Quantifying Focal Disruptions in Axonal Microtubules

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***Abstract*—Focal disruptions in axonal microtubules are a common consequence of Traumatic Brain Injuries (TBIs), and the ability to quantify these disruptions is essential to evaluating *in vitro* injury models and therapies. Here, we develop and implement an algorithm to demonstrate the ability to quantify focal disruptions in the microtubules of cultured neurons. The algorithm is implemented using a combination of ImageJ for pre-processing and Matlab for axon analysis and tubulin breakdown identification.**

I. Introduction

Brain injury is one of the most common causes of disability and death in adults. Traumatic Brain Injury (TBI) affects approximately 270,000 people a year and costs the U.S. more than $48 billion annually. Around 70,000 people die each year from head injuries, but many of those who manage to survive are left with long-term disabilities as a result of their injuries[1]. While there have been many promising therapeutic treatments for TBI, clinical trials have not been successful. *In vitro* studies can provide insight into the cellular mechanisms leading to neuronal death and degeneration after TBI and aid in developing more effective treatments by isolating components of the injury pathway to investigate the connection between mechanical trauma and resulting pathology.

Focal Disruptions in microtubules are a common consequence TBI[2]. Microtubules are thought to be disrupted as a result of protease and calpain activation that occurs after increased intracellular calcium in TBI. The ability to identify and quantify these disruptions is important for validating *in vitro* models of injury and for evaluating potential therapeutic treatments. Here, we demonstrate the ability to quantify focal disruptions in microtubules by developing and implementing an algorithm primarily using Matlab. Microtubule disruption is induced by increasing intracellular calcium levels in neurons and a simultaneous fixation-extraction method was used for visualizing the cytoskeleton. Focal disruptions in microtubules were quantified using a semi-automated thresholding and segmentation of the axon followed by ordered intensity measurements and filtering to identify local breakdowns in tubulin.

II. Materials and Methods

A. Neuronal Culture

Glass coverslips were coated overnight with a solution of 0.1 mg/ml of poly-DL-lysine in sodium borate buffer. Embryonic day 8 chick forebrain neurons were dissected, dissociated and plated at a concentration of 1.5 x10^4 cells/cm^2. Cultures were maintained in M199 medium and incubated at 37°C and 5% CO_2 for 5 days before experimentation.

B. Microtubule Disruption

Microtubule disruption was achieved by increasing intracellular calcium concentrations using calcium ionophore A23187. High calcium concentrations are known to activate proteases such as caspases and calpains which degrade the cytoskeleton[3]. Cultured neurons were incubated in 10μM of A23187 for 30 minutes.

C. Visualization of the Cytoskeleton

A combined fixation and extraction method was used to visualize the breakdown of microtubules while removing free tubulin monomers [4]. Experimental cultures were fixed in PHEM buffer with 1% Triton X-100 and 0.05% glutaraldehyde for 15 minutes and then treated with 2 mg/ml of sodium borohydride to quench autofluorescence and stained with FITC-conjugated DM1A anti-tubulin (1:100) to stain tubulin.

D. Quantifying Focal Disruptions

The quantification of focal disruptions in microtubules involved manual segmentation pre-processing in ImageJ, the automatic creation of a profile plot in Matlab, and further filtering analysis and thresholding in Matlab to identify focal disruptions in microtubule intensity. ImageJ was used for the segmentation part of the analysis due to the ease and speed of the user interface, which could then be integrated into the Matlab code (Fig. 1). The image was imported into ImageJ and rotated so that the soma would be oriented towards the left of the screen. This was important because the Matlab code used later would identify the left-most point of the axon in order to find the anterograde-retrograde direction. The image was then cropped so that only the axon was included, background subtracted using a 50-pixel rolling ball radius, and converted to an 8-bit image using a custom macro. The image

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was then thresholded so that the axon could be segmented out of the original image. This axon was then imported into Matlab for further processing.

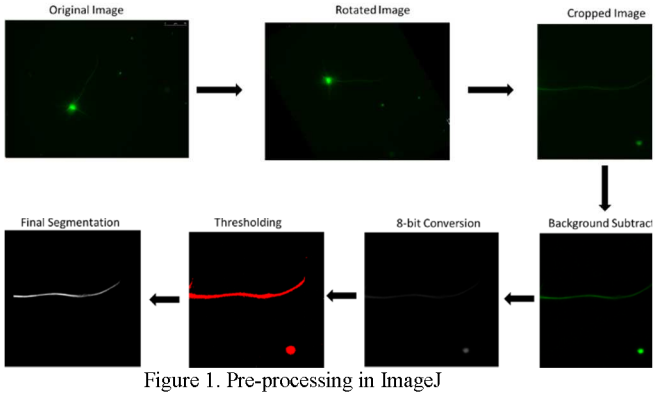

Figure 1. Pre-processing in ImageJ

The final segmented axon was imported into Matlab and the plot profile of axonal intensity was created by finding and pruning the spine, finding the slope at every point on the spine, and using the overlapping line perpendicular to the slope to find the width of the axon at that point (Fig 2). The average fluorescence intensity of all pixels along that overlapping line was used to find the average intensity at that point. The values were oriented from proximal to distal and were used to create the plot profile of tubulin intensity along the axon.

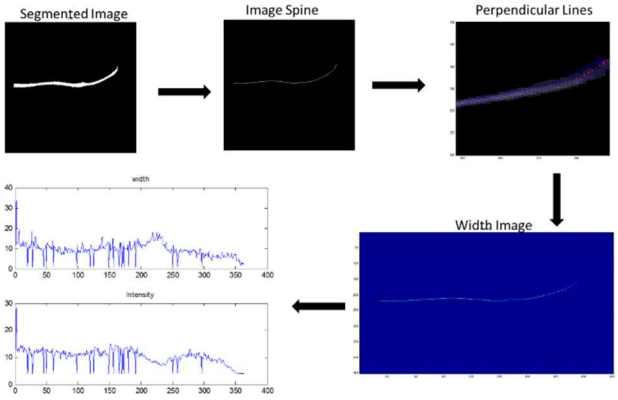

Figure 2. Plot Profile creation in Matlab

The values for the plot profile were then analyzed in a separate program to find focal disruptions by finding local minima in fluorescence intensity. The intensity of the values along the axon was smoothed and filtered using a low-pass filter. All local minima and maxima were identified, and areas of microtubule disruption were chosen from the local minima based on a manual threshold. The final graphs are then presented to the user along with an image of the axon with the focal disruptions highlighted.

III. Results and Discussion

The output of the program results in clearly identified focal breakdowns in microtubules(Fig 3 A-B). When used to analyze the entire population of cells, cells treated with calcium ionophore A23187 had more focal disruptions in microtubules than control cells (Fig 3 C-D).

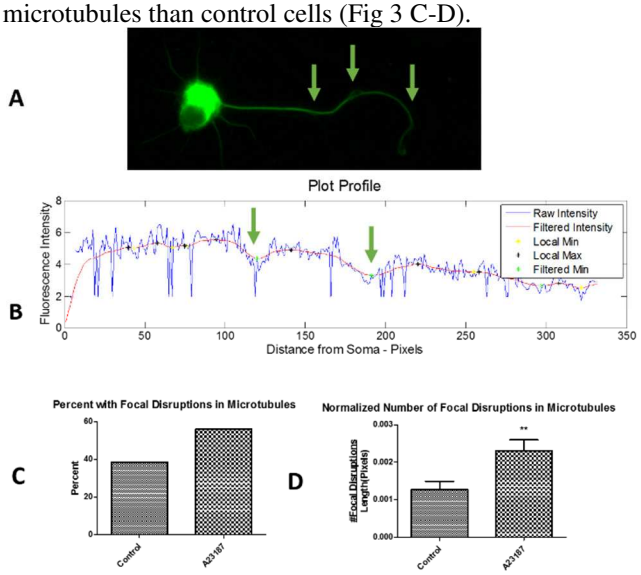

Figure 3. Cytoskeletal changes after increasing intracellular calcium with calcium ionophore A23187..A, Representative image of A23187-treated neuron stained for tubulin. Arrow denotes focal disruption of microtubules. B, Plot Profile of intensity of representative neuron, arrow corresponds with area identified as having disrupted microtubules. C, A23187 treated cells have a higher percentage of cells with focal disruption in microtubules. D, A23197 treated cells have more focal disruptions in Microtubules. One-tailed mann-whitney test, $p<0.005$. N>80 cells from three coverslips for each condition. Error bars represent SEM.

These results show that increased cytosolic calcium can induce focal disruptions in microtubules, but more importantly they demonstrate our ability to identify and quantify these focal disruptions. This is important both for furthering our understanding of the TBI pathway and for evaluating therapeutic treatments

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