Published in final edited form as:

Methods Mol Biol. 2013 ; 1018: 261–268. doi:10.1007/978-1-62703-444-9_24.

Semi-automated Analysis of Dendrite Morphology in Cell Culture

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Summary

Quantifying dendrite morphology is a method for determining the effect of biochemical pathways and extracellular agents on neuronal development and differentiation. Quantification can be performed using Sholl analysis, dendrite counting, and length quantification. These procedures can be performed on dendrite-forming cell lines or primary neurons grown in culture. In this protocol, we describe the use of a set of computer programs to assist in quantifying many aspects of dendrite morphology, including changes in total and localized arbor complexity.

Keywords

neuron; dendrite; axon; sholl; morphology; tracing; computer assisted

1. Introduction

Precise dendrite patterning is important for correct neuronal function. Normal communication, signal processing, and action potential propagation are all dependent on correct dendrite morphology (1–4). Alterations in the dendrite arbor have been associated with diseases such as Alzheimer's disease, epilepsy, and Rett syndrome (5–10). Understanding how factors affect the dendrite arbor is crucial to understanding how neurons function in both healthy and disease states.

There are several ways to quantify dendrites in growing cells. Sholl analysis is a measure of dendrite complexity that is typically performed on the whole dendritic arbor of a cell. Concentric, equidistance circles are drawn radiating from the cell body. Marks are noted on the circles where dendrites pass through the circles, and a graph can be drawn to show dendrite number at specific distances from the cell body. Measurements of dendrite type can be made by counting the number of dendrites that extend directly from the cell body (primary), dendrites that branch from a primary (secondary), and dendrites that branch off of

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any other type of branch (tertiary, quaternary, etc.). Length is typically measured from the cell body to the tip of the dendrite. Sholl analysis, dendrite number, or dendrite length measurements performed in the normal fashion, by hand, must be done separately and require repeat analysis to capture to all of the information present in a neuron image. The use of the Bonfire program allows capture of all data discussed above while only requiring one set of manipulations to be done on the images. Bonfire also separates the data for specific sub-regions of the dendrite, such as length of secondary dendrites. The resulting data make very complex analysis of dendrite branching possible, and many changes that alter the arbor can do so without changing the total arbor measurements (11).

2. Materials

2.1 Images

1. 8-bit images TIFF images (*see Note 1*).
2. Invert color scheme of images to black dendrites on white background for ease of tracing (*see Note 2*).

2.2 Software

The Bonfire program requires the use of several open source programs to perform the quantitation in a semi-automated fashion

1. Image J – <http://rsbweb.nih.gov/ij/>
2. Neuron J plugin for Image J – http://www.imagescience.org/meijering/software/neuron_j/
3. NeuronStudio – <http://research.mssm.edu/cnic/tools-ns/html>
4. Bonfire Program – <http://lifesci.rutgers.edu/~firestein>
5. MATLAB – www.mathworks.com/products/MATLAB

2.3 Organized File Structure

The Bonfire program requires very specific information and folder layouts to work properly. Please follow directions precisely (Fig. 1).

1. Experiment folder (containing multiple conditions folders and bonfire folder)
2. Condition folder (containing all images for analysis of that condition) (*see Note 3*)
3. Bonfire folder (containing Bonfire MATLAB scripts)

3. Methods

3.1 Perform computer-assisted tracing of dendrites using Neuron J

1. Load the Neuron J plugin into Image J (*see Note 4*).
2. Select the ‘Set parameters’ button and choose ‘Dark’ as the dendrite appearance.

3. Open the image to be traced using the 'Open' button in Neuron J (*see Note 5*).
4. Adjust the brightness/contrast and zoom as needed to properly visualize dendrites during tracing. (*see Note 6*)
5. Select the 'Add traces' button on the Neuron J toolbar and trace the perimeter of the cell body.
6. Select the 'Label tracings' button and then select the cell body trace. Label the tracing with Tracing ID: 'N1' and Type: 'Type 06'. This is the only trace that will be labeled.
7. Trace all the dendrites using the 'Add traces' button. It is recommended that you start at the cell body and trace outward. These traces should not be labeled in any way. (*see Note 7*).
8. Save the tracing by selecting the 'Save traces' button and save the trace (*.ndf) in the same directory where the image is stored in the 'Condition folder'.
9. Select the 'Export tracings button' and export a tab delineated text file with a separate file for each tracing. Let Neuron J name these files for you (*see Note 8*).
10. Select the 'Measure tracings button' and select 'Display tracing measurements' (*see Note 9*). Run this command and save the file as "*_info" where * is your exact image file name. If your computer attaches the extension '.xls' then delete it (*see Note 10*).
11. Complete this process for each neuron in all the conditions to be analyzed. All of the images and generated files for a single condition should be contained in that 'Condition folder'.

3.2 Convert tracings into an arbor connection map using Bonfire

1. Use the Bonfire program in MATLAB and run the script 'bonfire_load' to reorganize the file structure of the 'Condition folder' (*see Note 11*). This folder contains the image files and Neuron J-generated files above. Each 'Condition folder' should now contain a folder for each cell (*see Note 12*).
2. Use the Bonfire program in MATLAB and run the script 'bonfire_ndf2swc' to create the arbor connection map from the trace file (*see Note 13*).
3. Select the condition folder that you wish to convert and allow the program to convert all of the .ndf files to .swc files. This script will convert all of the files in the condition folder but needs to be run once for each 'Condition folder'.

3.3 Correct errors in the arbor connection map using NeuroStudio and Bonfire

1. Open the image file associated with the arbor connection map to be modified in NeuroStudio by selecting File -> Open and choosing the appropriate image.
2. Select Run -> settings and change the X, Y, and Z boxes in the 'Voxel size' window to 1.

3. Select File -> import SWC to open the appropriate SWC file and overlay it on the image opened in step 1.
4. Use the tools in NeuroStudio to correct the branches and endpoints of the SWC file (*see Note 14*).
5. Follow these rules to ensure proper reading of the arbor map: 1. Each branch point (yellow dots) may only have 2 daughter branches and one mother; 2. There should only be one cell body (red dot) (*see Note 15*).
6. Save the dendrites in the same folder as the image and SWC file by selecting File -> Save neurites. Use default name and do not rename.
7. Using Bonfire in MATLAB run the script 'bonfire_trace_check'. This script will check all of the arbor maps in a single conditions folder. Select the condition you wish to analyze and repeat analysis for each condition (*see Note 16*).

3.4 Collect and Analyze data from arbor maps using Bonfire

1. Using Bonfire in MATLAB, run the script 'bonfire'. This script will analyze one condition folder. Select the condition folder you wish to analyze and select run (*see Note 17*).
2. The script 'bonfire_results' will generate graphs that can give you an idea of the results of the experiment but no statistics are possible from these graphs so it is only recommended for use as an indicator.
3. Repeat for each condition.
4. Using Bonfire in MATLAB, run the script 'bonfire_export' to export the results to excel for further analysis (*see Note 18*).

4.1 Data assembly in Excel

This section contains recommendations for analysis raw data after the Bonfire program has compiled it. These methods are recommendations only and other scientifically valid methods may be used.

1. Bonfire generates a single book in excel for each condition of an experiment. Each book contains multiple sheets with data on specific regions of the arbor as well as total arbor differences
2. It is recommended that you compile statistics for each condition within the excel book created by Bonfire. These condition statistics are then copied to a new book that combines all conditions of an experiment. This allows for easier graphing and exporting to other statistical analysis software.

4.2 Further data analysis

1. Statistical power for branching analysis is generated by the number of neurons that are analyzed. Try to image >15 images for each condition, in each of 3 trials, since the variability in neurite number from cell to cell can be significant. It is

not recommended that you use the number of dendrites as the 'N' for your experiment.

2. Bonfire generates a large amount of data that may or may not be relevant to the question that you are trying to answer. However the large amount of data that is generated can draw out interesting phenomena. For example a reduction in secondary branching may be compensated for by an increase in tertiary branches.
3. When analyzing the data generated by the Sholl analysis it can be useful to consider portions of the curve separately instead of the curve as a whole. For example: a total sholl graph could be broken into distance groups like 1-100 μm , 100-200 μm , etc... or method such as two-way ANOVA can be used to compare the effect of condition and distance on the branching behavior (Fig. 2). Deciding where to place the edges of the bins with the grouping method can be somewhat arbitrary and caution should be taken with the two-way ANOVA since it is not an appropriate test for a continuous variable. This analysis method does not consider the branches at a certain distance from the soma to be continuous since areas of significance tend to cluster together in large groups.

4.3 Graphical presentation

1. Dendrite number, length, and tip number are typically presented as a simple column graph.
2. Sholl analysis is typically presented as an X-Y coordinate graph with '# of Intersections' as the Y-axis and 'Distance from soma' as the X-axis (**Fig. 2.**).

4. Notes

1. Note 1: Images used in development of protocol were taken at 200X measuring 512×640 pixels with a $\mu\text{m}/\text{pixel}$ ratio of 1.5/1. This $\mu\text{m}/\text{pixel}$ number is hard coded into the program but can be changed by replacing the value of the variable 'pix_conv' with the desired value. The program is size independent but if consistent trouble is encountered later a last resort may be to try adjusting your files to match these conditions.
2. Note 2: Black dendrites on a white background are normally easier to trace. You may use white dendrites on black background but be sure to select the 'Light' dendrite tracing in Neuron J.
3. Note 3: This folder should not contain any other folders when you start the analysis. Only the images for analysis of that condition should be included.
4. Note 4: The Neuron J plugin for Image J must be installed in Image J before use in this protocol.
5. Note 5: you must use the open button or the image will load using Image J and not the Neuron J plugin

6. Note 6: when adjusting brightness and contrast make sure that you are able to see the feature you examining without overexposing the edges and making accurate tracing difficult.
7. Note 7: While not required that you stop at each branch point and start a new trace, doing so can help if unknown errors occur in later steps. Trace whatever neurites are visible with a 2x zoom; the program will eliminate all neurites below a certain size as those are considered filopodia and not neurites.
8. Note 8: It is important that the files all be named based off of the image name. Neuron J will do so and add important numbering data to the name.
9. Note 9: If you only wish to examine total dendrite length, then you can select 'display the group measurements' and manually make the μm to pixel conversion. This, however, is not recommended because many of the changes that occur in dendrite arbor patterning are often seen only in more complex dendrite relationships. You may also capture specific information about type of dendrites using Neuron J by labeling each dendrite properly, but this is extremely time-intensive and very error prone.
10. Note 10: For Mac users, bring up the file info menu to delete the extension; otherwise, some versions of the OS will not completely delete the extension.
11. Note 11: To run a script in MATLAB you must first select the 'Bonfire folder' as your master folder. The Bonfire folder is located in your 'Experiment folder'. After loading the correct master folder, type the name of the script as written into the MATLAB command window and follow the prompts and protocol. You only need to select this once unless you restart/close MATLAB.
12. Note 12: If the load command does not work double check the file structure and check that the extensions have been deleted of the '*.info' file.
13. Note 13: The SWC file is a file that is readable in NeuroStudio. This will allow you to view all the connections and correct any errors in the automated conversion from the trace file to the SWC file.
14. Note 14: It is recommended that you become familiar with the dendrite tool in NeuroStudio before you begin analysis.
15. Note 15: As you connect various dendrites to the cell body and change the connections between branch points, the cell body should remain red. The rest of the red dots should change to the appropriate color. If at any time in the process your cell body changes color to a different color, it is recommended that you start the correction process again with that arbor map.
16. Note 16: If there are errors, reopen the SWC file with NeuroStudio, correct the errors, and re-save the SWC file. Re-run 'bonfire_trace_check' again until all errors are gone for all conditions.
17. Note 17: This script creates a *.mat file which can only be read by MATLAB and must be exported to excel for analysis.

18. Note 18: This will export the data from one condition and must be repeated for each folder. Bonfire does not perform statistical calculations.

Acknowledgements

This work was supported by NSF grants IBN-0919747 and IBN-0548543, March of Dimes Foundation grant 1-FY08-464, and NJ Governor's Council for Medical Research and Treatment of Autism grant 10-406-SCH-E-0.

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Fig 1.

Initial folder structure. Initial folder structure as called for in section 2.3

Fig. 2.

Example graph of Sholl analysis data. Significance in this graph was determined by using two-way ANOVA and indicated p value given to the group of distances that showed same significant difference from control. This data was generated from N = 45 neurons.