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Stereological estimation of cholinergic fiber length in the nucleus basalis of Meynert of mouse brain

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Phillip Steindel, Ph.D.
Review Editor
JoVE
1 Alewife Center, Suite 200, Cambridge, MA 02140

August 20, 2019

Dear Dr. Steindel,

Please find our revised manuscript entitled “Stereological estimation of cholinergic fiber length in the nucleus basalis of Meynert of mouse brain”.

We are thankful to reviewers for providing many positive comments and suggestions to improve the manuscript. We have incorporated most of the suggestions of the reviewers in the manuscript. Also, we have highlighted about 2.5 pages of protocol which we believe important for making video.

Thank you for your consideration.

Yours sincerely,

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TITLE:

Stereological Estimation of Cholinergic Fiber Length in the Nucleus Basalis of Meynert of the Mouse Brain

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KEYWORDS:

choline acetyltransferase (ChAT), nucleus basalis of Meynert (NBM), immunohistochemistry (IHC), stereology, fiber length, space ball

SHORT ABSTRACT:

Neuronal fiber length within a three-dimensional structure of a brain region is a reliable parameter to quantify specific neuronal structural integrity or degeneration. This article details a stereological quantification method to measure cholinergic fiber length within the nucleus basalis of Meynert in mice as an example.

LONG ABSTRACT:

The length of cholinergic or other neuronal axons in various brain regions are often correlated with the specific function of the region. Stereology is a useful method to quantify neuronal profiles of various brain structures. Here we provide a software-based stereology protocol to estimate the total length of cholinergic fibers in the nucleus basalis of Meynert (NBM) of the basal forebrain. The method uses a space ball probe for length estimates. The cholinergic fibers are visualized by choline acetyltransferase (ChAT) immunostaining with the horseradish peroxidase-diaminobenzidine (HRP-DAB) detection system. The staining protocol is also valid for fiber and cell number estimation in various brain regions using stereology software. The stereology protocol can be used for estimation of any linear profiles such as cholinoceptive fibers, dopaminergic/catecholaminergic fibers, serotonergic fibers, astrocyte processes, or even vascular profiles.

INTRODUCTION:

Quantitative estimates of length and/or density of nerve fibers in the brain are important parameters of neuropathological studies. The length of cholinergic, dopaminergic, and serotonergic axons in various brain regions are often correlated with the specific functions of the region. Because the distribution of these axons is generally heterogeneous, design-based stereology is used to avoid bias during sampling. The space ball probe of stereology has been designed to provide efficient and reliable measures of line-like structures such as neuronal fibers in a region of interest[1]. The probe makes a virtual sphere that is imposed systematically in the tissue to measure line intersections with the surface of the probe. Because it is impossible to put sphere probes in the tissue for analysis, the commercially available software provides a virtual three-dimensional (3D) sphere, which is basically a series of concentric circles of various diameters that represent the surface of the sphere probe.

Selective cholinergic neurodegeneration is one of the consistent features of Alzheimer's disease (AD)[2-4]. Dysfunctional cholinergic transmission is considered a causative factor for cognitive decline in AD. Cholinergic dysfunction is also evident in many other mental disorders such as Parkinson's, addiction, and schizophrenia. Different aspects of cholinergic neurodegeneration are studied in animal models (e.g., reduction in acetylcholine[5], ChAT protein[6], cholinergic fiber neurodegeneration in the vicinity of amyloid plaques[6], and decrease in cholinergic fibers and synaptic varicosities[7,8]). Fiber degeneration is believed to take place earlier than neuronal loss, because cholinergic neuronal loss is not always observed in studies. Most of the cholinergic neurons are in the basal forebrain and the brain stem, and their axons project to various brain regions such as the cortices and hippocampus. NBM is situated in the basal forebrain and found to be one of the commonly affected brain areas in AD.

The fractionator method of stereology is based on systematic random sampling of a tissue at multiple levels. Section sampling fraction (SSF) is the non-computer based systematic sampling of sections for the fractionator method of stereology. Area sampling fraction (ASF) is fractionation of an area of the region of interest in the section. Thickness sampling fraction (TSF) is the fractionation of the thickness of a section. The space ball probe allows us to quantify profiles of interest in a 3D sphere at fractionated locations. Here we use a space ball probe for estimation of the total length of cholinergic fibers in the NBM of mouse brain to illustrate the procedures. The current protocol provides details on tissue processing, sampling methods for stereology, immunohistochemical staining using the ChAT antibody, and unbiased stereology to estimate cholinergic fiber length and fiber density in the NBM of mouse brain.

PROTOCOL:

All procedures for using these animals have been approved by the Kansas City Veterans Affairs Medical Center Institutional Animal Care and Use Committee. Eighteen-month-old mice overexpressing Swedish mutant beta-amyloid precursor protein (APPswe) and their C57/BL6 WT littermates were used for the experiments. Details of breeding and genotyping is given in He et al.[8].

1. Perfusion and tissue processing

1.1. Anesthetize mice using an intraperitoneal injection with ketamine (100 mg/kg) and xylazine (10 mg/kg). Pinch toes to confirm a lack of response before continuing[9].

1.2. Transcardially perfuse with ~50 mL of ice cold 0.1M Dulbecco's phosphate buffered saline (DPBS) followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB)[10].

CAUTION: PFA is toxic. Use personal protective equipment (PPE) when working with PFA.

1.3. Remove brains[10] and immerse in 4% PFA in 0.1 M PB at 4 °C for postfixation for 24 h. Wash with DPBS 3x.

1.4. Change to 15% sucrose solution in 0.1 DPBS overnight and then 30% sucrose solution in 0.1 M DPBS for another 48 h at 4 °C.

1.5. Remove the tissue from the sucrose solution and freeze in a cryotome chamber preset to a temperature of -20 °C. Frozen samples can be stored in sealed tubes at -80 °C until sectioning.

1.6. Embed tissues in optimum cutting temperature embedding medium (see **Table of Materials**) and mount on a specimen disc. Cut serial 30 µm thick sections in a coronal plane using a cryotome and collect all the sections consequently in 24 well culture plates filled with cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% DPBS; **Figure 1A**–**C**). Store in a -20 °C freezer.

NOTE: Thicker sections (e.g., 50 µm) are preferable when feasible. Make sure that the sections are kept in cutting order and all sections are completely in cryoprotectant. A 96 well plate can be used as an alternative to 24 well plates to collect sections.

1.7. Cover the wells with plate sealer to prevent evaporation and drying. Store plates at -20 °C until further use. Sections stored at -20 °C are stable for several months for ChAT immunohistochemistry.

2. Systematic section selection for IHC

NOTE: A pilot study should be done to know the total number of sections required to achieve an acceptable coefficient of error (CE). The CE value is an expression of the total amount of error in the sampling procedure. The lowest value represents the minimal error and a CE value lower than 0.1 is considered acceptable by the software used here (see **Table of Materials**)[11].

2.1. Identify the first and last sections for each brain by comparing morphological features with a standard mouse brain atlas such as Franklin and Paxinos. NBM begins at bregma -0.0mm and ends at -1.6 mm. Therefore, about 50 sections contain NBM. The total number of sections is one of the parameters required during stereology. The selection of every 8th section (SSF = 1/8) gave a total of 6–7 sections for the analysis and yielded an acceptable CE for both volume estimation and fiber length estimation in our pilot study.

2.2. Randomly start with one of the first eight sections and then systemically sample every 8th section until the last posterior section containing NBM (**Figure 1C**).

3. Immunohistochemistry

3.1. Transfer the cryopreserved sections to room temperature in cryoprotectant and then 0.1M phosphate buffer (PB) in 6 well plates.

3.2. Wash 3x in PB.

3.3. Incubate in 0.3% H_2O_2 in methanol for 15 min.

3.4. Wash 3x in Tris-buffered saline (TBS).

3.5. Incubate in 0.25% Triton X-100 in TBS for 30 min.

3.6. Block with 10% normal bovine serum (NBS) in 0.1% Triton X-100 in TBS for 30 min.

3.7. Incubate with a 1:1,000 dilution of goat anti-human ChAT primary antibodies (see **Table of Materials**) in TBS with 0.1% Triton X-100 and 1% NBS for 48 h at 4 °C.

3.8. Wash 3x in TBS at room temperature. Perform all further incubation and washing at room temperature.

3.9. Incubate with biotinylated bovine anti-goat secondary antibody (see **Table of Materials**) for 1 h.

3.10. Wash 3x in TBS.

3.11. Incubate with the avidin-biotin–peroxidase complex (see **Table of Materials**).

3.12. Wash 3x in TBS.

3.13. Develop using an enhanced DAB peroxidase substrate solution (see **Table of Materials**) according to the manufacturer's recommendations.

CAUTION: DAB is a suspected carcinogen. It is toxic by contact and inhalation. Use PPE when working with DAB.

3.14. Wash the section a couple of times with distilled water and keep in Tris pH = 7.6.

3.15. Mount the sections on the gelatinized slides. All sections from one tissue can be put on the same slide. Air dry the sections, dehydrate 2x for 5 min each with 70%, 90%, 95%, and 100%

ethanol, and then clear the sections with two 10 min xylene washes. Coverslip the sections using mounting medium (see **Table of Materials**).

NOTE: The processing time of dehydration and clearing affects the thickness of the sections. Therefore, the same conditions should be used for all sections. In the current study, the mean value for the final thickness was 21.11 ± 0.45 µm.

3.16. Keep the sections in the fume hood to dry. The dry sections are ready for stereology.

4. Stereology

NOTE: See the **Table of Materials** for the microscope and software used. An immersion objective with a numerical aperture (NA) > 1.2 will be useful and should be used if required. Slides should be grouped according to genotype or treatment group and coded. The complete stereology for one study should be performed by the same person and the person performing the stereology should be blind to the identity of the individual slides or group examined[1,12,13].

4.1. Open a new study in the software. (**File** > **New Study**). A '**Study Initialization**' dialog box will open. Fill out the study information, using **Multi-level** (**Fraction Based**) (**Figure 2A**).

4.2. Double click on '**Volume**' under **Parameters**, which will open the **Volume** dialog box. Name the feature of interest and select '**Region Point Counting**' probe. Click **Next**.

4.3. Double click on the next parameter, '**Length**'.

4.3.1. Provide a name of the feature (e.g., '**L**') Select '**Sphere**' probe and click **Next**.

4.4. Next, click on the '**Study Initialization**' dialog box, which will open the '**Case Initialization**' box. Fill in the case information. Groups must be coded before starting the study. The total number of sections is the number of sections starting from the first section containing the area of interest to the last section containing area of interest (see step 2.1). The section sampling interval is eight, because every 8th section was selected for the IHC staining.

4.5. Clicking **Next** opens the '**Probe Initialization**' dialog box, which will automatically set the lowest magnification for the region selection and volume estimation. Confirm the settings and check if the region of interest can be identified at the selected lower magnification. Double click on '**Volume'** and fill 50,000 µm^3 per point for the region volume fraction. Click **Done**.

4.6. Under **Object (High) Magnification**, set **Length** at 63x or 100x. Double click on '**Length**' to open the **Length-Sphere** dialog box and set the diameter of the sphere to 10 µm. Click **Done**.

NOTE: The guard zone should be determined based on the actual thickness of the sections. The operator must check the thickness of the sections at multiple sites to avoid any damage. If necessary, adjust the guard zone thickness based on section thickness.

4.7. Set appropriate values for frame area, frame height, guard zone, and frame spacing.

NOTE: These values depend on the heterogeneity of the object profiles (fibers) in the region of study. A frame area of 400 μm^2, frame height of 10 µm, guard zone of 2 µm, and frame spacing of 300 µm gives acceptable CE values for fiber length estimation in NBM. A pilot study must be performed with these values before heading to the next case.

4.8. Follow the instructions provided by the software after each step. The instructions appear either as a dialog box and/or at the bottom of the screen.

4.9. Insert Section 1.

4.10. At low magnification (5x), define the region of interest by making an arbitrary boundary around the NBM. Click the **Next** button at the left corner of the video window. Follow the instructions and confirm all green points are in the NBM. The points can be included or excluded by clicking on the points.

NOTE: There is no definite, set boundary around the NBM. The selection is mostly investigator-defined. The ChAT+ cholinergic neurons of the NBM can be observed in the internal capsule (ic) and globus pallidus (GP). In this protocol, the ic with ChAT+ cholinergic neurons and their projections (fibers) and whole GP is included in the NBM (**Figure 1D**).

4.11. Follow the instructions and change to 63x for fiber measurements at the current fraction. The '**Section Thickness**' dialog box appears on the screen. Set the top and bottom surface of the section to measure the actual thickness of the section. The manual Z axis movement should be used. Click on **Done**.

NOTE: If there is no fiber in the area, the step can be skipped to go to the next fraction location.

4.12. Move the Z axis slowly from top to bottom in the frame height of the section and mark all intersecting fibers on the surface of the virtual sphere probe (**Figure 3**). When done, click **Next** to go to the next location.

4.13. After completing all the fractions, the software will ask to insert the next section. Repeat steps 4.9–4.12 for all six or seven sections of the tissue.

4.13.1. At the end, the software will generate a result for the case showing the CE values (**Figure 4**). If the CE is acceptable, proceed to the next case. **File** > **New Case**. If the CE is not acceptable (**Figure 4A**), the software provides recommendations to change some of the parameters. In many cases, decreasing the frame spacing decreases the CE values to an acceptable range (**Figure 4B**).

4.14. After completing all the cases, generate results for each case and group (**File** >**Results**).

5. Analysis and statistics

5.1. The software provides data for each sample and each group. The software itself calculates fractions such as SSF, ASF, and TSF, and provides the total reference volume (V_{REF}) and total length (L) of the fibers in the reference region (in this case, NBM) (**Figure 4B**). Export the data and save or copy to the statistical software of choice for between group analysis. **Table 1** shows typical result data for statistical analysis. Analyze fiber density (Lv) by dividing the total fiber length with the reference volume.

REPRESENTATIVE RESULTS:

Representative results are shown in **Table 1** and **Figure 5**. Group C, which was decoded as APPswe group (APP), had significantly lower fiber length (**Figure 5B**) and fiber length density (**Figure 5C**) compared to their wild type (WILD) littermates. The results showed that there was no significant difference in the volume of the NBM between the two groups analyzed (**Figure 5A**).

FIGURE AND TABLE LEGENDS:

Figure 1: Illustration of tissue processing and sampling used in the present study. Sample preparation and sampling. (**A**) The cerebellum and olfactory bulb are removed before mounting on the specimen disc. (**B**) Orientation of the brain on the specimen disc for section cutting. A shallow longitudinal incision (marked with a line) on the one hemisphere helps to identify the side of the brain in the sections. (**C**) Schematic diagram of a 24 well plate showing the systematic selection of every 8^{th} section from the 24 well plate (marked as 'X'). (**D**) Schematic diagram of coronal sections delimiting the borders of NBM in the systematically selected six sections. (**E-G**) Representative images of coronal sections immunostained for ChAT and location of NBM (outlined). (**H**) A high magnification image showing the NBM boundaries. LV = lateral ventricle; VL = ventrolateral thalamic nucleus; ic = internal capsule; GP = globus pallidus; CPu = caudate putamen (striatum); NBM = nucleus basalis of Meynert (represented as 'B' in Franklin and Paxinos mouse atlas). Scale bar = 1 mm (**E-G**), 200 µm (**H**).

Figure 2: Screenshot images. (**A**) '**Study Initialization**' (**B**) '**Case Initialization**', and (**C**) '**Probe Initialization**' dialog boxes.

Figure 3: Sphere probe using an optical dissector. Representative screenshot images showing the four planes of a sphere and the marking of intersecting fibers.

Figure 4: Representative results. (**A**) shows an unacceptable CE and (**B**) shows an acceptable CE for length estimation. The ASF, SSF, and TSF for each case is also presented in the results.

Figure 5: Representative data and analysis. Graphical representation of volume (**A**), length (**B**), and length density (**C**) in the NBM of two groups. The data were analyzed within two different groups using the Student t-test. *$p < 0.05$.

Table 1: Representative data and analysis. Volume and length values were directly copied from

the results provided by the stereology software. Length density (Lv) was calculated by dividing the length values by the volume values of each case. p values were calculated using Student's t-test.

DISCUSSION:

Here we demonstrate a method to estimate the density of cholinergic fibers in the NBM using a space ball (sphere) probe. This probe estimates the total fiber length in the region of interest. The total length can be divided by the volume of the region to get the fiber density. To estimate the volume of the region, the Cavalieri point count method was used. The Cavalieri point count method is an unbiased and efficient estimator of a 3D reference volume for any region. The method calculates an estimate of the area on a cut surface of a section by counting points (representing area fractions) and then multiplying by the distance between two sections analysed[11]. The method does not require labor intensive, accurate tracing of the perimeter of the region of interest. It is used in conjunction with optical fractionators to estimate the density of cells and fibers.

Stereological analysis requires a precise sampling method. The brain region of interest should be properly defined with staining. The NBM sits between the AP bregma -0.0 mm to -1.6 mm per the Franklin and Paxinos mouse atlas. For immunohistochemistry, sections should be systematically, randomly chosen, which means that the first section should be selected randomly and then other sections should be chosen systematically. For an adult mouse brain, the NBM consists of about 1,600 µm (anterior-posterior), which means about 53 coronal sections 30 µm thick. Then, if every 8^{th} section is selected, there will be 6−7 sections required to stain for stereological analysis. In our usual procedure, 6−7 sections are enough for estimating the total number of cholinergic fibers in the NBM. Analysis of the CE is suggested for verification at the beginning of the methodological optimization[12].

A proper staining methodology is the basic requirement for a study. ChAT staining for cholinergic fibers can be challenging, and many antibodies stain some of the cellular parts but not the distant axodendritic processes. Please refer to our previously described protocol for the relevant details regarding ChAT staining[8].

The method essentially requires thick sections because histological processing causes shrinkage in the tissue. Ideally, sections of more than a 20 µm post-processing, final thickness (often thinner than the initial tissue section thickness) is required for space ball probes. Therefore, a section thickness of 50 µm is recommended. The shrinkage is generally uneven, and it can affect volumetric distortions within the tissue and therefore change in Lv value. For example, a multifold difference was observed in capillary length density when it was analyzed in vivo using multiphoton imaging[14,15]. Considering this issue, it is more effective to report length per region instead of length per volume.

Although the given values in the protocol worked perfectly, a pilot study for an individual study is always advised. After completing a single tissue sample case, the software provides a CE value for the chosen sampling design. The CE value is used to estimate the precision of the estimate

and can be calculated by several formulas. The software used here uses Gunderson's 1999 formula to analyze the CE and considers it acceptable if the values are less than 0.1[1,16]. The sampling design scheme should be adjusted until the CE value becomes acceptable before the protocol is adapted for other samples. In general, an increased number of sampling (by reducing the frame interval) reduces the CE value. Practically no structure in the biological system is an ideal line profile, but ribbons or cylinders. Therefore, deciding the exact intersecting feature at a focusing point varies from person to person. Thus, the stereology of all samples of a particular study should be performed by same person. To avoid possible bias, the stereology operator should be blind to the sample identifier.

Reproducibility is a major concern in this method. One factor is the tissue shrinkage and deformities during sample processing which can be overcome to some extent by using in vivo confocal microscopy[13-15]. The space ball requires high contrast staining and good imaging resolution to visualize structures. As fibers are not usually in linear form, the determination of intercepts remains mostly the operator's decision. Automated segmentation of histological features has been proposed to overcome this problem. However, this is not available yet[13].

The advantage of using stereology is that it provides an unbiased scheme to analyse structures in a 3D tissue. The space ball probe provides isotropic fractions within tissue samples and therefore offers an unbiased approach to quantify fiber length. An alternative method to analyze fiber density is measuring the optical density of a histochemical staining. The staining intensity-based methods provide semiquantitative estimation of the staining density and may or may not be sensitive enough to differentiate the changes of cholinergic neurons and fibers in the NBM. Stereological methods using the space ball (sphere) probe uses the determination of fibers based on their visual characteristics and provides estimation of the real length of the fibers. The protocol can also be used to analyze other linear profiles in the brain, such as cholinoceptive fibers (using acetyl choline esterase histochemistry), dopaminergic or catecholaminergic fibers (tyrosine hydroxylase immunostaining), serotonergic fibers (serotonin immunostaining), vascular structures (CD31 immunostaining), or astrocyte processes (glial fibrillary acidic protein, GFAP, immunostaining)[17-21].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

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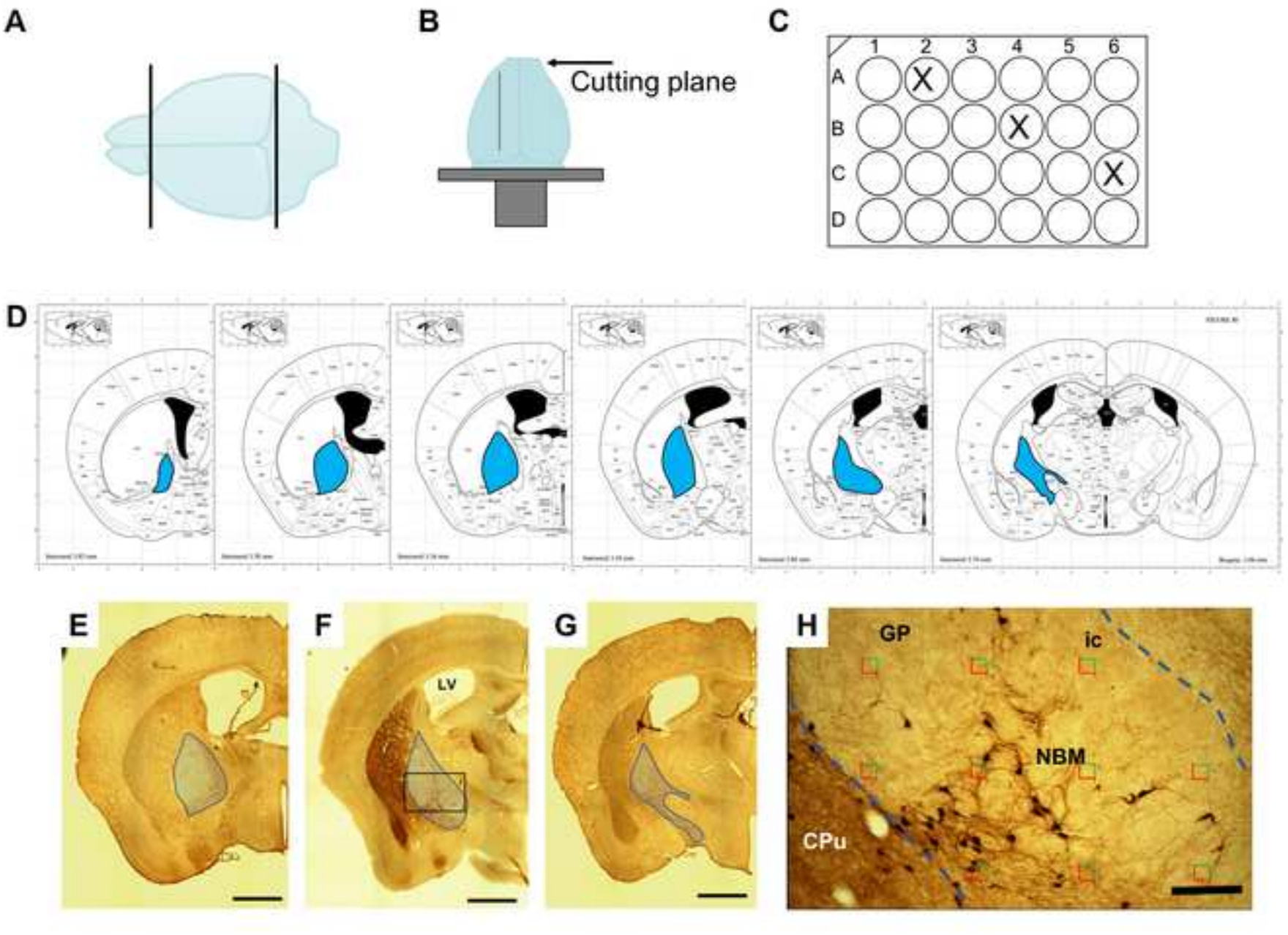

Figure2

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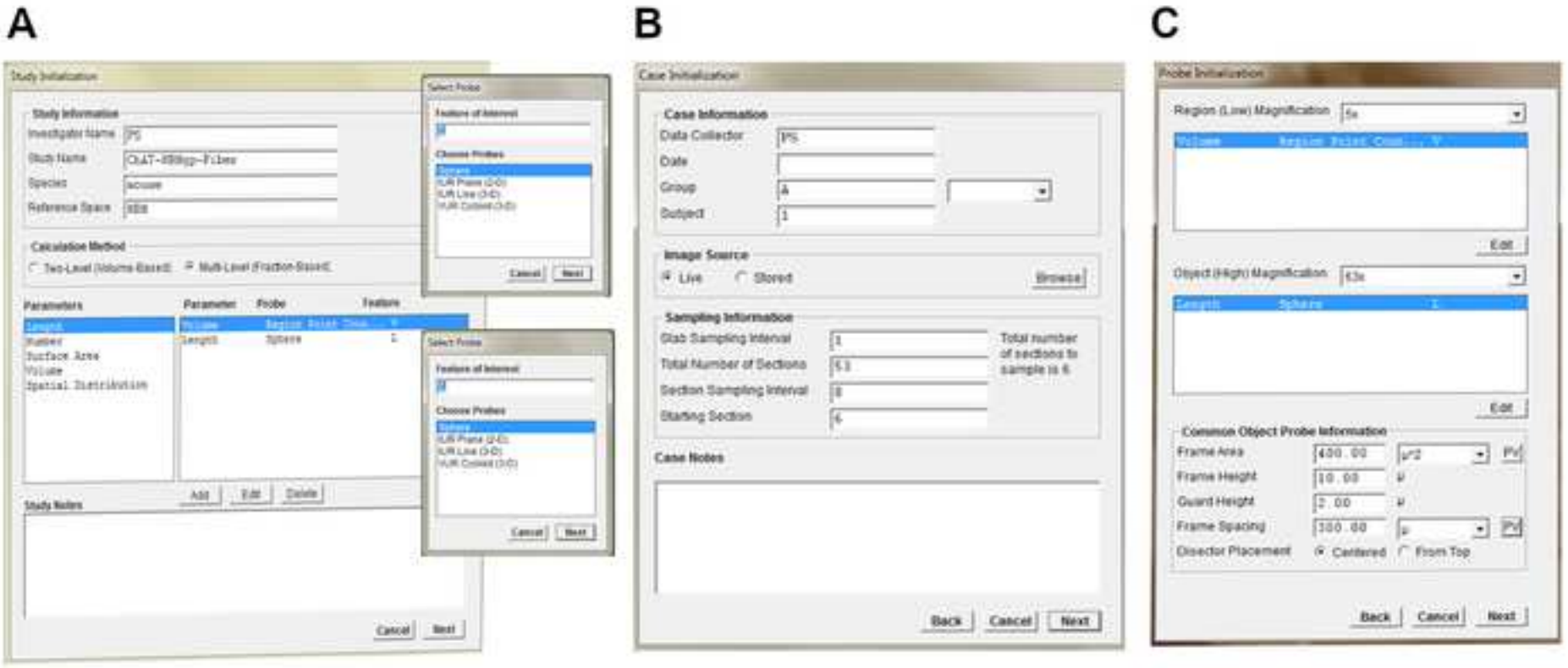

Figure3

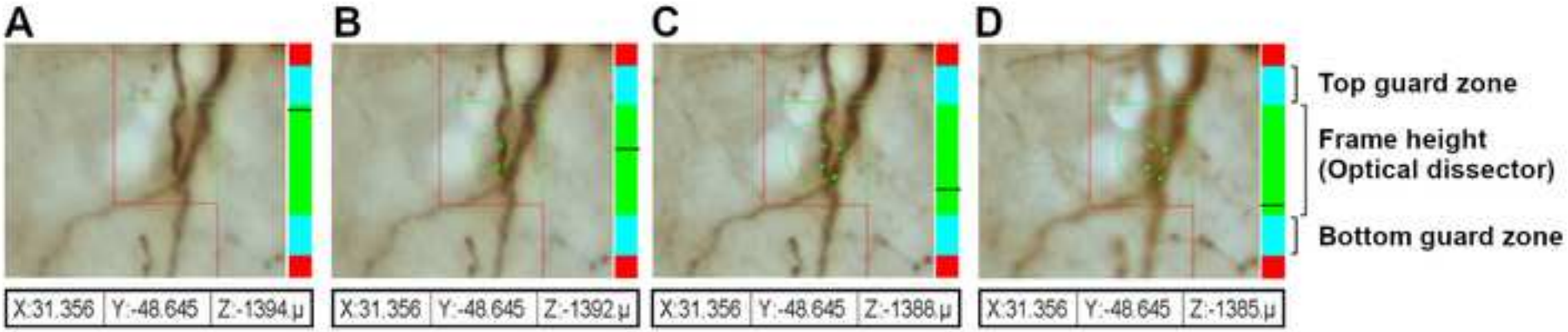

Figure4

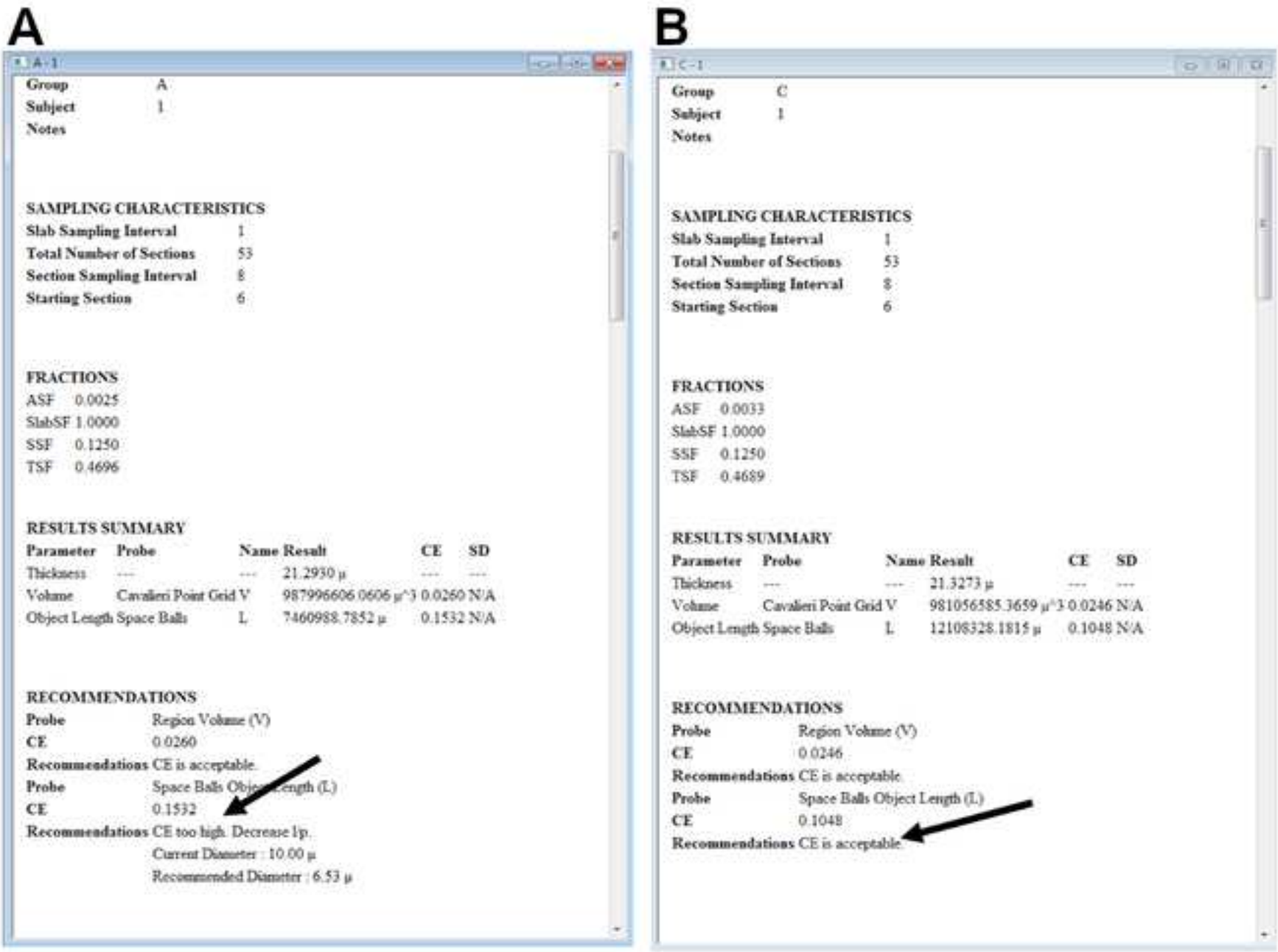

Figure5

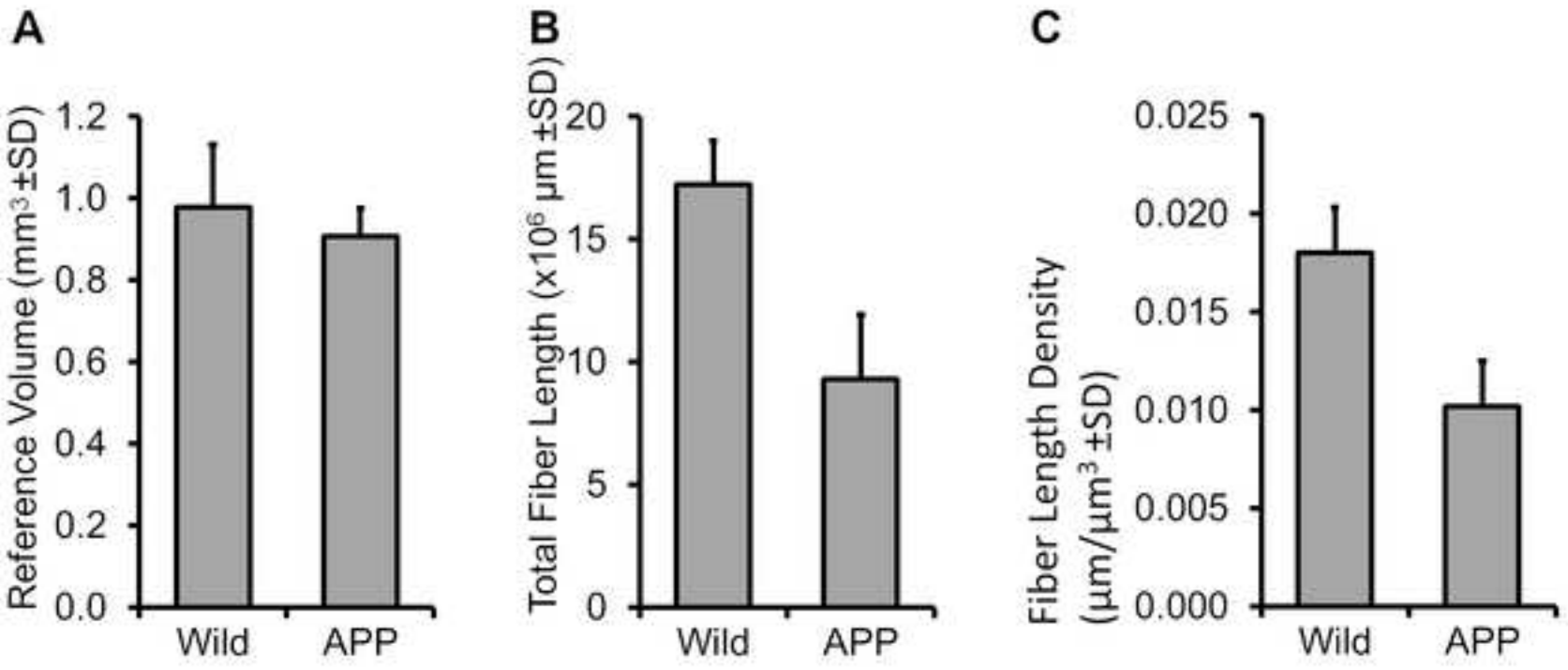

Table 1

Group	Case #	Volume (µm^3)	Length (µm)	Lv (µm/µm^3)
B	1	926885302	16446282	0.018
B	2	856582400	19254528	0.022
B	3	1150520830	15980131	0.014
C	1	981056585	12108328	0.012
C	2	894169486	6905567	0.008
C	3	998618871	10359766	0.010
Statistics				
Mean Group B		977996177.3	17226980.33	0.018
SD Group B		153490036.6	1771309.218	0.004
Mean Group C		957948314	9791220.333	0.010
SD Group C		55927744.89	2647567.494	0.002
TTEST B vs C		0.84	0.02	0.049

Name of Material/ Equipment	Company
ABC kit	Vector Laboratories
Anti-ChAT Antibody	Millipore, MA, USA
Bovine anti-goat IgG-B	Santacruz Biotechnology
Bovine Serum, Adult	Sigma-Aldrich, St. Louis, MO, USA
Cryostat	Lieca Microsystems, Buffalo Grove, IL, USA
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich, St. Louis, MO, USA
Ethylene Glycol	Sigma-Aldrich, St. Louis, MO, USA
Glycerol	Sigma-Aldrich, St. Louis, MO, USA
Hydrogen Peroxide	Sigma-Aldrich, St. Louis, MO, USA
Immpact-DAB kit	Vector Laboratories
Ketamine	Westward Pharmaceuticals, NJ, USA
Microscope	Lieca Microsystems, Buffalo Grove, IL, USA
Optimum cutting temperature (O.C.T.) embedding medium	Electron Microscopy Sciences, PA, USA
Paraformaldehyde	Sigma-Aldrich, St. Louis, MO, USA
Permount mounting medium	Electron Microscopy Sciences, PA, USA

Stereologer Software	Stereology Resource Center, Inc. St. Petersburg, FL, U
Triton X-100	Sigma-Aldrich, St. Louis, MO, USA
Trizma Base	Sigma-Aldrich, St. Louis, MO, USA
Trizma hydrochloride	Sigma-Aldrich, St. Louis, MO, USA
Xylazine	Bayer, Leverkusen, Germany
Xylenes, Histological grade	Sigma-Aldrich, St. Louis, MO

Catalog Number	Comments/Description
PK6100	
AB144P	
SC-2347	
B9433	
D5652	
324558	
G2025	
H1009	
SK4105	Enhanced DAB peroxidase substrate solution
0143-9509-01	
AF6000	Equipped with motorized stage and IMI-tech color digital camera
62550-12	
P6148	
17986-01	

Stereologer2000	Installed on a Dell Desktop computer.
T8787	
T1503	Tris base
T5941	Tris hydrochloride
Rompun	
534056	

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Author(s):	Prabhakar Singh, David Peng, William Suo

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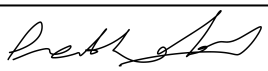
Signature:	
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Point-by-point response to the comments:

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have checked and tried to correct these issues.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Response: We have reformatted it as required.

3. Please define all abbreviations before use; e.g., APPswe.

Response: We have updated all abbreviations.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of 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: TissueTek, Leica, Millipore, ABC Elite, ImmpactDAB, Permount, Stereologer, etc.

Response: We have updated the manuscript to avoid using the commercial symbols and descriptions.

Protocol:
1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: We have highlighted important steps of the protocol for filming.

2. For each protocol step, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: We have edited manuscript and provided reference as required.

Specific Protocol steps:
1. 1.1: Please explain how proper anesthesia is confirmed.

Response: We have edited the step and provided a citation with details of the method to confirm anesthesia.
2. 1.3: How exactly are brains removed?

Response: We have added a JOVE reference for detailed method of perfusion and brain removal.
3. 3.12: Do you wash in TBS again?

Response: Yes. Corrected.
Figures:
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Response: Data presented is not used in any publication.
2. Please include a scale bar, defined in the legend(s), for all images taken with a microscope.

Response: Corrected.
3. Figure 5: What statistical test was used here?

Response: We have mentioned in the figure legend that "Data analyzed within two different groups using student t-test" (line 257).

Discussion:
1. Please also include any limitations of the technique as well as its significance with respect to existing methods (with references) in the Discussion.

Response: We have added major advantages and limitations of the method in discussion (lines 309-322).
References:
1. Please do not abbreviate journal titles.

Response: We have used Endnote software with Jove Style for references. We have checked again each of the references manually. Please let us know if we need to correct any specific reference.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Response: We have updated the Table of Materials.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript describes the protocol for software-based stereology to estimate total length of cholinergic fibers in the NBM of the basal forebrain. They have used the space ball probe of the Stereologer software for length estimate using immunostaining with horseradish peroxidase - Diaminobenzidine (HRP-DAB) detection system for ChAT. The protocol can be used for many other fibers in nervous system, which makes it excellent one.

The article is written in a very good manner and suitable for publication in its current form (with minor changes).

Major Concerns:
None

Minor Concerns:
Line 36: If suitable, please use full form of NBM again here in long abstract as short abstract may not be uploaded on PubMed.

Response: We have added full form of NBM in the long abstract.
Line 85: Please check the dosage of ketamine. Is it 285 mg/Kg?

Response: It should be 100mg/kg. We have corrected the dose in the manuscript.
Line 94: 1.5 Remove tissue from the sucrose SOLUTION and ..... Use the word solution in the sentence to make it more clear.

Response: Corrected.

Reviewer #2:
The authors present a methodological approach for using a design-based estimator of fiber length, the Spaceballs probe, to evaluate cholinergic fiber length in a brain region relevant to the pathophysiology of Alzheimer's disease. The manuscript describes the mechanical process of tissue preparation, sampling, measuring/counting, and analyzing the results. It does this in a rather simple way that will make it difficult for the reader to understand the logic of the approach and modify the procedure for different circumstances.

A number of specific points are listed below. If addressed, that response would make the manuscript have a much greater impact. However, there are two larger concepts that the authors do not address. The first is the missed opportunity to explain why one would undertake this procedure rather than simply measure optical density of staining (also noted below in comments on the discussion). The second is discussing the value of obtaining the estimate of fiber length in the nucleus where these projection neurons reside (NBM) in the absence of also estimating the neuronal population. Fiber length alone in this region provides little context to interpret the biology. Furthermore, this is in the region of projection. Obtaining the fiber length in the region of terminal projection would provide data of much more relevance for this study that could be compared with the region of projection and the number of underlying cholinergic projection neurons.

Specific comments on the protocol:

1.4 It should be noted that prolonged incubation in 30% sucrose can lead to substantial tissue shrinkage. As the Spaceballs probe is a local estimator, it is highly sensitive to changes in volume. Even if the reference volume is individually calculated for each specimen to adjust values, it is recommended to keep the exposure time identical for all specimens.

Response: We have specified a duration in revised manuscript.

1.5-1.6 Sections could equally well be prepared on a sliding freezing microtome. In any case, as there will be substantial tissue section thickness shrinkage upon processing and there will need to be sufficient height to employ the Spaceballs probe, it would be recommended to maximize section thickness, perhaps to 50 µm.

Response: We have added a note in the protocol and recommended thick sections. Also, we have provided more details about its importance in the discussion section.

1.6 Multiwell plates in general should be stated as most investigators utilize 96 well plates for tissue collection and storage.

Response: We have added a note in the revised manuscript to use 96well plate as a, alternative of 24well plate.

2.1-2.2 Systematic sampling and its relationship to the coefficient of error is the most critical point of this process. The rationale of this should form the main point of the paper and it should be clearly explained what CE is and what is considered acceptable. The discussion section touches only superficially on this point.

Response: We have added more details about CE and determination for statistically acceptable results. (Lines 111-114, 300-302)

3.7 and 3.9 The secondary antibody must recognize the species in which the primary antibody is raised. This will not work as described. Is the primary antibody really goat anti-human ChAT?

Response: Thank you for catching the error. We have corrected it in the revised manuscript.

3.15 The procedure described will substantially reduce mounted section thickness. Data on the final mounted section thickness should be provided.

Response: We have provided mean value of the measured thickness in the revised manuscript.

Section 3 Many labs have moved away from brightfield staining to using fluorophore-labeled secondary antibodies and detection by fluorescence microscopy. An alternative protocol for this should be provided.

Response: Fluorescent method is a good alternative of brightfield staining for most antigens but not for ChAT. At least, this is our experience. We have tried many times with fluorescent staining of ChAT, and the quality of the staining was only good enough for labeling the cell body and major processes. The thin distal processes of the cholinergic neurons could not be clearly documented with fluorescent method, and the limited resolution of partial cholinergic fiber profiles was deemed inappropriate for quantifying the cholinergic fiber density. Therefore, we are unable to include fluorescence stating as an alternative to the current protocol.

4.7 and 4.12 The authors are correct that the indefinite boundary of the NBM should not be calculated from a drawn contour defining the area. It appears they are describing the Cavalieri Estimator here. That

should be specified and some explanation of the Cavalieri Estimator provided. The rationale for the point density should be discussed.

Response: We have added more details about Cavalieri estimator in the discussion section. (Line 269-275)

4.8 and 4.9 The rationale for all of these parameters should be explained to be meaningful for anyone trying to implement this approach. There is concern that there would remain adequate mounted section thickness to implement the probe with a suitable guard zone, despite the assurance statement in the discussion. Here again, a thorough statistical explanation of the CE is required and the logic behind determining what value is "acceptable". Clearly the investigator needs to make this decision and not this on a software cue.

Response: We have added more information about thickness and determining guard zone in section 4.8. Determination criteria of acceptable CE has been provided (lines 112-14 and 300-302).

4.13 The rationale for the lens selection should be provided. In fact, the magnification is much less relevant than the axial resolution, so an immersion objective, with an NA ≥ 1.2 is probably necessary. Brightfield imaging has another resolution disadvantage here that could be overcome by using an oil condenser with proper Köhler illumination.

Response: We agree that axial resolution is more relevant than magnification. We have included reviewer's suggestion and added recommendation to use immersion objective. (lines 160-162).

4.18 See comments above on the explanation of "acceptable" CE :

Response: Information added to the revised manuscript (lines 112-14 and 300-302).

Representative Results This section could also benefit from a discussion of the need to match these values to the underlying cholinergic neuron population. These data are needed to inform if length differences observed could be explained by a different base population of projection neurons. It would be appropriate to extend the discussion of fractionated sampling to include the optical fractionator probe.

Response: Manuscript is focused on immunohistochemistry for cholinergic fibers and use of Sphere probe of Stereologer software. We have performed this experiment in NBM because cholinergic fibers in this region has been found affected very early in the mouse models of Alzheimer's disease (Stokin et al 2005). Optical fractionator protocol for cell counting has already been published in JOVE. Therefore, we have not discussed about the population of neurons and their projections. We have revised representative results section to make more explicable.

Discussion This section stands out from the rest of the manuscript in using very poor English. It requires editing. The discussion also misses the opportunity to explain why taking a stereological approach is superior to simply measuring staining intensity as a surrogate for fiber density.

Response: We have revised the discussion section and added more details about advantages and limitations of the method.

Reviewer #3:
Manuscript Summary:
This method paper might be helpful for other studies on any linear profiles.

Minor Concerns:
The following issues need to be addressed.

1. In fig. 3, scale bar needs to put into, and in the figure legend of fig. 3, the magnification needs to be stated.

Response: Added.
2. It is difficult to define the boundary of NBM. Figure 1 C illustrates the schematic diagram of coronal sections delimiting the borders of NBM in the systematically selected 6 sections. We suggest the authors to increase the relative immunohistochemical sections in the levels of schematic diagram of figure 1 C so that the readers know hoe to define NBM.

Response: We have added two more representative images of ChAT stained sections.

3. The current study estimated the NBM volume. The authors had better to increase the figure illustration for the volume estimation.

Response: Software provides option to do volume estimation simultaneous with the fiber analysis. As same sections are used for region point counting for the volume estimation, we don't expect any confusion to the users during volume estimation. We have edited protocol section to make it clearer (Section 2.1).
4. In figure 4A, CE is too high, and the authors stated that the software gave suggestion for how to decrease CE. It is better for the authors to change the experiment as the software suggested and compare the new result to the previous result.

Response: Figure 4 is provided to show format of results and how to check acceptance of CE values. Decreasing frame spacing provides more sites of analysis and therefore more representation of the sample. With our experience, first we reduced frame spacing and found the CE values are acceptable and reported in this manuscript.
5. Why did the authors use group B to represent wild type littermates and use group C to represent APPswe group. This is not the normal way to do. Please correct them.

Response: We are sorry for the confusion created due to codes of the groups. We used group A for our pilot studies. And then, we used blinded group names B and C for two experimental groups. We have corrected group names as WILD and APP in results and figure 5 to avoid confusion.

Reviewer #4:
Manuscript Summary:
The ms gives a clearcut advice how the study cholinergic fiber length in mouse basal nucleus.

Major Concerns:
There are very few references.

Response: This is a protocol article therefore we have provided citations related with protocol. In our introduction and discussion, we have cited many appropriate review articles which can be used for further reading. We have added few more references in the revised manuscript.

Minor Concerns:
Generally, the article is sound and of high level.

I only have few concerns that should e considered.

1.3: state how long the brains are (or can be) post-fixed:

Response: Information added in the revised manuscript.

2.1.: please give are more actual edition of the Atlas used.

Response: Edited.
2.1: please give reference why 8 systematically sampled out of 50 sections are enough.

Response: Reason to choose this number has been clarified in revised manuscript. Reference has been provided in discussion.
3: all steps on a shaker at which temperature? (cold room?)

Response: information added to 3.8.
3.12. end with a .

Response: Corrected.
General: use ce or CE throughout the ms.

Response: Rechecked and corrected.

General: use the official abbreviation of the basal nucleus of Meynert according to Franklin + P. = B
Line 227, 228: there is 2x (C) -please correct.

Response: "B" abbreviation was making confusion at many places in the manuscript therefore we have used NBM which is also a widely used abbreviation. We have clarified Franklin and Paxinos abbreviation in the figure legend.
Fig 1, second C , D and E: please CLEARLY line out the respective area under study - thus I can see also problems doing so.

Response: We have added more representative images and outlined the area (Figures 1E to 1H)

General: the word process is used - what is meant exactly: axons, dendrites ?

Response: We have corrected the sentence.

Please discuss possible problems of the diameters of structures under study.

Response: We have included the problem in the discussion.