Short Technical Reports

A neurite quality index and machine vision software for improved quantification of neurodegeneration

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Current methods to assess neurodegradation in dorsal root ganglion cultures as a model for neurodegenerative diseases are imprecise and time-consuming. Here we describe two new methods to quantify neuroprotection in these cultures. The neurite quality index (NQI) builds upon earlier manual methods, incorporating additional morphological events to increase detection sensitivity for the detection of early degeneration events. Neurosight is a machine vision-based method that recapitulates many of the strengths of NQI while enabling high-throughput screening applications with decreased costs.

Introduction

Neurodegenerative diseases, such as amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease, share morphological features of axonal degradation that precede the death of the cell body as an early event in disease (1–3). Presently, there are limited methods to quantify protection afforded by candidate molecules that may prevent, delay, or slow the degradation process. Current methods for quantifying neurodegeneration—such as measurement of longest remaining neurite, halo area, or percentage of degenerated axons (4–6)—can be subjective, are time-consuming, and are impractical for high-throughput drug screening.

Our focus into neurodegenerative disease began with the finding that activation of SIRT1 by resveratrol was linked to neuroprotection (7). Subsequently, several laboratories showed that application of exogenous nicotinamide adenine dinucleotide (NAD) protected axons from degradation (6–7). Araki and colleagues demonstrated that NAD's protective effect was circumvented by SIRT1 RNAi; however, the precise mechanism(s) of protection has

not been conclusively elucidated (5–8). Having identified many new and potent SIRT1 activators in high-throughput in vitro enzyme assays, we wanted to develop the capacity to rapidly evaluate these compounds in a cell-based assay for neuroprotection. As the best of several systems tested, we chose the dorsal root ganglion (DRG) model to assess neuroprotection by NAD. In this set of experiments, we performed a dose-response experiment with varying concentrations of NAD over 90 h and scored neurodegeneration using either the previously described remaining axons or, as we have termed it, “intact neurites” (IN) method (7,8), the neurite quality index (NQI), or Neurosight (for high-throughput analysis) from images collected at different time points.

We elaborate on the details of each of these new methods herein and discuss their potential advantages. Furthermore, we propose the use of a new metric for the quantification of neuroprotection based on novel biological insight revealed by these new methods.

Monitoring neuronal demise after transection of the cell bodies from radiating neurites in a DRG explant culture is a classic model used for the

study of neurodegenerative processes. The typical morphological changes that neurites undergo following trauma or toxic exposure are known as Wallerian degeneration (9). This process begins with a latent period following the trauma or toxic insult during which time no morphological changes can be detected and then progresses with swelling of the axolemma and disintegration of the cytoskeleton, forming bead-like elements along the nerve fibers (10, 11). These fibers gradually fragment and become more irregular until only remnants remain (Figure 1A). Selected images from our neurodegeneration assay are shown (Figure 1B), highlighting these changes. Historically, several methods have been developed to assess Wallerian degeneration, including the counting of intact neurites and the measurement of neurite halos. For our purpose, we wanted the ability to benchmark our assays against one of these established methods and, because of its simplicity, chose IN, a binary criterion that has been widely used to quantify neurodegeneration (7,8).

Materials and methods

Generation of DRG explant cultures (day 1)

A single dose-response assay was performed to compare three different analytical methods to quantify neuroprotection afforded by treatment with NAD. Six DRG cultures were initially selected for each concentration of NAD to be evaluated, for the cut vehicle control and for the un-transected (uncut) vehicle control. Following culture, treatment, and transection, three to six DRGs were photographed and analyzed at each time point for each treatment group. Experimental conditions were based on the protocol utilized by Araki et al. (7). Neurites obtained by this method are mostly axons as shown by positive immunostaining for tau and negative map2 stain (data not shown) (12).

Each well of the 24-well culture plates was pre-coated with collagen (C7661; Sigma, St Louis, MO, USA) and filled with 1 mL of pre-warmed Neurobasal medium (Cat. no 21103049; Invitrogen, Carlsbad, CA, USA) freshly supplemented with B27 supplement (Cat. no. 17504–044, Invitrogen), penicillin-streptomycin (Cat. no. 15070–063, Invitrogen), and 50 µg/mL mouse nerve growth factor (mNGF, 2.5S, Cat. no. 11362348001; Roche, Indianapolis, IN, USA). DRGs were collected from six CD1, E13.5 mouse embryos (Charles River Laboratories, Wilmington, MA, USA) and placed individually in

pre-coated wells of the culture plates. Each DRG was centered within the well using a slightly bent 23-gauge needle on a 1-mL syringe barrel, and cultures were incubated at 37°C with 5% CO₂. The DRG cultures were allowed to mature for 12 days, during which interval one-half of the culture medium was replaced every third day with freshly supplemented medium. On day 12, fields were selected for observation using a Zeiss Axiovert 200 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA) with a mechanized stage and Axiovision software (Carl Zeiss Microimaging, Inc.). After recalling specific orientation calibration settings for each plate, the 20× objective with phase-contrast was used to identify and mark (by saving *x* and *y* coordinates) at least four distinct fields around each DRG at approximately equal distances from the center of the culture.

On day 13, the DRG cultures received their respective treatment, NAD (0.125, 0.25, 0.5, 1, 2, and 4 mM NAD) or water (vehicle control in both cut and uncut control DRG cultures). The NAD stock solution [N1511 (Sigma), 100 mM in water, buffered to pH 7.0] was diluted in freshly supplemented neurobasal medium to the desired final concentration, and old culture medium was replaced with 1 mL of the respective drug solution.

Transection (day 14, 0 h)

After 24 h pre-incubation with NAD or with vehicle (water), all but the uncut control DRGs were transected. All of the cell bodies were cut away from the radiating neurites and removed using a 23-gauge needle, while the culture plate was mounted on a Nikon 100 inverted microscope (Nikon Instruments, Inc., Melville, NY, USA) using 10× magnification. Each well was then covered with 500 μ L of sterile mineral oil (M5310; Sigma) for better imaging throughout the remainder of the experiment. Previous experiments had established that this treatment had no effect on the health or rate of neurodegeneration of the DRG cultures. Each previously designated DRG field was immediately photographed at 0 h post-transection, and cultures were returned to the incubator.

Photography (0–90 h)

At specified time points post-transection (0, 6, 12, 24, 48, 72, and 90 h), the DRG culture plates were removed from the incubator to the microscope stage. Using a Zeiss inverted microscope (Axiovert 200) with a mechanized stage, the Axiovision

Figure 1. Wallerian degeneration. (A) Sketch of a DRG culture undergoing Wallerian degeneration. At 0 h, fibers of all thicknesses are smooth. By 24 h post-insult, the fibers become rough in appearance, but only the thin fibers are fragmenting and some have disappeared altogether. With the passage of time fibers of all calibers assume a more beaded appearance and eventually fragment as seen at 66 h. (B) Morphological features of mouse DRG neurites (primarily axons) undergoing Wallerian degeneration, photographed at 20× using phase microscopy with a Zeiss Axiovert 200 inverted microscope. Each image is 1000 × 1000 pixels (1 μ m = 0.7 pixels). (a) Thick fiber, intact and smooth along entire length. (b) Thick fiber, intact with moderate granularity. (c) Thick fiber, very rough with slight fragmentation. (d) Thin fiber, very rough and largely fragmented. (e) Fiber remnants. (f) Sites of fragmentation.

imaging software allowed the stage to be moved to each previously designated field where a photograph could be taken after minor focusing when necessary. Each image was identified by plate, field position, and time post-transection and bore a unique identifying number. The images were saved as Axiovert files and later converted to other image formats for analysis.

Analysis by manual methods

All images were scored by two manual methods. For one method, the percentage of IN was determined by scoring a minimum of 100 fibers as fragmented or intact. The second method, the NQI, required scoring each photograph by comparison to general descriptions of

neurites in progressive stages of neurodegeneration (Table 1).

Automated analysis by Neurosight

All photographs were batch-converted to tagged image file format (TIFF) using the Axiovision export function. Each series of photographs was analyzed for similarity to the 0-h image using proprietary machine vision software—Neurosight—and follows the steps detailed in the “Results and discussion” section and the workflow depicted in Figure 2. A sample of raw data generated by the program can be found in the Supplementary Materials.

Statistical analysis

All data was analyzed and graphed using Microsoft Excel 2003. Two-way ANOVA

Figure 2. Workflow steps in Neurosight acquisition and data processing. Photographs were taken of neurites from mouse DRG cultures at specific time points following transection and analyzed by Neurosight to quantify the degradation relative to the time zero image. (A) Images in this study have been registered to initial time point. Registration correction varied by up to 10% of total visual field (8% as shown in this example). (B) A masking function was implemented to normalize background between samples due to varying cell density between DRG cultures. (C) Self-similarity index in which subsequent time points are compared with 0-h image was calculated and scaled such that 1 corresponds to identity and 0 to nonidentity.

Table 1. The neurite quality index key

Score	Description of DRG culture image
10	Thick, intermediate, and thin fibers present, intact along entire length and all mostly smooth.
9	Thick, intermediate, and thin fibers present, intact along entire length, mostly smooth, but some fine granularity present primarily in the thin fibers.
8	Thick, intermediate, and thin fibers present with fine granularity; moderate granularity and slight fragmentation of thin fibers.
7	Thick and intermediate fibers present with moderate granularity; rare fragmentation of intermediate fibers; thin fibers very rough and moderately fragmented.
6	Thick and intermediate fibers show marked (heavy) granularity; rare fragmentation of intermediate fibers; thin fibers very rough and moderately fragmented.
5	Thick fibers show marked granularity but rare fragmentation; intermediate fibers very rough and slightly fragmented; thin fibers very rough and largely fragmented.
4	Thick fibers predominate with marked granularity and slight fragmentation; intermediate fibers very rough and moderately fragmented; thin fibers very rough and largely fragmented.
3	Thick fibers predominate with extremely heavy granularity and moderate fragmentation; intermediate fibers very rough and largely fragmented; thin fibers are remnants only.
2	Thick fibers predominate with extremely heavy granularity and abundant fragmentation, some still appear intact; all other fibers are now mostly remnants.
1	A few thick fibers may exist with extremely heavy granularity and abundant fragmentation; all other fibers are now remnants.

was performed utilizing Bonferroni post-hoc analysis.

Calculation of latent phase

Latent phase was defined as the period of time before which the first derivative of the degradation curve produced a negative integer. Calculations were performed on each sample with groups formed and queried using two-way ANOVA.

Results and discussion

Our premise in developing the NQI was that early subtle changes in neurite morphology such as focal swelling of the axon membrane and granulation along the nerve fibers were not adequately captured using existing schema, potentially missing early cues in degeneration that may have use in neuroprotectant screening (Figure 1B). In order to address these issues, the NQI was developed based on a 10-point scale encompassing more detailed cellular morphometric criteria than fragmentation alone (Table 1). In an image of 1000×1000 pixels (1 pixel represents $\sim 0.7 \mu\text{m}$) the relative thickness of the fibers allowed them to be classified as thick ($\sim 14 \mu\text{m}$ or greater in diameter), thin ($\sim 3.5 \mu\text{m}$ or less in diameter), and intermediate ($\sim 3.5\text{--}14 \mu\text{m}$ in diameter). Therefore, NQI accounts for the fact that smaller diameter axons have shorter latent periods prior to onset of fragmentation and faster rates of degradation than axons of larger caliber (9,13,14), adding a potentially critical metric to this screen.

While better reflecting the visual hallmarks of neural degeneration, time, observer fatigue, cost, and subjectivity remain significant issues when scoring neurodegradation based on the NQI. To overcome these limitations and enable higher throughput drug screening, we collaborated with Reify Corporation to develop Neurosight, a machine vision application based on their Visible technology (15) designed to quantify morphometric changes observed under visible or fluorescent conditions. Through the production of aggregate change indices (15), Neurosight measures the progression of visible biological phenomena throughout neurodegeneration. The utility in producing change indices centers on the capacity to generate self-similarity measurements for a series of images taken of the same field over time. Several critical aspects of Neurosight's design allow it

to approach the sensitivity of human scoring (Figure 2). First, it utilizes robust image registration algorithms for precise alignment of images from different time points to compensate for misalignment. In the series of assays which comprise the data set for this report, images were misregistered by up to 120 pixels but more typically by no more than several in the x and y axes. As depicted in Figure 2, misregistration of up to 10% was handled by the program. Second, to account for potential differences in cell density between cultures, an image mask was created to distinguish the area of interest for self-similarity analysis to assure that the self-similarity calculation was derived from neurite-populated areas only. Additionally, it eliminates spurious data due to new growth and assures contribution to the similarity index from the disappearance of thin fibers. This is not possible in either of the two methods previously described. Finally, the masking feature has potential to be applied to dispersed neuronal cultures, thereby expanding screening opportunities.

As expected, the plots resulting from IN, NQI, and Neurosight show typical degradation curves similar to previous reports (7,8) (Figure 3, A–C). Additionally, recapitulating earlier findings (7,8), IN, and the other scoring methods, demonstrated a significant ($P < 0.01$) protective effect from the lowest dose of NAD used compared with the untreated sample after 35 h. Furthermore, the neuroprotective effects of NAD treatment cluster roughly into two groups with NAD doses less than 1 mM showing a weaker neuroprotective effect than the higher three doses. Statistical inspection of this grouping shows that there are no statistically relevant differences in any scoring system between doses in the group spanning 1 mM and higher until 66 h and later. For the purposes of screening candidate neuroprotective molecules, these late time points are of questionable relevance. In the group of doses under 1 mM, NQI was the only method able to distinguish between NAD levels, perhaps due to the additional early hallmarks that comprise its score. Similarly, NQI was the first quantitative method to detect statistically significant differences between NAD treated and untreated samples (15 h). Neurosight was able to distinguish significant differences in these groups at 25 h and IN at 35 h.

To explore other potential degeneration events that could form the basis for high-throughput screens, we derived

Figure 3. Post-transection latent phase and decay plots of DRG cultures pretreated with varying doses of NAD. (A and D) Plots are based on analysis of the percentage of intact neurites in images of DRG cultures taken at different time points. (B and E) Plots are based on analysis of neurite images by the NQI. (C and F) Plots are derived from Neurosight analysis of neurite culture images. Statistical analysis (see “Materials and methods”) on degradation curves (A–C) demonstrated a significant ($P < 0.01$) protective effect from the lowest dose of NAD used compared with the untreated sample (0) after 35 h in all measurement assays. Additionally, data clusters roughly into two groups with NAD doses less than 1 mM showing a weaker neuroprotective effect than the higher three doses and NQI as the most sensitive scoring system for early degeneration events. In measuring the latent period preceding degeneration (D–F), all methods demonstrated a statistically relevant difference between 4 mM NAD and the positive control ($P < 0.01$), but only Neurosight demonstrated a relevant difference between all treated groups versus untreated samples.

the length of the latent period prior to degeneration for each treatment group by each analytical method (Figure 3, D–F). All scoring methods show a trend toward correlation between NAD dose and the length of the latent period. Not unexpectedly, given that fragmentation is a later-occurring event in degradation, the latent period is somewhat longer in IN than in NQI and Neurosight analyses. It is noteworthy that all methods demonstrated a statistically relevant difference between 4 mM NAD and the positive control ($P < 0.01$), but only Neurosight found a relevant difference between all treated groups versus controls. Neurosight did not demonstrate significance between doses, however. Whether this is a true limitation of the model or the analytical methods used remains to be elucidated.

Intradose comparisons, however, would require additional sampling around the end of the latent phase to confirm or refute significance. To our knowledge, this is the first demonstration of a correlation between drug dose and an effect on the latent phase. To further assess the veracity and potential of this metric in drug discovery, it would be instructive to over-sample time points surrounding the latent phase. Further, the general differences in slope of the curves post-latent phase have lead us to postulate that an additional effect of NAD in this system may be to alter the rate of decay. Here too, additional research on this trend including more data points after the onset of degradation will be required to confirm this hypothesis.

A final distinction between these methods, not apparent from the plots,

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is the time taken to derive the metric for each image. Analysis of by IN and NQI each require 1–3 min per image, whereas Neurosight analysis takes ~1 s. In this experiment where an average of four replicates per treatment (eight treatments) were evaluated over 10 time points, the time spent per image difference resulted in 10 h of investigator's time for analysis on average for IN and NQI versus minutes of computer time for analysis by Neurosight.

In conclusion, we have shown that the NQI more accurately reflects the biological phenomena of degradation than IN by encompassing a broader spectrum of visual phenotypic change taking place within neurites undergoing degradation. However, it is still extremely time-consuming and subjective. Neurosight, through its production of self-similarity indices, provides an objective, rapid and flexible analysis that is scalable to medium- and high-throughput formats. As seen herein, Neurosight is not presently as sensitive as the NQI manual scoring method. Analysis by Neurosight using 16-bit image capture rather than the 8-bit used in these experiments could improve registration and sensitivity. Nonetheless, the software's ease of use in capture and time saving provides the only viable option, to our knowledge, for high-throughput drug screening and can be improved in future versions. Based on the data presented here, we propose that NAD acts to delay the onset of decay and may also affect the rate of degradation post latent-period. Furthermore, pending verification, the length of the latent period may be considered as a new metric for neurodegradation as it would make assays much faster. However, it is possible that other therapeutic agents could act independently to either delay degradation onset or slow the rate of decay. More work is needed to address these intriguing questions.

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Competing interests

T.M. and A.M.G are employees of Reify Corp., the company that developed and currently licenses Neurosight software.

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