Use of a Custom Macro in Analysis of Rat Sciatic Nerve Sections

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Abstract

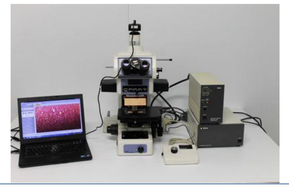
Introduction: Technology is a powerful analytic tool. This abstract outlines the use of a custom macro to analyze rat sciatic nerve histology sections as part of a larger study to evaluate functional and histologic outcomes after segmental nerve injury. The problem facing analysis of nerve sections is that the microscopic features of a nerve, such as the individual axon, cannot be viewed on the same scale as the entire nerve bundle. The goal of this analysis was to extrapolate the average total values of axon count, axon density, fiber diameter, and myelin thickness on a nerve section image taken at 400x magnification to the total area captured at 40x magnification.
Methods: Images of nerve sections stained with toluidine blue were captured using a microscope with a mounted camera and image acquisition software (Nikon Eclipse E800; Nikon DS-Ri1; NIS Elements BR 3.10 SP3 Hotfix5). Multiple images at different light intensities were used to determine a contrast and saturation that would grant the best accuracy. From these images, a custom macro using Image-Pro Premier (Version 9.1.5262.28) was developed to compute the desired measurements (Media Cybernetics, Rockville MD).
Results: At 400x magnification, circular shape recognition was used to determine each axon myelin sheath pair in the selected area. Area analyzed was within the "circle of good definition". After an axon myelin pair was recognized, multiple diameter measurements were taken, and these values were the key to calculations of axon area, fiber diameter, and myelin thickness. Two images taken at 400x magnification for each sample were used to calculate an average for each measurement. An image of the entire nerve slice at 40x was taken, and this calculated area was used extrapolate measurements to the entire nerve section.
Discussion: The utility of this software and macro is exciting due to its reproducibility, accuracy, and efficiency. Not only does it take human processing and potential error out of the equation, but it solves the scale discrepancy when trying to analyze microscopic parameters on a larger scale. This procedure is versatile and can be implemented in future histologic analysis of nerve sections.

Introduction

Computer analytics software grants us powerful processing power for which the extent of application is not yet reached. This presentation explains the use of image analysis software to solve many issues involved in manual analysis of nerve sections such as scaling, inefficiency, and reproducibility. Each one of these problems was addressed in the design of a custom macro for image analysis software Image Pro Premiere (Version 9.1.5262.28 Media Cybernetics, Rockville MD). Software was programmed to analyze axon diameter, axon area, fiber diameter, and myelin thickness. Scaling presented an issue because the size of an individual axon (captured at 400x magnification) could not be viewed on the same magnitude as the whole nerve section (captured at 40x magnification). Multiple images had to be reconciled with a scale conversion for each nerve studied. Human processing of a single image could take upwards of three hours. The use of a custom macro shortened this time to be nearly instantaneous. Although the entire area of a given section was not analyzed, the accuracy of this program gave confidence that extrapolation of the average measurements of two images captured at 400x magnification could be applied to the total area captured at 40x magnification. Lastly, anytime a human is performing a process there is concern for error that affects reproducibility. Use of a macro ensured that every axon and myelin sheath pair of every image was analyzed the exact same way.

Methods and Materials

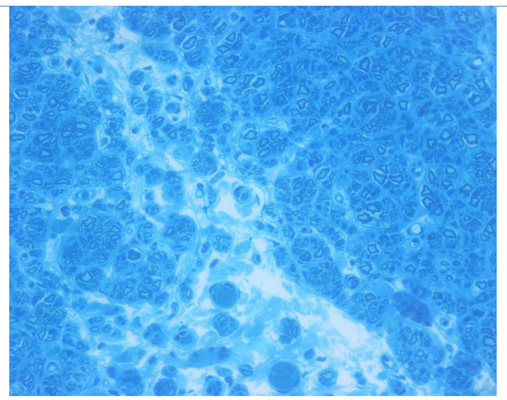
Nerve Section Preparation: Nerve samples harvested from rat sciatic nerves were fixed in 4% paraformaldehyde and stored in a 4^{o}C freezer. Sections were fixed in glyceraldehyde, mounted in epoxy resin, and sectioned at 10um. Final staining was performed with toluidine blue to give contrast to both the axon and the myelin sheath.
Image Capture: Two images of each section in different areas of the slice were taken at 400x magnification. One image of the entire section was taken at 40x magnification. Images were captured using a microscope with a mounted camera and image acquisition software (Nikon Eclipse E800; Nikon DS-Ri1; NIS Elements BR 3.10 SP3 Hotfix5). Initially, many different images at different light intensities were taken and used in Image Pro Premiere to find the correct saturation and contrast that would grant the best accuracy of the analysis.



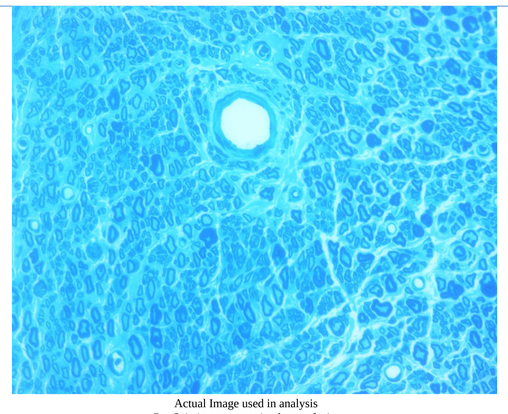
Stock photo of microscope setup used. Nikon Eclipse E800

Results

After proper contrast was determined, the pixel to distance ratio was used to unify the scales of each image. For any given section of nerve, three total images were used in the calculations. Two images at 400x magnification were used to get average measurements in a given area. At 400x magnification, only the part in true focus, the "area of good definition," was used in analysis. From within this area, circle recognition technology was able to delineate each axon myelin sheath pair. The diameter determination was the key calculation from which all other calculations came. By assuming that each shape was a perfect circle, calculation of area of the axon was done using the average of the largest and smallest measurements of diameter. Diameters were also measured from the edges of the myelin sheath. The same calculation gave an output that was the total diameter of the axon plus the myelin, or fiber diameter. The smaller area (axon only) subtracted from the larger area (axon and myelin) gave the myelin area. Likewise, diameter of the inner circle (axon edge) subtracted from the larger diameter (myelin edge) divided by two gave the myelin thickness. Finally the third image at 40x magnification of the entire slice area gave a measurement of total area. Given averages were then extrapolated to the larger area. Final outputs consisted of total axon count, axon density, fiber diameter, and myelin thickness.



Actual Image used in analysis
Rat Sciatic nerve mid graft site



Actual Image used in analysis
Rat Sciatic nerve proximal to graft site

Discussion

Using a macro to quickly analyze, calculate, and extrapolate measurements between two different scales is an extremely powerful tool. Its precision and reproducibility based on algorithm is a feat that manual analysis cannot begin to approximate and use of custom macros is exciting for the future of all histology, but this technology is not without its limits. Finding the proper saturation and contrast was a very difficult balance that depended both on the pathologist preparing the slides as well as the proper camera settings. This problem limits the application of any specific macro to other systems but ensures that, if executed to protocol, multiple images can be rapidly analyzed. Additionally, artifact among the slides presented an issue with analysis. The program was not able to determine artifact from axon/myelin at times and slides had to be re-prepared. Given the issues faced, designing similar macros that follow the guide of this procedure will allow for future versatility.

Conclusions

Use of a custom macro is helpful to unify the scales of images taken at different magnifications by using the pixel to distance ratio. Macros ensure reproducibility and also reduce human error while expediting processing exponentially. Although a given macro is specific to certain saturation and contrast parameters, the concepts, tools, and program all can be applied to a vast number of different tasks. The true capability of this computing power has not yet been reached, but its potential is exciting.

Future Directions

The concepts and calculations in this macro were effective but could be elaborated on in the future. The power of this analysis software would allow for more data points to be collected as well as more calculations. Future analysis should focus on increasing the accuracy of diameter measurements. Additionally, calculations based on ovals may be of use due to the shape of many axon myelin pairs.

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Acknowledgements

Scott Grainer- For slide preparation and staining
Matthew Batchelor- For extensive coding of the macro