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Morphometric Analysis of Peripheral Myelinated Nerve Fibers through Deep Learning

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Abstract

Irrespective of initial causes of neurological diseases, these disorders usually exhibit two key pathological changes - axonal loss or demyelination or a mixture of the two. Therefore, vigorous quantification of myelin and axons is essential in studying these diseases. However, the process of quantification has been labor intensive and time-consuming due to the requisite manual segmentation of myelin and axons from microscopic nerve images. As a part of AI development, deep learning has been utilized to automate certain tasks, such as image analysis. This study describes the development of a Convolutional Neural Network - based approach to segment images of mouse nerve cross sections. We adapted the U-Net architecture and used manually-produced segmentation data accumulated over many years in our lab for training. These images ranged from normal nerves to those afflicted by severe myelin and axon pathologies, thus maximizing the trained model's ability to recognize atypical myelin structures. Morphometric data produced by applying the trained model to additional images was then compared to manually obtained morphometrics. The former effectively shortened the time consumption in the morphometric analysis with excellent accuracy in axonal density and g-ratio. However, we were not able to completely eliminate manual refinement of the segmentation product. We also observed small variations in axon diameter and myelin thickness within 9.5%. Nevertheless, we learned alternative ways to improve the accuracy through the study. Overall, greatly increased efficiency in the CNN-based approach out-weighs minor limitations that will be addressed in future studies, thus justifying our confidence in its prospects¹.

Keywords

axon; myelin; peripheral neuropathy; morphometric analysis; deep learning

Introduction

A majority of neurological diseases result in two types of pathological changes - demyelination or axonal loss. Some may have a mixture of the two with variable degrees of severities from each. Physical disabilities from neurological diseases are often correlated

¹All the relevant code is freely available at <https://neurology.med.wayne.edu/drli-datashairing>

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with the severity of the pathological changes (Krajewski et al., 2000; Trapp et al., 1998). Therefore, efforts have been made to quantify these pathological changes in neural images, human nerve biopsies or nerve tissues from animal models. Quantified results may serve as biomarkers to track the severity and progression of the diseases.

Morphometric analysis of peripheral nerves has been utilized to quantify myelin and axon pathology for decades. This is typically done on semithin (1 μ m) sections stained with Toluidine Blue. Images captured under light microscopy or electron microscopy (EM) are processed to quantify the axon diameter, density, or myelin thickness.

This type of analysis has proven to be highly labor intensive. Traditionally, analysis of microscopic images requires segmentation of each image. Experimenter has to color-label specific subjects to be quantitatively measured. For nerve semithin sections, segmentation mainly colors myelin to separate them from axons and the background. The process of segmentation allows the interested object to be clearly isolated, so measurements can be accurately obtained. Image segmentation has been performed manually, despite it being time-consuming. Manual segmentation has also suffered from inter-experimenter variability.

Over the past 15 years, computational approaches to microscopic image segmentation have emerged (Ta et al., 2009); however each approach has been rather specific in its application and not easily generalizable to other types of image. With respect to myelinated axon image analysis, the trend has been away from manual and towards multiple-step semi-automatic segmentation like AxonSeg (Zaimi et al., 2016). In addition, microscopic image analysis has been taken advantage of recent breakthroughs in deep learning and the use of convolutional neural networks (CNNs) (Litjens et al., 2017). However, there have not been many applications of CNNs to the analysis of myelinated nerve fiber images. A recent study has applied the CNN approach to analyze peripheral nerve axons and myelin with a higher level of automation (Zaimi et al., 2018). However, the methodology has not been applied to various pathological changes in peripheral nerves, thus limiting its application.

In this study, we implemented a CNN-driven approach to quantify myelin and axon morphometrics in mouse sciatic nerves. Our laboratory has been studying the mouse sciatic nerves using morphometric analysis for decades. Images manually segmented in the past made the machine learning feasible by providing abundant training data. Moreover, our lab has been investigating peripheral neuropathies in many mouse models exhibiting a variety of pathological changes. These resources have enabled the CNN machine learning to recognize and quantify these pathological changes.

Materials and Methods:

1. Semithin section production and microscopic images

Nerves used for imaging both the training and testing of our model were collected from mice as a part of a non-survival procedure. The extracted nerves were put in fixative, a solution of 4% paraformaldehyde, 0.5% glutaraldehyde in PBS. Nerve tissue was osmicated in 1% OsO₄ solution for one hour. The tissue was dehydrated by submersing in ethanol at increasing concentrations from 30% to 100%, after which it was embedded in epoxy resin.

Once the epoxy blocks were solidified by an overnight 60°C incubation. A Leica ultrathin microtome was used to cut 1µm thick sections. Sections were then mounted on Fisher Superfrost slides and stained with Toluidine blue for five minutes. Images were acquired using a Leica microscope at 100x optical magnification. All images used for training and testing were saved as 8-bit TIFF image files at a resolution of 1392 × 1040. Training segmentation masks corresponding to training images were saved as binary images in TIFF format.

2. Manual segmentation

The aim of image segmentation was to outline myelin and axons, so they were clearly isolated from background. For images segmented manually, we preprocessed each image to increase the contrast between myelin and the background. Any myelin sheath that was not fully within the image frame was erased. Using ImageJ's image processing functions such as the Fill, Pencil and Brush tools, all non-myelin features were removed and all myelin was highlighted. Occasionally the 2px-wide pencil tool was used to separate blended fibers, but overall the use of "drawing" tools was kept to a minimum. The image was then thresholded, converted to a binary TIFF file, and split into two files: one for the outer myelin sheath outlines and one for the inner ones. ImageJ's analysis tool was used to measure areas and coordinates of both groups of objects. The coordinates of each inner element were matched to those of an outer one in an Excel template (Fig. 1). From the difference of inner and outer areas of each sheath, approximate inner and outer radii were calculated using the formula $R = \sqrt{\frac{A}{\pi}}$ and subsequently, myelin sheath thickness and g-ratio (defined as a ratio of the inner radius to the outer $g = \frac{R_{inner}}{R_{outer}}$ measurements were obtained. Axon density was also calculated for each image by dividing the number of axons with the area of a rectangular space that encased all nerve fibers (Fig. 2).

3. Network architecture

We used Keras, a computational open-source of deep learning library for deep learning-based methods. Keras utilized CNNs arranged in a U-Net architecture. As aforementioned in Introduction, CNNs are a type of computational neuronal network that was adapted for processing images. In Keras, CNNs are structured in a shape of "U" for its process flow of data computation. Because the "U" shape, it has been called U-Nets. It has been mainly used for image segmentation (Ronneberger et al., 2015). This architecture has yielded high-quality results for biomedical image segmentation. The unique feature of the architecture is that it consists of two paths: the contracting path and the expanding path. The contracting part of the architecture resembles a standard convolutional encoder, and encodes the image into feature maps at several levels. Each level in this path consists of two convolutional layers followed by a 2×2 pooling layer. As a result of each pooling operation, the number of encoded features is doubled (see the left part of Figure 1 for further illustration). The expanding path of the U-Net architecture in essence reverses the process of the first half: each step consists of an up-sampling of the feature maps, which halves the feature amount, followed by 2 convolution layers. At each step of the expanding path, the feature map is

concatenated with the corresponding encoding from the contracting path. This allows better localization and learning of features (Fig. 3).

Our implementation used 4 contracting steps, which takes the encoded feature number to 512. ReLU activation was used for all layers, and Adam optimization algorithm was used. Both of them are typical steps for deep learning projects. All convolutional layers used Keras' "same" padding option which allows keeping a layer's output and input lengths identical. The remaining parameters were left at default values.

4. Data augmentation and model training:

Training data consisted of 280 original microscopic images of mouse sciatic nerve cross-sections paired with their respective segmentation masks. The segmentation masks were manually obtained as part of previous studies on sciatic nerves in hereditary neuropathy mouse models. To augment the training data, horizontally and vertically flipped images were added to the training, thereby multiplying the amount of training materials by four.

Images and masks were converted to NumPy array, normalized, and resized to 1024×1024 pixel resolution. Batch size was set to one image at a time due to GPU memory size limitation. Training was performed for approximately 1000 epochs with a 0.2 validation split. The Dice coefficient was selected as the loss of function, due to its usefulness in measuring spatial overlap. In our case, it measured how well the validated segmentation results were aligned with the image respective training segmentation maps (Zou et al., 2004). The Keras checkpoint function was used to save the best iterations of the model as measured by the dice coefficient (Fig. 4). The training process achieved a training accuracy which plateaued at a dice coefficient of 0.91, while the validation dice coefficient varied between 0.81 and 0.85. The trained model and accompanying code is freely available at: <https://neurology.med.wayne.edu/drli-datashairing>

5. Mouse breeding and genotyping:

All mice used for this project were on a C57BL/6 background. Conditional knockout mice were made by utilizing the Cre/loxP system to generate tissue-specific knockouts. Specifically, we crossed *Mpz^{Cre}* mice with *Pak2^{fl/fl}* or *Fig4^{fl/fl}* mice to obtain conditional knockouts having the *Pak2* or *Fig4* genes removed exclusively in Schwann cells. For genotyping, tissue was obtained by ear punch, and digested using DirectPCR Lysis Reagent (Viagen, Los Angeles) and proteinase K. PCR was performed with forward primer and reverse primer (Table 1). The PCR products were run on 2% agarose gel to be visualized for determination of genotypes.

Pmp22^{+/-} mouse and genotyping have been described previously (Adlkofer et al., 1995). The mouse was created in the mixed background of C57BL/6 and 129. It was subsequently backcrossed with C57BL/6 over 10 generations to produce a congenic strain (Guo et al., 2014).

Results

We have adapted U-Net network architecture based on its demonstrated efficiency for biomedical image segmentation. The existing sciatic nerve images were acquired for segmentation from multiple genotypes of mice. In order to expose the model to a broad range of pathological changes in peripheral nerves, we included severely diseased nerves from *Pak2* Schwann cell knockouts (*ScPak2^{-/-}*) which exhibit greatly reduced axon fiber density and severe dysmyelination (myelin fails to form during development); whereas nerves from *Pmp22* heterozygous knockout mouse (*Pmp22^{+/-}*), a mouse model for hereditary neuropathy with liability to pressure palsies (HNPP) present a particular type of pathology called tomacula. On semithin images tomacula present as nerve fibers with excessively folded myelin sheath in paranodal regions. Sometimes, it may also be present in juxtaparanodes or internodes (Adlkofer et al., 1995; Bai et al., 2010). Details of the mice of varying genotypes used in the study are as follows: 5 *Pmp22^{+/-}* mice, 5 *Pmp22^{+/+}* (6 months old); 10 *Fig4* Schwann cell conditional knockout (*ScFig4^{-/-}*), 10 controls (*ScFig4^{f/f}*) (9 months old); 4 *Pak2* Schwann cell conditional knockout (*ScPak2^{-/-}*), 4 controls (*ScPak2^{+/+}*; 1 month old). On average 5 to 6 images were collected per nerve in order to cover the entire cross-sectional area. By using images with highly variable pathologies, we aimed to prepare the model for future segmentation of mutant mouse or human biopsy nerve images.

About 60% of images used appeared morphologically normal under light microscopy. Training the model for ~1000 epochs brought the training prediction accuracy to 0.91 expressed as dice coefficient.

The trained model was then tested in 5 *Pmp22^{+/-}* and 5 *Pmp22^{+/+}* mouse images that were not used for the training. Prediction time averaged several seconds per image using Keras with GPU-accelerated Tensorflow backend. In addition, we performed segmentation of the tested images manually to enable direct comparison of the two methods. Each process was timed and final measurements were compared.

After the model was trained, we applied it to the test images which produced the segmentation mask of myelin vs. background, labeling myelin white and background black. However, some manual tools had to be used to separate occasional conjoined sheaths and remove false positives in order to take measurements using ImageJ (Fig. 5). On average, 20 to 30% of axons required at least some manual correction or separation from its neighbors. The degree of manual correction employed also depended highly on the axonal density of any given image. Despite our inability to completely eliminate manual intervention, it took only 15-25% (10-15 minutes versus 50 to 60 minutes) of the time of the corresponding steps spent for the manual segmentation. If a sheath was segmented incompletely (an incomplete sheath was defined as <80% of myelin circumference was outlined, forming a crescent, instead of a circle), this would be removed from further analysis. The image then was thresholded and converted to a binary image. Axonal measurements were taken in the same manner as for the manually-segmented images.

Measurements obtained from the 10 test images were compared between the two methods. Specifically, we looked at the axon density in each image, outer diameter of nerve fibers, thickness of the myelin sheath, and the g-ratio of each myelinated fiber. We found a 2.5% decrease of nerve fiber density as measured by automatic segmentation, compared with the manual method. *Pmp22*^{+/-} nerves were affected more - exhibiting a 4.0% decrease, compared to a 1.0% decrease for *Pmp22*^{+/+} images. The most notable disparity was observed in comparing the axonal diameters: our method produced an average diameter that was 4.2% lower than that of the manual method. Axonal diameters in *Pmp22*^{+/-} nerves were found to be 9.5% smaller as assessed by measurement of automatic segmentation than by those of manual segmentation. The axons in *Pmp22*^{+/+} nerves were 1.6% larger on average according to the trained model than by manual measurement. Average myelin thickness was found 2.0% larger by automatic processing. In *Pmp22*^{+/-} images, myelin thickness was decreased 3.2% (possibly hinting that our methods still are undertrained with regards to tomacula, a distinct *Pmp22*^{+/-} pathology). By contrast, in *Pmp22*^{+/+} nerves, myelin thickness measured 9.4% thicker by trained model than by manual segmentation. G-ratio on average decreased 2.6%, only 0.5% lower in *Pmp22*^{+/-} images and 4.3% lower in *Pmp22*^{+/+} images (Table 2).

After noticing the differences in diameter measurements we were concerned that the fiber analysis could be intrinsically skewed. To address this we performed a distribution analysis of myelinated fiber diameters (Fig. 6). This analysis produced a distribution highly similar to distributions resulting from manual segmentation. This suggests no particular type or size of myelin is disproportionally affected by the automatic method.

In our deep learning-based method, the majority of time required was utilized during the training stages. Once the model was fully trained, this method took 10-15 minutes per image. Semi-manual segmentation required about 65-76 minutes per image, where the bulk of time was used to manually remove the background, artifacts and separating myelin sheaths.

Discussion

Implementation of our segmentation method has resulted in several important improvements. It has allowed our lab members to reduce the time necessary to analyze nerve morphometrics. This is particularly helpful when a large cohort of animals needs to be evaluated.

Our lab has accumulated numerous manual segmentation masks over the years. We have been able to leverage this data to train the computational model in implementing a deep-learning CNN-based approach. As we segment more images with the new method, the corrected masks can be used as additional training data, which would lead to further improvements in its accuracy. As discussed below, this work also identifies specific areas that we can modify to improve the accuracy of morphometric analysis in the near future.

The knowledge gained during the development of this project has provided a basic framework to develop deep learning-based approaches to analyze other images collected in

our lab, as well as pathological images from human nerve samples. For the latter, multi-center collaboration would be the most effective way to build up sufficient training data. Our lab alone does not have a sizeable collection of human sural nerve biopsies, which have been commonly used for pathological diagnosis in patients with peripheral nerve diseases. However, a large amount of human sural biopsies and images can be gathered across multiple institutions. This should allow us to evaluate this method in human nerve samples and perform additional training of the model.

We believe a similar approach can be used for analyzing non-myelinated nerve fibers in Remak bundles. However, these non-myelinated nerve fibers cannot be visualized under light microscopy, but they can be readily imaged under electronic microscope. These fibers can also be measured by using the deep-learning based method.

While our CNN-based approach is an improvement compared to the manual segmentation method, we had identified several important areas to be improved.

Limitations in the training data:

In the process of manual segmentation during our previous studies, we erased all myelinated fibers from the images' edges if the fibers were partially cut. The resulting images were subsequently used as the training data, which could have potentially biased the model, since it ignored these myelin segments along edges. Our training data were constructed exclusively from images from mouse sciatic nerve semi-thin sections. It is unknown how the model would perform on images of mouse spinal cord or human biopsy nerves.

We were only able to use segmented images from our previous morphometric studies as training data, so our pool of mouse genotypes was limited. We expect that using more morphometrically diverse images for additional training would improve the model's segmentation capacities further. We are well-suited to segment sciatic nerve images from mice with additional genotypes and pathological changes, and will continue training the model as we acquire new segmentation masks during the course of our research activities.

It may have been beneficial to train our model to differentiate between 3 classes (background, myelin, axon), instead of 2 (myelin vs non-myelin). We may implement this in the future, but it would require restructuring of the training data as well as some changes to the network architecture.

As seen in comparing the results of our automated model with the manual approach analyzing images of WT and *Pmp22*^{+/-} nerves, our model is still not completely accurate in segmenting myelinated axons with diameters under 2μM. Because the accuracy of our method is in theory not size dependent, we suspect that inaccurate analysis of small fibers was a result of thin myelin sheaths being a slightly darker colored than thicker myelin sheaths. More advanced pre-processing methods may help this issue. The model also occasionally failed to fully separate fibers that are tightly clumped. We believe better pre-processing and more training data can improve this issue as well.

Limitations independent of the segmentation process:

Independent of the segmentation process, we used the same ImageJ tools to conduct the measurements. Since the ImageJ particle measurement tools only work with images in binary format we had to threshold the segmented images and thus introduced additional variability to the final results. As per our method, two measurements were taken: one for inner axons and one for outer myelin. This resulted in two sets of objects that contain a pair of x,y coordinates and the area. We exported the two lists to excel, where a macro matched each inner object to the closest outer one. This process is not instantaneous and currently involves multiple rounds of copying and pasting. To improve the general workflow we hope to make this process mostly automated, to avoid copying and pasting tables after every image is measured. Also, since ImageJ cannot measure the diameter of an object we used an approximating calculation that assumes each object is circular and calculates the radii from the area.

It should be clarified that our technique by no means will substitute the qualitative visual analysis of the nerves by a trained neuropathologist, but a complementary tool. The pathologist may still identify different pathological changes that would not be quantified by our CNN-based method. For instance, inflammatory cells infiltrated into nerves would not be quantified by our method, but are important for diagnosis.

In conclusion, we have developed a CNN-based approach to segment images of mouse nerve cross sections. We adapted the U-Net architecture that was utilized for our project. We trained the network using available segmentation data produced manually by our lab, including images of nerves with severe pathological changes, to maximize the trained model's ability to recognize atypical myelin structures. This model significantly decreased the time required to perform the morphometric analysis. However, we were not able to completely eliminate the need for manual refinement of the automated segmentation product. We evaluated the performance of our method by comparing measurements acquired from manual segmentation and our CNN method. This comparison showed our method was accurate in measuring axon density and g-ratio, but varied in axon diameter and myelin thickness by 9.5%. More importantly, we have learned alternative ways to improve the accuracy through the study. Despite a few limitations, we were able to greatly increase the efficiency of morphometric analysis and are overall optimistic about its prospects.

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Figure 1.

Manual segmentation: i) Contrast was enhanced through ImageJ default tools. ii) All myelin sheaths that were not fully present within the frame and all non-myelin objects were removed. Myelin was labeled colored white on black background. iii) Image was converted to binary and separated into two files for measurements: inner and outer outlines. Measurements of each object's area and centroid coordinates were taken and exported to Excel. iv). An Excel formula was used to match inner and outer objects. Radii of each object was approximated and used in calculating myelin thickness and g-ratio.

Figure 2.

Axonal density measurement: To account for incomplete sheaths erased over the image edges, the effective area of the image was calculated as a rectangle with sides $X_{max} - X_{min}$, $Y_{max} - Y_{min}$, where X_{max} is X coordinate of the rightmost object, Y_{max} is Y coordinate of the object closes to the upper edge of the image and so on.

Figure 3.

U-Net architecture: Each rectangle represents a data tensor. Tensor sizes are indicated on the side of each row and on the top of each layer. The figure is adapted from Convolutional Networks for Biomedical Image Segmentation by Ronneberger O, et al (2015).

Figure 4. Model Training: i) Original images paired with ii) their respective manual segmentation masks were used to train the model. Keras checkpoint functionality was used to save the best performing model in hdf5 format.

Figure 5.

Applying a trained model: i) Saved model weights, and ii) test images are loaded into the network (iii) to produce the automated prediction (iv). Some manual refinement (v) is usually required to separate conjoined sheaths and remove the false positives. After the manual corrections are implemented the image is split into (vi) inner and outer objects to be measured and analyzed in the same manner as was done after manual segmentation.

Figure 6.

Frequency distribution of axonal diameters: As seen by comparing the distributions between the two methods, the CNN approach performed adequately. However, a noticeable difference is observed for axons with diameter ≥ 2 nm, which is consistent with our experiences of the model missing some smaller fibers.

Table 1:

Primer sequences: Primers used to genotype the mice are listed in this table. All PCR was performed with One Taq® Quick-Load® 2X Master Mix with Standard Buffer. The PCR products were analyzed on 2% agarose gels.

Genotype	Forward primer	Reverse primer
<i>Pmp22</i>	CAGCCACCATGCTCCTACTC	WT: CAGCCCTTGCTCACTGTCTAC KO: GCAGCGCATCGCCTTCTATC
<i>Pak2^{fl}</i>	ATCTTCCCAGGCTCCTGACT	TGAAGCTGCATCAATCTATTCTG
<i>Fig4^{lox}</i>	TCATTGAGTAAGAAAGGGGTCA	TCAGAGAAGACAGGGCAGAAA
<i>Fig4^{fl}</i>	GGACGTTTAGACAATTACAGCTATT	GACTGACTTCCCACCGTCAT
<i>DHFR^{re}</i> or <i>Mpz^{Cre}</i> PCR	ACCCTGTTACGTATAGCCGA	CTCCGGTATTGAAACTCCAG

Table 2.

Comparison between manual and automated methods

HNPP images.	Manual	CNN	Difference (from manual)
Axon Density	47029.9 axons/mm ²	45162.89 axons/mm ²	−4.0%
Diameter	2.05±0.82 μm	1.86±0.47 μm	−9.5%
Myelin thickness	0.96±0.42 μm	0.93±0.47 μm	−3.2%
G-ratio	0.53±0.11 μm	0.53±0.10 μm	−0.5%
WT images.	Manual	CNN	Difference
Axon Density	46249.6 axons/mm ²	45776.02 axons/mm ²	−1.0%
Diameter	1.88±0.82 μm	1.91±0.82 μm	+1.6%
Myelin thickness	0.65±0.32 μm	0.71±0.35 μm	+9.4%
G-ratio	0.65±0.07 μm	0.62±0.08 μm	−4.3%
Overall	Manual	CNN	Difference
Axon Density	46638.31 axons/mm ²	45463.5 axons/mm ²	−2.5%
Diameter	1.97±0.82 μm	1.88±0.82 μm	−4.2%
Myelin thickness	0.81±0.42 μm	0.82±0.43 μm	+2.0%
G-ratio	0.59±0.11 μm	0.58±0.10 μm	−2.6%