Microglial Sholl Analysis

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Method Article

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Abstract

This is an effective tool to measure the process complexity of microglia in order to detect subtle changes in microglia morphology. Confocal Z-projections using a single antibody on fixed tissue can be used to detail microglia profiles in comparison to various conditions including brain region, disease models, and aging.

Introduction

This profiling technique can be used to compare microglial morphology in response to injury, age, genetic background, or for regional comparisons. Sholl profiles detailing process length, complexity, and soma size may be obtained by following this procedure.

Reagents

-4% PFA fixed floating brain sections -0.5% Triton in PBS -PBS \(0.1M) -Normal chicken serum \(Vector Labs) -Bovine serum albumin -Aquamount -Polyclonal Rabbit anti-Mouse Iba1 antibody \(Biocare Medical) -Alexa Fluor® 647 Chicken anti-Rabbit IgG \(Life Technologies) -DAPI nuclear stain \(Sigma)

Equipment

-Confocal microscope \(Leica SP8 used in all of our studies) -FIJI software \(Variant of ImageJ)

Procedure

1.Sacrifice an animal with an i.p. injection of a lethal dose of ketamine. 2. Transcardially perfuse the mouse on a low speed \(~3 minutes) with 15 mL of ice-cold 0.1M PBS containing 5U/mL heparin. \(the right atrium is clipped with scissors shortly after perfusion to ensure effective clearing of the brain). 3. Next infuse 25 mL of ice cold 4% PFA. 4. Remove the brain and store in 4% PFA at 4 degrees C for 72 hours of post-fixation with gentle rocking in 20 mL scintillation vials. 5. Remove excess PFA by dabbing the brain on a kimwipe and then transfer to a scintillation vial containing a 30% sucrose solution at 4 degrees. 6. Once the brain has fully sunk \(~2 days) the brain can be snap frozen in isopentane on dry ice or mounted immediately for freezing on chucks for slicing. 7. Mount the brain on a cryostat chuck using the cut bulb of a transfer pipette and OCT. 8. Place the empty cylinder of the transfer pipette bulb on to the chuck pre-stored in the cryostat Quick Freeze bar. 9. Place a layer of OCT on the chuck and orient the frozen or fresh brain flush with the bulb for coronal sectioning. 10. Place 40 um coronal sections in 0.1% sodium azide in PBS for storage at 4 degrees C. 10. Briefly wash slices in PBS to remove sodium azide. 11. Wash in 0.5% PBST \(Triton X-100) for 15 minutes at room temperature for permeabilization. 12. Wash in two rinses of PBS for 5 minutes at room temperature. 13. Block for 2 hours at room temperature in 5% normal chicken serum. 14. Incubate in primary antibody \(Biocare Medical polyclonal rabbit-anti mouse Iba1) at 1:300 in 0.5% BSA in PBS overnight at 4 degrees C. 15. Wash 3x for 5 minutes in PBS at

room temperature 16. Place in secondary antibody \(Alexa Fluor® 647 Chicken Anti-Rabbit IgG) diluted 1:800 in 5% normal chicken serum in PBS for 2 hours at room temperature. 17. Wash for 5 minutes in DAPI diluted 1:20,000 in PBS at room temperature. 18. Wash 2x for 5 minutes in PBS at room temperature. 19.Mount on slides using Aquamount and let dry in the dark at room temperature for at least four hours. 20. Using the 63x objective on the confocal microscope, utilize the UV wide-field laser and DAPI fluorescence to select three fields from a given slice to represent that brain region. Three fields from each slice with three slices per mouse with at least three mice per experimental condition should be used to ensure a representative sampling of microglia \(n > 30 microglia). 21. Once the focus is set on the eyepieces, switch to the optimal laser for Iba1 fluorescence and range the z-stack on the microscope so that an entire microglia is located within the z-stack \(~30 um per stack). 22. While imaging preferences should be determined by each user, our lab prefers to use HyD detectors in counting mode on the SP8 Leica confocal microscope. This combined with a 3-frame average provides the best resolution for obtaining clear Z-stacks. 23. Capture each Z-stack at a minimum resolution of 512x512, and enable your stack to make one slice per micron. 24. Load images on to FIJI using the drag and drop method. 25. Prepare a maximum intensity Z-stack projection 26. Make the image binary by thresholding to include microglial processes. 27. Isolate a single microglia by removing surrounding processes with the eraser tool. 28. Using the line segment tool, draw a single line from the center of the microglia soma to its longest process. 29. Using the Sholl analysis plugin, define the first shell to be 10 um outside of the cell body \(to exclude the soma from Sholl analysis) and set each step to be 5 um. Select linear profile to conclude the analysis. 30. Copy the Sholl profile and enter in to Excel. Two complete microglia per field should be used for analysis. Avoid incomplete microglia or those with processes on the margin of the field. 31. Processes can then be erased and the microglia soma size recorded. 32. Once profiles are compiled for each mouse, total profiles for each experimental condition are pooled and imported in to statistical software. A two-way ANOVA should then be performed with the number of intersections from soma center and experimental variable used as the factors.

Timing

Tissue Collection- ~2 hrs Tissue Fixation and Cryoprotection- ~4days Tissue processing and immunohistochemistry - user defined Imaging and quantification- \(~1 day for a cohort of 6 mice)

Anticipated Results

Properly prepared tissue and a quality microscope should ensure excellent and reproducible microglial imaging with regards to the Iba1 antibody. CX3CR1-GFP mice may also be imaged for Sholl Profiling in a similar way.

References

1. Morrison, H. W., and Filosa, J. A. A quantitative spatiotemporal analysis of microglia morphology during ischemic stroke and reperfusion. J. Neuroinflammation \(2013)

Acknowledgements

This profiling technique was implemented and adapted from the Morrison and Filosa paper cited previously.

Figures

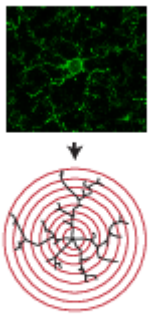

Figure 1

Figure1. Schematic of Max-projection Z Stack and Sholl shells