Approaches to quantify axonal morphology for the analysis of axonal degeneration

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Morphological hallmarks of axonal degeneration (AxD): Axons transmit signals from one neuron to another through the proper communication in the nervous system. Therefore, the disintegration of axons, a process named AxD, has detrimental consequences and plays a key role in many neurological diseases.

AxD is characterized by the formation of two morphological hallmarks, namely axonal fragments and axonal swellings. Whereas axonal fragments are separated segments of the axon resulting from axonal breakdown, axonal swellings are spherical structures that may emerge along the axon prior to the occurrence of AxD (Palumbo et al., 2021). Quantifying these morphological hallmarks enables researchers to follow AxD over time, better understand its mechanisms, and anticipate and assess candidate treatments.

Here, we give a brief overview of the different approaches to quantify AxD based on axonal morphology and explain how recent machine learning tools may be used to decipher the progression of AxD to further our understanding of this process and develop novel therapeutic interventions.

Image binarization and manual labeling to quantify AxD: A widely applied approach to analyze AxD is the thresholding of axons/axon fragments from the background by converting the original image to a binary image (Yang and Wang, 2020; Yang et al., 2018). As is the case for image binarization, a threshold of the gray image is selected. The selected threshold value is supposed to be the gray value of the background, axons or axonal fragments. Structures that are continuously connected are counted as axons, whereas axonal fragments are identified as non-connected structures and counted using the particle analyzer module of Imagej. Further measures of AxD are: i) the distance between lesion site and axonal tip, ii) the distance between fragments, iii) the length of the axon axonal frag lengths, and iv) fragmentation rate. The above-described approaches can be applied in vitro, ex vivo, and in vivo (Kerschensteiner et al., 2005; Knöferle et al., 2010; Gerdis et al., 2015; Canty et al., 2020). For the latter, fluorescently labeled axonal tracts using transgenic mice or viruses are required. One advantage of binarization is that it is sensitive enough to recognize thin axons, which are then either omitted or misinterpreted as axonal fragments. Another drawback of image binarization is that it cannot be used to quantify axonal swellings.

Approaches to investigate the occurrence of axonal swellings in vitro, ex vivo, and in vivo experiments involve calculating i) the ratio of the number of axonal swellings compared to the axon length by manual labeling of regions of interest using Imagej (Yang et al., 2019) and ii) the axonal swelling density measuring pixel intensity per axon length using MATLAB (Canty et al., 2020). As is the case for image binarization, these approaches are semi-automatic and subjective, because they require manual annotation, and thus are time-consuming. Hence, a tool to analyze axonal morphology including both hallmarks of AxD—axonal swellings and axonal fragments—would be of high relevance and subject of future development.

Principle of deep learning to classify structures in microscopic images: Recent progress in the field of machine learning has opened novel ways of how to quantify axonal morphology on microscopic images. Convolutional neural networks (CNNs) represent a deep learning approach to classify specific structures in microscopic images (Arrázola et al., 2019). A CNN consists of three different layers, namely input, hidden, and output layer, containing several nodes whose connection strengths are defined by weights. In the input layer, the CNN receives information about the dimensions of the images as input pixel values. The backbone of a CNN is the extraction of pixel information from different convolutional operations to recognize edges, lines, specific shapes, and gradient orientation. Convolutional operations are based on the application of different filters, also known as kernels, that slide as a small window region over the image and calculate the sum of pixel values.

To minimize the computational power necessary to process the data by reducing the image size, further pooling operations are employed for each area of the image covered by the kernel. During pooling, either the maximum or the average pixel value of the kernel coverage is selected. Each pixel value originating from different kernels is then multiplied by an associated weight and transformed by a mathematical function to activate the node as it incorporates several weighted inputs. The activated node then propagates the information to the subsequent nodes in the next layer which differently weigh the input. These weight-activated node activations eventually determine which nodes are most strongly connected.

In the output layer, the probabilities of classifying specific structures in the microscopic image are calculated leading to the assignment of each pixel to one of the classes (e.g., axons, axonal fragments) as output. The resulting segmented image containing the classified pixels is then compared to the original image. The distance between the original image and the resulting image is called “backpropagation” is eventually used to re-adjust the weights among the nodes in each layer to ensure that the output prediction of the CNN corresponds to the original image.

This learning process can be performed by the CNN itself or be taught via supervised learning (Yang and Wang, 2020). In the case of supervised learning, several microscopic images are annotated by experts. The original microscopic images and their annotations are “fed” into the CNN and used for output computation. Finally, the performance of a CNN to match the original image should be validated using independent datasets and compared against expert raters.

Deep learning-based approaches to quantify AxD: Several CNNs have recently been developed for the recognition of axonal morphologies (Box 1). TrailMap facilitates the 3D visualization of axonal projections in cleared fixed tissue (Cheng et al., 2019). The authors adapted the AdipoClear protocol to reduce the autofluorescence of myelin and axons of healthy mice to generate and share datasets to train CNNs that eventually determine which nodes are most strongly connected.

Based on the acquired data on the changes of morphological hallmarks of AxD over time, we were able to detect four morphological patterns of AxD (granular, retraction, swelling, and transport degeneration) in a model of AxD in hemispheric stroke (Cheng et al., 2019). The TrailMap tool cannot only assess AxD by quantifying the morphological hallmarks of AxD, but also determine the morphological hallmarks of AxD in tracking AxD in its early stages. This versatility may allow to expand our understanding of AxD in the context of neurological diseases. Therefore, TrailMap could be used to determine AxD on 3D images. Features such as branching, lengths, and diameters, as well as tracking of axonal cargo transports are also of high relevance and subject of future development.

One of the main limitations of the currently available deep learning-based approaches is that they cannot quantify AxD in vivo. In the future, it is thus important to go back and share the trained CNNs that include in vivo microscopic recordings of axons undergoing AxD over time, which will enable the analysis of AxD progression with time. In addition, one of the general limitations in training neural networks is the availability of training data, which must be annotated manually by experts. To reduce labeling costs, active learning approaches can be applied to identify the images that yield the best results (Grüning et al., 2020), thereby saving time to implement or modify existing deep learning-based approaches.

Conclusions: Recent deep learning-based approaches have significantly improved our abilities to quantify axonal morphological changes ensuring objectivity and standardization in determining the need for manual annotation and thresholding. This is of high importance to allow systematic investigation of the mechanisms underlying AxD. Together with enhanced throughput test systems, this will form a platform for the thorough evaluation of the authors’ studies to prevent and treat AxD in many neurological diseases.
A microscopic image containing axonal features (axons, axonal swellings, and axonal fragments) is introduced to the CNN as an input image. Each node of the input layer forwards dimensionless pixel values to the hidden layers for structural information extraction via convolutional and pooling operations. In each of the hidden layers, nodes employ different filters (e.g., cyan filter for edges, orange filter for gradients, yellow filter for shapes, and purple filter for curvatures) and propagate the information to the nodes of the subsequent layer until reaching the output layer. In the output layer, all information is integrated to fully segment the input image. To correct potential errors in the output prediction of the CNN (dark grey = axons, light grey = axonal swellings, white = axonal fragments), backpropagation alters the connections among the nodes by readjusting the weights. Scale bar: 100 µm. AxD: Axonal degeneration; CNN: convolutional neural network. Unpublished data.

**Box 1 Comparison of different deep learning-based approaches to quantify features of AxD:**

| Deep learning-based approach | Specimen | Microscopy | Networks | Learning strategy | Recognized structures | Outcome parameters |
|-----------------------------|----------|------------|----------|-------------------|----------------------|-------------------|
| TraitMap (Friedmann et al., 2020) | Whole tissue ex vivo | Light sheet microscopy | 3D CNN based on u-net | Supervised and unsupervised | Axons, dendrites, and somata | Axon density |
| CMN (Schubert et al., 2019) | Tissue slices ex vivo | Electron microscopy | Cellular morphology neural networks based on multiview CNN | Supervised | Axons, dendrites, and somata | Reconstruction of volume and localization of axons, dendrites, and somata |
| AxonDeepSeg (Zaïmi et al., 2018) | Tissue slices ex vivo | Electron microscopy | CNN based on u-net with ResNet-50 | Supervised | Axons and myelin | Number of axonal swellings |
| DeepBouton (Cheng et al., 2019) | Whole tissue ex vivo | High-resolution stage-scanning confocal microscopy | Ensemble of CNNs, based on u-net with ResNet-50, recurrent neural network | Supervised | Axons, axonal swellings, and axonal fragments | Area of axons, axonal swellings, and axonal fragments, degeneration patterns |
| EntireAxon (Menon et al., 2020; Palumbo et al., 2020) | Spatially isolated axons in microfluidic device in vitro | Phase-contrast and fluorescence microscopy, time-lapse imaging | CNN based on u-net | Supervised | Axons, dendrites, and myelin | Number of axonal swellings |

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