Axon flex 2 user manual

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The manuscript is accompanied by a Supplementary Note that presents the software in further detail. Introduction Skeletonized hippocampal CA1 cell (juvenile mouse) in which apical and basal dendrites have been analyzed separately and color coded according to their Sholl profile. Warmer hues indicate higher number of Intersections (N). Critical radius (rc) and Mean value (Nav) are indicated. The Sholl technique1 is used to describe neuronal arbors. This plugin can perform Sholl directly on 2D and 3D grayscale images of isolated neurons. Its internal algorithm to collect data is based upon how Sholl analysis is done by hand — it creates a series of concentric shells (circles or spheres) around the focus of a neuronal arbor, and counts how many times connected voxels defining the arbor intersect the sampling shells. The major advantages of this plugin over other implementations are: Installation After installing SNT, Sholl commands can be accessed through the Plugins › Neuroanatomy › Neuroanatomy Shortcut Window, or the SNT icon in the ImageJ toolbar. Direct Analysis of Images In this mode (bitmap analysis), the plugin requires a binary image or a segmented grayscale image (2D or 3D) containing a single neuron. Segment the neuronal arbor using Image › Adjust › Threshold... (shortcut: ⇧ Shift + T). When using multichannel images, you will have to set the its display mode to Grayscale using Image › Color › Channels Tool... (⇧ Shift + Z), because images displayed as Composites cannot be thresholded. Define the center of analysis using a valid startup ROI. Run Analysis › Sholl › Sholl Analysis..., adjusting the default Parameters in the dialog prompt. Press More » Cf. Segmentation to visually inspect the two thresholded phases: arbor and background. Problems? Read the FAQs. Some of the illustrations of this manual pertain to older versions of the software. Most prompts have meanwhile been simplified. However, descriptions remain relevant to current use. Startup ROI The center of analysis can be specified using one of three possibilities: Straight line: A Straight line from the focus of the arbor to its most distal point using the Straight Line Tool. The advantages of using line selections are twofold: 1) Center of analysis and Ending radius are automatically set, and 2) Horizontal/vertical lines (created by holding ⇧ Shift while using the Straight Line Selection Tool) can be used to restrict analysis to sub-regions of the image. Single point: A single point marking the focus of the arbor using the Point Selection Tool. With single point selections, only the center of analysis is defined. Thus, this option is suitable for batch processing of images with different dimensions with undefined Ending radius. Multi-point selection:A Multi-point selection (multi-point counter) in which the first point marks the center of analysis while the remaining points mark (count) the number of primary branches required for the calculation of ramification indices). Suitable for cases in which inference from starting radius is not effective. Three types of ROIs expected by the plugin when analyzing images directly. Left: Line defining center of analysis (focal point), hemisphere restriction and ending radius. Middle: Single point defining center of analysis. Right: Multi-point selection in which the first point defines the focal point while the remaining points (2 to 5) serve as counters for primary neurites. Cf. Segmentation Press More» Cf. Segmentation to visually confirm which phase of the segmented image will be sampled. This command highlights foreground from background pixels and is particularly useful when analyzing black and white (binary) images or when using the B&W lookup table in the Threshold Widget (Image › Adjust › Threshold… ⇧ Shift + T). Cf. Segmentation allows you to ensure that you are measuring neuronal processes and not the interstitial spaces between them. Here is an example using an axonal arbor of a Drosophila olfactory neuron from the DIADEM dataset2: Segmented image Cf. Segmentation output Intersections mask Top row: Image properly segmented: Arbor is sampled. Bottom row: Aberrant segmentation (inverted image): Background is sampled. Note the reversal of Cf. Segmentation output and how the intersections mask no longer decorates the axonal processes but the interstitial spaces between them. The consequences of the phase inversion are twofold: 1) the program oversamples (cf. hue ramps on upper left of Intersections mask) and 2) the program detects artifacts induced by the edges of the image (cf. top-right and bottom-right corners of mask where intersections are sampled in the absence of real axons at those locations). Also, note that the initial black and white image would look the same under an inverted lookup table (Image › Lookup Tables › Invert LUT). With binary images, Sholl Analysis treats zero intensities as the background, independently of the image lookup table or the state of the Black background option in Process › Binary › Options.... As with any other ImageJ routine , confusing background with foreground pixels will lead to aberrant results, including: 1) overestimation of branches and 2) artifacts at distances intersecting the boundaries of the image canvas. Analysis of Traced Cells Main prompt (v3.6.8) when input is traced data In this mode the plugin analyzes reconstructed arbors, which is particularly relevant for stainings that do not allow single-cell resolution or proper segmentation. Traced cells are better analyzed through SNT's main interface, as it offers more options for defining the center of analysis, tagged branches, etc. However, dedicated prompts are also available (Plugins › Sh › Sholl Analysis (Tracings)...) for cases in which viewing traces is not necessary, or when batch processing is needed, since such prompts are macro recordable and/or themselves created by scripts. You can use analyze reconstructed data from any software capable of SWC, not just SNT. Third-party software includes Py3DN, Neuromantic , NeuronStudio , neuTube , or Vaa3D). In addition, L-Measure , NLMorphologyConverter or Neuron can also be used to convert several file formats (including proprietary formats from closed-source commercial software such as Neurolucida®, MicroBrightField, Inc.) into SWC. Run Sholl Analysis (Tracings)... and specify a tracing file (swc, eswc or traces extension). If needed, you can also specify the image associated with the reconstruction. This will allow the plugin to use the image's metadata to determine spatial units and x,y,z spacing. Choose the center of analysis using the drop down menu in the main prompt listing SWC tags (axon, dendrite, soma, etc.). Note that if your tracings are not tagged you can do so in SNT Adjust the default Parameters Problems? Read the FAQs Analysis of Existing Profiles Linear plot for CA1 cell described above. Using the soma as center, image was sampled twice using the Restrict analysis to hemicircle/hemisphere option in order to segregate apical from basal dendrites. For convenience, distances for basal branches were assigned negative values. For clarity, the binary image of the arbor was rotated, scaled and overlaid (in green) over the plot canvas. Note that it is also possible to restrict curve fitting to a sub-range of distances once data is collected. Input data: Any tab or comma delimited text file (.csv, .txt, .xls, .ods) can be used. You can drag & drop these files into the main ImageJ window, import data from the clipboard, or use data from any other table already opened by ImageJ. Restricting input data: To restrict measurements to a range of distances (see related example), select the range of distances you want analyze. You can click the first row in the range, and then drag the mouse to the last row, or by holding down ⇧ Shift while selecting the last row in the range. Then, in the prompt, activate the Restrict analysis to selected rows only checkbox. Calculation of Radius step size: Radius step size is calculated from the difference between the first two rows in Distance column. This is mainly relevant when choosing Annulus/Spherical shell as normalizer. Parameters The majority of parameters is shared by all the Analysis commands in theAnalysis › Sholl › submenu. However, some settings are specific to the type of data used as input: A segmented image, a tracing, or a previously obtained profile. When analyzing images, input values take into account the scale information of the image (which can be set using the Analyze › Set Scale... or Image › Properties... (⇧ Shift + P), the type of image (2D or 3D), and its active ROI. Definition of Shells Starting radius The radius of the smallest sampling circle/sphere, i.e., the first distance to be sampled. Ending radius The radius of the largest (last) sampling circle/sphere. It is automatically calculated if a line ROI is used. Note that the specified distance may not be actually sampled, if Radius step size is not a divisor of Ending radius-Starting radius. In this case, the program will choose the largest possible distance smaller than the specified value. You can clear Ending radius or set it to NaN ("Not a Number") to sample the entire image. This is particularly useful when batch processing images with different dimensions. Radius step size The sampling interval between radii of consecutive sampling circles/spheres. This value may be set to zero for continuous (1-voxel increment) measurements. For stacks with anisotropic voxel size, setting Radius step size to zero, sets the step length to the dimension of the matching isotropic voxel, i.e., the cube root of the product of the voxel dimensions (3D images) or the square root of the product of the pixel dimensions (2D images). Restrict analysis to hemicircle/hemisphere This option is only available when an orthogonal radius has been created (by holding ⇧ Shift while using the Straight Line Selection Tool). It can be used to limit the analysis to sub-compartments of the arbor. For horizontal lines, this option instructs the algorithm to measure intersections at sites equidistant from the center that have y-coordinates above/below the drawn line. For vertical lines, it instructs the plugin to measure intersections at sites equidistant from the center that have x-coordinates to the left/right of the drawn line. Main prompt (v3.4.1) when input is a segmented image Multiple Samples/Noise Reduction Samples per radius (2D images only) Defines the number of measurements to be performed at each sampling circumference. These measurements are then combined into a single value according to the chosen integration method. This strategy, a break from previous approaches, increases the accuracy of non-continuos profiles by diluting out the effect of processes extending tangent to the sampling circumference. Visually, this option can be imagined as the "thickness" of the sampling circumference: e.g., for a radius of 100 pixels and a value of 3 Samples per radius, the final number of intersections would integrate the measurements sampled at distances 99, 100 and 101. Note that it would not make sense to increase the number of samples beyond the length (in pixels) of Radius step size. For this reason, this option is limited to a draconian (and arbitrary) maximum of 10 samples. Samples integration (2D images only) The measure of central tendency used to combine intersection counts when multiple Samples per radius are used. Options are Mean (the default), Median or Mode. Ignore isolated (6-connected) voxels (3D images only) - If checked, single isolated voxels intersecting the surface of sampling spheres are not taken into account, which may allow for smoother profiles on noisy image stacks. However, it should be noted that connectivity in the stack volume may not reflect connectivity on the surface of a digitized sphere. Indeed, in certain contexts, it is possible (though unlikely) to obtain higher intersection counts when this filtering option is active. Keep in mind that this is just a refinement feature, and you should not expect it to mitigate major artifacts derived from poor segmentation. Descriptors and Curve Fitting Enclosing radius cutoff The number of intersections to be used in the definition of Enclosing radius. Number of primary branches The number of primary branches (i.e., those originating directly from cell soma when the center of analysis is the perikaryon) to be used in the calculation of Schoenen ramification indices. It is automatically populated using multi-point counts if a multi-point ROI is detected at startup. Set it to zero (or NaN) to disable calculations of ramification indices. Infer from starting radius If checked, the Number of Primary branches is inferred from the count of intersections at Starting radius. Main prompt (v3.4.3) when input is tabular data Fit profile and compute descriptors If checked, data is fitted according to the chosen method and appropriate metrics calculated automatically. If unchecked, only sampled data is plotted. Show fitting details - Choose this option to have all of the parameters of the simplex fitting printed to the Log window. The coefficient of determination (R2, a measure of goodness of fit) is always stored in the Sholl Results table even when this option is not selected. Choice of Methods The type of profile(s) to be obtained. Linear (profile without normalization), or normalized profiles: Linear-norm, Semi-log, or Log-log. Polynomial Specifies the degree of the polynomial to be fitted to the Linear profile3. While the polynomial of best approximation, or "best fit", should be empirically determined for each analyzed cell type, it is possible to ask the plugin to predict the order of the fitting polynomial (or at least try) using the choice Best fitting degree. In this case, the plugin will loop through all the available choices of polynomials, perform each fit in the background and choose the one with the highest coefficient of determination. Most informative Select this option when you cannot predict which type of normalized profile best describes the dataset. If chosen, the plugin will use the Determination ratio to determine which of Semi-log or Log-log methods is more appropriate. Linear-norm is not performed. Best fitting degree and Most informative choices are obviously more computer-intensive and can be monitored by activating the Show fitting details checkbox. Normalizer The property of the sampling shell to be used in the normalization of Linear-norm, Semi-log, and Log-log profiles. It is described below. Output Options Create intersections mask If checked, a 16/32–bit maximum intensity projection of the analyzed image is generated in which the measured arbor is painted according to its Sholl profile. The type of data (Raw, i.e., sampled or Fitted) is displayed in the image subtitle and can be specified in Analysis › Sholl › Metrics & Options… or using the Options… command in the More» drop-down menu. The default Lookup Table (LUT) used by the mask can be changed using Image › Lookup Tables. The background color [gray level: 0 (black) to 255 (white)] can also be set in Metrics & Options..., or at any later point using Image › Color › Edit Lut... WYSIWYG versions (RGB images) of these masks can be otained using by pressing ⇧ Shift + F (Image › Overlay › Flatten) or by running Analyze › Tools › Calibration Bar... Intersection points and sampling shells can be retrieved as ROIs. Intersection points are placed at edges of detected clusters of foreground pixels, not their center. Overlay sampling shells and intersection points (2D images only) If checked, two sets of ROIS are added to the image overlay: 1) concentric shells matching sampled distances (circular ROIs or composite ROIs when using hemicircles); and 2) Multipoint ROIs at intersection sites between shells and clusters of foreground pixels. Save results to If checked, all the results (with the exception of the Sholl Table) are saved to the specified directory. These include: 1) Sholl plots (saved as PNG images), 2) A table containing detailed data and 3) The Sholl mask. Files are named after the image filename and analysis method. Saving options can be specified in Analysis › Sholl › Metrics & Options… (Options… command in the More» drop-down menu). Do not display saved files If checked, saved files are directly saved to disk and are not displayed. Activate this option when batch processing files. Sholl Plots Linear, Linear-norm, Semi-log and Log-log profiles for the ddaC cell (File › Open Samples › ddaC Neuron), version 3.0. Most of the retrieved metrics are automatically highlighted by the plugin. Linear profile: Mean value (horizontal grid line) and Centroid (colored mark). Logarithmic profiles: The Sholl regression coefficient (also known as Sholl decay) can be retrieved by linear regression using either the full range of data (blue line) or data within percentiles 10–90 (red line). For this particular cell type, the Semi-log method is more informative when compared to the Log-log method. (1) Linear N = a + br + cr2 + dr3 + er4 + fr5 + ... + xrn Outputs a N vs Distance profile. Data is fitted to a polynomial function4. Critical radius, Critical value and Mean value of function are calculated (2) Linear-norm N/S = a rb Outputs a N/S vs Distance profile. Points are fitted to a power function. It is an intermediate representation of the data that can be used to gauge the choice of normalizer. Once plotted under a logarithmic scale the Linear-norm curve is similar to the Semi-log profile (3) Semi-log log(N/S) = -k r + m Outputs a log(N/S) vs Distance profile. A linear regression is fitted to the sampled data. The Sholl regression coefficient (k) is calculated (4) Log-log log(N/S) = -k × log(r) + m Outputs a log(N/S) vs log(Distance) profile. Data is also fitted to a straight line. This is an alternative approach5 of obtaining a relevant regression coefficient, when the Semi-log method returns a poor fit N For 2D images, the Number of clusters of pixels (8–connected) intersecting the circumference of radius r For 3D images, the Number of clusters of voxels (26-connected) intersecting the surface of the sphere of radius r r Distance from center of analysis (radius of Sholl circle/sphere) log Natural logarithm, the logarithm to the base e SThe chosen property of the sampling shell to be used in the normalization of Linear-norm, Semi-log, and Log-log profiles. For 2D images, the Perimeter of the sampling circumference (2πr) or the Area of the corresponding circle (πr2) For 3D images, the Surface of the sampling sphere (4πr2) or its respective Volume (4/3πr3) A third normalization option is also available when performing non-continuous sampling: Annulus/Spherical shell. In this case, the normalization is performed against the area of the annulus formed between the circumferences at r ± Radius step size/2 (2D images), or against the volume between the two spheres at r ± Radius step size/2 (3D images). Metrics Morphometric descriptors and other properties of the arbor are printed to a dedicated table named Sholl Results. Output is fully customizable using Analysis › Sholl › Metrics & Options… or using the Options... command in the More» drop-down menu. The first columns log analysis parameters: Image Directory, filename and voxel unit, Channel, Lower and Upper Threshold levels, X,Y (in pixels) and Z (slice number) coordinates of center of analysis, Starting and Ending radius, Radius step, Number of Samples per Radius, etc. Other parameters are described below. Metrics based on sampled data Intersecting radii The number of sampling radii intersecting the arbor at least once. Sum of intersections (Sum inters.) The sum of all intersections. Mean of intersections (Mean inters.) Sum inters. divided by Intersecting radii. See also Mean value, FAQ Median of intersections (Median Inters.) The median value of sampled intersections. Skewness The skewness of the sampled data, an indication of how symmetrical the distribution is around its mean. Positive values indicate an asymmetrical distribution with a longer tail to the right. Negative values indicate data with a longer tail to the left. A popular rule of thumb considers that if the skewness is greater than 1.0 (or less than -1.0), the distribution may be considered far from symmetrical. See also Skewness (fitted data) Kurtosis The kurtosis of the sampled data, which quantifies whether the shape of the distribution matches that of a Gaussian distribution, assuming that a Gaussian distribution has a kurtosis of 0. A distribution more peaked than a Gaussian has a positive kurtosis while a negative value indicates a flatter distribution. See also Kurtosis (fitted data) Highest count of intersections (Max inters.) The maximum value of sampled intersections, i.e., the maximum in a linear [N vs Distance] profile, reflecting the highest number of processes/branches in the arbor. See also Critical value. Radius of highest count of intersections (Max inters. radius) The distance at which the Highest count of intersections occurred, reflecting sites of highest branch density. Note that if the same maximum occurs multiple times, only the first distance is considered. See also Critical radius Schoenen Ramification index (Ramification index (sampled)) A measure of ramification4: the ratio between Max inters. and the number of primary branches. It is only calculated when primary branches is valid and not zero. See also Ramification index (fit) Centroid radius The abcissa of the centroid (i.e., the geometric center or barycenter) of the linear profile. It is highlighted on the N vs Distance plot. Centroid value The ordinate of the centroid (i.e., the geometric center or barycenter) of the linear profile. It is highlighted on the N vs Distance plot. Enclosing radius The last (thus, the widest) of Intersecting radii to be associated with the number of intersections specified by Enclosing radius cutoff. For a cutoff of 1 (the default) Enclosing radius is the widest of Intersecting radii. It reflects the Feret length of the arbor. Metrics based on fitted data Determination ratio The ratio of the coefficient of determination for the semi-log method and that for the log–log method5. If the semi-log method is better relatively to the log–log method, the Determination ratio becomes larger than 1. It is the parameter used by the plugin to silently predict the normalization method that is the most informative. The prediction can be monitored by activating the Show fitting details checkbox. Sholl regression coefficient (Regression coefficient) The slope (multiplied by -1) of the linear regression described in (3) and (4), i.e., k, a measure of the rate of decay of the number of branches with distance from the center of analysis. Higher k values reflect larger changes in the function log(N/S). To optimize the fit, the plugin retrieves a second linear regression centered around the median distance, excluding distances at the edges of the profile. Details of this second fit are also registered on the Sholl table under dedicated columns, e.g., Sholl regression coefficient [P10-P90], when data within the 10th–90th percentile is used. Regression intercept The y-coordinate m described in (3) and (4). Regression R2 (Regression R^2) The coefficient of determination of the linear regression described in (3) and (4). Critical value The local maximum of the polynomial fit, i.e, N at Critical radius in (1). Abbreviation: Nm. See also Max inters. Critical radius The distance at which Critical value occurs. By default (see Advanced Usage), it is calculated with a precision of 1/1000 of Radius step size. Abbreviation: rc. See also Max inters. radius Nomenclature: Previous authors have used different terms to describe the largest value taken by the Sholl profile, including Dendrite maximum. Since the Sholl technique is not restricted to dendritic arbors and can be applied to any tree-like structure such as axonal arbors, mammary ducts or blood vessels (cf. List of citations), Here we adopt the term Critical radius, renaming Dendrite maximum (Nm) to Critical value. Mean value The mean value3 of the fitted polynomial function (1), representing the average of intersections over the whole area occupied by the arbor. Abbreviation Nav. On the Sholl plot, it is highlighted as the height of the rectangle that has the width of Enclosing radius − First intersecting radius and the same area of the area under the fitted curve on that discrete interval. It is analogous to Mean inters., the arithmetic mean of sampled intersections throughout the arbor (cf. Metrics based on sampled data). By default (see Advanced Usage), it is calculated with a precision of 1/1000 of Radius step size. Schoenen Ramification index (Ramification index (fit)) Schoenen Ramification index retrieved from fitted profile: The ration between Critical value and Number of primary branches. See also Ramification index (sampled) Skewness (Skewness (fit)) The skewness of the fitted polynomial distribution between Starting radius and Ending radius. Kurtosis (Kurtosis (fit)) The kurtosis of the fitted polynomial distribution between Starting radius and Ending radius. Polynomial R2 (Polyn. R^2) The coefficient of determination of the polynomial fit described in (1). SNT provides several scripts and commands that faciliate all type of Sholl-related analyses. You'll find them through the Plugins › Neuroanatomy › Neuroanatomy Shortcut Window, as well as in Script Editor's Templates › Neuroanatomy menu. Here are some examples: Sampled data from the ddaC cell being fitted to polynomials of varying degree (BAR script) Combine Sholl Profiles... aggregates individual profiles into a single plot, obtaining the average profile (with standard deviation) of a group of cells (v3.6.12 snapshot) The Convex Hull as Center script exemplifies how to adopt arbitrary focal points, in this case the centroid of the neuron's convex hull (v4.1.2 snapshot) In addition, several BAR commands – installed by subscribing to the BAR update site – were designed to complement the first release of Sholl Analysis. While, some of these tools have meanwhile become outdate, some remain relevant. These include: Segmentation tools Thresholding, shape-based masking and edge-detection routines (full list) Find Peaks Retrieves local maxima under several filtering options such as peak amplitude, peak height and peak width. Can be used to retrieve secondary sites of branch density Fit Polynomial Fits a polynomial of any degree to sampled data. Features an heuristic algorithm for guessing a polynomial "best fit". Expands the built-in repertoire of polynomial fits up to 50th order functions. Create Boxplot Allows direct comparison of metrics between groups or sets of data (specially useful when tagging images with the Comment field in Analysis › Sholl › Metrics & Options…) Interactive Plotting Whole-purpose routine that plots data from imported spreadsheets. Pre-processing This section discusses some aspects that should be taken into account when segmenting neuronal arbors to be processed by Sholl Analysis. Since image segmentation (i.e., the partitioning of images into analyzable parts) is vulnerable to noise and background fluorescence, it is not possible to generalize universal routines that efficiently binarize grayscale images. This means that any procedure that tries to appropriately describe the original fluorescence image with a binary mask must be tailored to the characteristics of individual datasets. As mentioned in Complementary Tools, several routines listed here as distributed through the BAR update site. Noise Noise can be mitigated through the usage of processing filters. Specially useful are edge-preserving filters: Rolling Ball or "Top hat" filters, e.g., Process › Subtract Background… Median Filtering (2D/3D), e.g., Process › Filters, Plugins › 3D Anisotropic Diffusion, Plugins › Process › Anisotropic Diffusion 2D Sobel Edge Detection, e.g., Process › Find Edges Shen-Castan Edge Detector (BAR plugin), BAR › Segmentation Frequency filters, e.g., Process › FFT › Bandpass Filter… Uneven Illumination Uneven illumination problems, typically associated with wide field microscopy, do occur in confocal microscopy when signal from deep layers of the tissue is not captured as bright as with superficial layers. This signal attenuation along the Z-axis will generate a shaded gradient across the stack that histogram-based segmentation will need to take into account. While these problems are better tackled during acquisition (e.g., using laser ramping), it is possible to mitigate this effect using histogram-normalization techniques. Examples: Bleach Correction, Image › Adjust Attenuation correction Segmentation It is possible to adopt more sophisticated segmentation algorithms when global thresholding methods do not yield satisfactory results. Examples: Batch Processing The Script Editor's Templates › Neuroanatomy menu lists demo scripts that perform batch operations. For sake of completeness, here is a small tutorial on how to write a macro from the ground up: Tutorial: Batch Analysis of Images using IJM Languages Any macro must set a center, or allow the Sholl Analysis plugin to access a ROI marking it. One could instruct ImageJ to read the coordinates of pre-existing ROIs from a text file, store a list of line selections in the ROI Manager, or write a morphology-based routine that detects the center of the arbor. However, marking the center of analysis is probably something that you will want to do manually. Here is a workflow: Place all the .tif images to be processed in a single folder. Select the Point Selection Tool in the main ImageJ window. With 3D images, make sure Set stack positions is active in the Image › Overlay › Overlay Options... prompt. Open the first image and press ⇧ Shift + T to activate the Threshold widget (Image › Adjust › Threshold...). Adjust threshold levels. Press the Apply button of the Threshold widget to create a binary image. Select the z-slice containing the center of analysis. Click over the center with the Point Selection Tool and press B (shortcut for Image › Overlay › Add Selection...). This will add the point ROI to the image overlay. Save the image as .TIFF by pressing S (File › Save As... › Tiff...). Repeat the last 2 steps until all images are marked, using ⇧ Shift + O (shortcut for File › Open Next) to iterate through all the images. When working with ROIs, it is critical that you work with .tif files because only this format keeps track of image overlays. IJ's Process › Batch › Convert... command allows bulk conversion between image formats. Now that all the images are marked, we could use the Macro Recorder (Plugins › Macros › Record...) and run Sholl Analysis on one of the images to find out how to call the plugin with suitable parameters. In this tutorial, we will use the ImageJ macro language. The single line of code that appears in the recorder window will look something like this: // Recording Sholl Analysis version 3.4.3 run("Sholl Analysis...", "starting=10 ending=400 radius_step=0 infer fit linear polynomial=[8th degree] semi-log normalizer=Volume create save do"); As you may have noticed, ImageJ plugins are controlled by a single lowercase sentence in which arguments are separated by a space. Input fields and choice lists appear as keyword=value pairs, active checkboxes by a single keyword. Options that are not needed can be omitted. This makes it easier to edit code blocks: start = 10; // variable that controls starting radius end = 200; // variable that controls ending radius step = 2; // variable that controls step size // Run the plugin run("Sholl Analysis...", "starting="+ start +" ending="+ end +" radius_step="+ step +" infer linear save do"); Now we just need to assemble a working macro to be pasted in the Process › Batch › Macro... prompt: // Get the number of ROIs of the image overlay nROIs = Overlay.size; // We cannot proceed if no ROI is available (safety check) if (nROIs==0) exit("No ROI was found. Cannot proceed."); // Select the last ROI of the overlay. Because we have activated the "Set stack positions" option, // this will automatically activate the Z-slice in which the ROI was created (3D images) Overlay.activateSelection(nROIs-1); // We now call the plugin as detailed by the Macro Recorder. We'll set 'Ending radius' to a non-numeric // value (NaN, "Not a Value") to make sure the maximum length for each individual image is used run("Sholl Analysis...", "starting=10 ending=NaN radius_step=0 infer fit linear polynomial=[8th degree] semi-log normalizer=Volume create save do"); Of course you can also automate any preceding steps. However, do not forget to ensure that the center of analysis will be available when the plugin is called: // Impose spatial calibration run("Properties...", "unit=um pixel_width=1.5 pixel_height=1.5 voxel_depth=3.0"); // Subtract background run("Subtract Background...", "rolling=50 sliding stack"); // Apply a favorite threshold setAutoThreshold("Huang dark stack"); // >>>> Make sure the initial point selection remains available

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