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Quantifying Levels of Dopaminergic Neuron Morphological Alteration and Degeneration in Caenorhabditis elegans.

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Title:

Quantifying Levels of Dopaminergic Neuron Morphological Alteration and Degeneration in *Caenorhabditis elegans*

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Summary:

In this article, we showcase how to use a seven-point scoring system to consistently quantify changes to dopaminergic neuron dendrite morphology in *C. elegans*. This system is intended for analyses of dopaminergic neurodegeneration assays utilizing genetic, chemical, and age-based models of neurodegenerative disorders.

Abstract:

Dopamine neuron loss is involved in the pathology of Parkinson's Disease (PD), a highly prevalent neurodegenerative disorder affecting over 10 million people worldwide. Since many details about PD etiology remain unknown, studies investigating genetic and environmental contributors to PD are needed to discover methods of prevention, management, and treatment. Proper characterization of dopaminergic neuronal loss may be relevant not only to PD research, but to other increasingly prevalent neurodegenerative disorders.

There are established genetic and chemical models of dopaminergic neurodegeneration in the *Caenorhabditis elegans* model system, with easy visualization of neurobiology supported by the nematodes' transparency and invariant neuronal architecture. In particular, hermaphroditic *C. elegans'* dopaminergic neuron morphological changes can be visualized using strains with fluorescent reporters driven by cell-specific promotors such as the *dat-1* dopamine transporter gene, which is expressed exclusively in their eight dopaminergic neurons.

With the capabilities of this model system and the appropriate technology, many laboratories have studied dopaminergic neurodegeneration. However, there is little consistency in the way the data is analyzed and much of the present literature use binary scoring analyses that capture the presence of degeneration but not the full details of the progression of neuron loss. Here, we introduce a universal scoring system to assess morphological changes and degeneration in *C. elegans*' cephalic neuron dendrites. This seven-point scale allows for analysis across a full range of dendrite morphology, ranging from healthy neurons to complete dendrite loss, and considering morphological details including kinks, branching, blebs, and breaks. With this scoring system, researchers can quantify subtle age-related changes as well as more dramatic chemical-induced changes. Finally, we provide a practice set of images with commentary that

can be used to train, calibrate, and assess the scoring consistency of researchers new to this method. This should improve within- and between- laboratory consistency, increasing rigor and reproducibility.

Introduction:

Parkinson's disease (PD) is an increasingly common neurodegenerative disease affecting up to 10 million individuals worldwide[1]. Males and older individuals are at a higher risk for developing PD; the average age of onset for the disease is 60 years, and PD incidence climbs from a 0.3% incidence in the general population to 3% in individuals over 80 years of age[1,2]. Although the details of PD pathology are not fully understood, this progressive disorder involves the loss of dopaminergic neurons in the substantia nigra region of the midbrain. Hypothesized mechanisms of this neuronal loss involve mitochondrial dysfunction, oxidative stress, and inflammation[2]. The causes and risk factors for the disease are still being explored, but involve a combination of environmental and genetic factors[1]. For example, studies have found positive associations between lifelong pesticide use and PD, as well as genetic susceptibility to familial PD[1,3].

The *C. elegans* model system, originally developed in part for neurobiology research[4], is well suited for evaluating dopaminergic neuron loss *in vivo*. Nass and colleagues pioneered the use of *C. elegans* for dopaminergic neurodegeneration[5], and many groups have since adopted the worm as a successful model for PD and dopaminergic dysfunction[6-20]. *C. elegans* are good neurodegenerative disease models for many of the same reasons that they are such a popular model organism for other areas of biology; their transparency allows for *in vivo* study of cellular processes, genetic manipulation in worms is relatively quick and easy, they have a short generation time of about three days, and they are easy to maintain[21]. Most PD worm models fall into one of three categories: age-based models, chemical models, and genetic models. The ability to synchronize a population of worms allows for the study of age-related neurodegeneration for an age based-model of neurodegenerative diseases associated with aging, such as PD[22]. Chemical exposures inducing PD-like neuronal defects have been established using a variety of chemicals including 6-hydroxydopamine (6-OHDA), rotenone, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[22]. Worms are also successfully used as genetic models of PD; strains with select neural gene knockouts can model various neurodegenerative diseases[1,4]. Combinations of genetic and environmental factors, or "gene-environment interactions," which likely play a major role in PD[2,17,23-28], have been examined by several groups using *C. elegans*. Finally, age-related dopaminergic neurodegeneration has also been observed[29,30]. If using an appropriate neural transgenic strain in fluorescent imaging, any of these PD worm models may be used to study dopaminergic neurodegeneration.

Quantifying changes to neuronal morphology is a critical component of neurodegenerative research. In *C. elegans*, many fluorescent reporter strains have been used to visualize morphological changes and loss of neurons. Strains suitable for neuronal imaging feature a fluorescent protein associated with cell-specific promoters. For dopaminergic neurodegeneration assays, our laboratory has used the BY200 [vtIs1 (*dat*-1p::GFP, *rol*-6)] strain, which has a green fluorescent protein (GFP) tag in the *dat-1* gene, expressed in the

dopaminergic neurons. Note that the BY200's roller phenotype has a very low penetrance and is rarely observed. Other common strains used for this type of imaging include BY250 [*dat*-1p::GFP], BY273 [baEx18[dat-1p::GFP+*dat*-1p::WT α-syn]], BZ555 [egIs1 [*dat*-1p::GFP]], and several others available from the Caenorhabditis Genetics Center (CGC) or upon request from specific laboratories[1,21,22,29]. These strains typically allow for visualization of all three classes of dopaminergic neurons: cephalic (CEP), anterior deirid (ADE), and postdeirid (PDE) neurons. *C. elegans* does not naturally express the alpha synuclein protein, but strains such as BY273 have been engineered to express it. However, we note that the scoring system we present was developed using BY200, which does not express alpha synuclein, and would need to be validated with that strain (or any other new strain) prior to use. Additional dopaminergic neurons are present in males but are rarely considered because males normally comprise <1% of a *C. elegans* population. Here, we focus on the four CEP dopaminergic neurons found in the head region of *C. elegans*. This set of neurons is easily located under fluorescent microscopy, is present in both hermaphrodite and male worms, does not typically overlap with other areas of auto-fluorescence, and is commonly reported on in worm studies. A healthy set of CEP dendrites typically displays as relatively straight, uninterrupted lines. Degenerated dendrites may show any combination of irregularities and signs of damage, including pronounced dots called blebs along the line of the dendrite and breaks in the line of the dendrite. Examples of CEP neurons at varying levels of degeneration can be seen in **Figure 1**.

Although dopaminergic neurodegeneration is being studied by a growing number of *C. elegans* laboratories, there has been a large variation in analytical methods of quantifying dopaminergic neuron damage[29,31-34]. Many published studies have reported on the presence or absence of CEP soma with a binary scoring system of degenerative versus typical or wild type neurons[31,32]. These scoring methods can identify certain stressors that induce neurodegeneration but cannot quantify the details of the progression of more subtle neuronal damage, or easily detect differences between neurodegeneration as induced by unique chemicals or other variables. Additionally, scoring systems focused on the cell bodies may not be sensitive to less severe levels of damage or to neuronal damage affecting only part of the cell, such as the dendrite. Since the dendrite appears to have the largest range of consistently detectable morphologic changes in response to chemical stressors, we have selected them as the basis for our analysis. The scoring system we present here is modified from dendrite morphology based multi-point scales that have been previously used in our lab[29,33]. This system expands these five- and six-point scales into a seven-point scale to account for age-related morphological changes, such as higher expected numbers of kinks in older adult dendrites, and to differentiate between severe damage and complete dendrite loss. The purpose of introducing this scoring system is to provide the ability to capture a comprehensive picture of neurodegeneration at all levels of neuronal damage and provide a universal system to support consistency across *C. elegan* dopaminergic neurodegeneration research. Because scoring is inherently subjective, it is critical to maximize consistency between individuals scoring, and to blind the scorer to the identity of the images using manual blinding or an automatic blinding program[35]. To improve consistency, we present a series of training images and utilize JoVE's video capabilities to demonstrate our scoring system in detail. We recommend using a system that both permits automated blinded scoring and allows the scorer to quantify her or his scoring consistency by re-scoring a subset of

images. This is particularly important when combining or comparing data from multiple scientists, or training scientists new to scoring.

Protocol:

1. Prepare Worms for Imaging

NOTE: See related JoVE video article[31]: https://www.jove.com/v/835/

1.1. For each experimental group, pipette or pick 20 to 30 worms to an imaging platform compatible with the imaging microscope. Most common platforms include 2% agarose gel pads mounted on glass slides with a coverslip[31] and 96-well plates containing well volumes at or less than 100 µL of liquid medium.

1.2. Paralyze the worms by adding 30-90 mM sodium azide (NaN_3), 2.5-8.5 mM levamisole HCl, or other paralyzing agent to the worms. If paralyzing in liquid, use a higher concentration of paralyzing agent than if paralyzing on agarose pads. Tap imaging platform gently to mix.

1.3. Allow worms to paralyze completely.

NOTE: This may take several minutes.

2. Image Dopaminergic Neurons

2.1. Locate worms' head regions under single-color GFP fluorescence using imaging microscope capable of taking z-stacks.

2.1.1. Be mindful of exposure and aperture settings; avoid overexposure of dendrites and keep settings consistent across trials. Make the dendrites as bright as needed for clear visualization; this typically results in overexposure of the soma.

NOTE: Images included in this protocol were captured using 400x magnification.

2.2. Scroll through the focus to find upper and lower bounds where the dendrites are clear. Set these as upper and lower bounds for a z-stack image capture.

2.3. Click to capture z-stack images for each worm.

NOTE: All following steps may be performed at any time.

3. Prepare Dopaminergic Neuron Images for Scoring

3.1. For each z-stack, open the image file using either the microscope software or an external image analysis software, load the stack in the software, and compress the stack into a single flattened image.

3.2. Blind images between and within treatment groups manually or using automatic blinding software.

4. Score Dopaminergic Neuron Dendrites

4.1. Work with one neuron image at a time. Choose one of the four CEP dendrites to assess for blebs, breaks, and irregularities including bends, kinks, and curves. Scoring from the left to right when the nose is at the top of the image is recommended to ensure repeatability in scoring.

4.2. Using the following guidelines, assign one score value to the dendrite. See **Figure 1** for representative scoring image examples.

0- no damage, "perfect" neurons
1- irregular (kinks, curves, etc.)
2- <5 blebs
3- 5-10 blebs
4- >10 blebs and/or breakage removing <25% of total dendrite
5- breakage, 25-75% of dendrite removed
6- breakage, >75% dendrite removed

4.2.1. If multiple criteria are met within a single dendrite (i.e., kinks and blebs), assign the highest applicable score.

4.2.2. Do not score dendrites that are not clearly visible, due to issues with image capture, overlapping with other dendrites, etc. If zooming in on a flattened z stack image, be mindful of enlarged pixels resembling false blebs.

4.3. Repeat for each dendrite. Repeat for all images.

4.4. Record all scores. Scores may be un-blinded at this time.

5. Prepare and Present Data

5.1. Calculate the total number of dendrites in each treatment group assigned to each neurodegeneration score. Calculate the total number of scored dendrites in each treatment group.

5.2. Divide the neurodegeneration score tallies by the total number of dendrites scored in the treatment group. Present data as a proportion of dendrites within a treatment group at each neurodegeneration score.

6. Perform Statistical Analysis

6.1. Using a programming software or manually, run a chi-squared test for independence between all treatment group pairs to be compared. When appropriate, apply a Bonferroni correction of the p-value according to the number of compared experimental groups to account for multiple comparisons.

NOTE: This test will determine significant differences between two groups, but details of the type of difference must be qualified by eye.

6.1.1. Select comparisons between experimental groups. This will vary based on experimental design.

NOTE: In our experiments, typically, controls are compared to their respective treatment groups, all controls are compared, and all treatments are compared.

7. Practice Scoring with Practice Set of Dopaminergic Neuron Images

7.1. See **Supplementary File 1** for a set of neuron images presenting across the full range of our scoring system with commentary and score key. This practice set is intended to train researchers new to this method and ensure inter-rater reliability.

8. Consider Alternate Protocol Options

8.1. Instead of capturing z-stacks, complete scoring at the microscope, without saving or stacking images.

NOTE: This option reduces requirements for technology capabilities, but removes the option for creating an archive of neuron images to return to at a later time, requires manual blinding, and permits blinding only between and not within treatment groups.

8.2. Instead of creating a single compressed image per stack, complete scoring by scrolling through the images of each z-stack.

NOTE: This option may be easier for some scorers and it reduces the risk for seeing false blebs on worms that moved during imaging and allows for scoring overlapping dendrites, but requires manual blinding and permits blinding only between and not within treatment groups.

Representative Results:

The scoring system described here was used to assess neurodegeneration in L4 larval stage BY200 [vtIs1 (*dat*-1p::GFP, *rol*-6)] *C. elegans* after rotenone exposure. Results of this experiment are shown in **Figure 2** and represent our scoring system's ability to detect and quantify variable levels of dopaminergic neuron damage. Rotenone is a naturally occurring electron transport

chain complex I inhibitor used in some pesticides, piscicides, and insecticides[36,37]. Note that working with toxic chemicals such as rotenone is inherently dangerous, and all labs should comply with all use and disposal regulations set by their institutions. In this experiment, liquid rotenone exposures at two doses, 0.03 µM and 0.5 µM, along with a control group, were begun immediately following a 0.5 M sodium hydroxide/1% sodium hypochlorite lysis to harvest eggs[38]. Eggs hatched in complete K-medium[33,39] with 0.25% dimethyl sulfoxide (DMSO), and worms remained in liquid for ~48 hours until mid-L4 larval stage, at which point they were removed from chemical exposure, and prepared, imaged, and, using **Figure 1** as a reference, scored according to the protocol steps above. For the higher dose of 0.5 µM rotenone, eggs were harvested 24 hours in advance to account for a rotenone-induced developmental delay and ensure all worms were stage synchronized at the time of imaging.

Figure 2 further demonstrates how our laboratory visualizes data collected using this scoring system. In this figure, a dose dependent neurodegeneration response can be appreciated, and the specific breakdown of the score distribution is displayed clearly. These particular results showcase how neuronal damage can present in different ways. For example, the 0.03 µM rotenone-exposed group has a decreased proportion of healthy neurons with a score of 0, as compared to the control group, yet also has a decreased proportion of 5 scores. Detecting this detail about the score distributions between experimental groups highlights the sensitivity of our seven-point scoring system. This data was analyzed for statistical significance according to the protocol, using a chi-squared test for independence with a Bonferroni correction.

Figure and Table Legends:

Figure 1. Dopamine neuron morphological alteration and degeneration scoring system representative images. This consolidated chart contains examples of neurons at each score and is intended to be used as a reference for scoring. Here, each labelled score corresponds to the most damaged dendrite in each worm, as indicated by the arrow in each panel. These images were taken using the protocol described in this paper with BY200 *C. elegans*. Please see **Supplementary File 1** for a set of scored images with commentary to be used for training those new to this scoring method.

Figure 2. BY200 L4 dopamine neuron morphology and degeneration scores after rotenone exposure. This figure shows representative results analyzed using the scoring methods described here. The visualized greater proportions of damaged dopaminergic neurons with higher rotenone exposure concentrations were statistically analyzed using chi-squared tests for independence. Both rotenone treatment groups yielded statistically significant p-values when compared to the control group. Different letters indicate statistical difference.

Discussion:

This protocol demonstrates how to use the seven-point scale developed in our laboratory to quantify levels of dopaminergic neuron morphologic alteration and degeneration in *C. elegans*. We created and shared this scale as a tool to standardize analysis of dopaminergic neurodegeneration work in worms. Recognizing the importance of studying pathways involved in highly prevalent neurodegenerative diseases, many investigators take advantage of the *C.*

elegans model's suitability for neurobiology visualization to study neurodegeneration[29,31-33]. However, there has yet to be an effort to reduce the large variation in how neuron damage is quantified across neurodegeneration research in worms. The scoring system presented here is thus intended to promote consistency in analyses and allow for comparison between studies.

Our scoring system may be used to analyze data derived from *C. elegans* experiments that use cell-specific fluorescent reporters that allow for visualization of dopaminergic neurons – specifically the CEP dendrites. Specifically, strains tagged at the *dat-1* gene for GFP visualization of the dopaminergic neurons are compatible with this scoring protocol, though many other related transgenic models of PD do exist. It is possible that this scoring system would also be useful with those models; however, this should be validated prior to using them. In particular, it is possible (but not tested to our knowledge) strains with mCherry may not be well suited for this protocol as mCherry aggregation may be indistinguishable from blebs or lead to cell stress. Rather than providing a commentary on all specific models of PD and related neurodegenerative disorders, we focus on the scoring of neurodegeneration data itself. Additionally, this protocol focuses only on neuronal morphology and does not consider fluorescence levels of the soma. Neurodegeneration assays may be performed alongside behavioral assays relevant to neurodegenerative diseases, such as locomotion, lifespan, and health-span experiments. Levels of degeneration in established chemical, age-based, and genetic models of PD can also be confirmed and detailed using this scoring system. Measuring models, contributors, and pathways associated with PD and other neurodegenerative diseases can add to the scientific knowledge about these disorders and point towards how to manage the growing population of affected individuals. Having comparable neurodegeneration results across literature is key in supporting this goal.

To interpret the results derived from this scoring system, we propose considering each dendrite scored as n=1, because different neurons within the same worm often respond differently to treatment. This allows the score spread of experimental groups to be displayed as proportions of the total number of dendrites scored in each group. This method, used for the representative results shown here, allows for easy comparison across treatment groups, accounts for differential responses within the same worm, and is easily analyzed with a Chi-squared test complimented by a Bonferroni correction for multiple comparisons. An example template for recording neuron scores and calculating percentages can be found in **Supplementary File 2**. We have considered two alternate methods for data analysis and identify flaws in each. The first option is averaging the scores of the four CEP neurons for each worm. This parametrizes the data; however, it assumes a linear relationship with increasing score and loses information about any variations in response to treatment within the same worm. The second option is to sum the scores of all four CEP neurons for each worm, which also parametrizes the data. This still assumes a linear relationship between scores, however it more capably accounts for differences within each worm than averaged scores by expanding the parameters of possible scores. However individual researchers decide to display their data, the results should be considered alongside experimental variables such as strain and worm age; for example, older worms have a higher expected baseline level of degeneration.

As these neurodegeneration score results are interpreted, researchers should also be aware of a few caveats and limitations of the scoring method. First, certain technological requirements are necessary to capture images suitable for scoring. The imaging microscope must support fluorescence channels and magnification and exposure settings that allow for clear visualization of CEP dendrites. As noted in the protocol, technological requirements may be reduced by protocol adjustments like scoring live images through the microscopic field rather than capturing images to be archived and scored at a later time. Second, possible statistical analysis methods for this data are limited as the data is non-parametric. The scoring scale is presumed to be progressive, but cannot be considered numeric since there are discrete score options and score increases are not necessarily proportional to each other with respect to biological function. For these reasons, chi-squared tests for independence are best suited for this type of data, meaning the statistical analysis depends on the observer to determine the direction of any statistical significance. Notably, the chi-squared test also only analyzes for differences in score distribution and is unable to provide evidence of differences in specific scoring categories. Finally, the functional significance of the morphological changes quantified by this scoring system have yet to be studied.

The future directions prompted by the development of this scoring system involve determining biological bases and correlations with individual neuron scores. Studying the functional significance of all points on the scoring scale will inform how to better translate results to conclusions applicable to understanding the causes and consequences of neurodegenerative diseases and developing prevention and treatment options. Future research on neurodegeneration in worms should aim to discover connections to other morphology, such as worm shape and size. Additionally, neurodegeneration research can be supported by studying other reporter *C. elegans* strains to measure endpoints such as bioenergetics, reactive oxygen species production, and mitochondrial morphology.

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Disclosures:
The authors have no disclosures.

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Figure 1

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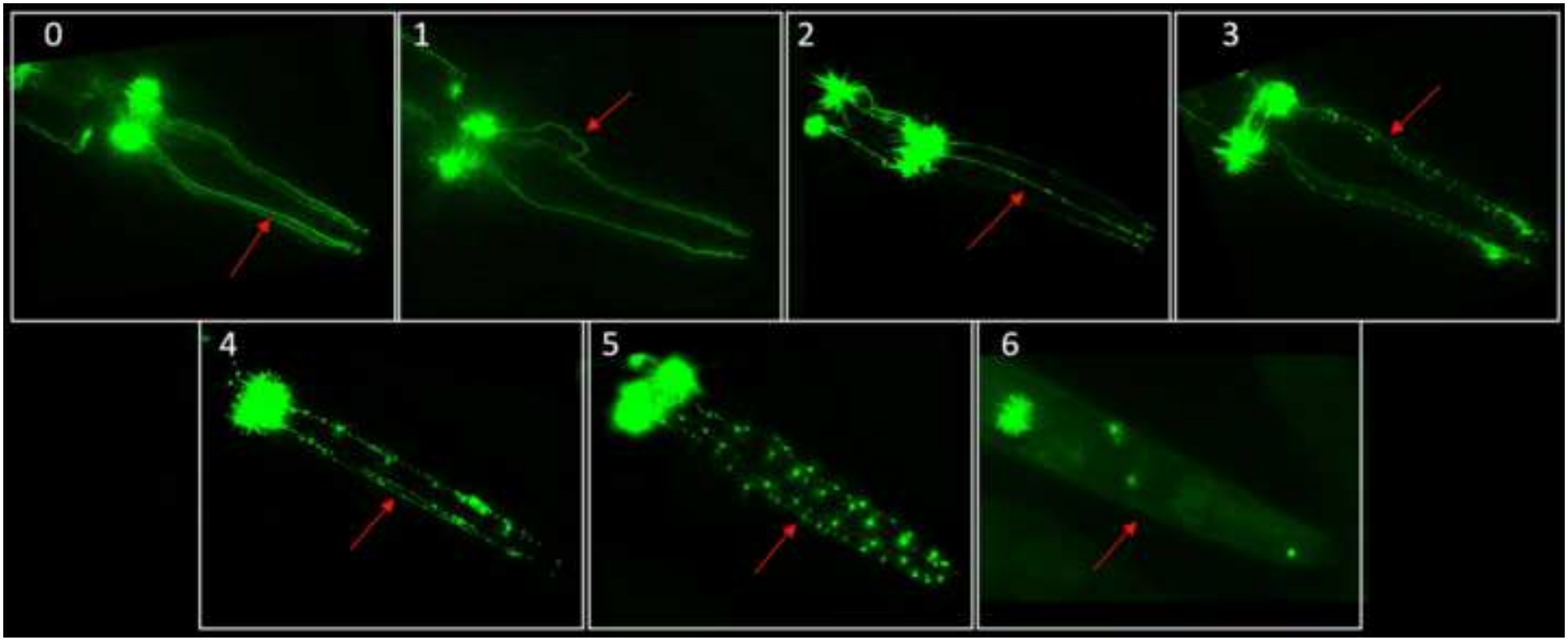

Figure 2

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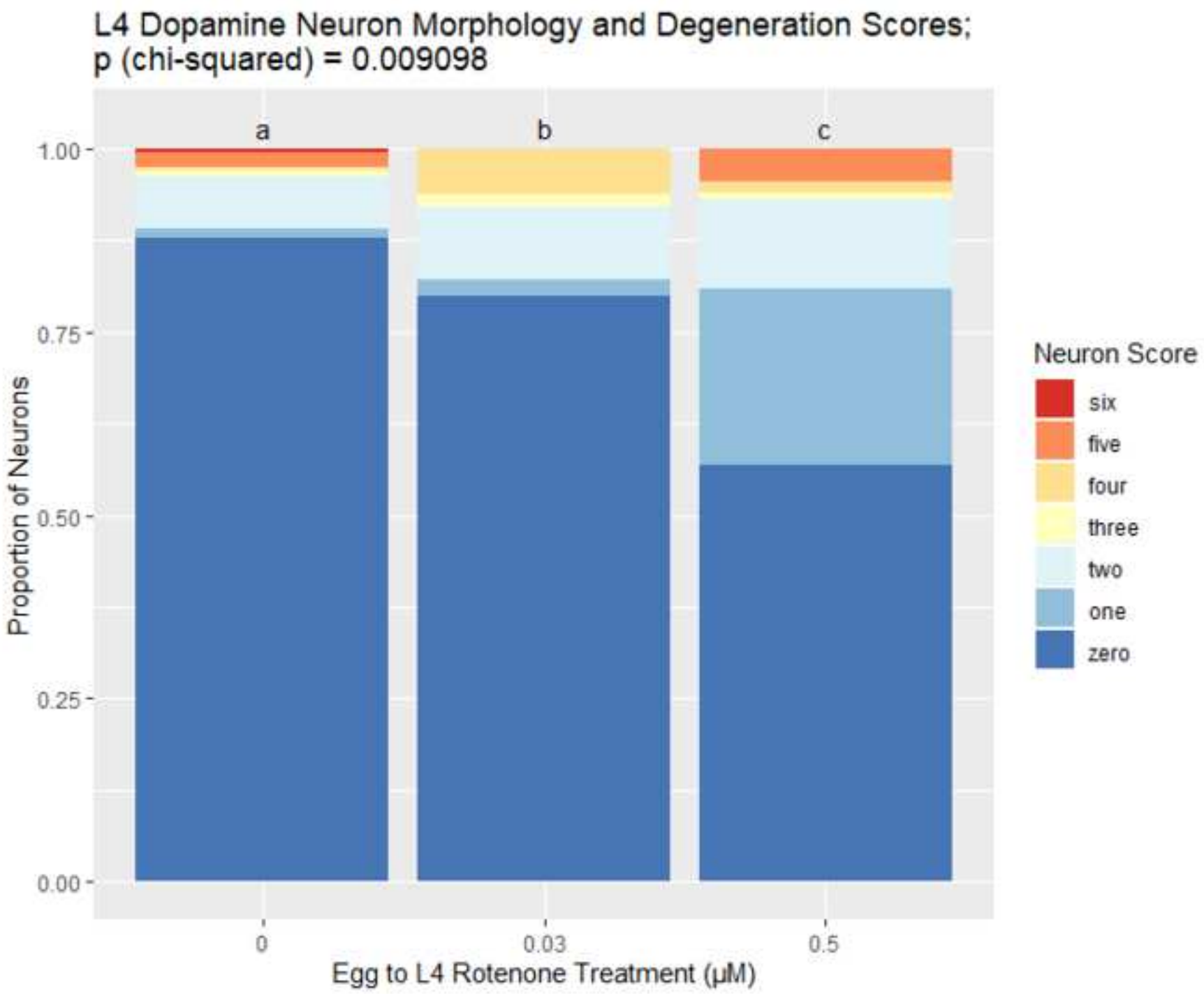


Dear Dr. Bajaj,

Thank you for reviewing our manuscript "Quantifying Levels of Dopaminergic Neuron Morphological Alteration and Degeneration in *Caenorhabditis elegans*" and providing the opportunity to submit our revisions. We have reviewed the careful feedback we received and done our best to address all the points brought up by the three reviewers and the editorial and production team. Please find new details, clarifications, and edits in our revised submission, highlighted with the "track changes" feature. Please also look below for responses to specific comments from each reviewer. In addition to addressing these comments and concerns, we also took this time to ensure the manuscript is well proofread and follows all JoVE formatting requirements. We very much appreciate the time spent by you and all our reviewers to ensure that our manuscript is in the best possible shape. We look forward to hearing back from you regarding our submission and are more than happy to address any further questions and comments you may have.

Respectfully,

Shefali Bijwadia, Katherine Morton, and Joel Meyer

Editorial and production comments:

Thank you for providing this feedback. We appreciate the attention to detail and the notes to ensure our manuscript is well received and fits the goals of your journal and its audience. We note below how we have responded to each of your comments.

Changes to be made by the Author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Manuscript has been thoroughly proofread by all authors.
2. Please make the title concise: Quantifying Levels of Dopaminergic Neuron Morphological 2 Alteration and Degeneration in Caenorhabditis elegans. Our title has been shortened as suggested.
3. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol. We have reviewed the abstract to ensure it is within word limit and contains the rationale, relevance, and goal of the protocol.
4. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Keyence BZ-X710 All-in-One Fluorescence Microscope, Keyence image file, Blinder, etc. We have removed all cases of commercial language from the manuscript. Specific commercial products we used are referenced in the table of materials.
5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." The protocol section has been amended to ensure formatting and phrasing follows JoVE requirements. Any text not written in imperative mood has been revised or moved to a "note".

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? This can be done by including mechanical actions, knob turns, button clicks in the software, etc. We have added key details to improve clarity in the protocol section. In particular, we have added specific actionable instructions such as "pipette or pick," "tap," and "click."
7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. We have reviewed the protocol section to ensure conciseness. Additionally, we have moved alternative protocol options, previously embedded within different steps of the protocol, into their own protocol step (step 8: Consider Alternate Protocol Options). This was intended to reduce instances of longer text throughout the key steps of the protocol.
8. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step. All protocol steps have been reviewed to confirm they are no more than 2-3 sentences.
9. Only one note can follow one step. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Notes have been checked to ensure they contain appropriate information and are each individually placed after one step.
10. Please ensure the Representative Results are described in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included. We have reviewed the Representative Results to ensure they are described with the presented protocol in mind. Please note that the first paragraph in this section describes the methods of the experiment used to generate the representative results, as the nature of our protocol has to do with the data collection and analysis rather than the experimental details themselves. A note has been added to mention **Figure 1** so that the text refers to all figures.
11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." The data presented in this manuscript is currently unpublished thus does not require copyright permission.
12. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

Please find these details in the paragraphs of the discussion section listed below:
a) Critical steps within the protocol Paragraph 3 (discussion of statistical analysis)
b) Any modifications and troubleshooting of the technique Paragraph 3 (presents two alternate options for analysis, along with caveats and suggestions)
c) Any limitations of the technique Paragraph 4
d) The significance with respect to existing methods Paragraphs 1 and 2
e) Any future applications of the technique Paragraph 5

Changes to be made by the Author(s) regarding the video:
1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.
2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

The protocol section of the video has been rearranged and re-recorded so that the narration reads word for word the written protocol, with minor deviations where needed to ensure clarity.
3. Introduction/Conclusion: Instead of having slides it will be nice to show this section as a person being interviewed on the camera or include some video shots. We have added video shots to both the introduction and conclusion section of the video.
4. Please do not show/narrate commercial terms in the video, VWR, Keyence, Blinder, etc. These narrations have been removed from the video. Any images containing commercial terms have been cropped or edited so as not to show the commercial terms. Please note that Blinder is non-commercial (though in the manuscript text and video narration we write/say "blinding software" to emphasize that any such software may be used).
5. 2:54: If possible, show worm images/ video shots for different scores. An additional worm image has been added at 2:30.
6. The representative results section should only show what are results obtained from the protocol being shown in the video. Any other details associated with the protocol should be in the protocol section. 5:03 this part needs to be moved to the protocol instead. Also instead of just showing the chemical please include a video shot of how it is used in the protocol. We have shortened this section to minimize the details of the rotenone exposure protocol, since these methods are relevant only to the particular experiment done to generate the representative results, and not to the protocol presented in the manuscript/video. We have chosen not to include these details in the protocol section to avoid confusion; any experiment using an appropriate strain can be performed and processed/analyzed according to our protocol. This is conveyed in the Introduction and Discussion.
7. Video Editing Content:
• Please downconvert the video maximum resolution for JoVE videos is 1920x1080 for 16:9 or 1440x1080 for 4:3. Videos larger than 1920x1080 is not acceptable. Video has been down-converted to 1440x1080 resolution.
• 00:05 - 00:49 Consider adding some video clips or image to pair with what is being explained in the narration. it will be easier to understand. Video clips have been added.
• 00:48 - 03:24 Consider adding some video clips or multiple image to pair with what is being explained in the narration. it will be easier to understand. This section's narration has been re-recorded in order to better reflect the manuscript, and video clips have been paired accordingly.
• 05:37 - 06:19 Consider adding some video clips or multiple image to pair with what is being explained in the narration. it will be easier to understand. Video clips have been added.
8. Audio Editing and Pacing:
• Audio are quite low. Please boost the audio level & ensure audio level peaks average around -9 dB. Audio levels throughout the video have been increased to 9 dB.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Bijwadia et al, provide a detailed protocol describing a scoring system for anterior dopaminergic neurons in C. elegans. I agree that this is an important contribution and fully support publication.

I have several points that can be improved, the most important one me urging the authors to better describe (highlighting key features by arrows) and extending the corresponding figure legend

degenerating neurons in their example Figures. Ideally, the video should be extended the narrator describing all example pictures. The second major comment relates to safe handling of compounds used for inducing neurodegeneration.

Dear reviewer #1,

Thank you for taking the time to review our submission. We have carefully reviewed your notes and made every effort to address all concerns and consider all recommendations. We appreciate your support for publication and are committed to using your thoughtful review to make this submission a successful contribution to the scientific community. Please find our specific responses to your point per point comments below.

Here my point per point comments:

IMPORTANT: I think the authors should comment on (and or provide a reference to) the safe handling (and inactivation) of the drugs used for inducing dopaminergic loss especially on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which passes the blood brain barrier and causes neurodegeneration. I think that this is one of the most dangerous drugs, that requires excessive safety procedures, and accidents handling MPTP occurred in the past. To my best knowledge 6-hydroxydopamine (6-OHDA) is much less critical it not passing through the blood brain barrier. Rotenone? (I assume no major safety concerns). It is important to ensure that ill equipped and unexperienced labs are not encouraged to use MPTP.

Thank you for recognizing the importance of working safely with toxic compounds and mentioning how we can better express this in our manuscript. We have added a general safety statement in the representative results section, where we describe our use of rotenone, a mitochondrial toxin. However, we kept this statement general, because researchers are testing more and more chemicals for their capacity for dopaminergic neurodegeneration. It is impossible to provide handling instructions for all that might be tested.

Please note that in most lab us use BY200 [vtIs1 (dat-1p::GFP, rol-6)] which largely does not show a roller phenotype (this phenotype having a very low penetrance). Please comment on this to avoid confusion.
It is out of the scope of this review, but I think that there is a case for generating a genome edited version of this reporter.
Please specifically mention that BY273 carries an alpha synuclein fusion, and that it thus should not be used for compound scoring!
Please mention that BY200 and BY273 (and other strains mentioned) are available at the GCG strain collection.

Thank you for sharing your thoughts and concerns pertaining to the mentions of specific strains in this manuscript. We agree that a genome edited strain would be useful. A mention of the low penetrance of the roller phenotype in BY200s has been added to the introduction section. We have also noted that scoring BY273 should not be done without validation, given that this strain carries an alpha synuclein protein (as a side note, we believe it may be interesting to test how a-syn expression might combine with chemical exposure in a gene x environment interaction; however, this is indeed beyond the scope of this manuscript). Please find the source of the BY200 strain, used for our experiments, listed in the List of Materials. To our surprise, it does not appear to currently be available from the CGC; therefore, we have

offered to make it available upon request. We have noted that many strains are available from the CGC, or upon request from specific laboratories.

At the end of the introduction section please add 1-5 lines describing a picture of normal and degenerating anterior dopaminergic neurons and refer to the first figure where this is shown. This way reading the methods section will become much easier.
Line 124-135 (and within the extended intro section (point above) please refer to your first Figure.

Details of typical normal and degenerated ADE neurons have been added to the end of the third paragraph in the introduction, with a reference to **Figure 1**.
Line 152 Scoring from the left to right when the nose is at the top of the image is recommended to ensure

indicate left right and 'nose in the first picture'.

We have added a left- and right-side label into the corresponding section of the video (2:36) to ensure clarity.

Line 200 Eggs hatched in complete K200 medium33,39 with 0.25% dimethyl sulfoxide (DMSO), and worms remained in liquid until mid-L4 larval stage, at which point they were removed from chemical exposure, and prepared, imaged, and scored according to the protocol steps above.

Can you improve the above sentence to better indicate to the non C. elegans expert reader that exposure was done for ~2 days from L1 stage larvae onwards. Do I assume correctly that ref 33 and 30 fully describe the K200 medium needed to grow and culture worms in liquid medium.

This sentence has been edited to contain a note that the exposure was done for approximately 48 hours. You are correct in your interpretation of reference 33 and 30.

Figure 1, panel 2 please change arrow such that it points to a 'blob'.
Figure 1, in the figure legend please indicate in all cases what the arrows point at. In panel 4 are all 4 dendrites still around? Panel 5 please comment as to why dendrites are scored as present.sc

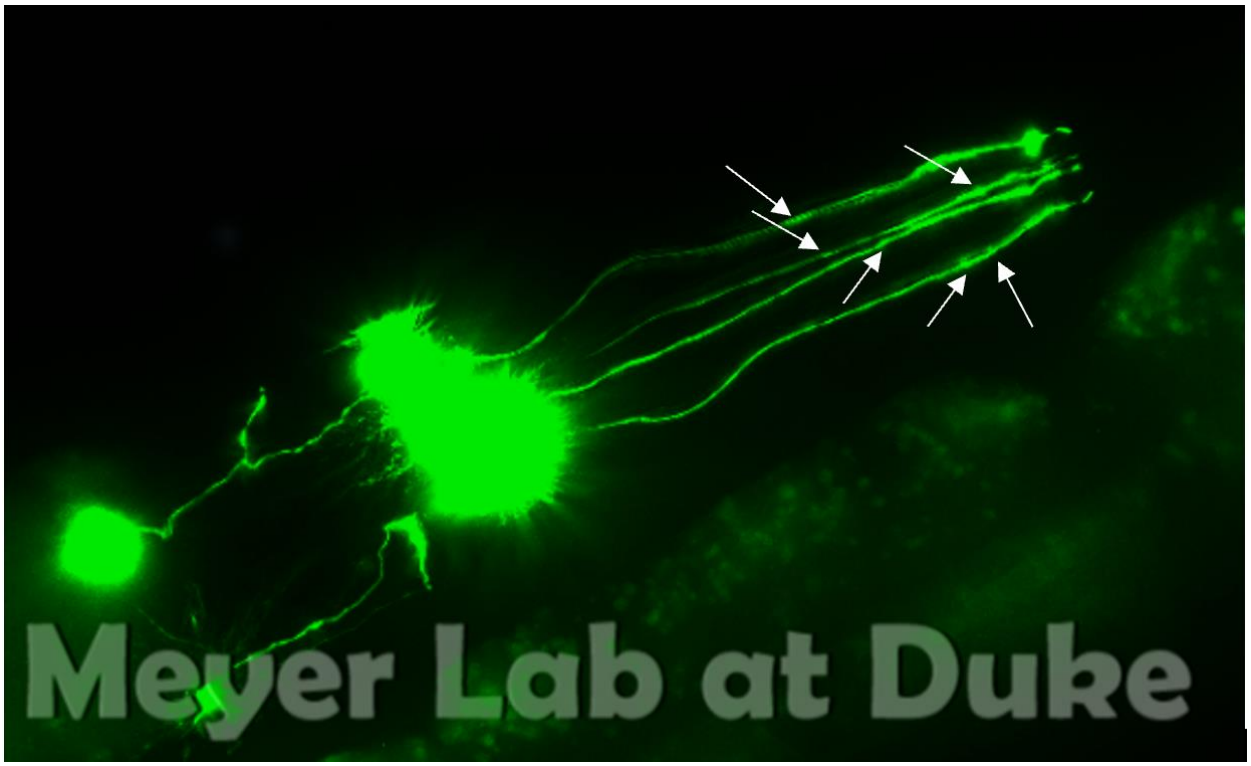
Thank you for these notes. We have clarified in the figure legend that the arrows in **Figure 1** point at one dendrite at the score value labelled in each panel. We do not point at specific blebs with the arrows because we consider the entire dendrite in our scoring system. In panel 4, all four dendrites are still present, but show clear damage. In panel 5, the dendrite indicated by the arrow is scored as present because 25-75% of the dendrite remains visible. Please note that line this dendrite can still be traced confidently (it presents as a dotted line due to the blebbing).

I strongly encourage to describe all the example Figures in the video. In many cases I was not sure if I agree on the score. Thus, extending the video to describe all sample pictures is important, I think.
Video: 3 image with only three dendrites scored. It appears to me that one neuron contains blobs.

We have rearranged the video narration so that the narration explaining the score categories plays as the viewer sees **Figure 1**. This change is intended to help the viewer associate the score value requirements with example images in real time and provide ample time to consider the image. In the first two walk-through example images, only 3 dendrites are scored because the images contained one un-scorable dendrite (which we explicitly state in the narration). These images were chosen specifically because we

felt it valuable to show examples of overlapping dendrites and limitations of z-stack captures. Please note that criteria for un-scorable dendrites are included in the protocol and mentioned in the video during the walk-through scoring.

Supp Figure 1) TS_01 All neurons are scored as 2, can you show the up to 5 blobs for each neuron by arrows?

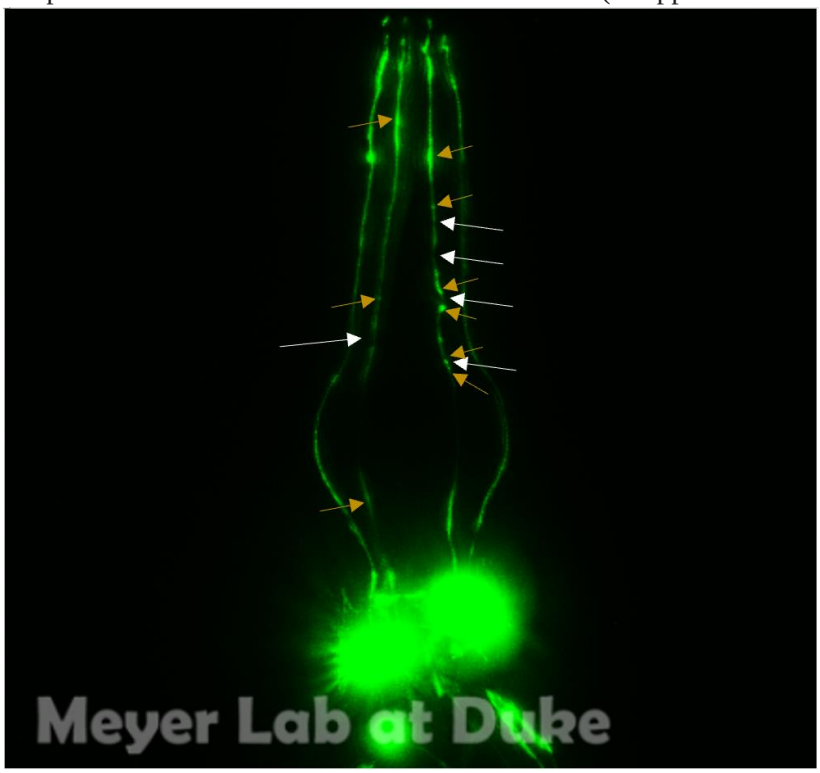


Please start with TS_21 as a wild type reference without any degeneration. In TS_21 is this picture underexpose compared to other pictures? (eg TS_16, 09, 13 etc)
Moved TS_21 to first slot, renamed TS_00, renamed TS_22 to TS_21 to keep numbering cohesive. The exposure on this image may be slightly lower, or the worm may have lower GFP expression. We chose deliberately to include worms with varying appearances to show how we score them despite the variability in image appearance.

TS_22, it appears that the worm moved during image acquisition. Remove?.
It is not uncommon for the projection of the z-stack to have high background depending on the projection method; it is also possible the position of the two less visible dendrites was not optimal for imaging. However, as the full length of the dendrite remains visible and trackable we still include the image in our analysis.

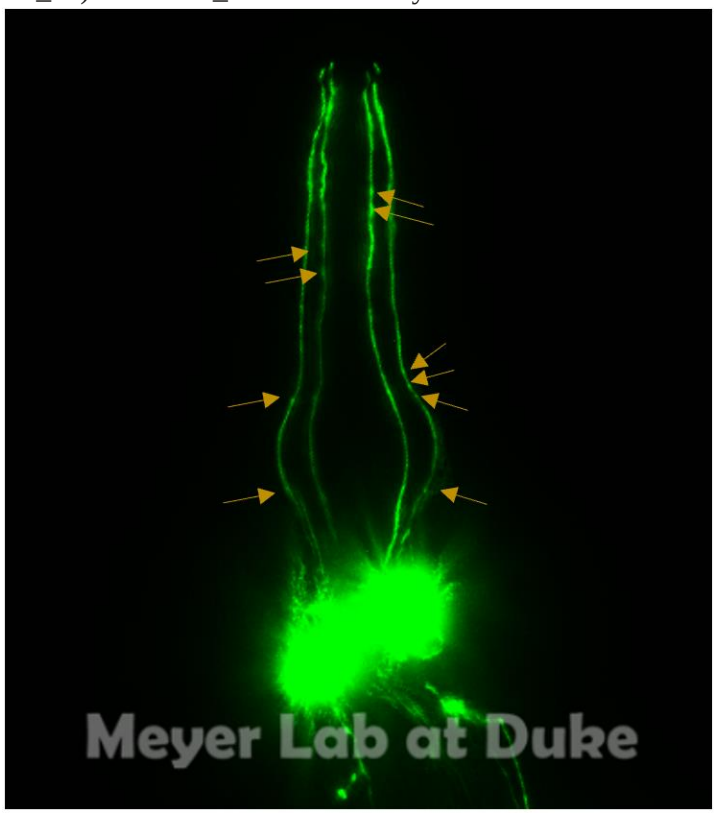
TS_02) why is D2 scored as 4? I do not see blobs. For D3 which is scored as 5, it appears that the part of

the posterior section of the dendrite is out of focus (as opposed to be absent or broken)



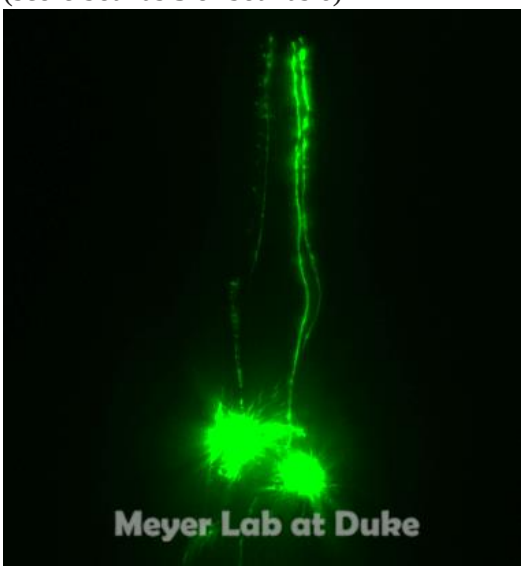
The white arrows indicate breaks, the orange arrows indicate blebs. As D2 has a break, it is scored a 4 as the break is more severe damage than blebs. D3 has multiple breaks, causing it to be scored a 5.

TS_03) As for TS_01 is it not really clear what a blob is.



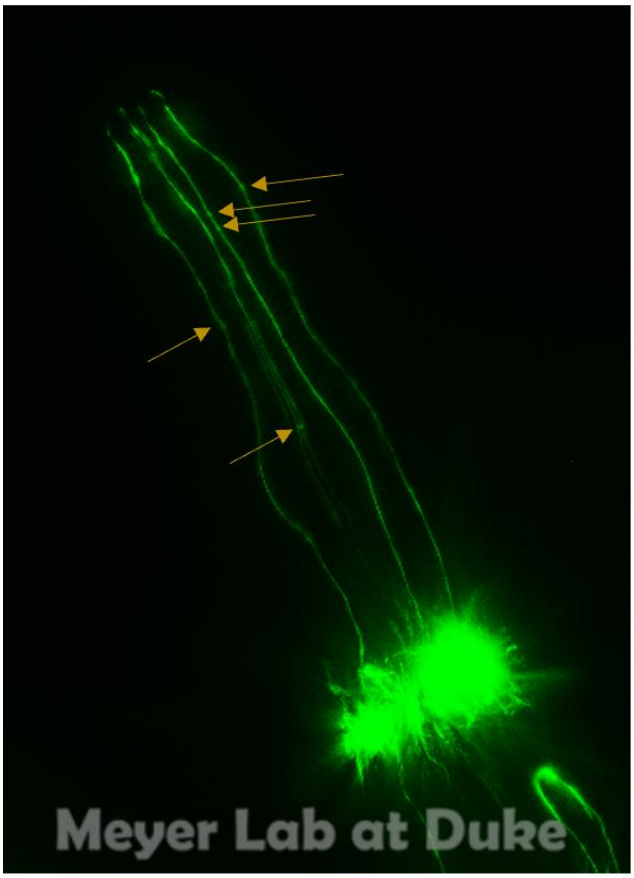
The orange arrows indicate blebs.

TS_08) It is not clear why one dendrite scores 5 the other 6. To me the both look equally degenerated (score both as 5 or both as 6)



Thank you for pointing this out. We have reviewed and re-scored this image. The first two dendrites have been both scored as a 5, due to breakage removing 25-75% of the dendrite in each case.

TS_11) Where are the blobs.



Blebs are indicated by the orange arrows.

Reviewer #2:

Manuscript Summary:

In this manuscript, authors developed a universal scoring system in order to assess morphological changes and degeneration in C. elegans' cephalic neuron dendrites. The described protocol represents a very useful tool to improve laboratory consistency and reproducibility when attemting to measure degeneration of dopaminergic neurons in this specific animal model. The method is well described and standarized so that other researchers can reproduce it thoroughly. Moreover, I find the training set of images and table very useful in order to achieve more consistent results over time.

Minor Concerns:

I recommend the authors including additional experiments analyzing at least one transgenic C. elegans model of PD expressing the protein alpha-synuclein in dopaminergic neurons (i.e. BY273 or UA44 strains). Neurodegeneration of cephalic neurons in these strains occurs concomitantly with age due to aggregation of alpha synuclein specifically in these neurons, so it would add more value to the protocol if they could show reproducible results with these models as well. Also, more detail of the statistic treatment

of data, adding analysis examples in their protocol would be very valuable as well.

Dear reviewer #2,

Thank you for your thoughtful feedback and for highlighting areas of our submission that can be clarified and improved. We agree that transgenic strains of PD expressing alpha-synuclein may produce interesting and important data, however our scoring protocol has not been validated with, and is not intended to account for, all PD *C. elegans* models. We focus instead on scoring itself rather than on specific models. To ensure this point is clearly conveyed in the manuscript, we have added clarification in the third paragraph of the introduction and in the second paragraph of the discussion section. Thank you for noting that more detail may be helpful for statistical treatment. We have added additional details into protocol step 6, on statistical analysis.

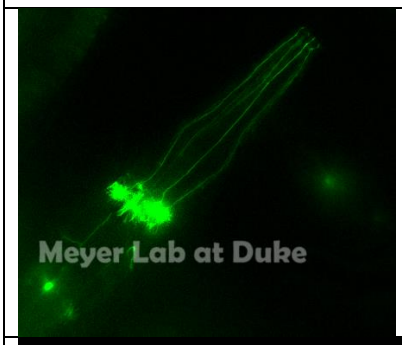
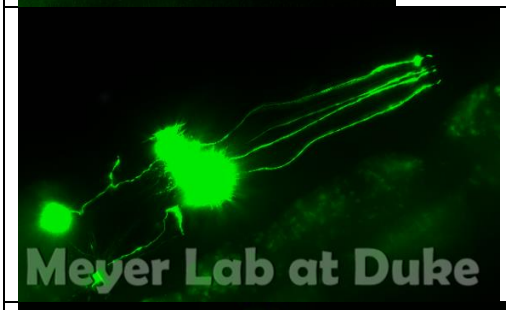
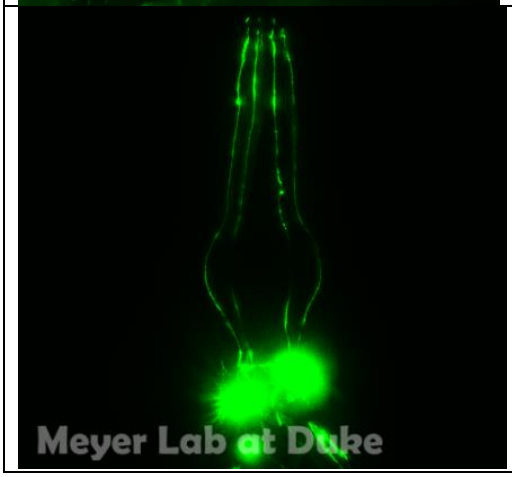
Reviewer #3:
Manuscript Summary:
Bijwadia et al. present a detailed protocol for the detailed quantification of morphological changes and neurodegeneration in the dopamine neurons of hermaphroditic C. elegans. The authors provided an in-depth overview on the background of research surrounding dopamine neuron morphology and neurodegeneration of C. elegans and its role in C. elegans PD. To address some of the shortcomings that current dopamine neuron degeneration scoring methods have, the authors proposed a 7-point scoring method. The work provides a robust write-up for replicating the scoring consistently, with a set of reference images for calibration.

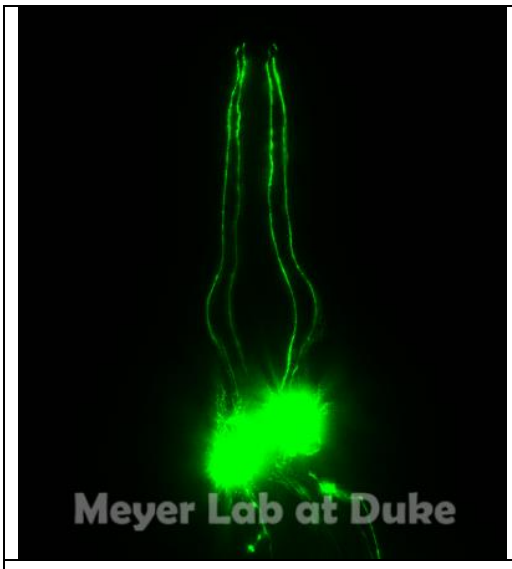
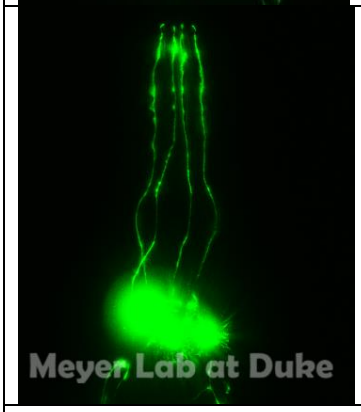
Minor Concerns:
* Having a criterion for each stage does help address some level of ambiguity (especially across different scorers), but there can still be some inconsistency during the image process and that can result in different interpretations, especially between stages 2-4. A good example is in the differences between images shown in figure 1 and in the Training set images. Should there be a directive set so that worms can be imaged consistently? For example, overexpose the soma, and make sure the processes fall within a certain GFP intensity range for scoring.
* Somewhat related to the previous point, the blebs in criteria 5-6 (from Figure 1) appear to be a lot more pronounced than ones shown in 2-3. How can we be sure that the blebs in 2-3 are not puncta, or a non-uniform distribution of GFP?
* Having a linear 0-7 score implies that there is a progression from healthy neurons (0) to dysmorphology (1) to blebbing (2-5) to degenerated (6). Can this progression be backed up with current literature?
* In the event where a process presents features from multiple criteria (ex. irregular + blebs), how can this be addressed consistently?

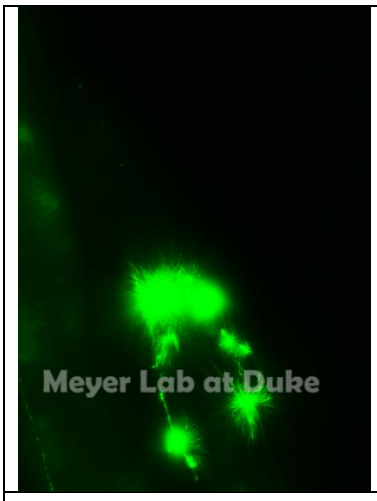
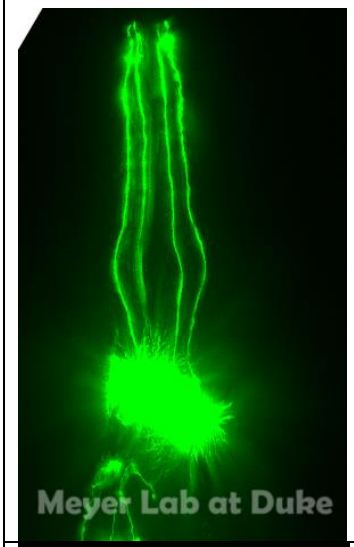
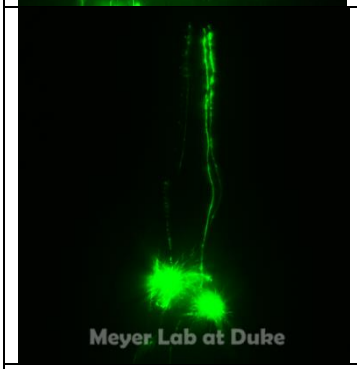
Dear reviewer #3,

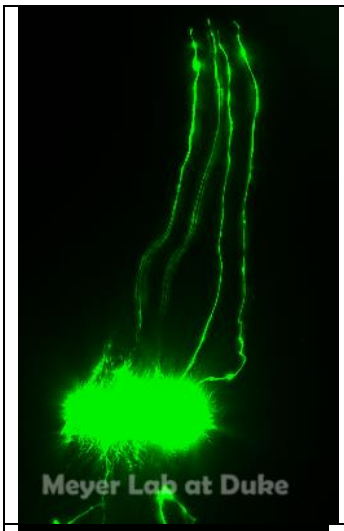
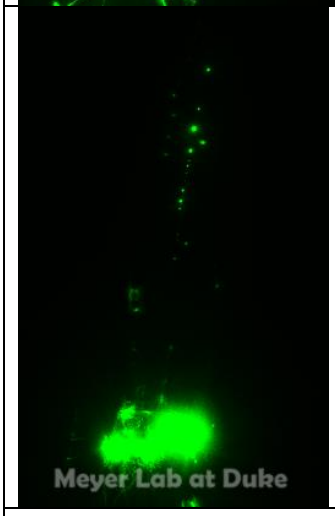
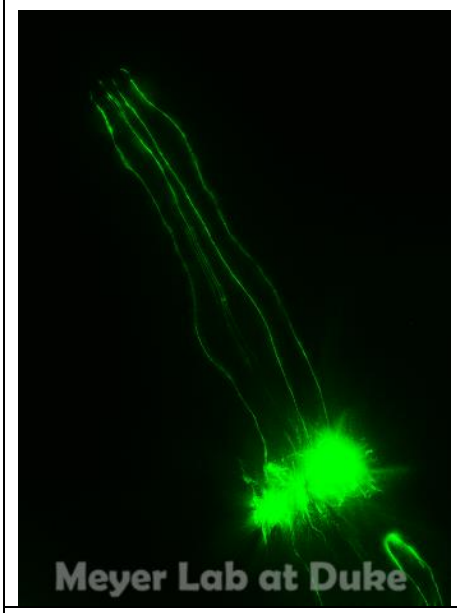
We appreciate your manuscript summary and the feedback you provided. We have taken your concerns into account as we have reviewed and revised our submission—please read on for our responses to your specific comments. First, we acknowledge that ideally all worms should be imaged consistently, but that perfect consistency is not possible with a score-based system. We do think that our new system, combined with a training set that all users will practice on, will significantly improve replicability. Unfortunately, because this protocol was designed for a variety of imaging instruments, we cannot provide one directive set due to the variation between instruments. We feel the generalizability of the

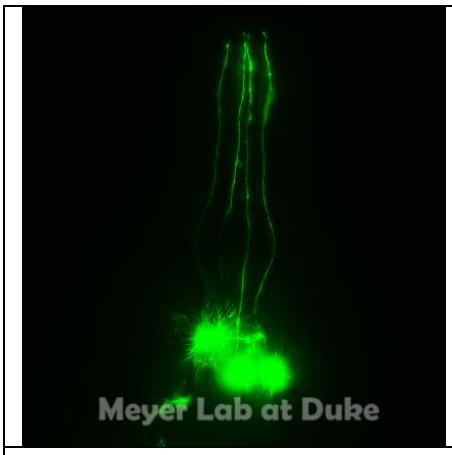
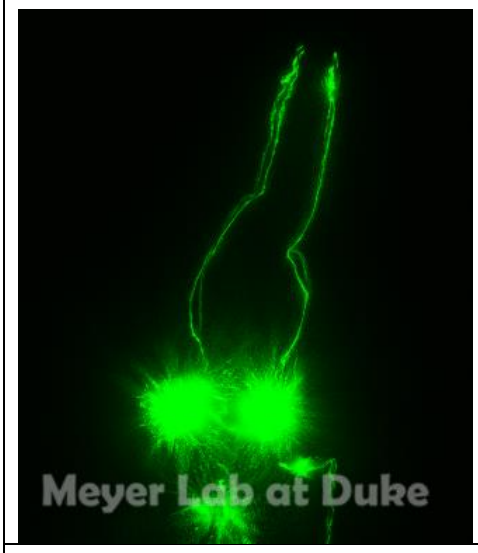
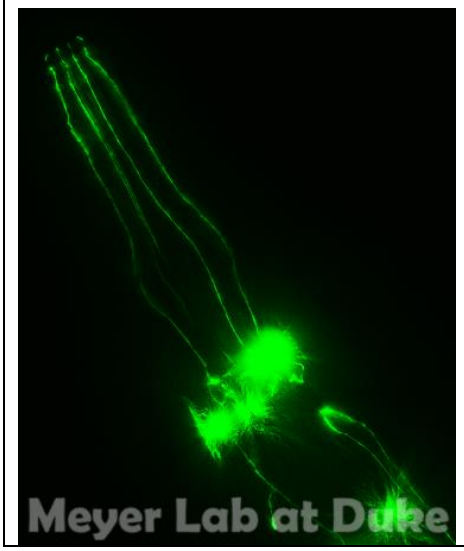
protocol to labs with different equipment availability is worth the trade-off in being able to prescribe instructions that might permit more nearly perfectly consistent image acquisition. Even so, we aim to maximize consistency in our protocol instructions, notably in protocol step 2. We have added a sentence (2.1.1: Be mindful of exposure and aperture settings; avoid overexposure of dendrites and keep settings consistent across trials. Make the dendrites as bright as needed for clear visualization; this typically results in overexposure of the soma.) in order to accomplish this goal. Please also see protocol step 4.2.2 to address concerns regarding false blebs and other notes to avoid mis-scoring dendrites. Second, we agree that the 0-7 score implies a progression from healthy to degenerated. While we use criteria (blebs, breaks) that have been used in previous literature as a sign of dopaminergic neuron damage, we agree that unfortunately, the progressive scale has not yet been validated in current literature. We note this as a most important area of research for future directions at the conclusion of the discussion section. We also note that while the scale presents as linear, the data is non-parametric, and provide discussion on how to best analyze the data with all these limitations in mind. Finally, thank you for pointing out the ambiguity surrounding scoring dendrites with multiple features, such as irregular + blebs. We have added instructions for this type of scenario in protocol step 4.2.1: If multiple criterion are met within a single dendrite (i.e. kinks and blebs), assign the highest applicable score.

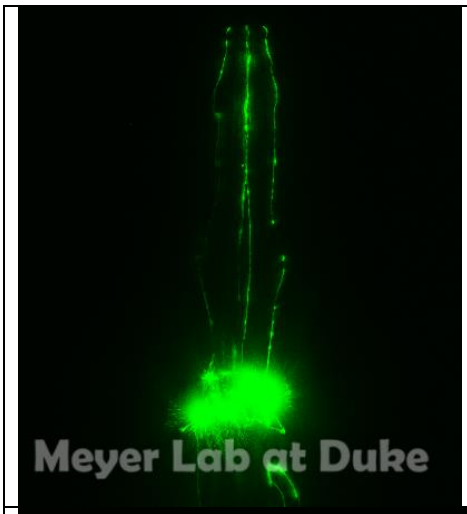
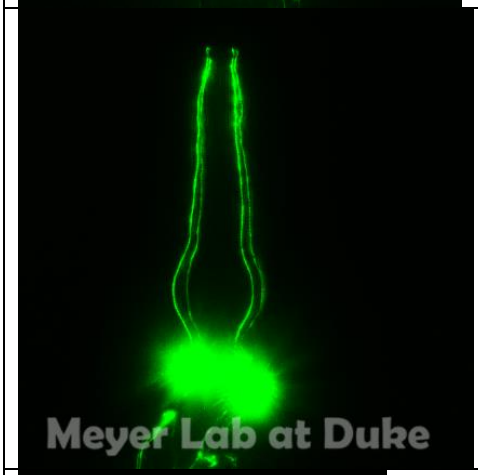
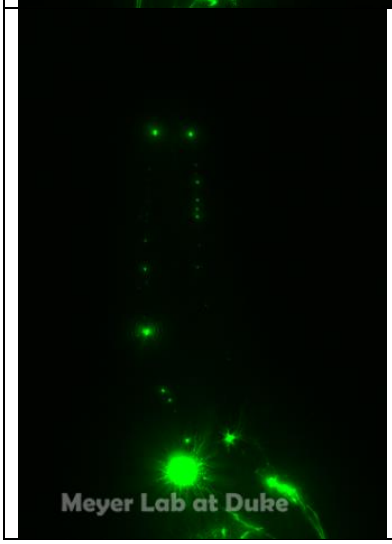
Training Set						
Image	Image Name	D 1	D 2	D 3	D 4	Notes
	TS_00	0	0	0	0	
	TS_01	2	2	2	2	
	TS_02	2	4	5	2	

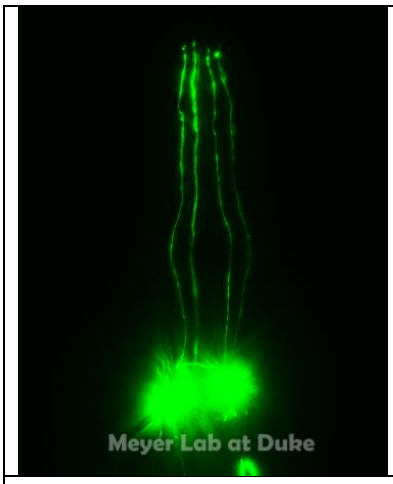
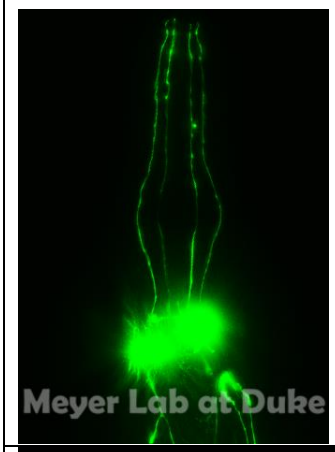
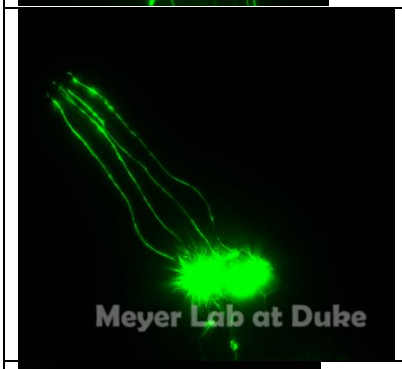
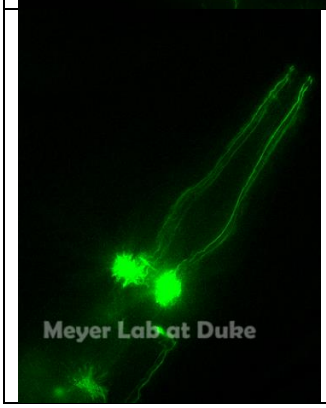
	TS_03	2	2	2	2	
	TS_04	4	NS	2	2	Dendrite 2 not scorable as over half of the dendrite was not captured in the z-stack.
	TS_05	2	3	3	2	
	TS_06	6	6	6	6	

						
	TS_07	2	2	0	2	
	TS_08	5	5	4	4	
	TS_09	2	2	2	2	

						
	TS_10	6	6	6	6	
	TS_11	2	2	2	2	
	TS_12	2	2	4	2	

						
	TS_13	1	2	NS	NS	Dendrites 3 and 4 were not scorable due to too much overlap
	TS_14	1	2	0	1	

	TS_15	4	NS	NS	4	Middle dendrites not scorable because they cannot be distinguished from each other
	TS_16	2	2	2	2	
	TS_17	6	6	6	6	

	TS_18	2	2	3	2	
	TS_19	3	3	3	3	
	TS_20	2	2	2	2	
	TS_21	1	2	0	0	

Notes: it is normal for the middle two dendrite to have lower fluorescence around the area of the pharynx; dendrites that are not distinguishable from each other or where over half of the dendrite is not clearly identifiable should not be scored

Supplemental File 2: Score Report Template

Click here to access/download;Supplemental File (Figures, Permissions, etc.);SupplementaryFile2.xlsx

Replicate	Experimen	Neuron Scc	Count	Proportion	# Neurons in Experimental Group
x	y	zero	#0	#0/n	n
x	y	one	#1	#1/n	n
x	y	two	#2	#2/n	n
x	y	three	#3	#3/n	n
x	y	four	#4	#4/n	n
x	y	five	#5	#5/n	n
x	y	six	#6	#6/n	n