Quantification of Beading Intensity in Cultured Neurons

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***Abstract*—We present an implemented method for automated quantification of neuronal beading. Beading is the "morphological hallmark" of axonal injury and is present in many neurological disease conditions as well. *In vitro* models of injury result in beading which has previously been difficult to quantify, and therefore difficult to use as a measurement of the degree of axonal injury. The beading program was designed to quantify beading intensity in single axons and output a unitless beading number that represents the size and number of beads in relation to the length and width of the axon. The program was used to analyze a sample set of images and was successful in demonstrating a quantifiable difference between injured neurons and sham controls. Bland-Altman analysis was used to assess agreement between the number of beads counted by the program and those counted manually.**

I. Background

Beading is a well-documented phenomenon in neurons that parallels neuronal damage. It is a term that describes focal bead-like swellings that have been shown in diffuse axonal injury after brain trauma and other neurological conditions such as epilepsy, amyotrophic lateral sclerosis, and multiple sclerosis[1]. In *in vitro* experiments, beading can be used as a morphological indicator of the extent of neuronal injury. However, difficulty in quantifying the degree of beading has made this problematic.

Quantification of beading has historically been very simplistic. Some papers merely observe the presence of beads[2]. Some present the percentage of bead-bearing neurons[1, 3, 4], and others quantify the intensity of beading for each neuron as the number of beads normalized by the length of the axon[5]. However, these methods can be very subjective in deciding what qualifies as a bead and only reveal information about the number of beads, not their size. Since beads are also associated with areas of disrupted transport and organelle accumulation[1, 2, 5-8], the human eye is biased towards dark swellings which may not actually have a significantly larger diameter than the rest of the axon. In an attempt to be less biased, some have elected to define beads as focal swellings that are at least two times the size of areas of the axon that appear health[9]. However, deciding what areas are "healthy" is also subjective, and ruling out smaller beads does not properly take into account the early stages of beading. The most advanced method of analyzing axonal beading to date[10] attempts to address this subjectivity but is unable to achieve satisfactory agreement between what is identified as a bead and what a human participant would choose. The program presented here addresses these issues.

Fig 1. A healthy neuron (left) and a neuron showing multiple beads (right)

II. Program Design

The beading program presented here was designed to remove as much user bias as possible and to output a unitless number that accounts for both the number and size of the beads in relation to the neuronal axon in order to allow for the comparison of beading intensity between different neurons. The program was written in Matlab, and takes a binary image containing only the structure of the axon as an input. In order to find the direction and curvature of the axon, the program then narrows the pixels representing the axon down to one pixel width, revealing the "spine" of the axon. For every pixel on the spine, a circle is created that best overlaps with the original axon. The diameter of this circle represents the width of the axon at that point. To find the average width of a non-beaded section of the axon, all potential beads are filtered out by removing all local maximums. The average of the remaining sections is taken as the width of the normal axon. The standard deviation of those sections is also calculated and used to decide which of the local maxima are significantly larger than the normal axon and identifies them as beads. The beading number is then calculated as a unitless number that represents the size and number of beads in relation to the length and width of the axon:

$$beading\ number = \frac{\sum_{i=0}^{n} {r_n}^2}{W * L} \quad (1)$$

where n is the total number of beads, r_n is the radius of bead n, W is the average width of the un-beaded axonal regions, and L is the length of the axon.

The program also outputs an image of the axon highlighting the areas that have been identified as beads to allow the user to remove swellings that are not beads. This is especially important since it is possible to have focal areas that are significantly larger than the rest of the axon but would not be considered beads.

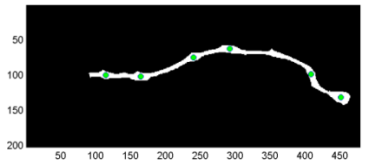

Fig 2. Example of beading program output highlighting identified beads and allowing the user to remove non-beads, as in the case of the last dot which is the growth cone and not a bead

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III. Materials and Methods

In order to validate the program as an effective method of measuring beading intensity, it was used to analyze images of injured axons. A previously established *in vitro* model known to induce beading [5, 11] was used to injure chick embryo neurons. 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[12]. Cultures were maintained in M199 medium and incubated at 37°C and 5% CO_2 for 5 days before experimentation.

A controlled shear stress device was used to apply uniform shear stress over the coverslips[13]. Sham controls were placed in the device but were not subjected to rotation of the cone. Injured cells were subjected to a shear impulse of 45 dyn/cm^2 with a 20 ms onset time. Neurons were fixed with 0.5% gluteraldehyde 35 minutes after injury, and phase contrast images were taken of individual neurons for analysis.

IV. Results and discussion

The beading program was used to analyze at least 30 neurons of each condition. Both a calculated beading number (1) and the number of beads counted were given for each individual axon. A blind manual count of the beads was also performed for comparison. In order for the program to be an appropriate tool for quantifying beading intensity, it must be able to successfully demonstrate a quantifiable difference between injured neurons and sham controls. There should be agreement between the number of beads counted by the program and the number of beads counted manually, but they are not expected to match perfectly because the program is less subjective than the human eye.

It can be seen from Figure 3 that the program is successful in demonstrating a quantifiable difference between injured neurons and sham controls.

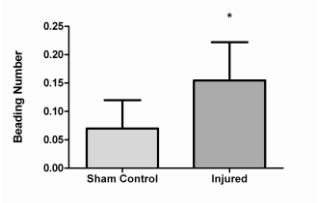

Fig 3. Graph of average beading numbers. The beading program was used to calculate beading numbers for both sham controls (n=36) and injured neurons (n=45). P <0.0001

When comparing the number of beads counted by the program and the number counted manually, the pearson correlation coefficient was calculated to be 0.8147 with a p-value<0.0001. This shows a strong correlation between human measurement and automated identification of focal swellings, but does not demonstrate agreement[14]. In order to show sufficient agreement, Bland-Altman analysis was performed (Figure 4).

The use of this program to measure beading intensity allows for a less biased and more quantifiable measurement than previous methods. The result of a unitless beading number potentially allows for the comparison of many different

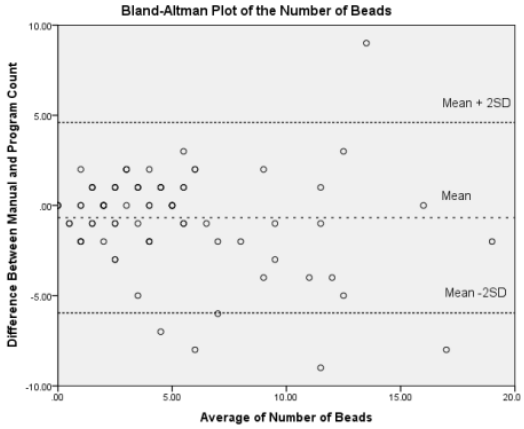

Fig 4. Bland-Altman graph demonstrating good agreement between the number of beads counted by the program and the number of beads counted manually

neurons. The implications of this method of quantification are that it allows for discrimination of neurons based on morphology as an indicator of neuronal health.

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