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TITLE:

Semi-Quantitative Determination of Dopaminergic Neuron Density in the Substantia Nigra of Rodent Models using Automated Image Analysis

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KEYWORDS:

alpha-synuclein, image quantitation, neurodegeneration, Parkinson's disease, stereology

SUMMARY:

Here we present an automated method for semi-quantitative determination of dopaminergic neuron number in the rat substantia nigra pars compacta.

ABSTRACT:

Estimation of the number of dopaminergic neurons in the substantia nigra is a key method in pre-clinical Parkinson's disease research. Currently, unbiased stereological counting is the standard for quantification of these cells, but it remains a laborious and time-consuming process, which may not be feasible for all projects. Here, we describe the use of an image analysis platform, which can accurately estimate the quantity of labeled cells in a pre-defined region of interest. We describe a step-by-step protocol for this method of analysis in rat brain and demonstrate it can identify a significant reduction in tyrosine hydroxylase positive neurons due to expression of

mutant α-synuclein in the substantia nigra. We validated this methodology by comparing with results obtained by unbiased stereology. Taken together, this method provides a time-efficient and accurate process for detecting changes in dopaminergic neuron number, and thus is suitable for efficient determination of the effect of interventions on cell survival.

INTRODUCTION:

Parkinson's disease (PD) is a prevalent neurodegenerative movement disorder characterized by the presence of protein aggregates containing α-synuclein (α-syn) and the preferential loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc)[1]. Quantification of dopaminergic neuron number is a vital part of PD research as it permits the evaluation of the integrity of the nigrostriatal system, thus, providing an important endpoint to assess the effectiveness of potential disease-modifying therapeutics. Currently, the standard for quantification of cell number is unbiased stereological counting, which utilizes two-dimensional (2D) cross-sections of tissue to estimate volumetric features in three-dimensional (3D) structures[2–4]. Modern design-based stereological methods employ comprehensive random sampling procedures and apply counting protocols (known as probes) to avoid potential artifacts and systematic errors, allowing for reliable detection of differences only slightly greater than inter-animal variation[5]. While stereology provides a powerful analytical tool for in vivo histological studies, it is time intensive, assumes uniform specimen preparation, and requires validation at several steps, which can impact the efficiency increasingly required for pre-clinical translational investigation.

Recent technological advances in digital science make it possible to adopt novel applications for more efficient assessments of pathology without a stereomicroscope, while filling a need as a surrogate of unbiased stereology. These methods increase speed, reduce human error, and improve the reproducibility of stereological techniques[6,7]. HALO is one such image analysis platform for quantitative tissue analysis in digital pathology. It comprises a variety of different modules and reports morphological and multiplexed expression data on a cell-by-cell basis across entire tissue sections using pattern recognition algorithms. The cytonuclear FL module measures the immunofluorescent positivity of fluorescent markers in the nucleus or cytoplasm. This allows for reporting of the number of cells positive for each marker, and the intensity score for each cell. The module can be adapted to provide individual cell sizes and intensity measurements, although this feature is not required for quantification of dopaminergic neurons.

The aim of this study is to verify this method with a previously validated viral vector-based α-syn rat model of nigral neurodegeneration[8–10]. In this model, human mutant A53T α-syn is expressed in the SNpc by stereotactic injection of adeno-associated virus hybrid serotype 1/2 (AAV1/2), resulting in significant neurodegeneration over a period of 6 weeks. The contralateral uninjected SNpc may, in some studies, serve as an internal control for the injected side. More commonly, injection of AAV-Empty Vector (AAV-EV) in a control cohort of animals is used as a negative control. We present a step-by-step guide to estimate the density of dopaminergic neurons remaining in the injected SNpc after 6 weeks using an automated image analysis software (**Figure 1**).

PROTOCOL:

All procedures were approved by the University Health Network Animal Care Committee and performed in accordance with guidelines and regulations set by the Canadian Council on Animal Care.

1. Stereotactic injection

1.1. Pair-house adult female Sprague-Dawley rats (250–280 g) in cages with wood bedding and ad lib access to food and water. Maintain the animal colony in a regular 12 h light/dark cycle (lights on 06:30) with constant temperature and humidity.

1.2. Perform unilateral stereotactic injection of AAV directly to the SNpc on the right side of the brain (right or left side, according to the preferences of each lab) as previously described[8,10]. Inject 2 μL of AAV1/2 at a final titer of 3.4×10^{12} genomic particles/mL.

2. Brain sectioning and immunohistochemistry (IHC)

2.1. Anaesthetize the rat with 5% isoflurane by placing in an anaesthetizing chamber for 3 min. Other approved methods may be used for this step after appropriate institutional review.

2.2. Once the rat has reached a surgical plane of deep anesthesia, transfer it to a nose cone firmly affixed to a necropsy table. Secure the rat's fore-paws using tape and use toe pinch-response method to determine the depth of the anesthesia. The animal must be unresponsive before continuing.

2.3. Make a lateral incision below the sternum and cut through the diaphragm along the entire length of the rib cage to expose the pleural cavity. Lift and clamp the sternum with a hemostat and place above the head.

2.4. Clamp the heart using forceps and insert a butterfly needle connected to a perfusion pump into the posterior end of the left ventricle. Perfuse rat transcardially with 150 mL of heparinized saline, or until the eyes and skin are clear. Perfusion with 4% paraformaldehyde (PFA), instead of saline, may be preferred to facilitate immunostaining with certain antibodies or thinner brain sectioning.

2.5. Once perfusion is complete, decapitate with a guillotine and extract the brain to a brain matrix, ventral surface facing up.

2.6. Using a fresh razor blade, make a cut in the coronal plane 2 mm rostral to the optic chiasm. Slide the blade from side to side to avoid warping the brain while slicing.

2.7. Immerse the posterior portion of the brain in a pre-labeled vial containing approximately 20 mL of 4% PFA for 48 h of post-fixation at room temperature. The anterior portion of the brain may be flash frozen in 2-methylbutane chilled to -42 °C before storage at -80 °C.

2.8. After 48 h, transfer the fixed brains to a labeled vial containing 30% sucrose in phosphate buffered saline (PBS) and store at 4 °C until they sink (48–72 h).

2.9. Prepare a microtome by placing dry ice in the trough of the specimen stage, followed by 100% ethanol. Once the stage has cooled, squeeze optimal cutting temperature (OCT) compound onto the stage until it forms a circle 2 cm in diameter and 0.5 cm thick. Once it has partially frozen, carefully lower the brain onto the mound of OCT, ensuring the striatal cutting surface remains parallel with the stage.

2.10. Add more dry ice to the stage to help the brain to freeze. Once the brain has turned a cream color, clear the stage of dry ice.

2.11. Poke a hole into the right side of the brain with a 25G needle to distinguish the right and left hemispheres. Take care not to pass the needle through anatomical structures of interest.

2.12. Serially cut 40 μm sections in the coronal plane beginning at bregma -3.8 and ending at bregma -6.8.

2.13. Store six series in labeled tubes with anti-freeze solution (40% PBS, 30% 2-ethoxyethanol, 30% glycerol). Each series should contain 12 brain sections.

2.14. Select one set of sections for immunohistochemical staining, and wash off anti-freeze solution with 3 x 10 min washes in 0.2% PBS-T.

2.15. Block for 1 h at RT with gentle nutation in blocking solution (10% normal goat serum (NGS), 2% bovine serum albumin (BSA) in 0.2% PBS-T). Follow this with incubation with rabbit anti-tyrosine hydroxylase (TH) antibody (1:500) and mouse anti-α-syn antibody (1:500) in 2% NGS in 0.2% PBS-T overnight at room temperature.

2.16. Wash off primary antibody with 3 x 5 min washes in 0.2% PBS-T, followed by 1 h incubation with goat anti-rabbit Alexa Fluor 488 secondary antibody (1:500) and goat anti-mouse Alexa Fluor 555 secondary antibody in 2% NGS in 0.2% PBS-T. Ensure the sections are protected from light and nutating gently.

2.17. Wash off secondary antibody with 3 x 5 min washes in 0.2% PBS-T and mount the complete set of sections on slides protected from light and dust using a narrow paintbrush. Coverslip with fluorescence mounting medium and seal with clear nail varnish.

3. Confocal microscopy and image acquisition

3.1. Capture IHC images using software coupled to a confocal microscope at 10x magnification. Open the pinhole to 1.5 AU to capture a wide plane totaling ~1.5 μm and set the focus on the injected side of the brain.

3.2. On the **Acquisition** tab, check the **Tile Scan** imaging option and set the dimensions to 10 x 4.

3.3. Under the **Acquisition Mode** panel, set the **Zoom** to 1.1. This helps to avoid any obvious stitching marks between tile scan images.

3.4. Set the **Frame Size** to 1024 x 1024 pixels and the **Averaging** to 2 to ensure high quality image acquisition.

3.5. In the **Channels** panel, set track 1 to Alexa488 and track 2 to Alexa555.

3.6. Load the slide onto the stage and choose a section with strong TH staining. Click on **Live** on the acquisition panel.

3.7. In the **Channels** panel, set the **Laser Strength** and **Gain** to levels that maximize signal and limit noise from the background. Use the range indicator to ensure that the signals are not overexposed (as indicated by a dark red overlay).

3.8. Repeat the above step with multiple slides to ensure staining is consistent between slides as the laser strength/gain cannot be adjusted between slides.

3.9. On the **Acquisition** tab, check the **Positions** box.

3.10. At this point, you are ready to begin imaging. Using the eyepiece, choose the first section showing positive TH staining, set the focus at the point of interest (i.e., SNpc) and then move the stage to the midline of the section. This saves the position in the x, y, and z axis and will image a tile scan capturing the whole section.

3.11. Repeat the above step for all sections throughout the SNpc giving a complete set of images of the SNpc. If detailed analysis of the uninjected side is required, steps 3.1 to 3.11 should be repeated by setting the focus on the uninjected side.

4. Image analysis and quantitation

4.1. Separate image files using appropriate software and import image files to automated image analysis software.

4.2. Define a region of interest by selecting the **Pen** annotation tool to draw an annotation around the SNpc.

NOTE: In sections which have a large amount of dopaminergic neuron loss, temporarily increasing the emittance/absorption can help to clearly define the SNpc (**Figure 2**).

4.3. Move to the **Analysis** tab and from the drop-down **Analyze** menu, select **Real-Time tuning**. This opens a separate window on the section image allowing for real-time modification of analysis parameters (**Figure 3**).

4.4. Under the **Analysis Magnification** section, select the appropriate image zoom.

4.5. Under the **Cell Detection** section, select nuclear dye as the dye used for TH staining (Alexa Fluor 488).

4.6. Adjust the **Nuclear Contrast Threshold**, **Minimum Nuclear Intensity**, **Nuclear segmentation Aggressiveness**, and **Nuclear Size** settings while carefully watching the Real-Time Tuning window.

NOTE: Accurate representation of each individual cell as a single cell in the Real-Time Tuning window is vital for accuracy. These settings are on an arbitrary scale depending on the software used, but correct adjustment is needed to allow the software to accurately differentiate between individual cells, and between cells and the background (**Figure 3**).

4.7. Repeat this process with a minimum of 10 separate samples to ensure a uniform agreement of what constitutes a cell across different sections.

NOTE: Additional cell markers (such as α-syn or NeuN) can be identified within the same analysis platform using the Marker 1 or Marker 2 sections on the analysis tab.

4.8. Once an appropriate number of images have been sampled and Real-Time Tuning has been adjusted accordingly, save the analysis settings in the **Settings Actions** drop-down menu.

4.9. Select all images to be analyzed and click on **Analyze**.

4.10. Choose the analysis setting you have just saved and in the **Region of Analysis** window, check the **Annotation Layer(s)** box. Then, check **Layer 1** and click on **Analyze**.

NOTE: For a single brain, the analysis typically takes about 5 mins. The completed result will clearly show each item that has been counted as a cell (**Figure 4**).

4.11. Once complete, export the summary analysis data for all sections. There is an option to export **Object Analysis Data**, which will give detailed data, including cell size of each individual cell detected. This dataset could be used to examine changes in cell size in response to a toxin/therapeutic.

4.12. Add the Total Cells from each section analyzed per animal and the Total Analyzed Area (mm^2). Divide the total number of cells by the total area analyzed to give the number of cells/mm^2 in the SNpc for each rat.

REPRESENTATIVE RESULTS:

By applying the above methods to brain tissue collected 6 weeks after AAV injections, we demonstrated that stereotactic injection of AAV expressing mutant A53T α-syn (AAV-A53T) in the SNpc of rat brain results in a significant reduction in the density of dopaminergic neurons compared to injection of empty vector AAV (AAV-EV) as a control (**Figure 5A,B**). The mean number of TH-positive neurons/mm^2 in the SNpc of rats injected with AAV-EV was 276.2 ± 34.7, and in the SNpc of rats injected with AAV-A53T was 41.2 ± 17 (*P* = 0.0003). Quantification of the number of dopaminergic neurons/mm^2 in the SNpc is similar to previously published reports[10, 11]. For the methods described here, 4 sequential sections per animal were analyzed. Previous studies have shown significant differences with as little as three sections, but analysis can be further increased up to 12 sections to encompass the whole SNpc depending on the model and intervention being studied by the investigator.

Unbiased stereology was also performed as previously described[12] on another set of brain sections from the same animals. Using this method, we also demonstrated that stereotactic injection of mutant A53T α-syn in the SNpc of rat brain results in a significant reduction in the estimated total number of TH-positive neurons in the SNpc, as compared to injection of EV-AAV (**Figure 5C**). Importantly, there was a strong correlation between the dopaminergic neuron numbers estimated using automated image analysis software and those estimated using unbiased stereology (r = 0.8819, *P*= 0.0007) (**Figure 5D**).

We also applied our methods using automated image analysis software to determine the number of TH-positive neurons/mm^2 on the uninjected side of rats injected with AAV-A53T or AAV-EV. The mean number of TH-positive neurons/mm^2 in the uninjected SNpc of rats injected with AAV-A53T was 123.2 ± 26.4, which was significantly greater than in the injected SNpc, which was 44.0 ± 15.8 (*P* = 0.0331) (**Supplementary Figure 1A**). The mean number of TH-positive neurons/mm^2 in the uninjected SNpc of rats injected with AAV-EV (215.6 ± 35.5) was not significantly different from the injected SNpc (276.2 ± 34.7), confirming there was no degeneration due to injection with AAV-EV (**Supplementary Figure 1B**). We calculated these results as a percentage of injected/uninjected and found that animals injected with AAV-A53T had a 69% reduction compared to the AAV-EV animals (**Supplementary Figure 1C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow of the method. Workflow demonstrating the steps required to inject AAVs, section and stain tissue, define a region of interest and optimize the software for counting of cells. Representative images of confocal tile scan, real-time tuning, and quantitation of cells. Scale bar = 100 μm.

Figure 2: Defining the region of interest. (**A**) Coronal brain section, including the SNpc immunostained for TH (green) from a rat injected with AAV-A53T α-syn. In rats with severe neurodegeneration (such as shown here), it can be difficult to identify the substantia nigra pars compacta. (**B**) Temporarily increasing the absorption of the image can identify the structure and allow an accurate identification of the region of interest. Scale bar = 1 mm.

Figure 3: Optimizing cell detection using the Cytonuclear method in HALO. Real-time tuning of the cytonuclear module allows the user to see changes in cell detection in real-time by altering Nuclear Contrast Threshold, Minimum Nuclear Intensity, Nuclear Segmentation Aggressiveness, and Nuclear Size. (**A**) Representative image with region of interest displayed. (**B**) Real-time tuning showing under-sampling in which the software does not detect all cells in the tuning window. (**C**) Over-sampling in which the software detects more cells than are evident in the tuning window. (**D**) Optimized tuning in which the correct number of cells are counted. Scale bar = 500 μm.

Figure 4: HALO optimized settings within a defined region of interest. Representative images of completed analysis using optimized settings for cytonuclear detection in HALO. Scale bar = 500 μm.

Figure 5: Expression of human mutant A53T α-syn in SNpc results in severe neurodegeneration at 6 weeks as quantified by HALO and unbiased stereology. (**A**) Representative images showing degeneration of TH-positive neurons in the SNpc 6 weeks after stereotactic injection of AAV-A53T α-syn at a titer of 3.4×10^{12} viral particles/mL. Immunofluorescent staining with anti-TH (green) and anti-α-syn (red) antibodies. Scale bar = 200 μm. (**B**) Quantification of the number of TH-positive neurons in the SNpc of rats injected with AAV-A53T α-syn or AAV-EV demonstrates that expression of mutant α-syn results in significant dopaminergic neuron loss. Unpaired *t*-test; n = 5 rats/group. Graph shows mean ± SEM, ***$P < 0.001$. (**C**) Representative images of colorimetric staining of dopaminergic neurons in the SNpc of AAV-A53T (left) or AAV-EV (right) injected rats used to perform unbiased stereology. Scale bar = 200 μm. (**D**) A significant correlation between HALO AI counting of TH^+ neurons/mm^2 (y-axis) and unbiased stereology cell numbers (x-axis). Pearson correlation (r = 0.8819, $P = 0.0007$).

Supplementary Figure 1: Significant unilateral neurodegeneration is observed in the SNpc of rats who received AAV-A53T injection. (**A**) Quantification of the number of TH-positive neurons in the injected or uninjected SNpc of rats that received a unilateral AAV-A53T stereotactic injection shows a significant decrease on the injected side. (**B**) Quantification of the number of TH-positive neurons in the injected or uninjected SNpc of rats that received unilateral AAV-EV stereotactic injection shows no significant changes. (**C**) Normalization to the uninjected contralateral side demonstrate a >50% decrease upon injection with AAV-A53T α-syn compared to AAV-EV. Unpaired *t*-test; n = 5 rats/group. Graphs show mean ± SEM, *$P < 0.05$, ***$P < 0.001$.

DISCUSSION:

The reliable assessment of the integrity of the dopaminergic system in pre-clinical models of PD is critical to determine the effectiveness of potential disease-modifying therapeutics. Therefore, it is important to control and minimize potential confounds that may reduce the reliability and reproducibility of histopathological data. Careful quantitative outcomes can provide more information than qualitative or semi-quantitative descriptions alone. At the same time, we must recognize that constraints in time and resources can make it difficult to perform unbiased stereological counting to quantitate pathological changes or loss of cells. However, with recent

advancements, many of these criteria can be fulfilled using computerized and automated imaging platforms[6].

This protocol describes a number of important steps in determining the stereological estimation of dopaminergic neurons/mm^2 in rodent brain. It should be noted that we used stereotactic injection to deliver AAVs to the SNpc, which places an importance on accurate delivery of these viruses in order to determine the effect of any treatment. The co-ordinates used for our study are bregma -5.2 mm (anterior-posterior), -2 mm (medial-lateral to the right) and -7.5 mm (dorsal ventral from the skull) on adult female rats weighing ~275 g. Correct delivery of the virus using these co-ordinates will ensure delivery of the AAVs to the SNpc.

In addition to this, care must be taken when preparing tissues for staining. Foremost, care should be taken to mark which side is the right/left. In our hands, the easiest and most reproducible way to do this was to use a 25G needle to poke a hole periodically in the left dorsal midbrain while cutting coronal sections to differentiate the uninjected side. An in-depth knowledge of the neuroanatomical regions of the rodent brain are important to differentiate when to begin cutting, and later to identify the SNpc when drawing a region of interest. It is important to note that TH staining does not exclusively stain neurons of the SNpc, and one should be able to differentiate between TH positive neurons in the ventral tegmental area and retrorubal field. A rat brain atlas is a useful guide for those lacking experience in rodent neuroanatomy. Antibody incubation times are uniform throughout the protocol and the focus is set in the minimum time possible to avoid photobleaching.

There is a low variability between samples once consistent neuroanatomical features are used to distinguish between sections. Having an experienced, blinded observer draw the region of interest is important to maintain consistency across the sections to be analyzed. Analysis of the uninjected side as shown in **Supplementary Figure 1** demonstrates there is a consistency to the method. However, care should be taken when interpreting data in this manner. Slicing brains consistently relies on comparing the presence of known neuroanatomical features on each side of the brain. Subtle differences may mean that the uninjected side on a given section may be more posterior/anterior than the injected side, and thus not an accurate direct comparison. Further analysis of the presented data highlights a limitation of this method. There is a large degree of variability in the dopaminergic neuron counts in the uninjected side (123.2 in the AAV-A53T group vs 215.6 in the AAV-EV), which one would not expect between similarly aged animals. There are a number of possible reasons for this discrepancy, including the low numbers per group used in this study and the above-mentioned anatomical differences from side to side. In addition to this, focus for fluorescent signal is set on the injected side, meaning a slight shift in the z-plane can leave the uninjected side out of focus, and thus some cells will not be detectable by the cell counting software. For this reason, it is recommended to compare the number of dopaminergic neurons/mm^2 on one side using known neuroanatomical features, and where focus has been set on the microscope specifically for this region. If a comparison to the uninjected side is needed, imaging should be repeated with the focus set on this side.

We present data obtained and analyzed with one of the several software packages that provide

quantitative image analysis with minimal user training[13]. Other programs are available and are increasingly being adopted to study neuropathology, including the quantification of dopaminergic neuron loss in experimental models of PD[6]. While some offer built-in algorithms to perform specific functions (e.g., amyloid plaque counting), many of the software packages share several common features that make them particularly useful for laboratories that perform repetitive analysis. In addition to region of interest identification, preprocessing steps that interfere with the signal (tissue folds, edge artifacts, ink marks, stains, etc.) can be removed from region of interest by manual and pattern recognition tools. Furthermore, the readouts provided can be used in conjunction with quantitative values obtained from protein, cellular, or behavioral studies to explore relationships and potential correlations between variables.

The method described above provides an accurate, semi-quantitative, and relatively inexpensive procedure for estimating neuron numbers in immunohistochemically stained brain sections. The real-time tuning feature allows for adaption of the method for all sections and ensures consistency even with slight variances in slicing methods. Having a blinded observer identify regions of interest in the SNpc mitigates confirmation or selection biases and the software provides a simple readout allowing calculation of neurons/mm^2. This method could be easily adapted to provide accurate counts of neurons in different brain regions while maintaining efficiency and cost advantages over more established stereological protocols. Advancement of slide-scanning technology for thicker sections would also allow for more efficient processing time for the above-described method.

Limitations

The above presented method has a number of distinct advantages in its speed and ability to detect large changes in neuron density, but also poses its own challenges. Unlike stereology, the method cannot provide an estimation of absolute cell numbers but instead calculates cell density within the SNpc. The estimates of cell density are highly, but not perfectly, correlated with cell numbers obtained using unbiased stereology. In addition, the method relies on tuning of cell size and shape by the user prior to beginning the analysis. This cannot guarantee that cells will not be missed due to being partially in the focal plane, or that cells of an unusual size and/or shape will be missed by the software. In the authors' experience, the method is particularly useful for examining the efficacy of potential disease-modifying therapeutics in pre-clinical rodent models. In conclusion, while stereological methods to quantify cell numbers remain widely used in neuroscience, the rapid acceleration of digitalization suggests that automated image analysis platforms will increasingly be adopted to study neuropathology, particularly as they continue to improve. It is important that the investigator understands the technical limitations of this approach and applies this methodology after careful consideration.

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DISCLOSURES:

The authors report no competing interests.

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Figure 1

Stereotactic Injection

↓

Perfusion, sectioning and Immunohistochemistry

↓

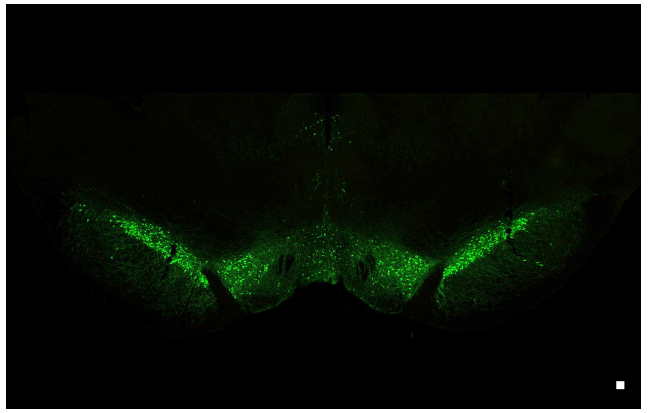
Confocal Imaging

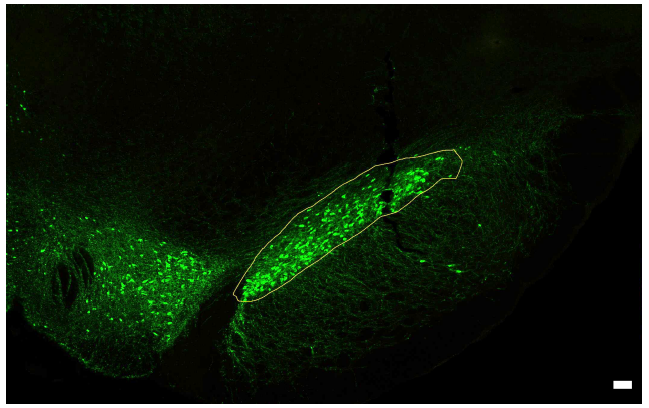
↓

Import into automated imaging platform and definition of ROI

↓

Automated counting of TH^+ neurons





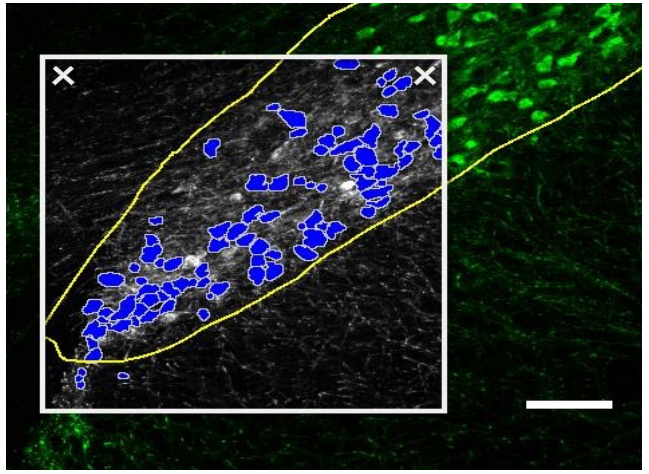


Figure 2

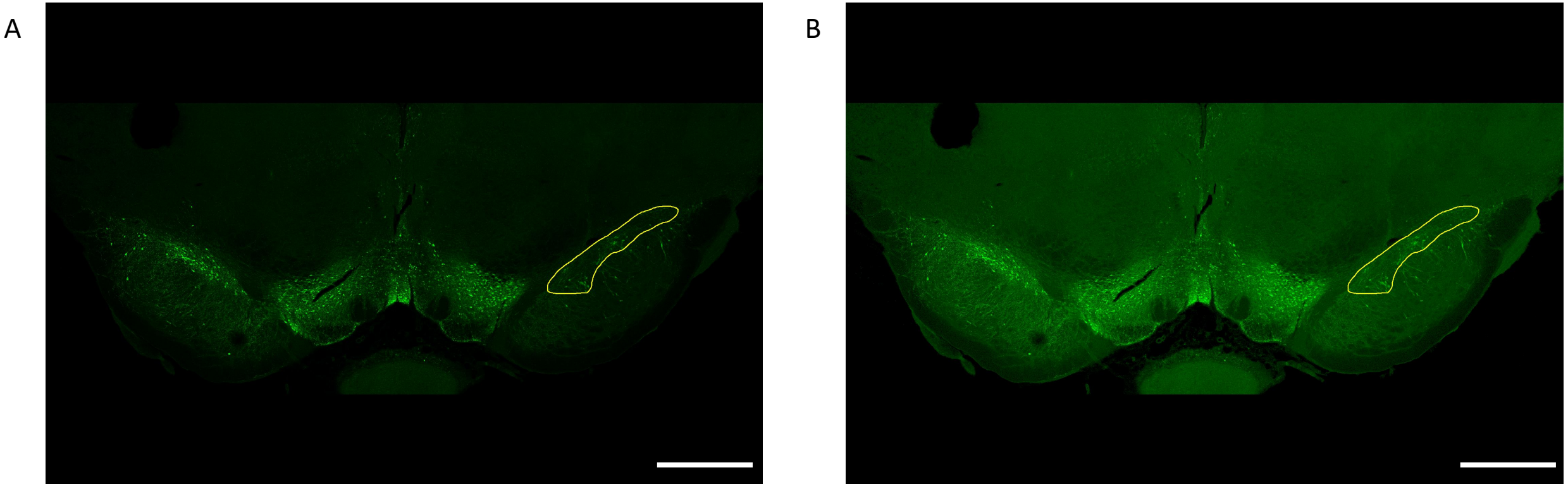


Figure 3

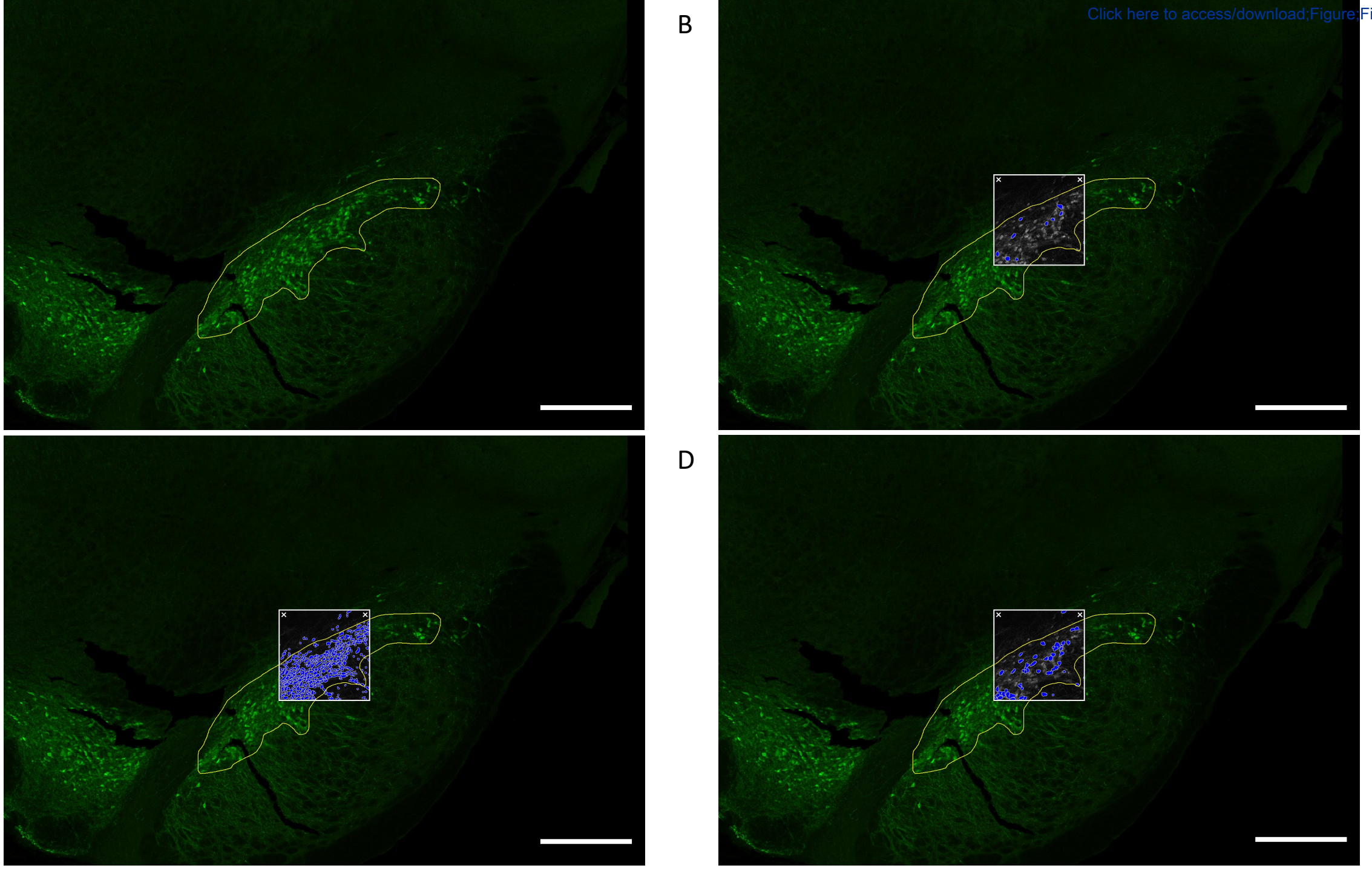
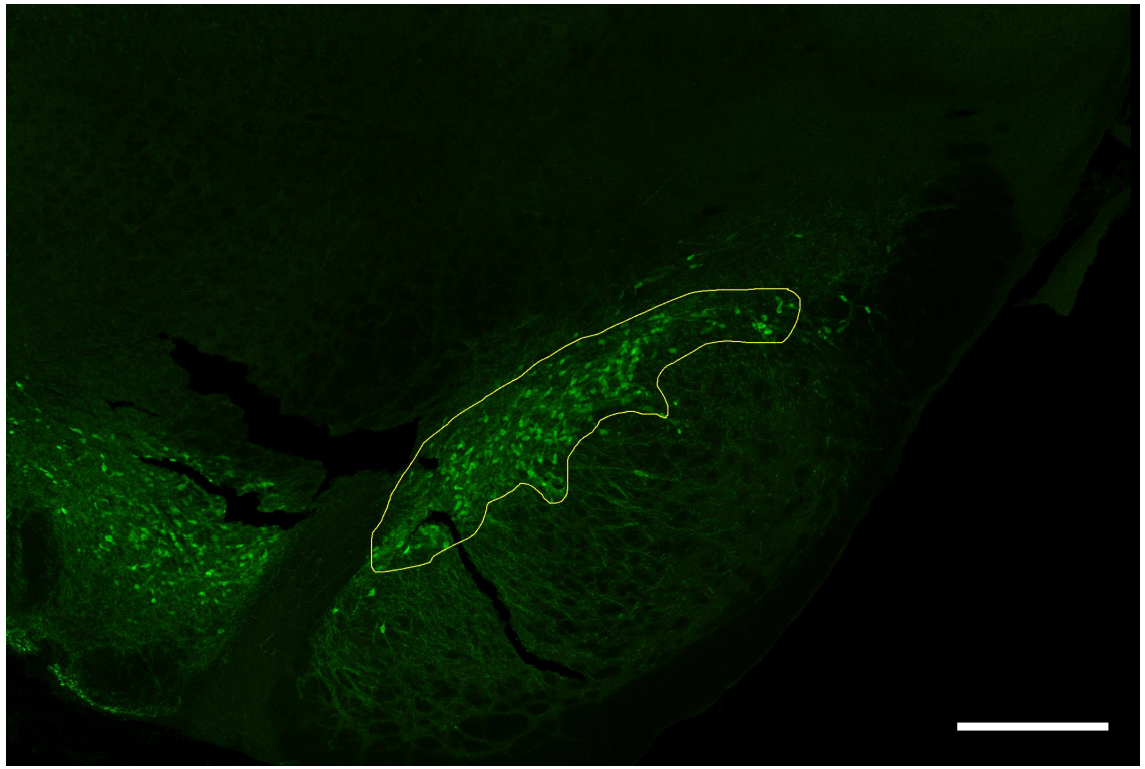


Figure 4

A



B

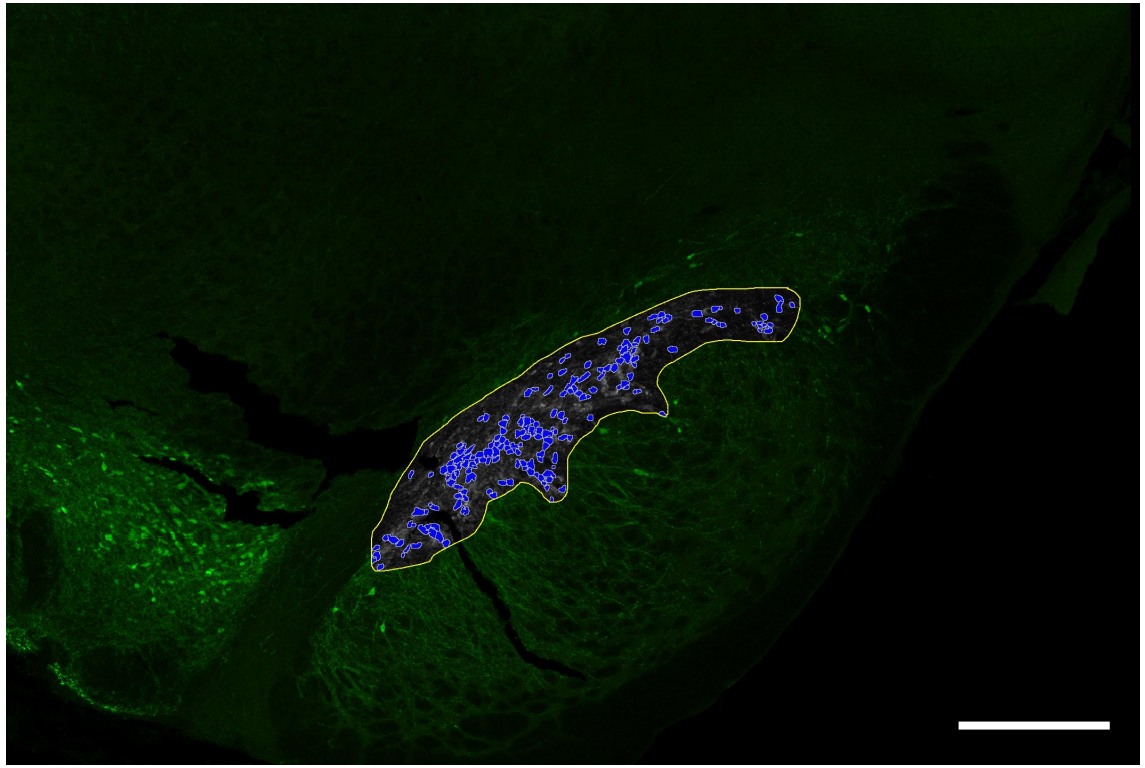
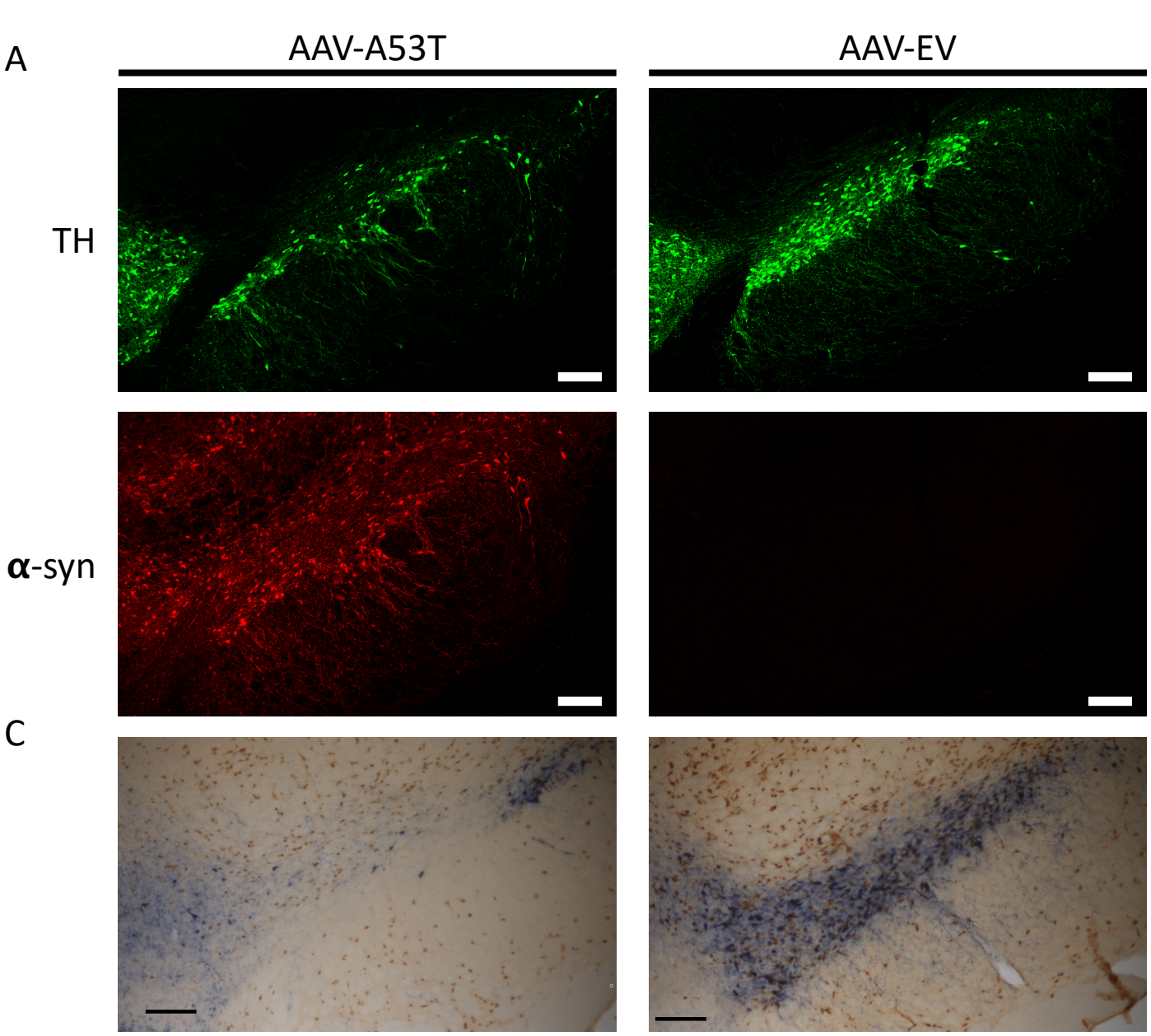
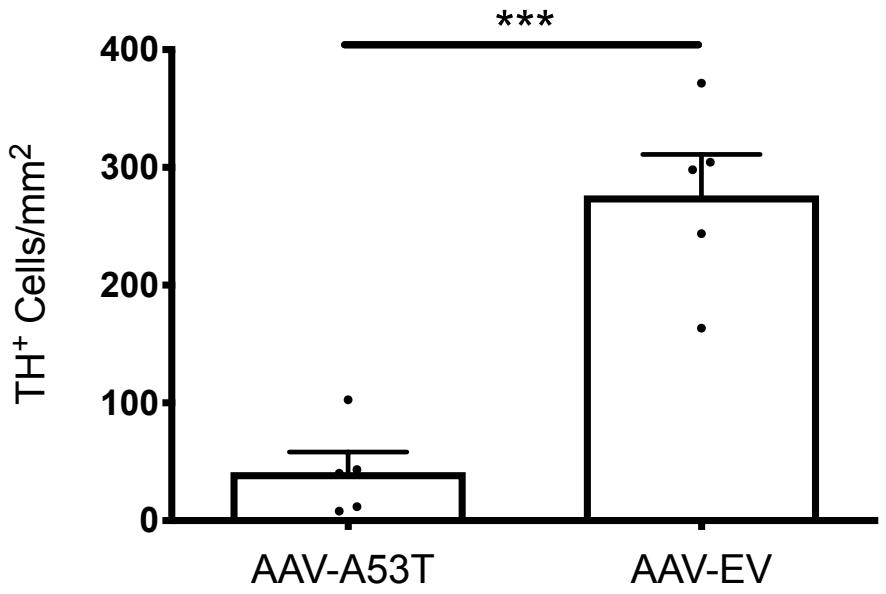
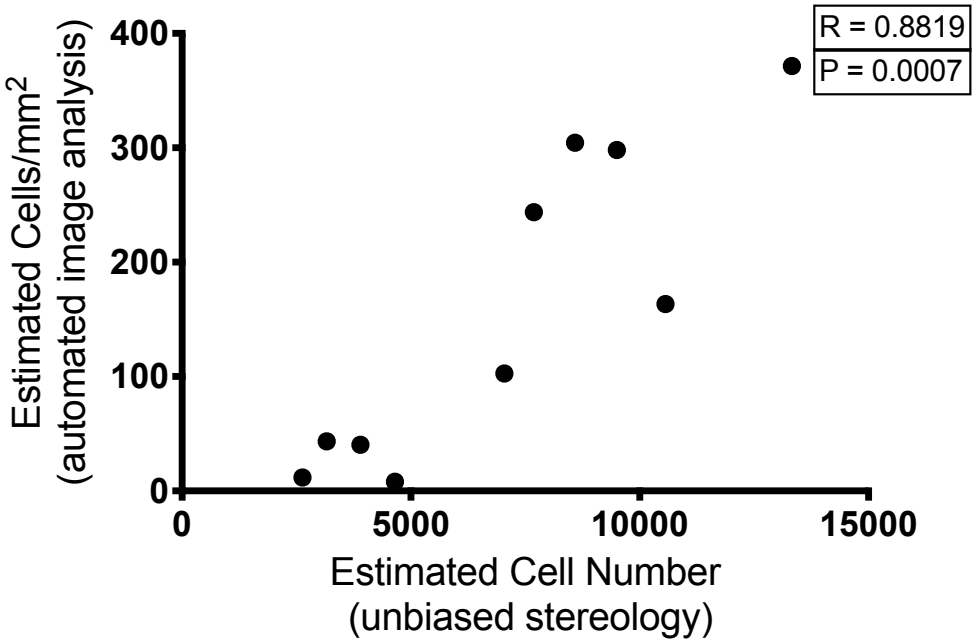


Figure 5

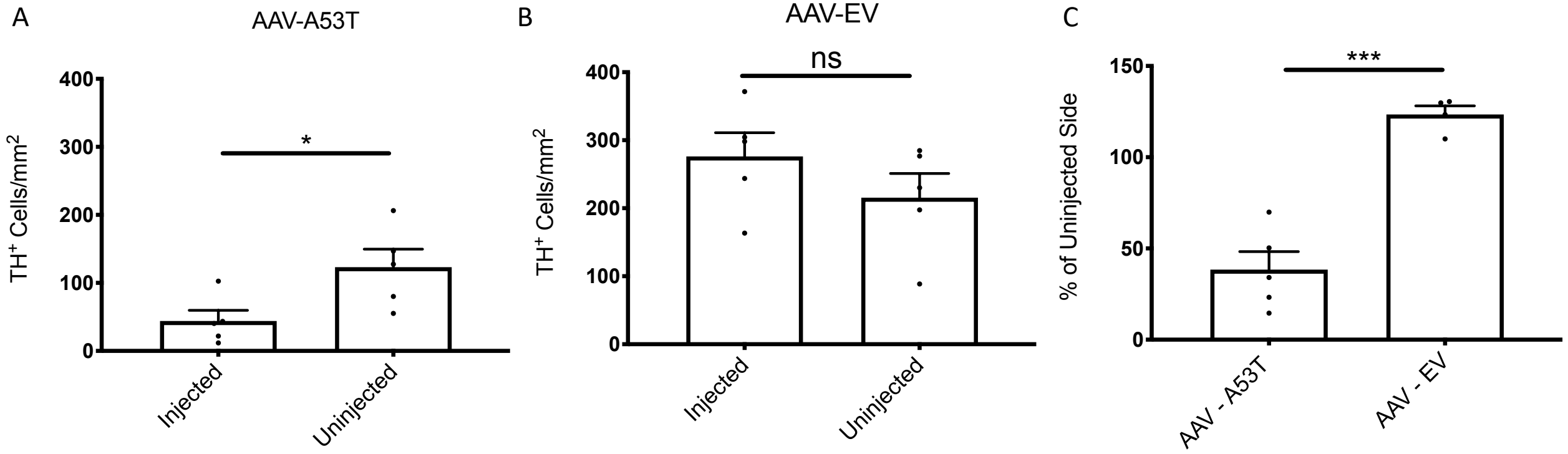






Supplemental Figure 1

Click here to access/download;Figure;Supp Figure 1.pdf



Name of Material/Equipment	Company	Catalog Number	Comments/Description
A-Syn Antibody	ThermoFisher Scientific	32-8100	
ABC Elite	Vector Labs	PK-6102	
Alexa Fluor 488 secondary antibody	ThermoFisher Scientific	A-11008	
Alexa Fluor 555 secondary antibody	ThermoFisher Scientific	A-28180	
Alkaline phosphatase-conjugated anti-rabbit igG	Jackson Immuno	111-055-144	
Biotinylated anti-mouse IgG	Vector Labs	BA-9200	
Bovine Serum Albumin	Sigma	A2153	
DAKO fluorescent mouting medium	Agilent	S3023	
HALO™	Indica Labs		
Histo-Clear II	Diamed	HS202	
ImmPACT DAB Peroxidase substrate	Vector Labs	SK-4105	
LSM880 Confocal Microscope	Zeiss		
NeuN Antibody	Millipore	MAB377	
Normal Goat Serum	Vector Labs	S-1000-20	
OCT	Tissue-Tek		
Paraformaldehyde	BioShop	PAR070.1	
Sliding microtome	Leica	SM2010 R	
Stereo Investigator	MBF Bioscience		
Sucrose	BioShop	SUC700	
TH Antibody	ThermoFisher Scientific	P21962	
VectaMount mounting medium	Vector Labs	H-5000	
Vector Blue Alkaline Phosphatase substrate	Vector Labs	SK-5300	
Zen Black Software	Zeiss		
Zen Blue Software	Zeiss		

Darren O'Hara
Post-Doctoral Fellow
Krembil Research Institute
UHN
Toronto

31st December 2020

Dear Dr. Nguyen,

Re: Manuscript JoVE62062

Thank you for the opportunity to re-submit our manuscript "*Semi-quantitative method to determine dopaminergic neuron density in the substantia nigra of rodent models using automated image analysis software*."

We have now addressed the key concerns raised by the reviewers and made the appropriate changes to our manuscript (see our detailed point-by-point response below). Specifically, we performed unbiased stereology to verify our findings according to the requests of two of the reviewers. Our results using unbiased stereology show a significant positive correlation with our initial results using the automated image analysis software. Furthermore, we have added a limitations section which addresses the valid concerns of the reviewers.

We hope that we have sufficiently addressed the reviewers comments and our work is now acceptable for publication in *JoVE*. We believe that our method has significant utility in providing a time efficient, semi-quantitative estimate of cell density, and we are confident that our manuscript will provide valuable information on assessing dopaminergic neuronal survival for the field of Parkinson's disease research and the wider field of neurodegeneration.

We thank you, and the reviewers for considering our work for publication.

Sincerely,

Darren O'Hara

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Authors' response: The manuscript has been proofread to ensure there are no spelling or grammar issues and have defined all abbreviations at first use.

2. Please make your title concise to fit it within 150 characters: Quantification of Dopaminergic Neuron Number in the Substantia Nigra of Viral Vector-Based α4 Synuclein Rat Models

Authors' response: The title has been amended: "Semi-quantitative method to determine dopaminergic neuron density in the substantia nigra of rodent models using automated image analysis software" which is 146 characters with spaces.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Authors' response: This change has been made in the revised manuscript.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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: HALO™ (Indica :Labs, Corrales, NM, USA); ThermoFisher P21962; Thermofisher 32-8100; Dako, Agilent Technologies, Inc., Santa Clara, CA, USA; Zeiss LSM 880; ZEN Black software (Zeiss, Oberkochen, Germany; ZEN blue (Zeiss) etc.

Authors' response: All commercial language has been removed in the revised manuscript and replaced with generic terms. All commercial products have been referenced in the Table of Materials and Reagents.

5. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

Authors' response: An ethics statement is now provided prior to the numbered protocol steps (Lines 93-95).

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your

protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Authors' response: We have added more specific details to the protocol steps in the revised manuscript.

7. 2.1: Please describe the process of anesthetization or cite a reference if this will not be part of the video.

Authors' response: More details on anesthetization are provided in Sections 2.1 and 2.2 (Lines 109-112).

8. 2.2: How is transcardial perfusion to be done? Please provide details.

Authors' response: More details on transcardial perfusion are provided in Sections 2.3 and 2.4 (Lines 113-120).

9. 2.7: Instead of "grape-sized amount", please provide a measurable quantity.

Authors' response: We have replaced "grape-sized amount" with "until it forms a circle 2cm in diameter and 0.5cm thick" on Line 133.

10. 3.7: How do you use the range indicator to ensure that the signals are not overexposed?

Authors' response: More details on the range indicator are provided in Section 3.7 (Lines 173-175).

11. 4.6: What settings you use for these parameters, and what is the goal of this adjustment?

Authors' response: More details on the goal of adjustments in the software are provided in Section 4.6 (Lines 200-206).

12. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Authors' response: We have highlighted the text to be filmed in yellow.

13. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Authors' response: Scale bars have been added to all images taken with a microscope and defined in the appropriate Figure Legend.

14. Discussion lines 265-267: Did you use stereotactic injection here in this study? If so, please clarify in the protocol. If not, please cite that reference.

Authors' response: We clarify that stereotactic injection was used in the study and cite a reference in Section 1.2 (Lines 100-103).

15. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Authors' response: Disclosures section is now included immediately after the Acknowledgements (Lines 396-397).

16. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references. Please do not abbreviate any journal names.

Authors' response: Reference formatting have been changed to adhere to these guidelines.

17. Please sort the Materials Table alphabetically by the name of the material.

Authors' response: Materials Table has been sorted in this manner.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes an image analysis method for estimation of cell numbers in a defined region of interest. The idea to speed up the process of dopaminergic SN neuron quantification is important and, as indicated by the authors, urgently needed.

However, I have major concerns regarding the scientific accuracy of this method described.

Major Concerns:

Although I like the idea that quantification of cell numbers can be done automatically by a software and thus can save precious time for the researcher, the method described is not sufficient to do so. It is rather a step backwards from what we nowadays know about cell quantification. There is a reason why design-based unbiased stereology is so complex and why neuroscience field has moved from 2-dimensional quantification (as here presented) forward to design-based unbiased stereology, because the reliability and reproducibility is superior for many reasons. The here presented method is inferior because of the following reasons:

a) not unbiased and I completely disagree with the authors claim that "having a blinded observer identify regions of interest in the SNpc removes all bias…" Does this mean that every blinded analysis is unbiased?

Authors' response: A blinded observer can mitigate confirmation or selection biases but we agree with the reviewer that it does not remove *all* bias from the analysis. Therefore, in the Discussion section in the revised manuscript, we have replaced the words "removes all biases" with "mitigates confirmation or selection biases" to make this more explicit (Lines 367-369).

b) does not give an estimated absolute cell number but a cell density,

Authors' response: We have highlighted this point in the Limitations section (Lines 375-389) we have now added to the revised version of the manuscript. We have also emphasized that HALO provides semi-quantitative measurements (eg. Line 364). To further emphasize this point and avoid any potential for confusion, we have changed the title of the manuscript to include "semi-quantitative".

c) cell size will matter with this method. This has already been taken into account 70 years ago by Abercrombie in 1946 (Estimation of nuclear population from microtome sections, Anat Rec., 1946). The method described here totally neglects this fact.

Authors' response: The method can and does take a minimum cell size into account. As mentioned in the Protocol, sections are 40µm thick and the pinhole is opened to allow a section of around 1.5µm to be imaged in a single plane in the middle of 40µm section (Section 3.1). Furthermore, it is possible with this approach to assess for changes in cell size which can be monitored as part of the analysis. Being able to assess for significant differences in cell size (within the limitations of this approach) will allow the investigator to understand if this contributes to the findings. We further re-

iterate that the benefit of this approach is its efficiency in testing potential therapeutics in the Limitations section (Lines 375-389) we have added to the revised manuscript.

d) The data from cell counts underline the inaccuracy and lack of efficacy of the method, since the authors find on the injected AAV-A53T SNpc around 40 cells/mm2, in the AAV-A53T uninjected side 123 cells and in the AAV-EV injected side 276, in the AAV-EV uninjected side 215 cells/mm2. I wouldn't expect a <50% reduction of the AAV-A53T uninjected side compared to EV and a difference of >30% comparing AAV-EV injected to non-injected side.

Authors' response: The reviewer raises an important concern and we do make the point that care should be taken when comparing the injected and uninjected sides. We believe a large part of the discrepancy arises from focus and not the quantification performed by HALO. The focus is set using the injected side and because of the section thickness, the uninjected side is often slightly out of focus. As such, the revised manuscript highlights comparisons between injected hemispheres between mutant A53T α-syn (AAV-A53T) empty vector AAV (AAV-EV) groups (Lines 228-238). We include the comparison between injected and uninjected sides (Lines 249-257) in order to highlight limitations in the Discussion section. We also make it clear that, due to these concerns, we recommend that each side should be imaged separately if labs wish to use this within-subjects comparison (Lines 349-350).

To verify that the method is at least as good as traditional counting methods such as unbiased design-based stereology the authors should verify if there is a significant difference between these methods (including all slices). For this, one needs to find a mathematical method to calculate from mm2 the absolute estimated number of neurons.

Authors' response: In the revised manuscript, we have performed stereological counts in sections from the same animals (Figure 5C,D). Correlational analysis between the absolute cell number quantification by stereology and the HALO cell number (cells/mm^2) yielded a significant positive correlation, suggesting that HALO can be used to provide a semi-quantitative assessment of cell numbers. As we emphasize in the revised version of the manuscript, we believe HALO and similar software platforms can provide semi-quantitative measures of dopaminergic neuron number (Line 364). In studies focused on determining the relative differences between groups or treatment conditions (and not necessarily quantifying absolute cell numbers), cells/mm^2 measurements have utility to account for differences in size of region of interest.

In which aspects is the described method advantageous over or at least equivalent to AI Aiforia that authors cite?

Authors' response: This comparison is outside of the scope of the current study. We are not stating that one program is superior to another. Each program is expected to have its own advantages and disadvantages, but the more salient point here is that programs in which you train the AI with annotated examples can provide semi-quantitative estimates of cell numbers using digital technologies that are adaptable to quantitate pathology. While our lab has not used AIFORIA, our understanding is that their platform is primarily AI based in the cloud whereas HALO is not. HALO

offers 5 AI networks that can be used for quantification at the cellular level using algorithms to analyze macro-cellular objects including the segmenting of nuclei and phenotyping them across a study, microcellular objects such as glomeruli, as well as modules for multiplex IHC or highplex FL that can quantify fluorophores, and provide outputs such as cell intensity in the nucleus, cytoplasm and/or membrane, cell area, total tissue/analysis area, etc. In the revised manuscript, we make reference to AIFORIA and cite a recent publication utilizing the software program to quantify dopaminergic neuron numbers in the SNpc (Lines 353-355). We have not included the name of the software program in the manuscript as we have been instructed by the editor that JoVE cannot publish manuscripts containing commercial language.

Minor Concerns:
In the introduction, the authors describe that the contralateral uninjected SNpc acts as control for the injected side. Contrasting this statement, the results part describes in the first part the comparison of AAV-A53T to AAV-EV as controls. This is shown in Fig 4. This is then followed by comparison of the injected side with the uninjected side in both AAV-A53T and AAV-EV rats. The authors should to modify their statement in the introductory part since it contains only part of what they have done.

Authors' response: The sentence in the Introduction has been amended in the revised manuscript to focus on the between-subject comparison performed between AAV-A53T to AAV-EV groups (Lines 85-87).

In line 256 authors describe that ' it is important to control and minimize potential confounds that may reduce the reliability '. How do they exclude possible alterations due to photobleaching or differences in staining intensity throughout the process of quantification? This information should be added.

Authors' response: We have included control samples that have been processed, stained and analyzed in a uniform fashion to rule out potential confounds that are introduced during the preparation of the tissue for analysis. To make this clearer in the revised manuscript, we have included the following sentence (Line 328-330): "Antibody incubation times are uniform throughout the protocol and the Focus is set in the minimum time possible to avoid photobleaching."

The AAV titer is weird. If it's the same AAV used by the Koprich/Brotchie group then it's around 1/1.000.000 concentration since the Brotchie group uses around 2.55-5.10 x 1012 gp/ml. The authors here use 3.4 x 106 gp/ml (since its an AAV it should be gp rather than vp) and interestingly find loss of neurons.

Authors' response: This was a typo and has been corrected in the revised manuscript (Lines 102-103).

Reviewer #2:
Manuscript Summary:
In this manuscript O'Hara et al. describe the protocol to quantify dopaminergic neurons in rat brain with an automated software (Halo), as an alternative to the time-consuming stereology methodology. The procedure is thoroughly explained from stereotactic injection of the AAV vector to the complete analysis using the Halo software.

Major Concerns:
** Figure 5 Lines (212 to 215). The average values for the non injected (123 for aSYN inj animals, 215 for EV animals) or EV injected (276) are quite variable (almost 50% variation) despite this should be the same. How can you explain this? One would like to see the raw numbers to be able to see these quantifications and how reliable they are. In stereology uninjected values remain with little variation between animals. This is important to measure a lesion. If you present this as % from the uninjected side, you will have an incorrect estimation if your uninjected side is so variable. One would like to have a constant number of cells in the uninjected side, otherwise lesion quantification is not reliable with this software.*

Authors' response: The reviewer raises an important concern and we do make the point that care should be taken when comparing the injected and uninjected sides. We believe a large part of the discrepancy arises from focus and not the quantification performed by HALO. The focus is set using the injected side and because of the section thickness, the uninjected side is often slightly out of focus. As such, the revised manuscript highlights comparisons between injected hemispheres between mutant A53T α-syn (AAV-A53T) empty vector AAV (AAV-EV) groups (Lines 229-239). We include the comparison between injected and uninjected sides (Lines 249-257) in order to highlight limitations in the Discussion section. We also make it clear that, due to these concerns, we recommend that each side should be imaged separately if labs wish to use this within-subjects comparison (Lines 349-350).

Minor Concerns:
Despite it is proposed as a good option to scan for therapeutic options for PD, the fact that you need confocal images makes it a less high-throughput option. If this could be done with chromogenic staining DAB and scanned with a slide-scanner instead it would allow to accelerate the quantification method. If chromogenic staining would not be an option, despite this is the preferred staining for stereological quantifications. The authors should specify if getting a single focal plein is needed (confocal image).

Authors' response: HALO is compatible with chromogenic staining with DAB and digitized whole slide images using a slide-scanner, but this is not recommended when working with 40µm sections as we are here, since we would need to manually set the focus for each slide. A sentence has been added in the Discussion (Lines 371-373) to account for future advancements in slide-scanning technology which will allow for better imaging of thick sections.

On top of this it would be good if the authors provide some more information regarding the following points:

* *Line 93, specify volume and total amount of viral vector genomes or particles injected.*

Authors' response: The volume and total amount of viral particles injected are specified in Section 1.2 in the revised manuscript (Lines 102-103).

* *Line 139, specify magnification used. Single z taken or several in each section? How is this selected (middle or depending on focus? This will change the number imaged per section. Do you select a specific Z per section quantified?*

Authors' response: Details regarding magnification (10x), pinhole size (1.5 AU) and selection (focus on the injected SN) are specified in Section 3.1 in the revised manuscript (Lines 161-163).

* *How many sections are captured per animal and needed?*

Authors' response: In the Representative Results section (Lines 236-239), we state that "…4 sequential sections per animal were analyzed. Previous studies have shown significant differences with as little as 3 sections, but analysis can be further increased up to 12 sections to encompass the whole SNpc depending on the model and intervention being studied by the investigator."

Variability would be more visual if individual animals are presented as dots in a graph instead of a bar. Additionally, specify if the error bars are SD or SEM. Additionally adapt the graphs axis from 5A and 5B same values.

Authors' response: We have included values from individual animals as dots in the revised figures. We indicate that error bars are SEM in the revised manuscript.

Reviewer #3:
Manuscript Summary:
O'Hara et al. describe the usage of the HALOTM image analysis platform as an automated method for the quantification of dopaminergic (DA) neurons in the substantia nigra of pre-clinical Parkinson's disease models, in particular, the AAV-alpha-synuclein A53T overexpression model. The development of a method that provides time efficient and accurate determination of DA cell number is relevant to the field of PD research in order to assess the potential of new disease-modifying therapeutics in a cost-effective and reliable manner (reducing human error and increasing reproducibility). The HALOTM image analysis platform was previously developed by Indica Labs and this study validates its utility in a pre-clinical PD model like the AAV-Syn model. The protocol is clearly written and generally very detailed, although some important information is missing regarding the image software analysis. In addition, comparison of their method with the gold standard method for quantification, unbiased stereology, should be performed in order to really evaluate the added value of the current protocol. The method here presented is promising but essential information is missing to really show it is an "accurate, quick and relatively inexpensive procedure".

Major Concerns:
Specific comments on the protocol:

1- Stereotaxic injection: indicate that the AAV injection is done unilaterally in the right side of the brain (right or left side, according to the preferences of each lab), in order to strengthen that the contralateral uninjected side is used as an internal control for the injected side.

Authors' response: More details on unilateral AAV injection are provided in Section 1.2 (Lines 100-103).

2- Point 4.6 refers to "accurate representation of each individual cell as a single cell … is vital for accuracy". Which criteria are used to define what a TH cell is? Is presence of TH immunostaining in focus the only criteria? Are cells where the nucleus excluded from TH staining is not visible also considered? Please consider adding a figure with representative images of the criteria used to show the system what a positive cell is.

Authors' response: Figure 3 in the revised manuscript contains several images displaying optimization of cell detection using the Cytonuclear method in HALO. Real-time tuning of the cytonuclear module permits altering Nuclear Contrast Threshold, Minimum Nuclear Intensity, Nuclear segmentation Aggressiveness, and Nuclear Size and is described in the revised text (Section 4.6) .

3- Point 4.7 indicates the process should be repeated with a minimum of 10 separate samples. How is this number determined? Have the authors performed the analyses using 5, 10 and 20 samples for example and estimated that with 10 samples the error between the estimate and the real nº of cells is less than 10%, as a universal criteria for acceptance of the error, and thus that sampling 10 samples is sufficient?

Authors' response: This number was determined by repeatedly sampling real-time tuning across a variety of sections, projects. In our hands, it has repeatedly been found that this represents a sample whereby the software has "learned" what a cell is, and manual counts by eye do not differ from those generated by the software.

4- Point 4.8 mentions additional cell markers like asyn or NeuN, please add the antibody reference and dilution for NeuN immunostaining.

Authors' response: The reference has been included in the Table of Materials.

5- Point 4.12: please indicate an estimate of the number of hours of analysis for a given number of images. This number is essential to evaluate if the method here proposed is really better in terms of time efficiency compared to other available methods.

Authors' response: The revised manuscript (Lines 217-219) includes an estimate of the number of mins of analysis time required for each brain (i.e., 5 min). So for an N = 10 as presented, it would take about 1 hour (Section 4.12).

6- Point 4.14: quantification of DA neurons in preclinical studies is usually given as the absolute number of DA cells in the SNpc, rather than as the nºcells/mm2. Please include this calculation in your protocol.

Authors' response: As we emphasize in the revised version of the manuscript (Lines 364-365), we believe HALO and similar software platforms can provide semi-quantitative measures as a surrogate of dopaminergic neuron number. In studies focused on determining the relative differences between groups or treatment conditions (and not necessarily quantifying absolute cell numbers), cells/mm^2 measurements have utility to account for differences in size of region of interest.

7- Is the HALO software freely available or is there a license to be paid? Is the license for one computer or several, annually or for a limited number of analyses (hours)? All this information seems important to determine if the method is "relatively inexpensive" as the authors claim.

Authors' response: HALO software requires a license to be paid per computer it is installed on. Our license agreement was negotiated by the University Health Network's Advanced Optical Microscopy Facility and Indica labs and is available to use on fee-for-use basis ($10/hr). Therefore, the overall cost will depend on the institutional resources available, but it can be relatively inexpensive when it is available through a core facility. We have not included this information in the manuscript as we have been instructed by the editor that JoVE cannot publish manuscripts containing commercial language.

8- Consider adding a figure with a graphical workflow of the procedure to better illustrate the different steps.

Authors' response: We have added a figure with a graphical workflow of the procedure (Figure 1) as suggested.

Specific comments on the representative results:

1- Quantification of DA neurons in preclinical studies is usually given as the absolute number of DA cells in the SNpc, rather than as the nºcells/mm2. To present the results in nºcells/mm2 makes it difficult to compare to other published results. Maybe data could be presented in both ways: as nºcells/mm2 and as absolute numbers.

Authors' response: Unlike stereology, the method cannot provide an estimation of absolute cell numbers but instead calculates cell density. We have included this point in the Limitations section we have now added to the manuscript (Lines 377-380). As we emphasize in the revised version of the manuscript, we believe HALO and similar software platforms can provide semi-quantitative measures of dopaminergic neuron number (Lines 364-365). In studies focused on determining the relative differences between groups (not differences in absolute cell numbers), cells/mm^2 measurements have utility to account for differences in size of region of interest.

2- Line 198: please indicate that samples were analyzed 6 weeks after AAV-injections, in this kind of progressive models it is important to know the time-point of analysis to contextualize the results.

Authors' response: The 6 week time-point is specified at the beginning of the Representative Results section (Line 229).

3- The protocol here described only used 4 sequential sections per animal instead of 12 sections to encompass the whole SNpc. This "simplification" of the protocol can introduce an important bias in the interpretation of the results specially in models dependent on the stereotaxic injection of AAVs above the SNpc. Despite the stereotaxic coordinates to target the SNpc are very well established, these types of procedures always carry some degree of inter-animal variability. The whole SNpc should be considered to improve the accuracy of the results.

Authors' response: Using the model presented here, we have not noticed a significant difference in our ability to discriminate groups when we have increased the number of sections analyzed from 4 to 6. This is not unexpected since the 4 sequential sections we typically analyze contain the densest proportion of TH+ cells in the SNpc and more importantly also contain the region immediately around the area of AAV injection where the effects of mutant α-syn are the most prominently noted. We have added a sentence in the revised text indicating that sampling the whole SNpc may need to be considered for other models (Line 238).

4- Comparison of the obtained results in AAV-EV and AAV-Syn A53T rats with their method and with the gold standard method for quantification, unbiased stereology, should be performed in order to really evaluate the added value of the current protocol. Correlation analyses would strengthen the utility of the proposed method.

Authors' response: Comparison between AAV-EV and AAV-Syn A53T rats and correlation between counts provided by HALO and unbiased stereology are included in the revised manuscript (Figure 5D). Correlational analysis between the absolute cell number quantification by stereology and the HALO cell number (cells/mm^2) yielded a significant positive correlation, suggesting that HALO can be used to provide a semi-quantitative assessment of cell numbers.

The authors might want to add a point in the discussion about other types of artificial intelligence systems that have recently been described for similar purposes (i.e. Aiforia, for example).

Authors' response: We have added the following sentence with a citation referring to a paper utilizing AIFORIA to count dopamine neurons in substantia nigra (Lines 353-355): "Other programs are available and are increasingly being adopted to study neuropathology, including the quantification of dopaminergic neuron loss in experimental models of PD (Penttinen, 2018)." We have not included the name of the software program in the manuscript as we have been instructed by the editor that JoVE cannot publish manuscripts containing commercial language.