019-0686-2.

[31] Q. Wang et al. "The Allen mouse brain common coordinate framework: a 3D reference atlas". In: *Cell* 181.4 (2020), pp. 936–953.

[32] J. Winnubst et al. "Reconstruction of 1,000 projection neurons reveals new cell types and organization of long-range connectivity in the mouse brain". In: *Cell* 179.1 (2019), pp. 268–281.

[33] M. Woo et al. *OpenGL programming guide: the official guide to learning OpenGL, version 1.2*. Addison-Wesley Longman Publishing Co., Inc., 1999.

[34] Yaoyao-Hao. *BrainMesh: A Matlab GUI for rendering 3D mouse brain structures*. https://github.com/Yaoyao-Hao/BrainMesh/. 2020.

[35] P. A. Yushkevich et al. "User-Guided 3D Active Contour Segmentation of Anatomical Structures: Significantly Improved Efficiency and Reliability". In: *Neuroimage* 31.3 (2006), pp. 1116–1128.

[36] B. Zingg et al. "Neural networks of the mouse neocortex". In: *Cell* 156.5 (2014), pp. 1096–1111.

[37] I. Zutshi et al. "Extrinsic control and intrinsic computation in the hippocampal CA1 circuit". In: *Neuron* 110.4 (2022), pp. 658–673.

Supplementary material

Supplementary table: 1. Comparison of anatomical tool kits

Functions/Software	HERBS	TRACER	Brainrender	Brainreg	SHARPTRACK
Year	2022	2021	2021	2020	2018
Python	✓	✓	✓	✓	
GUI	✓		✓	✓	✓
Histology data	✓	✓			✓
Whole-Brain data	**[1]		✓	✓	
Pre-surgical registration	✓	✓			✓
Post-surgical registration	✓	✓		✓	✓
3D Visualization tool	✓	✓	✓	✓	✓
In-built atlases	✓	✓	*[2]	*	✓

[1] In development
[2] Plugin required