Chapter 8

Histological Analysis of Neurodegeneration in the Mouse Brain

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Abstract

Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) are characterized by chronic and progressive neuronal loss. Being able to detect and quantify neurodegeneration is the first step to identify mechanisms underlying neuronal cell death and to develop novel therapeutic strategies. In this chapter, we describe a practical method for detecting and quantifying neurodegeneration in adult and aging mouse brains based on protocols developed in our laboratory over the last decade. We include protocols on sample preparation, immunohistochemical analysis, and stereological methods for counting neurons using examples of AD and PD mouse models. We also describe how to use Fluoro-Jade staining and terminal deoxynucleotidyl transferase dUTP nick end labeling to detect degenerating neurons and apoptotic cells, respectively, and how to use specific proteins as early markers of neurodegeneration.

Key words Neurodegeneration, Alzheimer's disease, Parkinson's disease, Mouse brain, Immunohistochemistry, Stereological neuron counting

1 Introduction

Neurodegeneration is characterized by a decrease in the total number of neurons in certain brain subregions. To determine whether animal models of neurodegenerative disorders such as AD and PD recapitulate degeneration of specific neuronal types as seen in human patients, it is first necessary to evaluate whether neurodegeneration occurs in animal models. It is then important to establish at which age and in which part of the brain neurodegeneration and frank loss of neurons begin and how quickly neurodegeneration progresses. In AD mouse models, a decrease in the total number of neurons is expected to occur primarily in the neocortex and hippocampus, whereas ideal PD mouse models should exhibit a decrease in the total number of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc).

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Stereology is a set of methods that allow objective estimation of the total number of objects such as neurons that distribute unequally in an irregular shape of a three-dimensional structure such as the cerebral cortex or SNc [1, 2], using the information in two-dimensional structures such as brain sections [1, 2]. Neurons vary in shape and size and are distributed unequally in different orientations in the brain. Design-based stereology eliminates the need for making assumptions regarding the size and shape of the neurons and their orientation, allowing an unbiased quantitative analysis [1, 2]. We have applied the optical fractionator stereological method in counting neurons to assess neurodegeneration in AD and PD mouse models for many years. Here, we describe how samples for stereology are prepared and how neurons are counted.

Cell death modes are classified as necrosis, apoptosis, or autophagic based on the cell's morphological features [3], although the mechanism and process underlying cell death are complex and vary among different diseases. While Fluoro-Jade dyes do not distinguish between the cell death modes (e.g., apoptosis vs. necrosis), they have been shown to label a wide range of degenerating neurons efficiently, with high resolution and contrast [4]. Apoptotic cells can be specifically detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) [5]. In addition, neurodegeneration in neurodegenerative disorders such as AD or PD is usually accompanied by a chronic inflammatory response [6–9], including glial cell [6, 8, 9] and complement activation [10] and the release of proinflammatory cytokines from glial cells [11]. We found Fluoro-Jade B-positive and TUNEL-positive cells and a chronic inflammatory response in presenilin conditional double-knockout (*PS* cDKO) mice [12–14]. Here, we present these findings and describe our method of immunohistochemistry, Fluoro-Jade B staining, and TUNEL.

2 Materials

2.1 Fixation of the Mouse Brain by Perfusion

1. Dissecting tools, blade, and brain matrix (Mouse 1 mm Coronal) (Braintree Scientific, Inc.).
2. Sodium pentobarbital (Nembutal) diluted with saline (40–50 mg/kg body weight).
3. 50 mL syringe connected to a winged needle (23 G) with plastic tube.
4. Kelly hemostatic forceps.
5. Ice-cold Ringer solution (or phosphate-buffered saline (PBS)) containing procaine and heparin; 500 mL Ringer solution (or PBS) + 2.5 g procaine hydrochloride + 125 mg heparin sodium salt.

6. Ice-cold 4 % (w/v) paraformaldehyde (PFA) fixative in PBS.
7. Ice-cold 20 % (w/v) sucrose in PBS and ice-cold 30 % (w/v) sucrose in PBS.
8. Ice-cold 1 × PBS.

2.2 Paraffin Processing, Embedding, and Sectioning of the Mouse Brains

1. Plastic tissue cassette and histology marker (solvent-resistant marker).
2. Tissue processor (SHANDON Hypercenter XP) and paraffin embedding machine (SHANDON Histocentre 2).
3. Metal base molds for embedding (38 × 25 × 12 mm).
4. Microtome and water bath for paraffin section mounting.
5. Glass slides (Colorfrost Plus Microscope Slides, 25 × 75 × 1.0 mm, Fisher Scientific).

2.3 Embedding and Sectioning of the Mouse Brains for Cryosections

1. OCT compound (Sakura Finetek) and disposable base mold for embedding (37 × 24 × 10 mm).
2. Cryostat.

2.4 Nissl Staining of the Mouse Brain Sections

1. Laboratory oven.
2. Histo-Clear (substitute for xylene, National Diagnostics), 50 % Histo-Clear/50 % EtOH (ethanol), 100 % EtOH, 95 % EtOH, 70 % EtOH, 50 % EtOH, and dH_2O.
3. 0.5 % cresyl violet solution: Add 2.5 g cresyl violet acetate (Sigma C-5042) to 500 mL dH_2O. Add ten drops of 10 % acetic acid, and dissolve completely by mixing and filtering twice. Wrap with foil. Use fresh one.
4. Diluted acetic acid: Add 2–3 drops of concentrated acetic acid to 100 mL dH_2O.
5. Glass coverslips (24 × 50-1, Fisher Scientific) and Permount (Fisher Scientific).

2.5 Immunostaining of the Mouse Brain Sections

1. Humidified chamber.
2. 0.3 % H_2O_2 in methanol: 198 mL of 100 % methanol + 2 mL of 30 % H_2O_2.
3. 1× Tris-buffered saline (TBS) (20 mM Tris, 150 mM NaCl, pH 7.4): For 1 L 10× TBS stock, dissolve 24.22 g Tris base (formula weight: 121.1 g) and 87.6 g NaCl in 900 mL dH_2O. pH to 7.4 with 12 N HCl. Add dH_2O to a final volume of 1,000 mL.
4. 0.1 % Triton X-100/TBS: 1 mL of Triton X-100 + 1,000 mL of TBS.
5. To make 1 L 10 mM citrate buffer:
   (a) Add 1.92 g anhydrous citric acid ($C_6H_8O_7$) to 900 mL dH_2O.

(b) Adjust pH to 6.0 with approximately 2.6 mL 10 N NaOH.

(c) Add dH_2O to a final volume of 1,000 mL.

6. 0.1 % Tween 20/TBS (TBST): 1 mL of Tween 20 + 1,000 mL of TBS.
7. 5 % normal goat serum (NGS)/TBST: 1 mL of NGS + 19 mL of TBST.
8. Primary antibodies: Mouse anti-NeuN (1:300, Millipore), rabbit anti-tyrosine hydroxylase (TH) (1:1,000, Millipore), mouse anti-MAP-2 (1:300, Sigma), mouse anti-synaptophysin (1:500, Sigma), and mouse anti-glial fibrillary acidic protein (GFAP) (1:500, Sigma).
9. Secondary antibodies: Biotinylated Goat Anti-Mouse IgG Antibody (BA-9200, VECTOR LABO), Biotinylated Goat Anti-Rabbit IgG Antibody (BA-1000, VECTOR LABO).
10. VECTASTAIN Elite ABC Kit (PK-6100, VECTOR LABO).
11. DAB Peroxidase Substrate Kit, 3,3 -diaminobenzidine (SK-4100, VECTOR LABO).
12. Harris Modified Hematoxylin (Fisher Scientific).

2.6 Immunofluorescence Staining of the Mouse Brain Sections

1. Alexa Fluor 488 or 568 Goat Anti-Rabbit IgG (H + L) (A-11034 or A-11036, Invitrogen).
2. Alexa Fluor 568 or 488 Goat Anti-Mouse IgG (H + L) (A-11031 or A-11029, Invitrogen).
3. 0.3 % Sudan Black (w/v) in 70 % EtOH (v/v); stir it in the dark (put aluminum foil around the container) at RT for 2 h. Filter it and store in the dark at 4 °C.
4. TO-PRO-3 (T3605, Invitrogen).
5. VECTASHIELD Mounting Medium (H-1000, VECTOR LABO).

2.7 Fluoro-Jade B Staining

1. Fluoro-Jade B (AG310, Millipore).
2. 0.01 % Fluoro-Jade B stock solution: 0.01 g per 100 mL dH_2O. Store at 4 °C wrapped in foil.
3. 0.1 % acetic acid (v/v): 500 μL per 500 mL dH_2O.
4. 5 % NaOH: 5 g pellets per 100 mL dH_2O.
5. 1 % NaOH/80 % EtOH: 100 mL 5 % NaOH + 400 mL EtOH.
6. 0.06 % $KMnO_4$: 0.12 g per 200 mL dH_2O. Note: TOXIC. Store at 4 °C, and use within 1 week.

2.8 TUNEL on Mouse Brain Sections

1. In situ cell death detection kit, fluorescein (# 11684795910, Roche), or In situ cell death detection kit, TMR red (# 12156792910, Roche).
2. To make the TUNEL reaction mix:

(a) Make this up 10 min or less before applying to slides.

(b) Add 19 parts labeling mix buffer to 1 part TdT enzyme (e.g., per 120 μL, use 6 μL enzyme + 114 μL labeling mix buffer); mix thoroughly.

2.9 Stereological Neuron Counting

1. Software: BIOQUANT stereology software (BIOQUANT Life Science, Nashville, TN) or Stereo Investigator (MBF Bioscience, Williston, VT).
2. Hardware: A conventional light microscope equipped with a motorized stage and focus control system, a color digital video camera, and a microcomputer.

3 Methods

Neurodegeneration is a process in which the normal brain undergoes a change in structure or function in a chronic and progressive manner, which differs from developmental abnormalities, in which structural or functional brain abnormalities occur from birth. To capture this process, which is characterized by chronic and progressive neuronal loss, it is important that brain samples are collected and examined at multiple time points (e.g., 2, 4, 6, and 10–12 months of age) so that we can be certain that the brain abnormalities observed later in life are not due to developmental defects. Thus, it is vital to begin the analysis at an early adult age, such as 2 months for mice, to ensure that there are no defects at this age. Only then, we can attribute defects observed later in life to neurodegeneration. Because aging is the most important factor that affects neurodegeneration, brain samples from aged mice (18–24 months old) should also be examined. Sex and genetic background also affect brain size and neurodegeneration; thus, the diseased and control samples, ideally age- and sex-matched littermates, should be processed and examined in parallel.

Histological examination of the brain consists of a number of steps, including fixation by perfusion, embedding, sectioning, and immunohistochemical analysis. Directly comparing the number of neurons, protein expression levels, and brain morphological features between the samples using immunostaining requires the exact same treatment and processing methods in performing the histological procedure. For example, the control and mutant samples should be placed in the same paraffin block and sectioned at the same time (Fig. 1). In addition, the immunohistochemical analysis of brain sections should be performed on the same glass slide (Fig. 1). To eliminate subjective factors, it is important that the investigator performing these procedures and analyses is blinded to potentially biasing factors, such as the mouse genotype and age.

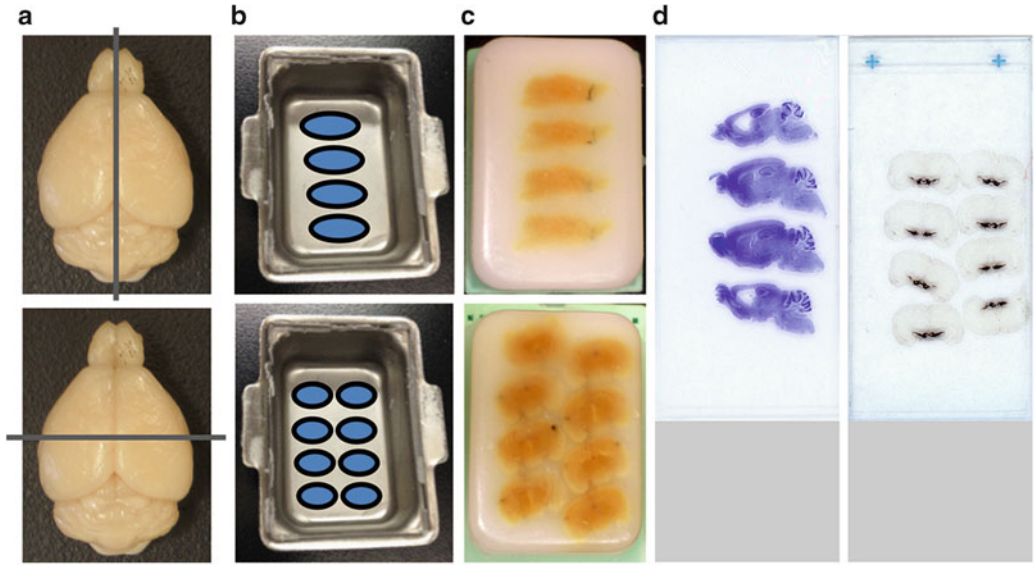

Fig. 1 Paraffin embedding and sectioning of mouse brains. (**a**) The *lines* on the images of brains indicate the positions of cutting plane for sagittal brain sections (*top*) and coronal brain sections (*bottom*). (**b**) Two diseased samples and two control samples, total four brain samples (indicated by *oval-shaped circles*) for sagittal brain sections are placed in the metal mold for paraffin embedding (*top*). Four diseased samples and four control samples, total eight brain samples (indicated by *oval-shaped circles*) for coronal brain sections are placed in the metal mold for paraffin embedding (*bottom*). (**c**) The images of paraffin blocks that contain four brains (*top*) and eight brains (*bottom*) for sagittal brain sections and coronal brain sections, respectively. (**d**) The brain sections on glass slides that are stained with cresyl violet (Nissl staining) (*left*) and anti-TH antibody (*right*). *Note*: Two *PS* cDKO (*top* and *bottom*) and two control brain sections (19 months old) are on the glass slide (*left*)

Various stains and antibodies can be used to examine different aspects of neurodegeneration. Nissl staining is a standard immunohistochemistry method that shows basic brain morphological features and allows comparison of brain architecture between samples and their controls (Fig. 2a–c). To visualize neurons in the brain or DA neurons in the SNc, immunostaining can be performed using a neuron-specific antibody (e.g., Neu-N) or a DA neuron-specific antibody (e.g., TH), respectively (Fig. 3a, d). After the staining, stereology can be performed to quantify the number of neurons in the cerebral cortex or nigral DA neurons in SNc, respectively.

Fig. 2 (continued) reactivity in hippocampal area CA1 of *PS* cDKO mice at 6 and 9 months of age. Scale bar: 50 μm. (**h**) Immunohistochemistry for GFAP, cathepsin S, complement component C1q, and CD45 on sagittal brain sections from control and *PS* cDKO mice at 6–7 months of age reveals high increases in immunoreactivity in the *PS* cDKO brain. Scale bar: 100 μm. This research was originally published in Beglopoulos, V., Sun, X., Saura, C.A., Lemere, C.A., Kim, R.D. and Shen, J. (2004) Reduced beta-amyloid production and increased inflammatory responses in presenilin conditional knock-out mice. J Biol Chem, 279, 46907–46914. © the American Society for Biochemistry and Molecular Biology. Images are adapted from refs. 12–14 with permission

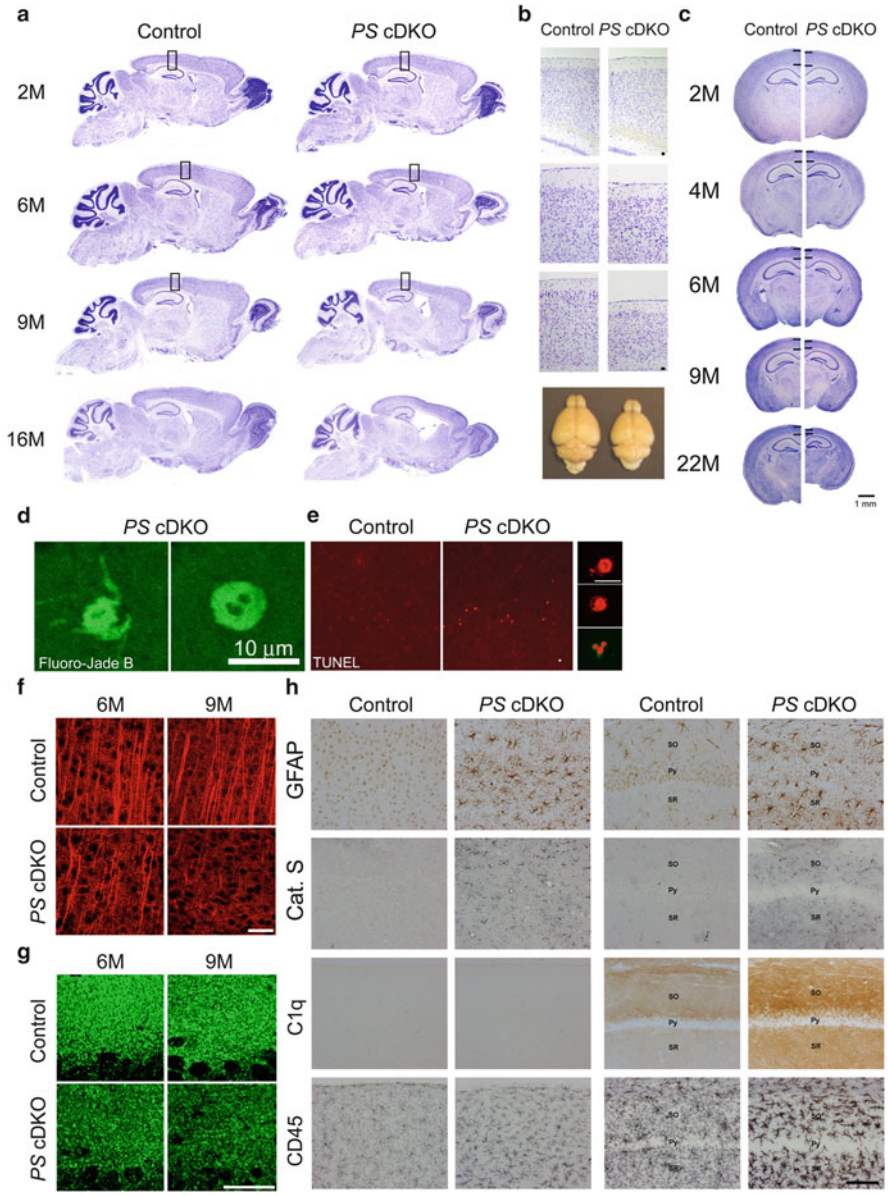

Fig. 2 Neurodegeneration in *PS* cDKO mice. (**a**) Nissl staining of comparable sagittal sections of *PS* cDKO and control brains at the age of 2, 6, 9, and 16 months demonstrates progressive loss of gray and white matter in the neocortex and hippocampus and enlargement of lateral ventricles. (**b**) Higher magnification views of the *boxed areas* in (**a**) show progressive thinning of cortical layers at 6 and 9 months of age. The image of brains at the age of 16 months demonstrates a gross cerebral atrophy in *PS* cDKO mice (*bottom*). Scale bar: 50 μm. (**c**) Nissl-stained images of coronal sections from age-matched control (*left*) and *PS* cDKO mutant (*right*) brains from 2 to 22 months of age are shown. *Black horizontal bars* delineate neocortical layers. At 2 months, no detectable difference is found in size or shape of the *PS* cDKO brain relative to control. However, subsequent ages reveal a gradual decrease in cortical thickness in *PS* cDKO mice. Scale bar: 1 mm. (**d**) Confocal images of Fluoro-Jade B labeling of degenerating neurons in *PS* cDKO brain sections. Scale bar: 10 μm. (**e**) *Left*: Confocal images of TUNEL-stained cells in the neocortex of control and *PS* cDKO mice. *Right*: Confocal images of individual TUNEL-positive cells. Scale bar: 10 μm. (**f**) Progressive reduction in MAP2 immunoreactivity in the neocortex of *PS* cDKO mice at 6 and 9 months of age. Scale bar: 50 μm. (**g**) Reduced synaptophysin immuno

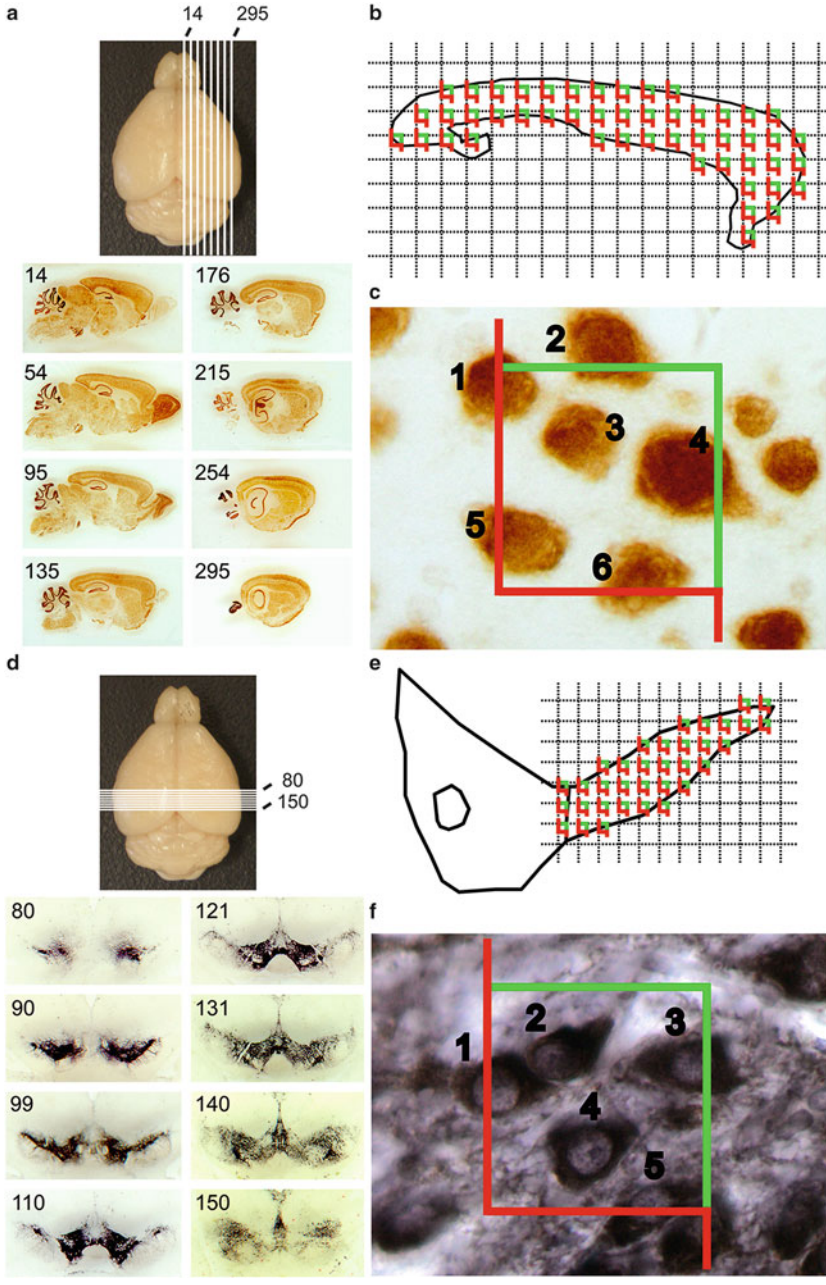

Fig. 3 Stereological neuron counting in the neocortex and SNc of mouse brain. (**a**) A series of 10 μm thick, NeuN-stained sagittal brain sections encompassing the entire cerebral hemisphere. The *lines* on the image of mouse brain (*upper panel*) indicate the location of the sagittal brain sections (*lower panel*). The *numbers* above the lines correspond to the section numbers of NeuN-stained brain sections. For stereological neuron counting the first brain section was selected randomly among the first sections and then every 40th section was selected systematically with a distance of 400 μm between the sections. (**b**) The area corresponding to the neocortex in the section 95 in (**a**) is superimposed with a *rectangular grid* that consists of grid areas (500 μm × 500 μm). This grid defines the positions of counting frames (50 μm × 50 μm). The counting frame is shown as a frame surrounded by *red* and *green* lines. (**c**) A high-magnification image of the section stained with anti-NeuN antibody is superimposed with a counting frame (50 μm × 50 μm) consisting of two exclusion lines (*red*) and two inclusion lines (*green*). Neurons, the nuclei of which are inside the counting frame, touching

Immunostaining for other neuronal markers (e.g., MAP2A and βIII-tubulin) can demonstrate the loss of soma, dendrites, and/or axons associated with neurodegeneration (Fig. 2f). Synaptic loss and dysfunction are early events in neurodegenerative diseases and can be revealed by immunostaining for synaptic markers (e.g., synaptophysin and PSD-95; Fig. 2g). Fluoro-Jade B staining and TUNEL of brain sections from an AD mouse model can reveal degenerating and apoptotic cells, respectively (Fig. 2d, e). Figure 2h shows glial and complement activation in brain sections from an AD mouse model.

To quantify the number of neurons in various brain structures, the optical fractionator, which is a combination of the optical disector and fractionator concepts [15–17], has been used extensively. The optical disector is a three-dimensional stereological probe that allows objects to be counted in a thick tissue section (Fig. 4) [15–17], whereas the fractionator is a statistically optimized spatial sampling concept that allows sampling of a known fraction of a structural component or region (e.g., the neocortex and SNc) in a systematic random fashion (Fig. 3a, b, d, e) [15–17]. In practice, the first brain section is sampled randomly and the following sections, separated by a fixed interval, are sampled systematically (Fig. 3a, d). Next, a grid is placed on each section at random by software (Fig. 3b, e). The grid has uniform distances between the lines in the *X*- and *Y*-directions and defines the position of the counting frame where the neurons are counted under a microscope (Fig. 3b, e). The optical disector approach is applied in each counting frame [15–17], and the neurons in the plane of focus in a certain vertical distance (disector height) through the *Z*-direction of the section are counted according to the counting rules, without any assumptions based on the size and shape of the neurons (Figs. 3c, f and 4) [15–17]. Guard zones are placed to avoid cutting artifacts at the upper and lower surfaces of the sections (Fig. 4) [15–17]. After all the sections are processed, the total number of neurons is calculated.

Fig. 3 (continued) the inclusion line, or crossing the inclusion line, are counted (No. 2, 3, and 4). Any neuron, the nucleus of which is touching the exclusion line or crossing the exclusion line, is excluded (No. 1, 5, and 6). (**d**) A series of 16 μm thick, TH-stained coronal brain sections encompassing the entire SNc. The *lines* on the image of mouse brain (*upper panel*) indicate the location of the coronal brain sections (*lower panel*). The numbers next to the lines correspond to the section numbers of TH-stained brain sections. For stereological neuron counting the first brain section was selected randomly among the first sections and then every tenth section was selected systematically with a distance of 160 μm between the sections. (**e**) The area corresponding to SNc in the section 99 in (**d**) is superimposed with a *rectangular grid* that consists of grid areas (100 μm × 100 μm). This grid defines the positions of counting frames (50 μm × 50 μm). The counting frame is shown as a frame surrounded by *red* and *green* lines. (**f**) A high-magnification image of the section stained with anti-TH antibody is superimposed with a counting frame (50 μm × 50 μm) consisting of two exclusion lines (*red*) and two inclusion lines (*green*). Neurons, the nuclei of which are inside the counting frame, touching the inclusion line, or crossing the inclusion line, are counted (No. 2, 3, and 4). Any neuron, the nucleus of which is touching the exclusion line, or crossing the exclusion line, is excluded (No. 1 and 5)

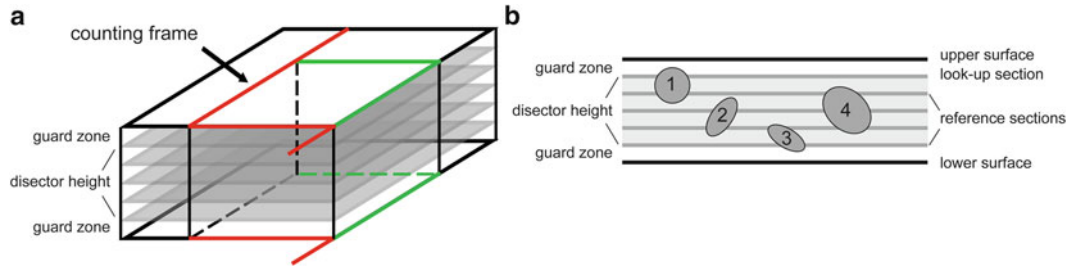

Fig. 4 Schemes of the counting frame in the *Z*-direction and the optical disector approach. (**a**) A schema of counting frame in the *Z*-direction of the section. In the optical disector approach, neurons in the plane of focus in disector height through the *Z*-direction of the section are counted according to counting rules without any assumptions on the size and shape of the neurons. Guard zones are placed to avoid cutting artifacts at the upper and lower surfaces of the sections. (**b**) A schema of four nuclei of neurons located in the alignment of planes through *Z*-direction of the section. From the top, they are upper surface section, look-up section, reference sections, and lower surface section. Four nuclei of neurons are located in the different planes of *Z*-direction. In the optical disector approach, the nucleus (No. 1) that is in clear focus in both the planes of look-up section and reference section is not counted. The nuclei (No. 2, 3, and 4) are in clear focus only in the reference sections and counted

The optical disector is a stereological method for counting objects in a thick tissue section (Fig. 4) [15–17]. Using this approach, only the objects in the plane of focus at disector height (i.e., the central part of the section) are counted (Fig. 4) [15–17]; however, some points must be considered regarding this type of sample preparation. First, sectioning by vibratome may cause distortion in the z-axis of the section [18], although this is still somewhat controversial [19]. Second, poor immunoreactivity in the center or other deep portions of the section may occur owing to poor penetration of the primary or the secondary antibody. If these issues are not overcome by a correction in the vibratome sectioning or pre-examination of the immunostaining, they might result in underestimation of the number of neurons when counting. To avoid these issues, cryosections are most frequently used for the optical disector method; however, in the practical analysis of the mouse brain to estimate neurodegeneration, paraffin sections of medium thickness are more convenient for handling most types of histological analysis. Once the paraffin sections are prepared, they can be kept for long periods and are useful not only for stereological neuron counting but also for other histological analyses. Thus, it is important to understand the issues involved in sample preparation for stereological methods and to analyze all the samples in the same manner. Several articles that describe the basis and process of stereological methods are available [1, 17].

3.1 Fixation of the Mouse Brain by Perfusion

It is important to perform the entire process of perfusion smoothly and properly, as incomplete perfusion often causes ischemic changes (ischemic neuronal death) in the brain. This can be achieved through repeated practice. Perfusion should always be performed in the hood. A brief description of the protocol follows.

1. A 50-mL syringe (60–70 cm in height) should be connected to a winged needle by a plastic tube and filled with 30 mL of ice-cold Ringer solution (or PBS) containing procaine and heparin. Fill the tube and needle with the solution, remove air bubbles from the tube, and clamp the tube with Kelly hemostatic forceps.
2. Anesthetize the mice by an intraperitoneal injection of sodium pentobarbital (40–50 mg/kg).
3. Open the thorax for access to the heart. Try not to damage the tissues, and avoid excessive bleeding from the tissues.
4. Insert a needle into the left ventricle, and start the Ringer solution flowing into the heart. Cut open the right auricle for drainage. The color of the liver should become pale (*see* **Note 1**).
5. Fill the syringe with 30 mL of ice-cold 4 % PFA fixative immediately before the Ringer solution runs out from the bottom of the syringe. The body, limbs, and tail should become stiff (*see* **Note 2**).
6. Dissect out the brain, including the olfactory bulb, if necessary. The color of the brain should be white if the perfusion was successful. The brain may then be weighed, and a picture of the brain may be taken with a ruler as a record.
7. Cut the brain into half using a brain matrix (Fig. 1a). For sagittal brain sections, transfer both brain halves into 20 mL of ice-cold 4 % PFA fixative and place them on ice. For coronal brain sections, including SNc, transfer the posterior part of the brain into 20 mL of ice-cold 4 % PFA fixative and place it on ice. Label the tubes with the mouse ID and the time. Mix the tube gently every 15 min.
8. After at least 2–2.5 h of postfixation, transfer half of the brain into 10 mL of ice-cold 20 % sucrose/PBS solution. The brain should float in the sucrose buffer. Keep the brain in 20 % sucrose/PBS solution at 4 °C.
9. After at least 3–3.5 h of postfixation, transfer the other half of the brain or the posterior part of the brain into 5 mL of ice-cold PBS solution (for paraffin processing). Keep the brain in the PBS solution at 4 °C until paraffin processing (*see* Subheading 3.2). Do not leave the brain in the PBS solution longer than 1 week.
10. After 24–48 h, the brain should sink toward the bottom of the 20 % sucrose/PBS solution. Once this occurs, transfer this half of the brain from the 20 % sucrose/PBS solution into 10 mL of ice-cold 30 % sucrose/PBS solution (for cryosections). After another 24–48 h, the brain should again sink toward the bottom. Keep the brain in 30 % sucrose/PBS solution at 4 °C until embedding in OCT compound (*see* Subheading 3.3).

3.2 Paraffin Processing, Embedding, and Sectioning of the Mouse Brains

1. Fix the mouse brain by perfusion, and put half of the brain in a postfixative and then in PBS.
2. Turn on the tissue processor, and select a program for mouse brain.
3. Put the fixed brain sample in the plastic cassette with a clear label of the mouse ID using a solvent-resistant marker.
4. Put all the cassettes in the chamber of the tissue processor. Start the tissue processor (usually programmed for delayed start and overnight processing).
5. On the next day, turn on the tissue-embedding machine a few hours before embedding.
6. Transfer the cassettes from the tissue processor to a hot paraffin bath in the tissue-embedding machine.
7. Place a metal mold on the hot plate, and pour hot paraffin in the metal mold (4–5 mm in depth).
8. Place the brain samples in the metal mold, with the cut surface of the brain down toward the bottom of the mold, using heated forceps (Fig. 1b). Note: Place two samples and two controls in the metal mold for sagittal sections. Place four samples and four controls in the metal mold for coronal sections.
9. Transfer the metal mold carefully from the hot plate onto the cold stage. Immediately after the paraffin on the bottom side of the metal mold is cooled and hardened, transfer the metal mold carefully from the cold stage and pour hot paraffin into the top of the metal mold.
10. Place the labeled tissue cassettes on top of the metal mold, and again pour enough hot paraffin to cover the top surface of the plastic cassette. Place the metal mold carefully onto the cold stage (approximately 30 min). If paraffin flows outside the cassette or shrinks with cooling, add hot paraffin.
11. When the paraffin is cooled and hardened, remove the paraffin block from the metal mold carefully with forceps (Fig. 1c). Wrap each paraffin block in a plastic wrap, and keep the paraffin blocks in a cool and dark place to avoid overdrying.

3.2.1 Sectioning

1. Label all the glass slides with the ID of the paraffin block and the slide number from 1 to 350 for the sagittal adult brain sections encompassing the entire cerebral hemisphere or from 1 to 350 for the coronal adult brain sections encompassing the SNc and locus coeruleus using a pencil or solvent-resistant marker for identification of the slides later.
2. Turn on a water bath equipped with a thermometer. Keep the temperature of the water bath at 45–50 °C (*see* **Note 3**).
3. Place a blade on the microtome, and set the thickness of the sections. Sagittal sections are usually cut at a thickness of 10 μm, and coronal sections, at a thickness of 16 μm.

4. Place the paraffin block onto the microtome chuck, and adjust the angle of the paraffin block for a proper cutting plane.
5. Cut the sections until you reach a section that includes the brain samples without chipping.
6. Cut the No. 1 section, spread it, pick up the section carefully with forceps or a fine paintbrush, and float the section on the surface of the water bath (*see* **Note 3**). Float the section onto the No. 1 glass slide, and stand the slide on a paper towel to remove excess water.
7. Cut the No. 2 section, and float the section onto the No. 2 glass slide. Cut the following sections in the same way. Keep all the sections on the glass slides even if the section is not complete (*see* **Note 4**).
8. Dry the glass slides on the bench overnight, and store them in the case in a cool, dark place.

3.3 Embedding and Sectioning of the Mouse Brains for Cryosections

1. Fix the mouse brain by perfusion, and put half of the brain in a postfixative solution, followed by 20 % sucrose/PBS solution and then 30 % sucrose/PBS solution.
2. Place the brains in a plastic tissue mold, with the cut surface of the brain facing the bottom of the mold. Add an OCT compound into the mold, remove air bubbles, freeze them on the metal shelf of a −80 °C freezer, and store them at −80 °C.

3.3.1 Sectioning

1. Label the glass slides with the ID of the block and the slide number, using a pencil or solvent-resistant marker.
2. Put OCT compound on the specimen disc, and then place the frozen brain samples on the specimen disc. Place the specimen disc with the brain samples in the cryostat chamber and freeze it with a weight on them.
3. Place the specimen disc with the brain samples onto the cryostat, and adjust the angle of the cutting plane of the sample.
4. Cut sections at a thickness of 5–15 μm, flip over the sections if necessary, and transfer the sections to room-temperature (RT) glass slides by touching the brain section toward the glass slide.
5. Store the glass slides in a suitable case in a −80 °C freezer until use.

3.4 Nissl Staining of the Mouse Brain Sections

1. Bake the paraffin sections at 55–60 °C for 1.5–2 h before use.
2. Deparaffinize and rehydrate the sections by sequential immersion in the following reagents. Shake slides up and down several times every 30 s in each buffer:
   (a) Histo-Clear (substitute for xylene) twice for 4 min each.
   (b) 50 % Histo-Clear/50 % EtOH for 4 min.
   (c) 100 % EtOH twice for 4 min each.

(d) 95 % EtOH for 4 min.

(e) 70 % EtOH for 4 min.

(f) 50 % EtOH for 4 min.

(g) dH_2O twice for 3 min each.

3. Immerse the glass slides in cresyl violet solution for 10 min. Shake slides up and down several times.
4. Rinse (three quick dips) the glass slides in dH_2O, and immediately go to the next step. If the staining is weak, go back to cresyl violet solution for additional immerse.
5. Rinse (three quick dips) the glass slides in diluted acetic acid.
6. Rinse the glass slides in dH_2O, and immediately go to the next step.
7. Rinse quickly in 50, 70, 95, and 100 % EtOH twice and then in 50 % Histo-Clear/50 % EtOH (three to five quick dips in each).
8. Rinse in Histo-Clear for 2 min.
9. Rinse in Histo-Clear for 5 min.
10. Put approximately five drops of Permount on the sections of the glass slide, and mount them with a coverslip. Flip the glass slide over, and press the slide onto a paper towel to remove any excess Permount. Remove small air bubbles by pressing the edge of the slide carefully.
11. Leave the glass slides in a well-ventilated place for 12–24 h.
12. Place the slides in the dark.

3.5 Immunostaining of the Mouse Brain Sections

When performing immunostaining with an antibody or a tissue section for the first time, it is important to have positive and negative control samples to distinguish real signals from staining artifacts. For positive controls, known tissue sections that express the target protein of the antibody can be used. For negative controls, processed tissue samples without primary antibody, secondary antibody, or both can be immunostained in parallel with samples processed using the normal staining procedure. To compare the expression level of certain protein in the immunostaining it is important to stain the sections in weak signals because it is difficult to compare the signal intensity in saturated dense signals. Therefore it is necessary to optimize the dilution of primary antibody.

3.5.1 Paraffin Sections

1. Bake the paraffin sections at 55–60 °C for 1.5–2 h before use.
2. Deparaffinize and rehydrate the sections by sequential immersion as described earlier (*see* Subheading 3.4).
3. Permeabilization and/or antigen retrieval may be necessary for certain antibody staining (*see* **Note 5**).

   (a) *Permeabilization*: Incubate the sections in 0.1 % Triton X-100/TBS for 15 min and then rinse in dH_2O two or three times.

(b) *Antigen retrieval*: Immerse the slides in 10-mM citrate buffer at pH 6.0 in a plastic microwaveable dish (e.g., Coplin staining jar), and bring the buffer to a boil five times (add a little fresh buffer on top after each boiling except the last boiling). Note that drying sections under microwave heating causes high-background immunostaining. Cool the slides to RT in citrate buffer for 15 min, and then rinse them in dH_2O two or three times.

4. Quench the endogenous peroxidase activity by incubating the sections in 0.3 % H_2O_2 dissolved in methanol for 15 min. Rinse the sections in an excess amount of dH_2O twice and then in TBS for 5 min each.

3.5.2 Frozen Sections (See Note 6)

1. Bring the glass slides to RT by leaving them on the bench for 5–10 min.
2. Rinse out OCT in TBS twice for 5 min each and then in dH_2O.
3. Quench the endogenous peroxidase activity as described earlier.

3.5.3 Staining with the Avidin–Biotin Complex

1. Tap off TBS on a paper towel, place the glass slide horizontally in a humidified chamber, and block nonspecific binding sites by covering all the areas of the sections with 5 % NGS/TBST solution (blocking solution, ~1 mL/slide) and incubating them for 60 min at RT (*see* **Note 7**).
2. Tap off the blocking solution on a paper towel, put primary antibody solution (diluted in 5 % NGS/TBST) on the sections, with the solution covering all the areas of the sections, and incubate them in a humidified chamber overnight at 4 °C. Note: Incubation at 4 °C for a few overnights gives better signals for some primary antibodies.
3. Tap off the primary antibody solution on a paper towel, and wash the sections in TBST three times for 5 min each.
4. Tap off the TBST on a paper towel, and add the biotinylated secondary antibody (5–10 μg/mL diluted in 5 % NGS/TBST). Incubate the sections in a humidified chamber for 60–90 min at RT.
5. Prepare the avidin–biotin complex (ABC) reagent (add two drops of reagent A and two drops of reagent B in 5 mL of TBS, mix the solution immediately, and allow the ABC reagent to stand for approximately 30 min before use).
6. Wash the sections in TBST three times for 5 min each.
7. Place the ABC reagent on the sections (~1 mL/slide), and incubate the sections in a humidified chamber for 30–60 min at RT.
8. Wash the sections in TBST three times for 5 min each and then in TBS.

9. Prepare the DAB substrate by adding two drops of buffer stock solution in 5 mL of dH_2O and mixing it well. Add four drops of DAB stock solution and two drops of hydrogen peroxide solution. Mix well. Put DAB substrate on the sections (~1 mL/slide), and incubate them in a humidified chamber for 5–15 min at RT.
10. Place the slides in dH_2O to stop the DAB reaction. Rinse the slides in dH_2O two or three times.
11. If necessary, perform counterstaining. Dilute Harris-modified hematoxylin five times in dH_2O. Dip the slides up and down in hematoxylin for 15 s, and wash them with running tap water. Check one of the slides under a microscope. If the staining is weak, repeat.
12. Dehydrate the sections by sequential immersion in the following reagents. Shake slides up and down several times every 30 s in each buffer: dH_2O, 50 % EtOH, 70 % EtOH, 95 % EtOH, 100 % EtOH twice, 50 % Histo-Clear/50 % EtOH, and Histo-Clear twice for 2 min each.
13. Mount the coverslips with Permount.
14. Leave the glass slides in a well-ventilated place for 12–24 h.
15. Place the slides in the dark.

3.6 Immunofluorescence Staining of the Mouse Brain Sections

3.6.1 Paraffin Sections

1. Bake the paraffin sections at 55–60 °C for 1.5–2 h before use.
2. Deparaffinize and rehydrate the sections by sequential immersion as described earlier (*see* Subheading 3.4).
3. If necessary, perform permeabilization and/or antigen retrieval (*see* Subheading 3.5).
4. Dip the slides in TBS.

3.6.2 Frozen Sections

1. Bring the slides to RT on the bench for 5–10 min.
2. Rinse out the OCT in TBS twice for 5 min each.

3.6.3 Staining

1. Place 5 % NGS/TBST solution on the sections, and incubate them in a humidified chamber for 1 h at RT.
2. Put primary antibody solution (diluted in 5 % NGS/TBST) on the sections, and incubate them in a humidified chamber overnight at 4 °C. Note: Mix the primary antibodies in 5 % NGS/TBST for double-immunofluorescence staining.
3. Wash the slides in TBST three times for 5 min each.
4. Put the appropriate secondary antibody (1:300 diluted in 5 % NGS/TBST) on the sections, and incubate them in a humidified chamber covered with aluminum foil for 60–90 min at RT. Note: Mix the appropriate secondary antibodies in 5 % NGS/TBST for double-immunofluorescence staining.

5. Wash the slides in TBST three times for 5 min each, and immerse slides in TBS twice for 5 min each.
6. Apply 0.3 % Sudan Black B/70 % EtOH to each slide for 10 min if necessary (*see* **Note 8**).
7. Wash the slides in TBST three times.
8. Put counterstaining solution (TOPRO-3 diluted 1:10,000 in TBS) on the sections, and incubate them in a humidified chamber covered with aluminum foil for 10 min at RT.
9. Wash the slides in TBST three times and then in TBS.
10. Mount the slides with a minimum amount of Vectashield (one or two drops) and coverslips. Place the slides in the dark at 4 °C.

3.7 Fluoro-Jade B Staining

1. Air-dry the frozen sections (20 μm thickness) on bench top.
2. Rinse the slides in PBS for 5 min on a rotating shaker.
3. Rinse the slides quickly in dH_2O twice.
4. Wick off excess dH_2O, and dry the slides on a 50 °C heat block for 5 min.
5. Incubate the slides in 1 % NaOH/80 % EtOH for 5 min on a rotating shaker.
6. Rinse the slides in 70 % EtOH for 2 min on a rotating shaker.
7. Rinse the slides in dH_2O for 2 min on a rotating shaker.
8. Incubate the slides in fresh 0.06 % $KMnO_4$ for 20 min on a rotating shaker.
9. Rinse the slides in dH_2O for 2 min on a rotating shaker.
10. Incubate the slides in 0.0004 % Fluoro-Jade B (4 mL 0.01 % FJB stock + 96 mL 0.1 % acetic acid) for 20 min on a rotating shaker. Cover them with aluminum foil. Note: Prepare this solution less than 10 min before using.
11. Rinse the slides in dH_2O for 1 min on a rotating shaker three times.
12. Wick off excess dH_2O, and dry the slides on a 50 °C heat block for 5 min. Cover with aluminum foil.
13. Immerse the slides in Histo-Clear for 3 min.
14. Mount the slides with Permount.

3.8 TUNEL on Mouse Brain Sections

3.8.1 Paraffin Sections

1. Bake the paraffin sections at 55–60 °C for 1.5–2 h before use.
2. Deparaffinize and rehydrate the sections by sequential immersion as described earlier (*see* Subheading 3.4).
3. Incubate the sections in 0.1 % TritonX-100/TBS for 15 min and then in dH_2O two or three times.
4. Immerse the slides in 10-mM citrate buffer at pH 6.0 in a plastic microwaveable dish, and bring the buffer to a boil five times

(add a little fresh buffer on top after each boiling except the last boiling).

5. Cool the slides to RT in citrate buffer for 15 min.
6. Rinse the slides in dH_2O two or three times and then in TBS.

3.8.2 Cryosections

1. Air-dry the slides on bench top for 5–10 min.
2. Rinse the slides in TBS for 10 min and then in dH_2O two or three times.
3. Immerse the slides in 10-mM citrate buffer at pH 6.0 in a plastic microwaveable dish, and bring the buffer to a boil three times (add a little fresh buffer on top after the first and second boiling).
4. Cool the slides to RT in citrate buffer for 15 min.
5. Rinse the slides in dH_2O two or three times and then in TBS.

3.8.3 Labeling

1. Place the blocking buffer (5–10 % NGS/TBST) on the sections, and incubate them for 60 min at RT in a humidified chamber. Note: If the background signals are high, use 10 % NGS and 3 % BSA/TBST as a blocking buffer.
2. Remove as much blocking buffer from the slides as possible on a paper towel, and place the slides horizontally in a humidified chamber. Apply at least 120 µL of the TUNEL reaction mixture to each slide, and put a small piece of parafilm on each glass slide. Wrap the humidified chamber with aluminum foil, and incubate it at 37 °C for 1–1.5 h.
3. Remove the parafilm, and rinse the slides in TBST three times and then in TBS for 5 min each.
4. Mount the slides with a minimum amount of Vectashield (one or two drops) and coverslip. Place the slides in the dark at 4 °C.

3.9 Stereological Neuron Counting

Please refer to the stereology software manual for the detailed instructions regarding the operation of the system.

1. Fix the mouse brains by perfusion, and perform the paraffin processing, embedding, and sectioning of the brains (*see* Subheadings 3.1 and 3.2). Note: Sagittal brain sections are usually cut at a thickness of 10 µm for cortical neuron counting, and coronal brain sections are usually cut at a thickness of 16 µm for nigral DA neuron counting. In principle, thicker sections are recommended for optimum stereological neuron counting.
2. Select a series of brain sections in which the target area for cortical neurons or nigral DA neurons can be counted (*see* **Note 9** and Fig. 3a, d).
3. Perform immunostaining for a neuronal marker (NeuN staining for cortical neuron counting and TH staining for nigral DA neuron counting; *see* Subheading 3.5 and Fig. 3a, d).

4. Turn on the microscope, instruments (video camera, controller, etc.), and the computer.
5. Start the stereology software, and place the first glass slide on the microscope.
6. Select the ×4 objective, and adjust the focus of the first sample in the live image mode.
7. Find and set a suitable landmark (reference) point.
8. Trace around the brain region (cerebral cortex or SNc) in which the number of neurons needs to be counted (Fig. 3b, e).
9. Select the size of the grid, and place the sampling grid randomly onto the brain region (Fig. 3b, e). The size of the grid is usually set at 500×500 μm for cortical neuron counting and at 100×100 μm for nigral DA neuron counting.
10. Add oil onto the slide, select the 100× objective, go to the position of the first counting frame, and adjust the focus of the section.
11. Select the size of the counting frame (Fig. 3c, f). The size of the counting frame is usually set at 50×50 μm for cortical and nigral DA neuron counting.
12. Set the positions of the top (upper surface) and bottom (lower surface) of the tissues. Record the thickness of the sections (Fig. 4a).
13. Select the size of the guard zones and the disector height (Fig. 4a).
14. Count the NeuN- or TH-positive cells in the clear focus of the multiple *Z*-direction levels of the section according to the counting rules (Figs. 3c, f and 4b).
15. Go to the following counting frames on the area, and count the cells.
16. Complete the counting in all the sections on all the glass slides in the same way.
17. The total number of neurons is calculated as follows [15–17]:

$$\text{Total number of neurons} = Q \times t / h \times 1 / \text{asf} \times 1 / \text{ssf}$$

where Q=the total number of cells actually counted in the counting frames of all the sections analyzed, h=the height of the disector, t=the mean thickness of the sections, asf=the area sampling fraction (the ratio of the counting frame area to the sampling grid area), and ssf=the section sampling fraction (the ratio of the number of sections analyzed to the total number of sections in the entire structure).

Using an example of neuron counting in the unilateral neocortex, 1 in 40 sections (every 40th section) is analyzed. The area of the counting frame is 2,500 μm² (50×50 μm), and the area

of the sampling grid is 250,000 μm² (500 × 500 μm). The height of the disector is set at 8 μm. If the mean thickness of the sections is 10 μm and Q=625, the total neuron number in the unilateral neocortex is estimated as follows:

t/h = 10 μm/8 μm, asf = 2,500 μm²/250,000 μm², and ssf = 1/40.

Total neuron number in the unilateral neocortex = $625 \times 10/8 \times 100 \times 40 = 3.125 \times 10^6$.

Total neuron number in the bilateral neocortex = 6.25×10^6.

In an example of DA neuron counting in unilateral SNc, one in ten sections (every tenth section) is analyzed. The area of the counting frame is 2,500 μm² (50 × 50 μm), and the area of the sampling grid is 10,000 μm² (100 × 100 μm). The height of the disector is set at 12 μm. If the mean thickness of the sections is 16 μm and Q=85, then the total DA neuron number in the unilateral SNc is estimated as follows:

t/h = 16 μm/12 μm, asf = 2,500 μm²/10,000 μm², and ssf = 1/10.

Total DA neuron number in unilateral SNc = $85 \times 16/12 \times 4 \times 10$ = 4,533.

Total DA neuron number in bilateral SNc = 9,066.

4 Notes

1. To use half of the brain for biochemical analysis, perfuse the animal with Ringer solution and dissect out the brain. After the dissection, cut the brain into halves and place one-half into a fixative for histological analysis. Use the other half of the brain immediately for biochemical analysis, or freeze it.
2. Keep watching, and make sure that the flow speed of the Ringer solution or the fixative is appropriate. If the speed is too fast, the solution may leak. If the speed is too slow, it may be because of a blockage. Check the line, and make sure that the top of the needle is properly positioned in the heart.
3. The temperature of the water bath and the floating time of the tissue sections on the water are important to ensure wrinkle-free sections. If a section does have wrinkles, raise the temperature of the water bath or extend the floating time of the tissue section on the water. On the other hand, if the paraffin of the section melts quickly on the water, lower the temperature of the water bath.
4. Humidity and the degree of dryness of the paraffin block influence the quality of the sections while cutting. Cutting the sections of a dry paraffin block often results in split or broken sections. In that case, placing wet cotton gauze toward the cutting surface of

the paraffin block for approximately 5 s before cutting can help ensure clear sections and a paraffin ribbon.

5. Permeabilization using detergents allows the antibody access to the inside of the cells to detect proteins in the cytoplasm, nucleus, or organelles, whereas antigen retrieval is used to increase the sensitivity of immunohistochemical detection of epitopes. However, these treatments sometimes cause nonspecific background staining. On the other hand, a higher detergent concentration, longer incubation of the sections in detergent, or excessive boiling during antigen retrieval can cause decreased immunoreactivities due to loss of protein structure or antigenicity. Therefore, it is important to use negative control experiments when immunostaining using these treatments.
6. In general, the antigenicity of a protein is better preserved and the sensitivity of the immunohistochemical detection of epitopes is higher in frozen sections than in paraffin sections. On the other hand nonspecific signals occur more often in the immunostaining using frozen sections compared to paraffin sections. It is important to put negative control in the immunostaining using frozen sections.
7. Placing the glass slides horizontally in the humidified chamber is important to ensure that all the tissue sections on the glass slides are being covered with the solutions. The solutions (blocking or antibody solution) should be put on the glass slides using a transfer pipette with a volume of at least 400 μL per glass slide. It is also important not to dry the tissue sections at any step in the immunostaining process. Drying the sections causes a failure of staining or a high background of staining. The tissue sections should be always covered with the solution (blocking solution or antibody solution, etc.) or in the washing buffer during the immunostaining process.
8. Autofluorescence could be observed in the mouse brain, especially in the brains of the aged mice. It is mainly caused by lipofuscin granules. The emission of autofluorescence is generated by a wide range of exciting wavelengths; thus, autofluorescence signals can be observed in multiple channels in fluorescence microscopy. It is necessary to distinguish the specific fluorescence signal of the antibody, whose emission is generated by exciting a certain wavelength, from autofluorescence, especially because strong autofluorescence interferes with the observation of specific signals of the antibody. Sudan black B is used for quenching autofluorescence [20].
9. Every 40th section is usually selected for counting cortical neurons. For example, starting from a random number between 10 and 19 (e.g., 12), sections 12, 52, 92, 132, 172, 212, 252, and 292 will be counted. Starting from a random number between 20 and 29 (e.g., 26), sections 26, 66, 106,

146, 186, 226, 266, and 306 will be counted. If any of the aforementioned sections are lost, it can be replaced with a neighboring one. For example, if section 202 is lost, then section 200, 201, 203, or 204 can be used instead. For counting DA neurons, every tenth section is usually selected and counted.

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References

1. Schmitz C, Hof PR (2005) Design-based stereology in neuroscience. Neuroscience 130:813–831
2. Sterio DC (1984) The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 134:127–136
3. Schweichel JU, Merker HJ (1973) The morphology of various types of cell death in prenatal tissues. Teratology 7:253–266
4. Schmued LC, Hopkins KJ (2000) Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res 874:123–130
5. Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, Brambilla C, Brambilla E (1996) In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem 44:959–968
6. McGeer PL, Itagaki S, Boyes BE, McGeer EG (1988) Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology 38:1285–1291
7. Hirsch EC, Hunot S (2009) Neuroinflammation in Parkinson's disease: a target for neuroprotection? Lancet Neurol 8:382–397
8. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL et al (2000) Inflammation and Alzheimer's disease. Neurobiol Aging 21:383–421
9. O'Callaghan JP, Jensen KF (1992) Enhanced expression of glial fibrillary acidic protein and the cupric silver degeneration reaction can be used as sensitive and early indicators of neurotoxicity. Neurotoxicology 13:113–122
10. Tenner AJ (2001) Complement in Alzheimer's disease: opportunities for modulating protective and pathogenic events. Neurobiol Aging 22:849–861
11. Lemere CA, Munger JS, Shi GP, Natkin L, Haass C, Chapman HA, Selkoe DJ (1995) The lysosomal cysteine protease, cathepsin S, is increased in Alzheimer's disease and Down syndrome brain. An immunocytochemical study. Am J Pathol 146:848–860
12. Saura CA, Choi SY, Beglopoulos V, Malkani S, Zhang D, Shankaranarayana Rao BS, Chattarji S, Kelleher RJ 3rd, Kandel ER, Duff K et al (2004) Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration. Neuron 42:23–36
13. Beglopoulos V, Sun X, Saura CA, Lemere CA, Kim RD, Shen J (2004) Reduced beta-amyloid production and increased inflammatory responses in presenilin conditional knock-out mice. J Biol Chem 279:46907–46914
14. Wines-Samuelson M, Schulte EC, Smith MJ, Aoki C, Liu X, Kelleher RJ 3rd, Shen J (2010) Characterization of age-dependent and progressive cortical neuronal degeneration in presenilin conditional mutant mice. PLoS One 5:e10195
15. Gundersen HJ (1986) Stereology of arbitrary particles. A review of unbiased number and

size estimators and the presentation of some new ones, in memory of William R. Thompson. J Microsc 143:3–45

16. Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B et al (1988) The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96:857–881
17. West MJ, Slomianka L, Gundersen HJ (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Rec 231:482–497
18. Ward TS, Rosen GD, von Bartheld CS (2008) Optical disector counting in cryosections and vibratome sections underestimates particle numbers: effects of tissue quality. Microsc Res Tech 71:60–68
19. Evans SM, Nyengaard JR (2004) Number: section introduction. In: Evans SM, Janson AM, Nyengaard JR (eds) Quantitative methods in neuroscience. Oxford University Press, Oxford, pp 61–89
20. Romijn HJ, van Uum JF, Breedijk I, Emmering J, Radu I, Pool CW (1999) Double immunolabeling of neuropeptides in the human hypothalamus as analyzed by confocal laser scanning fluorescence microscopy. J Histochem Cytochem 47:229–236