A Rapid and Sensitive Automated Image-Based Approach for In Vitro and In Vivo Characterization of Cell Morphology and Quantification of Cell Number and Neurite Architecture

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ABSTRACT

Stereological methods for tissue cell counting, specifically for neuron quantification, decrease systematic error and sampling bias; however, they are tedious, labor intensive, and time consuming. Approaches for cell (neuron) quantification in vitro are not accurate, sensitive, or subsequently reproducible. Neuronal phenotype is related to alterations in cell morphology and neurite pattern. The techniques currently available for quantification of these features present several limitations. In this unit, we provide validated automated procedures for in vivo and in vitro quantification of cell number, morphological cell changes, and neurite morphometry in a fast, simple and reliable manner. Our method counts up to 8 times as many neurons in less than 5% to 10% of the time required for stereological analysis (optical fractionator). In summary, this technology offers an unparalleled opportunity to examine features of cells at high resolution in a complex three-dimensional environment. These techniques provide an exceptional in vivo and in vitro system for neurotoxicity studies, disease modeling, and drug discovery. *Curr. Protoc. Cytom*. 68:12.33.1-12.33.21. © 2014 by John Wiley & Sons, Inc.

Keywords: Neuroprotection • neurodegeneration • neurotoxicity • rotenone • neuron • neurites • morphology • quantification

INTRODUCTION

Toxic impairment of cells can cause loss of essential cellular functions, resulting in individual cell death. Specifically, neurodegenerative disorders—and neurotoxin insult—are characterized by chronic and progressive neuronal loss and glial activation. Current efficient, quantitative methods to evaluate neurodegeneration are lacking or inadequate. For instance, design-based unbiased stereology is used to obtain accurate quantitative assessment from tissue sections in which cells are uniformly organized and distributed; the optical fractionator probe is the most sophisticated stereological method and merges the optical dissector with the fractionator (West et al., 1991). However, this technique is labor intensive and exceptionally time consuming.

Traditional stereology cannot be employed for in vitro cell counting, as cells show a random distribution in cultures. Flow cytometry, tritium uptake, and visual enumeration have been considered as an alternative analytical techniques for determining cell (neuron) counts or survival, but essential factors such as the structure of the cell or its health cannot

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be determined (Groszer et al., 2001; Salthun-Lassalle et al., 2004; Toulorge et al., 2011; Welsbie et al., 2013).

In addition, morphological alterations such as shrinkage and elongation represent additional features of cell death. Significant phenotype changes in size or shape have been described during apoptosis both in vitro and in vivo (Kermer et al., 2002; LaFerla et al., 1997), but current quantitative approaches show limited robustness, reproducibility, and accuracy.

For studies of neuronal development, neuroprotection, and neurotoxicity, parameters in addition to cell (neuron) count are necessary to characterize neuronal phenotype, such as quantitative evaluation of neurite morphogenesis. Quantification of neurites is exceptionally important because they play a central role in synaptic integration, neuronal connection, and information processing (Morita et al., 2006; Petrinovic et al., 2013). Neurites grow in three-dimensional networks; therefore, a high-efficiency neurite reconstruction has to operate in 3-D. Although several procedures are available (Zhang et al., 2007), the evaluations are beset with methodological drawbacks.

This unit focuses on in vivo and in vitro quantification of both neurons and glial (GFAP)-positive cells using an automated microscope platform equipped with a digital camera. The scanning is performed using a motorized XYZ stage capable of high-speed image acquisition and assembly of up to 400 images from four different light channels into a single high-resolution montage for analysis (see Basic Protocol 1 and Basic Protocol 2). NIS-Elements software (Nikon) is utilized as a counting tool. Furthermore, we provide a protocol to quantitatively measure morphological alterations using the MetaMorph package (see Basic Protocol 3). We also propose a protocol for neurite assessment using the FilamentTracer module of Imaris (Bitplane; see Basic Protocol 4 and Basic Protocol 5). For these protocols, basic understanding and expertise in animal handling procedures, cell culture techniques, and immunohistochemistry are required.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

BASIC PROTOCOL 1

IMMUNOFLUORESCENCE QUANTIFICATION OF NEURONS AND ASTROCYTES IN VIVO

To optimize system reliability and sensitivity to detect physiological changes in neurons, multiple-dimension (XYZ) automated digital image collection methods are applied. For this purpose, we have designed and engineered an operational automated upright microscope synchronized with a linear-encoded motorized stage that can quickly scan the entire surface of a specimen. This technology allows the acquisition of multiple channels over several stage positions, creating large-field-of-view data sets that are automatically stitched together to generate extremely high-resolution montages (Fig. 12.33.1).

We used the mitochondrial complex I inhibitor rotenone to induce degeneration of dopamine (DA) neurons in the substantia nigra (SN) of rats (Betarbet et al., 2000). Systematic administration of rotenone reproduces the main neuroanatomical, biochemical, and behavioral features of Parkinson's disease (Betarbet et al., 2000; Cannon et al., 2009).

Materials

6- to 7-month-old male Lewis rats
Rotenone (see recipe)
4% (w/v) paraformaldehyde in PBS (see *APPENDIX 2A* for PBS)\

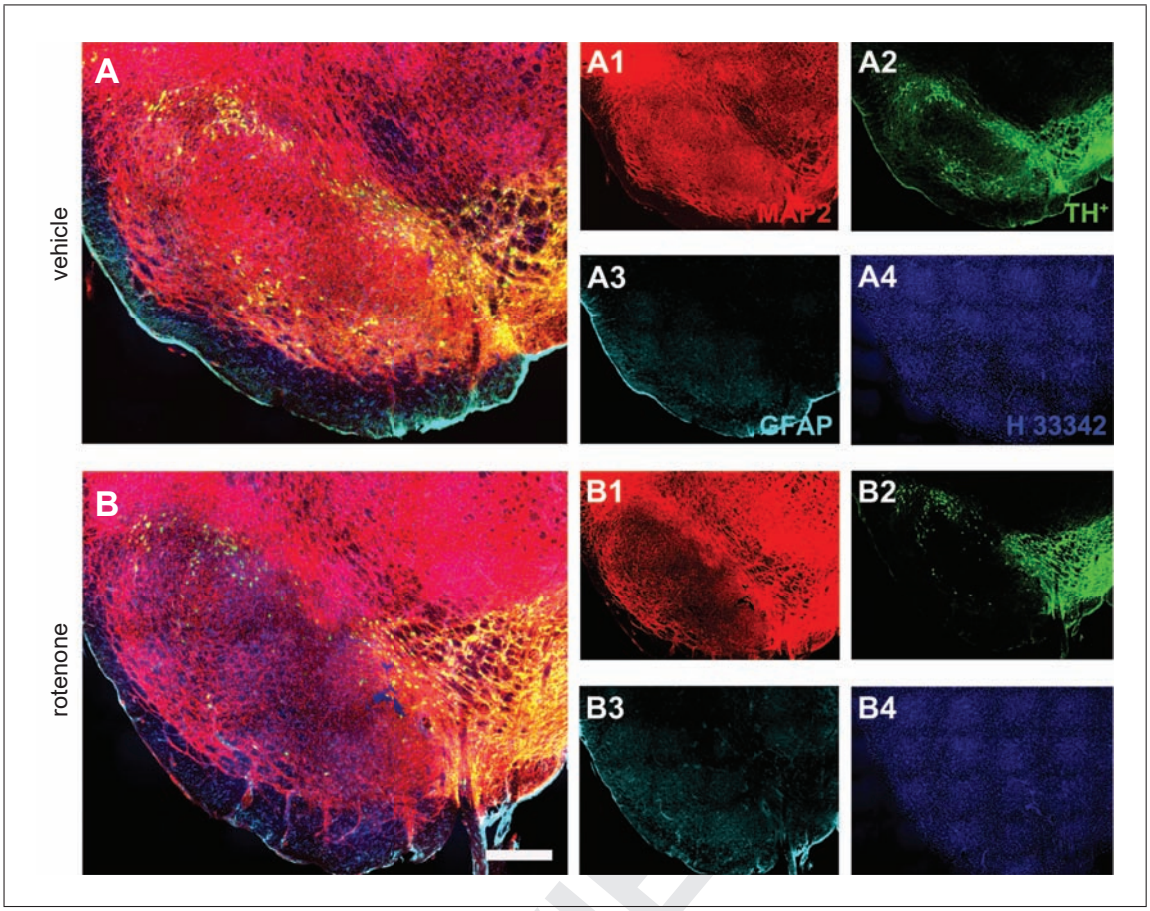

Figure 12.33.1 Reconstruction of brain regions from digitized images using the motorized stage setup. Serial sections from the substantia nigra brain (**A**-**B**) are fluorescently immunolabeled and acquired at 20× using the motorized stage approach. MAP2, TH^+, GFAP, and nuclei immunoreactivities are shown in non-treated rats (A1-A4). Qualitative analysis reveals a decrease in neuronal expression (both MAP2 and TH^+) and an increase in astrocyte content following rotenone treatment (B1-B4). Red: MAP2; green: TH^+; cyan blue: GFAP; navy blue: H 33342. Scale bar = 500 μm. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/cy1233*.

30% (w/v) sucrose
Cryoprotectant solution (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1% (v/v) Triton X-100 in PBS (see *APPENDIX 2A* for PBS)
Blocking solution: phosphate buffered saline (PBS; *APPENDIX 2A*) containing 10% (v/v) normal (donkey) serum and 0.3% (v/v) Triton X-100
Primary antibodies:
- Mouse monoclonal antibody for microtubule associated protein 2 (MAP2; Millipore, cat. no. MAB378)
- Sheep polyclonal antibody for tyrosine hydroxylase (TH; Millipore, cat. no. AB1542)
- Rabbit polyclonal antibody for glial fibrillary acidic protein (GFAP; Millipore, cat. no. AB5804)

Secondary antibodies:
- Biotinylated ?? antibody
- Cy3-conjugated anti-sheep antibody (Jackson ImmunoResearch, cat. no. 713–165–003)

Alexa Fluor 647–conjugated anti-mouse antibody (Invitrogen, cat. no. A31571)
Alexa Fluor 488–conjugated anti-rabbit antibody (Invitrogen, cat. no. A21206)

BisBenzimide H 33342 fluorochrome trihydrochloride dye (Sigma-Aldrich, cat. no. B2261)
?Vectamount?Aquamount? mounting medium
?Vectastain avidin-biotin complex (ABC) standard kit?
?3,3′-Diaminobenzidine (DAB) chromogen kit?
?Hydrogen peroxide (H_2O_2)?

Surgical instruments: scissors, forceps, #11 scalpel blade, spatula, razor blades
Glass petri dish
Freezing sliding microtome (*UNIT 12.15*)
24-well plates
6-well plates
Bench-top agitator
Plus-coated microscope slides (e.g., ??)
Glass coverslips
Automated upright fluorescence microscope (?Supplier?; cat. no.?)
Cooled CCD camera

Additional reagents and equipment for injection (Donovan and Brown, 2006a) and euthanasia (Donovan and Brown, 2006b) of rodents, cryosectioning (*UNIT 12.15*), and microscopy/data analysis (Basic Protocol 1)

Animals and treatment

1. Treat rats with daily intraperitoneal injections (Donovan and Brown, 2006a) of 3.0 mg/kg rotenone in a 1-ml volume.

   Animals are randomly separated into two groups of five animals each.

Histology and brain tissue processing

2. Sacrifice the animals by CO_2 asphyxiation followed by rapid decapitation (Donovan and Brown, 2006b) when a potentially debilitating phenotype is observed, i.e., when clear signs of akinesia, rigidity, and postural instability are evident.
3. Remove the brains and fix them in 4% PFA in PBS for 7 days.
4. Transfer the brains into 30% sucrose for a minimum of 3 to 4 days for cryoprotection.
5. Prepare 35-μm thick coronal sections using a freezing sliding microtome and collect them as free-floating sections in wells of 24-well plates (see *UNIT 12.15*).
6. Maintain the sections in cryoprotectant solution (prepared as described in Reagents and Solutions) at −20°C until ready to perform the subsequent assays.

   Sections may be stored for years.

Immunohistochemistry for fluorescence labeling

7. Wash the SN sections (with three to four sections per well of a 6-well plate) three times in PBS, each time for 10 min at ??ºC.
8. Incubate sections in wells with 1% (v/v) Triton X-100 in PBS for 5 hr at 4°C.
9. Wash sections three times in PBS, each time for 10 min at room temperature.

Table 12.33.1 Number of TH-Immunoreactive Cell Counts Using the Motorized Stage Approach[a]

Sample	In vivo		In vitro	
	Vehicle	Rotenone	Vehicle	Rotenone
#1	1818	1385	1326	407
#2	2061	1288	1374	374
#3	1953	1125	1220	407
#4	2098	1157	1651	611
#5	2343	995	1919	684

[a]For all, $n = 5$ samples for each treatment group (vehicle or rotenone).

10. Incubate in blocking solution (10% normal donkey serum and 0.3% (v/v) Triton X-100 in PBS) for 1 hr at room temperature.
11. Add primary antibody solution and incubate for 72 hr at 4°C.

    The primary antibody solution consists of a cocktail of the following antibodies in ??:

    Mouse monoclonal antibody for MAP2: 1:2000.

    Sheep polyclonal antibody for TH: 1:2000.

    Rabbit polyclonal antibody for GFAP: 1:2000.
12. After an additional incubation in ?fresh? primary antibody solution for 1 hr at room temperature, wash the sections in PBS three times, each time for 10 min at ??ºC, to remove unreacted primary antibodies.
13. Incubate with secondary antibody solution for 2 hr at room temperature.

    The secondary antibody solution consists of a cocktail of the following antibodies in ??:

    Cy3-conjugated anti-sheep antibody: 1:500.

    Alexa Fluor 647–conjugated anti-mouse antibody: 1:500.

    Alexa Fluor 488–conjugated anti-rabbit antibody: 1:500.
14. Wash sections twice in PBS, each time for 10 min at room temperature.
15. Incubate sections with H 33342 as a nuclear counterstain for 5 min at room temperature.

    Use H 33342 at a 1:3000 dilution in ??..
16. Wash sections three times in PBS, each time for 10 min at room temperature.
17. [?Development/visualization?] Mount the sections onto plus-coated slides and coverslip them using ?Vectamount?Aquamount? mounting media.

Motorized stage imaging analysis and cell counting (neurons and astrocytes)

We used an automated upright fluorescence microscope equipped with 5 fluorescent channels (blue, green, red, far red, and near IR) and high-NA plan fluor/apochromat objectives. Chronic rotenone administration (3.0 mg/kg per day) results in a bilateral lesion to the nigrostriatal DA system and leads to an inflammatory process (Cannon et al., 2009; Tapias et al., 2013). Our motorized stage approach is capable of quantitating different cell types and, additionally, is sensitive enough to detect physiological alterations (Tables 12.33.1 and 12.33.2). We have employed this method with TH and GFAP-positive cells in nigral rat sections (see Video *1 at http:/www.currentprotocols.com/protocol/cy1233*).

Table 12.33.2 Number of GFAP-Positive Cells Counted Using the Motorized Stage System[a]

Animal	Vehicle	Rotenone
#1	1847	3027
#2	2067	2607
#3	2767	3292
#4	2088	2480
#5	1610	2512

[a]Astrocytic evaluation is carried out in 5 adult animals per group using the motorized stage technique.

$$N = \sum Q^{-} \cdot \frac{1}{VF}$$

$$VF = ASF \cdot HSF \cdot SSF$$

Figure 12.33.2 Mathematical model for total cell quantification. The estimated number of objects (N) equals to the number of particles counted (ΣQ^{-}) by the reciprocal of the volume fraction. Three components constitute the volume fraction: the area sampling fraction (ASF), the height sampling fraction (HSF), and the section sampling fraction (SSF).

All slides were scanned under the same conditions for magnification, exposure time, lamp intensity, and camera gain. Neuronal counting was performed by a single trained investigator.

18. Use the 20× objective (0.75 NA) to perform the analysis.
19. Collect images using a cooled CCD camera and an encoded motorized stage.
20. Stitch images automatically.
21. Perform quantitative analysis on fluorescent images generated in four fluorescent colors (stained for MAP2, TH^{+}, GFAP, and H 33342).
22. After scanning the entire surface of the sample, delineate the SN as an active ROI.
23. For neuronal (MAP2 and TH^{+}) and astrocyte (GFAP) cell counts, stitch the images with NIS-Elements.
24. Subtract the background.
25. Threshold for each individual channel.
26. Use the colocalization function.
27. Setup the exclusion/inclusion parameters.
28. Determine the thickness of objects using the Z axis of the automated scope.
29. Calculate the height of the cells using the MetaMorph package.

Mathematical model for estimation of the total number of DA neurons

We generated a mathematical model to provide an unbiased stereological estimation of the total number of cells using the motorized stage approach (Fig. 12.33.2).

30. Calculate the estimated total number of cells (N) which, as described in Figure 12.33.2, is a multiplication between the cells counted ($CN = \Sigma Q^{-}$) and the reciprocal of the volume fraction, which in turn, is a multiplication of three factors: the

$$CE = \frac{\sqrt{\text{Total Var}}}{CN}$$

$$CN = \sum_{i=1}^{n} Q^{-}$$

$$\text{Total Var} = CN + VAR_{SRS}$$

$$VAR_{SRS} = \frac{3(A - CN) - 4B + C}{12}, \text{ m = 0}$$

$$VAR_{SRS} = \frac{3(A - CN) - 4B + C}{240}, \text{ m = 1}$$

$$A = \sum_{i=1}^{n} (Q_i^{-})^2,\ B = \sum_{i=1}^{n-1} Q_i^{-} \cdot Q_{i+1}^{-},\ C = \sum_{i=1}^{n-2} Q_i^{-} \cdot Q_{i+2}^{-}$$

Figure 12.33.3 Coefficient of error determination. The coefficient of error (CE) is calculated as the square of the total variance (Total Var) divided by the sum of the counted neurons (CN); VAR_{SRS} corresponds to the variance in the systematic random system where the *m* class can be either 0 or 1.

area sampling fraction (ASF), the height sampling fraction (HSF), and the section sampling fraction (SSF).

ΣQ^{-}: The total number of neurons determined in the delineated ROI from the SN.

ASF: The area sampling fraction and equals 1 (the entire SN was analyzed).

HSF: Due to the lack of an optical fractionator for the motorized stage system, we estimated the height of the cells (using MetaMorph software) relative to the thickness of the sample.

SSF: Corresponds to the section interval.

Coefficient of error determination for total neurons counted

The coefficient of error (CE) provides a measure of precision that represents random error generated by sampling, noise, and other measuring events. CE was measured by a single-sample prediction formula developed initially by Matheron (1971) and further elaborated upon by Gundersen and Jensen (1987) (Fig. 12.33.3). The coefficient of error (CE) Gunderson values must to be <0.1 for all animals when $m = 1$ (Tables 12.33.3 and 12.33.4).

31. Calculate the CE as the ratio between the square of the total variance and the total number of neurons counted ($CE = \sqrt{\text{Total Var}}/CN$).

The variance of the total area is defined as the sum of the counted neurons ($CN = \Sigma_{i=1}{}^{n} \cdot Q^{-}$) and the variance of the area in the systematic random sampling (VAR_{SRS}). In fact, these data give information on the section number required to obtain an appropriate variation for section samples. In the intersectional variability due to systematic random sampling, A is the sum of squares of all counts from all sections [$\Sigma_{i=1} (Q^{-}{}_i)^2$]; B is the sum of the product of the number of neurons counted in each section and the number of neurons counted in the next section [$\Sigma_{i-1} (Q^{-}{}_i \cdot Q^{-}{}_{i+1})$]; and C is the sum of the products of counts in section i and the counts in section i+2 [$\Sigma_{i-1} (Q^{-}{}_i \cdot Q^{-}{}_{i+2})$].

Table 12.33.3 CE Estimation for TH$^+$ Quantitation When $m = 0$ and $m = 1$ for Both Vehicle and Rotenone-Treated Animals[a]

	Vehicle				Rotenone			
$m = 0$	CN	VAR_{SRS}	Total Var	CE	CN	VAR_{SRS}	Total Var	CE
#1	1818	14893	16711	0.07	1385	19833	21218	0.11
#2	2061	7521	9582	0.05	1288	25351	26639	0.13
#3	1953	10647	12600	0.06	1125	1567	2692	0.05
#4	2098	7784	9882	0.05	1157	2798	3955	0.05
#5	2343	56215	58558	0.10	995	4846	5841	0.08
$m = 1$	CN	VAR_{SRS}	Total Var	CE	CN	VAR_{SRS}	Total Var	CE
#1	1818	744	2562	0.03	1385	991.68	2376	0.04
#2	2061	376	2437	0.02	1288	1267.58	2555	0.04
#3	1953	532	2485	0.03	1125	78.36	1203	0.03
#4	2098	389	2487	0.02	1157	139.93	1296	0.03
#5	2343	2810	5153	0.03	995	242.33	1237	0.04

[a]For all, $n = 5$ rats for each treatment group.

Table 12.33.4 Motorized Stage CE Estimation for GFAP$^+$ Cell Counts[a]

	Vehicle				Rotenone			
$m = 0$	CN	VAR_{SRS}	Total Var	CE	CN	VAR_{SRS}	Total Var	CE
#1	1847	11,267	13,114	0.06	3027	67,454	70,481	0.09
#2	2067	15,326	17,393	0.06	2607	53,732	56,339	0.09
#3	2767	34,355	37,122	0.07	3292	114,234	117,526	0.10
#4	2088	8498	10,586	0.05	2480	42,184	44,664	0.09
#5	1610	25,445	27,055	0.10	2512	133,686	136,198	0.15
$m = 1$	CN	VAR_{SRS}	Total Var	CE	CN	VAR_{SRS}	Total Var	CE
#1	1847	563	2410	0.03	3027	3372	6399	0.03
#2	2067	766	2833	0.03	2607	2686	5293	0.03
#3	2767	1717	4484	0.02	3292	5711	9003	0.03
#4	2088	424	2512	0.02	2480	2109	4589	0.03
#5	1610	1272	2882	0.03	2512	6684	9196	0.04

[a]Smoothness factor, $m = 0$ and $m = 1$. Data presented correspond to the average values obtained from 5 rats per group.

Hence, $VAR_{SRS} = (3(A - CN) - 4B + C)/12)$, where $\alpha = 12$ for a smoothness factor of 0 ($m = 0$) and $VAR_{SRS} = (3(A - CN) - 4B + C)/240)$, where $\alpha = 240$ for a smoothness factor of 1 ($m = 1$).

BASIC PROTOCOL 2

Automated Neuronal and Neurite Quantification and Cell Morphology Assessment

IMMUNOFLUORESCENCE QUANTIFICATION OF NEURONS IN VITRO

Traditionally, cell counts in vitro have been difficult to quantitate accurately; the traditional visual enumeration method, selecting representative fields of view and manually counting immunostained neurons (Caiazzo et al., 2011), is not a reliable process due to the arbitrary distribution of cells. We present here an implemented method for automated cell (neuron) quantification in cultures (Fig. 12.33.4).

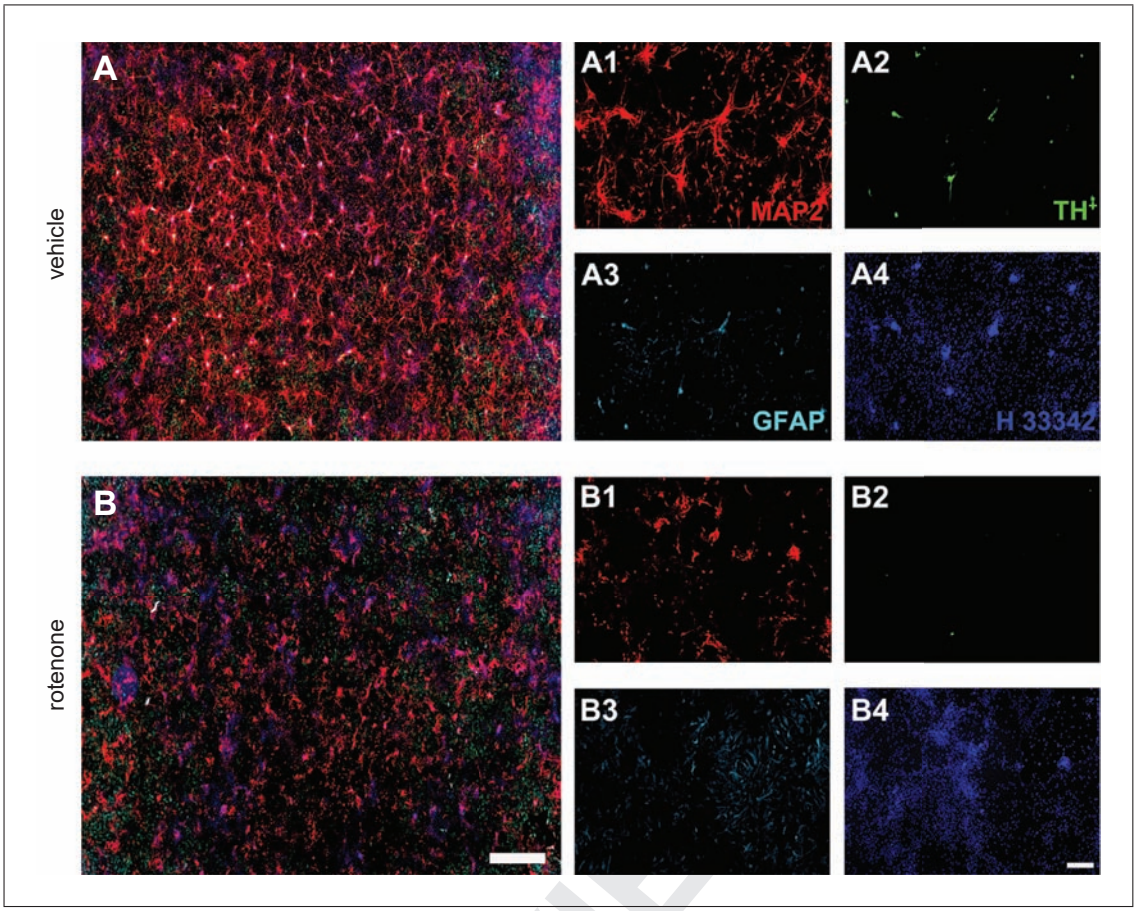

Figure 12.33.4 In vitro cell imaging using the motorized stage technique. Montage images from primary ventral midbrain cultures (E17 rats) are acquired at 10× objective using an automated microscope and stitched together using the NIS-Elements software (**A** versus **B**). Cells are treated with 50 nM rotenone at DIV 5 for a duration of 5 days. Pictures, especially zoomed-in images, show significant evidence of neuron loss and increased astrogliosis following rotenone administration when are compared to treatment with vehicle (B1 to B4 versus A1 to A4). MAP2 red; TH^+ neurons green; GFAP cyan blue; and nuclei navy blue. Scale bar = 1000 μm (panels A, B); scale bar = 200 μm (images A1 to A4 and B1 to B4). For the color version of this figure, go to *http://www.currentprotocols.com/protocol/cy1233*.

Materials

2- to 3-month-old ?pregnant? female Sprague-Dawley rats
Neurobasal medium (see recipe)
?? mg/ml trypsin/?EDTA?
0.1 mg/ml poly-d-lysine hydrobromide (PDL; Sigma, cat. no. P7280)
Complete minimal essential medium (complete MEM; see recipe)
Neurobasal medium (see recipe)
Glial cell–derived neurotrophic factor (GDNF)
Rotenone (see recipe)
4% (w/v) paraformaldehyde in PBS (see *APPENDIX 2A* for PBS) supplemented with 0.02% (v/v) Triton X-100 and 1 mM $MgCl_2$
10% normal horse serum in PBS (see *APPENDIX 2A* for PBS)
Vectamount?Aquamount? mounting medium
?Vectastain avidin-biotin complex (ABC) standard kit?
?3,3′-Diaminobenzidine (DAB) chromogen kit?
?Hydrogen peroxide (H_2O_2)?

Surgical instruments (scissors and forceps)
Petri dishes

Circular ?glass? coverslips
24-well tissue culture plates
Plus-coated microscope slides (?Supplier?; cat. no.?)
Automated upright fluorescence microscope (?Supplier?; cat. no.?)
Cooled CCD camera

Additional reagents and equipment for preparing primary ventral midbrain culture (Studer, 1997), counting cells with a hemacytometer (*APPENDIX 3A*), and determination of cell viability by trypan blue exclusion (*APPENDIX 3B*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Primary ventral midbrain neuron cultures

1. Dissect ventral midbrain tissues from E17 Sprague-Dawley rat brains.
2. After removal of the meninges, dissociate the pooled ventral midbrain tissues by mild mechanical trituration and enzymatic digestion using ?concentration? trypsin.
3. Evaluate cell viability and overall cell yield using the trypan blue assay (*APPENDIX 3B*) and a hemacytometer (*APPENDIX 3A*).
4. Prepare PDL-coated circular ?glass? coverslips by immersing the coverslips in 0.1 mg/ml PDL for ?? hr at ??ºC. Seed the resuspended cells on the coverslips pre-coated with PDL in 24-well culture plates in 0.5 ml complete MEM per well at a density of 5×10^5 cells/well.
5. Maintain the cultures at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.
6. Two days after the initial seeding, change the culture medium to fresh serum-free neurobasal medium. Add 50 ng GDNF per ml culture. Treat primary cell cultures with 50 nM rotenone or vehicle for 5 days beginning on the fifth day in vitro (DIV 5).

   Starting at DIV 5, the total treatment incubation time with rotenone or vehicle [?please describe vehicle control?] is 5 days (DIV 10).

Immunocytochemistry

7. At the end of the treatment period, fix the cells in 4% PFA, 0.02% Triton, and 1 mM $MgCl_2$ in PBS for 30 min.
8. Wash each well three times in PBS, each time for 10 min at room temperature.
9. Incubate in blocking solution (10% normal horse serum in PBS) for 30 min at room temperature.
10. Expose the cultures overnight at 4°C to the same primary antibodies and at equivalent concentrations in PBS with 1% normal serum that were used for immunofluorescence labeling in brain sections (see Basic Protocol 1, step 11).
11. Rinse the cells three times in PBS, each time for 10 min.
12. Incubate for 2 hr with the same secondary antibodies described above for immunofluorescent staining of tissue sections at a 1:1000 concentration (see Basic Protocol 1, step 13).
13. Rinse cells once in PBS for 10 min at room temperature.

14. Incubate sections with H 33342 as a nuclear counterstain for 5 min at room temperature.

    Use H 33342 at a 1:3000 dilution in ??..

15. Rinse cells once in PBS for 10 min at room temperature.

16. [?Development/visualization?]. Mount the coverslips onto plus-coated slides using Aquamount mounting media.

Fluorescence microscopy cell quantification using the motorized stage system in cultures

The same automated microscope described in Basic Protocol 1 can be utilized for these experiments. We acquire fluorescent images in four fluorescent colors (stained for MAP2, TH^+, GFAP, and H 33342). All slides are scanned under the same conditions for magnification, exposure time, lamp intensity, and camera gain (see Video 1 at *http://www.currentprotocols.com/protocol/cy1233*). Quantitative analysis is performed by an unbiased investigator. After scanning the entire surface of the sample, the central region of the coverslip—excluding the edges to eliminate some cell aggregation and fluorescence saturation—is used for in vitro examination (~75% of the total area).

Rotenone is utilized to study the sensitivity and utility of the motorized stage technique. We want to test whether our approach has the potential to accurately detect the neurotoxic effects of rotenone, which include cell death, shrinkage, or loss of processes, and alterations in cell morphology. Prior studies have revealed that systemic administration of rotenone induces neuronal death in DA neuron-glia cultures from ventral midbrain (Gao et al., 2011).

17. Use the 20× objective (0.75 N.A.) to perform the analysis.

18. Acquire images in four fluorescent colors (stained for MAP2, TH^+, GFAP, and H 33342), and perform quantitative assessments.

19. Perform background subtraction.

20. Set threshold for each individual channel.

21. Use the colocalization function.

22. Set up the exclusion/inclusion parameters.

CELL MORPHOLOGY ASSESSMENT IN VIVO

BASIC PROTOCOL 3

Morphological alterations including cell shrinkage or elongation, aflagellated ovoid shape, nuclear fragmentation, and pyknosis are associated with toxicity. In pathological processes such as neurodegenerative disorders, permanent structural changes are specifically important. Cells undergoing apoptosis display characteristic morphological modifications (Mattson, 2000) and their quantitative assessment could be worthwhile for the development of effective new neuroprotective therapies (Fig. 12.33.5).

For materials, see Basic Protocol 1.

1. Perform Basic Protocol 1.

   No additional materials, animals, treatments, or immunohistochemistry are needed because the brain sections that were previously used in Basic Protocol 1 for estimating the total number of neurons are also utilized to assess cell (TH^+) morphology.

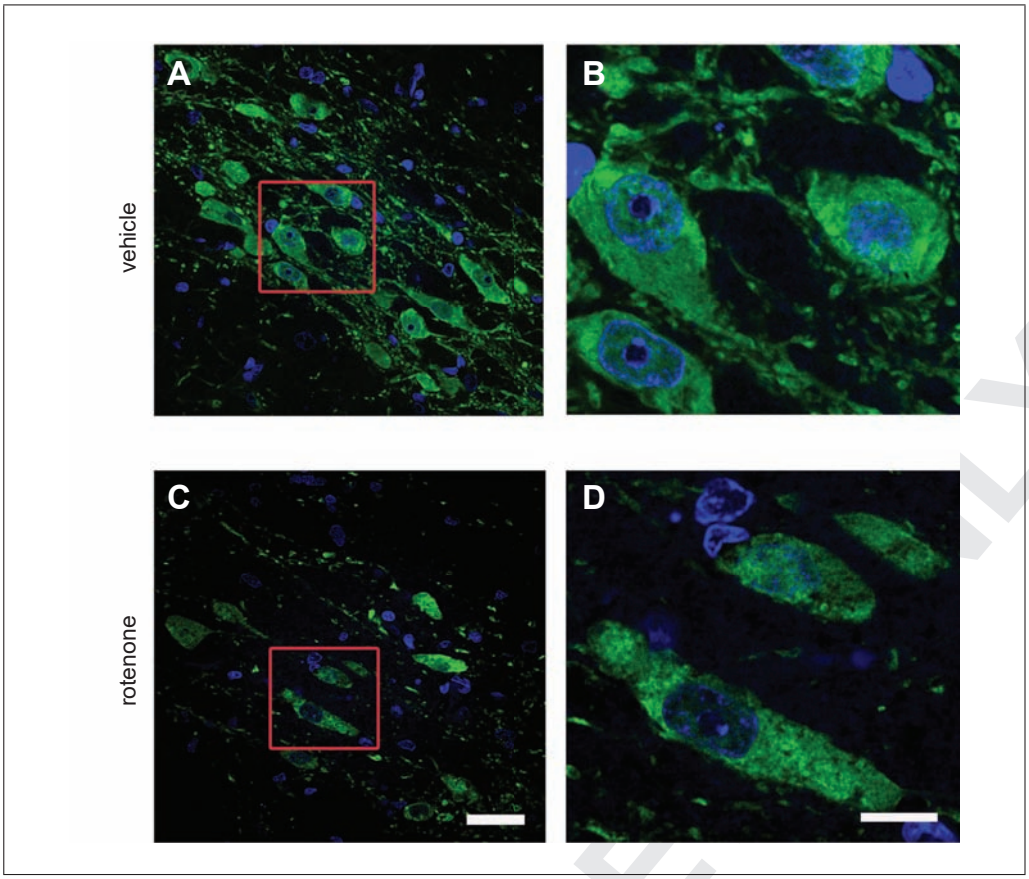

Figure 12.33.5 Neuron morphologic features. Micrographs stained for TH and acquired using confocal laser microscopy at 60× denote alteration in cell morphology after rotenone administration (**A** versus **C**). Insets of nigral sections illustrate morphological changes in terms of shape (shrinkage or elongation) in the rotenone-treated group (**B**) compared to the untreated group (**D**). MetaMorph software was used for quantification. For cell shape analysis, a shape factor of 1 represents a circular object while a 0 value indicates a straight line. Scale bar = 30 μm (A, C); scale bar = 10 μm (B, D). For the color version of this figure, go to *http://www.currentprotocols.com/protocol/cy1233*.

Cell shape and area factors

2. Acquire wide-field fluorescent images using an automated upright scope and a 60× oil-immersion objective (1.45 N.A.).
3. Save as a `.jpg` file.
4. To quantitate cell morphologic features (shape and area) open the `.jpg` file in MetaMorph software.
5. In the menu, go to "Display" and select "Color separate." Then, press "Separate."
6. Go to the "Measure" option, select "Calibrate distances" and then "Calibrate by region."

   Units should be given in μm.
7. Drag the calibration region bar into the original scale bar and choose "Apply."
8. In the menu, go to "Process" and select "Background and Shading Correction."

   Indicate the following factors: source, operation, parameters, and object size.
9. In the picture, press the "Look-up table" option and select "Monochrome."

10. Choose the "Threshold" option and select "Inclusive threshold."
11. In the menu, select the "Trace region" option for tracing the region of interest.
12. In the menu, select "Measure" and then "Integrated morphometry analysis."

    Determine the following parameters: area and shape, position, and orientation.

13. Press "Measure."
14. Exclude the undesired cells from the study using the limit function.
15. Select "Object data."
16. Save the data. Select "Configure log," then "Open log," select "Dynamic data exchange," and press "F9: log data."

    The shape factor value differs from 0 to 1, where 0 indicates a flattened object whereas 1 indicates a perfect circle.

ASSESSMENT OF NEURITE MORPHOMETRY IN VIVO AND IN VITRO

BASIC PROTOCOL 4

During development, neurons extend numerous processes (neurites) that differentiate into axons and dendrites. The function of the nervous system depends on communication between neurons via neurites. Neurite architecture analysis is particularly interesting and a useful endpoint in relation to neuropathological disorders, neurotoxicity, neuropharmacology research, and high-throughput screening. However, neurite quantification is a difficult task, significantly hampered by technical difficulties and by the lack of a highly reproducible, sensitive, and uniform method. Here, we provide a fast and reliable protocol for neurite quantification in tissue (SN) and cell cultures (Figs. 12.33.6 and 12.33.7).

For materials, see Basic Protocol 1 (for the "a" steps below) or Basic Protocol 2 (for the "b" steps below).

For in vivo quantitative analysis of neurite patterning

1a. Perform Basic Protocol 1.

    Materials, animals, treatment, histology, brain tissue processing, and immunofluorescence staining are the same as in Basic Protocol 1.

2a. Acquire 3-D image stacks to produce an extended depth-of-focus high-resolution image or maximum-intensity projection

    The same samples utilized for neuron counting are also employed to measure neuronal patterning and connections.

3a. Open the file using the FilamentTracer module of IMARIS (7.1.1 version).

4a. Select the channel of interest.

5a. Under the "Surpass" option, add a new filament.

6a. Go to "Create" and mark "Segment a region of interest."

7a. *Optional:* Select a color for the tracing.

8a. Determine the area of analysis.

    The region of interest (ROI) was defined using a sampling grid (of 8 squares); practically the entire SN pars compacta of the specimen was used for an unbiased neurite examination

9a. Define the starting points and the seed points.

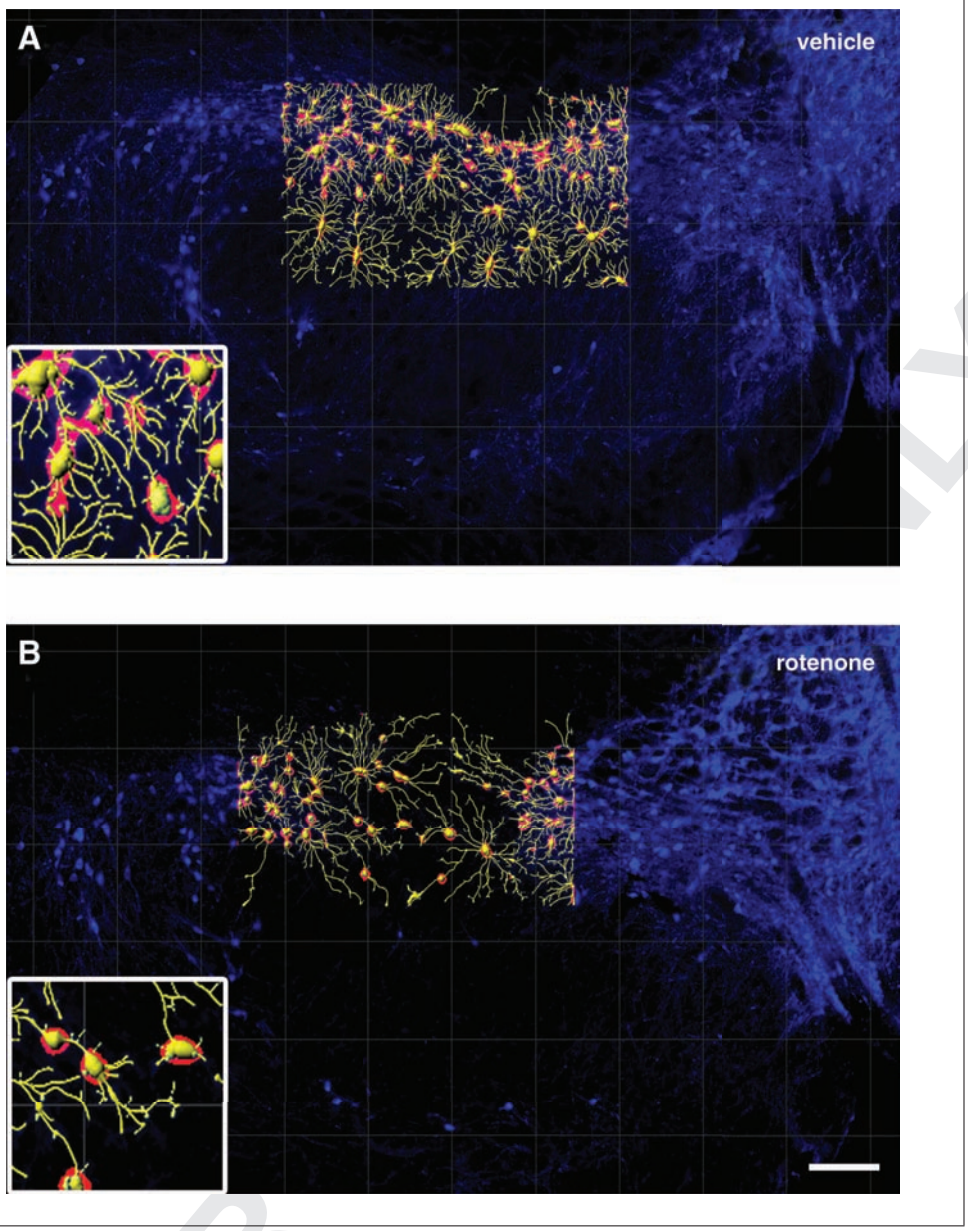

Figure 12.33.6 In vivo neurite quantification. The sections used for neuron estimation are also analyzed for neurite morphometry analysis. The Imaris FilamentTracer module is used for assessment of neurite outgrowth. TH^+ labeling in control (**A**) and rotenone-treated (**B**) animals within the entire SN pars compacta reveals a significant decrease in neurite density. Neurites are pseudocolored yellow, and DA neurons are labeled in red. Scale bar = 200 μm. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/cy1233*.

10a. In "Settings" mark the following options: show dendrites, beginning point, show spines.

Render quality = 50%.

11a. Establish the threshold for the starting points.

12a. Determine the threshold for the seed points.

13a. Select the diameter of the sphere region.

14a. Mark "Shortest distance from distance map."

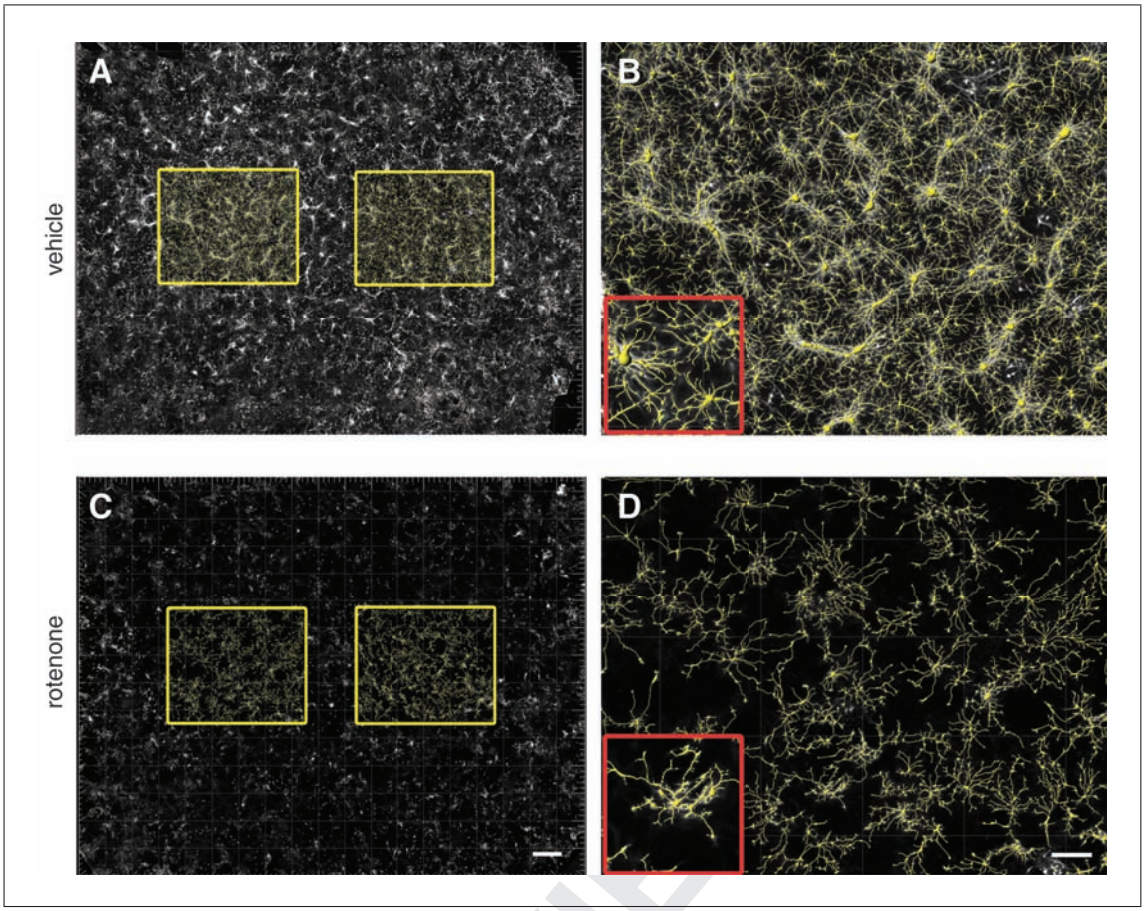

Figure 12.33.7 Neurite morphometry in cell cultures. Neurite architecture of primary ventral midbrain cultures was analyzed at DIV 10 using Imaris (Bitplane) software. To be unbiased, 2 squares (equivalent to the 15% of the total area of the coverslip) are selected in the center of the image (**A** and **C**). Dystrophic neurites are observed following rotenone administration compared to the vehicle-treated group, especially in zoomed-in images (**B** and **D**). Scale bar in A and C, 500 μm; in B and D, 200 μm. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/cy1233*.

15a. Go to "Detect spines" and repeat steps 8a to 13a.

16a. Choose "Additional regions" or "All done."

17a. Select "Finish: Execute all creation steps and terminate the wizard."

18a. Go to "Statistics" to generate detailed results and save the data as an Excel file.

Neurite parameters such as the length of processes, number of segments, and number of branches were obtained.

For neurite outgrowth quantification in vitro

1b. Perform Basic Protocol 2.

Materials, animals, treatment, midbrain neuron cultures, and immunofluorescence staining are the same as in Basic Protocol 2.

2b. Use the same procedure as outlined above for in vivo assessment.

The same samples analyzed for neuronal quantification are also used to measure the morphometry of neurites in vitro. For unbiased analysis, a large area equivalent to two squares (comprising 20 sub-squares each) should be consistently selected in the center of the image. Each sub-square corresponds to 0.5 mm; thus, the total area (A = XY) measured is determined to be 10 mm^2.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, sees* ***SUPPLIERS APPENDIX****.*

Cryoprotectant solution

1 ml 0.1 M ?Na??K?PO_4^{3-} buffer, pH 7.2
600 g sucrose
600 ml ethylene glycol
Store up to ?? at ??ºC

Minimum essential medium (MEM), complete

0.5 ml/well of MEM containing:
2% heat-inactivated fetal bovine serum
2% heat-inactivated horse serum
1 g/liter glucose
2 mM L-glutamine
1 mM sodium pyruvate
100 μM nonessential amino acids
50 U/ml penicillin
50 μg/ml streptomycin
Store up to ?? at ??ºC

Neurobasal medium

Neurobasal medium(e.g., Life Technologies) containing:
2% (v/v) B27 supplement (Life Technologies)
2 mM Glutamax I (Life Technologies)
0.5 mg/ml Albumax I (Life Technologies)
50 U/ml penicillin
50 μg/ml streptomycin
Store up to ?? at ??ºC

Rotenone

For rat treatment: Prepare rotenone (?Supplier?; cat. no.?) as a 50× stock dissolved in pure DMSO (2%) diluted in a medium-chain fatty acid termed Miglyol 812 N (98%; ?Supplier?; cat. no.?). Store up to ?? at ??ºC.

For primary cell cultures: Prepare the rotenone stock (5 mM) in pure DMSO, then dilute in neurobasal medium to a final concentration of 50 nM. Store up to ?? at ??ºC.

COMMENTARY

Background Information

Cell counting and quantification of morphological changes in cells (neurons) are essential for assessment of neuroprotection and neurotoxicity, although difficult. Unbiased stereology using the optical fractionator has become the recognized method for post hoc cell counting in tissue; nevertheless, the setup is exceptionally labor intensive and time consuming. In this work, we present novel automated techniques that are capable of analyzing approximately 8 times as many neurons in less than 5% to 10% of the time taken using the optical fractionator stereological method (Tapias et al., 2013).

To quantify features of interest, we have developed a mathematical model. Guard zones, which define the upper and lower limit of the sample on the *Z* axis for the counting frame (West et al., 1991), are unnecessary for the motorized stage approach because the images are collected in the middle of the section for each sample. In addition, application of guard zones could be inconsistent for the reason that tissue shrinkage may influence the thickness of the sample (Carlo et al., 2010). Furthermore, our system does not use an optical fractionator, but is not likely to provide redundancy in cell counting; by its very nature, the physical process of image collection using

the stage scanning system ensures a single count for every object. To certify that nothing is lost, there is a 15% overlap between frames; however, during computer stitching of the stacks, the overlaid regions are automatically removed, avoiding double counts.

The motorized stage system was specifically applied in the SN brain region for neuron and astrocyte estimation; nevertheless, the approach is also efficient and amenable to quantification of any cell type, not only in several major brain areas but also in sections from other tissues. Correspondingly, an extensive range of cell cultures can be analyzed using our methodology.

The precision of estimates is represented by the CE in order to elude methodological sampling error. The CE can be expressed as $m = 0$ and $m = 1$. Most biological tissues are a structural continuum without abrupt changes in structure, conventionally described by the $m = 1$ smoothing class (Gundersen et al., 1999).

Currently, there is a lack of sensitive tools to quantify the number of cells in vitro. Thus, our motorized stage method may represent a comprehensive framework to analyze and quantify neurons in culture. Importantly, the motorized stage instrument is capable of quickly scanning the entire surface of the sample, which greatly improves sensitivity and precision.

Morphological alterations of cells could be also determined through our motorized stage tool. Changes in morphology—abnormalities in the shape (elongation) of the cell as well as the soma staining intensity—may suggest functional cell impairment prior to cell death. Neuron morphologic features are measured using MetaMorph software. For the analysis, a shape factor of 1 represents a circular object while a 0 value indicates a straight line. A suitable background and shading correction as well as application of a median (smoothing) filter object are needed to minimize noise of the images, allowing for more accurate analysis of overall trends in elongation.

Neurite degeneration is a common (and the earliest) pathological feature (preceding neuronal death) of many neurodegenerative disorders (Saxena and Caroni, 2007). Several methods for neurite reconstruction have been proposed; the majority of the approaches are based on skeletonization algorithms (Cohen et al., 1994) or vectorial tracking methods (Al-Kofahi et al., 2002; Zhang et al., 2007). However, these procedures show important limitations.

Because neurons and neurites form a complex tree-like structure, a successful strategy for accurate tracing applications has to operate in 3-D. Software such as NeuronJ, Neurolucida, and NeuriteIQ provide 2-D reconstructions or use manual tracing, which is time consuming and error prone. The V3D-Neuron and the Simple Neurite Tracer applications are semi-automatic tracing tools in 3-D (Peng et al., 2010; Longair et al., 2011), but they can be time consuming (starting and successive points along the dendritic tree must be manually determined).

Since the bulk of manual or semi-automated tools available for neurite quantification are tedious, time consuming, and potentially subject to observer bias, the reconstruction is potentially nonreproducible. To overcome these challenges, we use the Imaris tracing algorithm, which uses 3-D image processing and visualization capabilities leading to further optimization, for better centering and branch-point placement (and diameter detection). The Filament tracer package enables optional improvement of neurite skeleton using a deformable curve algorithm that fits the path as nearly as possible to the center of the image and may lead to an optimal workflow that estimates with high precision the radius of the traced neurite along the boundaries of the components forming the skeleton. The only parameters that require manual introduction are the length and the size of the neurites. For parity, image evaluation must use identical grid dimensions.

In summary, we report that our system suitably overcomes many of the hurdles encountered in analyzing multidimensional tissues and cultures accurately and reliably. In contrast to contemporary methods, which are unsuitable or cumbersome, we describe here a simple, sensitive, and fast assay for quantifying cell counts and changes in cell morphology.

Critical Parameters and Troubleshooting

In order to obtain accurate and reproducible results from fluorescence imaging quantification, investigators should be particularly meticulous regarding some critical steps.

First, there is a key factor to be considered in obtaining quality data sets. It is extremely important to have exceptional staining. Staining of cell cultures is highly uniform, but in vivo it may be inconsistent. Independent of the type and affinity of the antibody, the fluorescence intensity between animals can

be somewhat irregular due to the biological variability of the specimen (about one or two animals out of every 20).

Furthermore, immunofluorescence can also be distinct between sections from the same animal; in particular, the number of sections per well may need to be limited to prevent immunodepletion (with a maximum of four sections per well in a 6 well-plate). Robust agitation during incubations is essential to prevent overlapping of tissue sections and to allow for uninhibited interaction with the antibody solution and optimal antibody penetration.

Although the assay is generally tolerant of minor non-uniformities in fluorescence intensities between sections, extreme variations in intensity may lead to unsuitable results.

In vitro, during all steps, do not disrupt the cells adhered to the coverslip (for example, the medium should be added very gently, the investigator should hold the edge of the coverslip with fine forceps, etc.). Cell death can be due to poor cell or tissue preparation. Poor culture conditions may lead to contamination of cell cultures.

When acquiring the micrographs, some caution is needed. If the levels of fluorescence are poor or nonexistent, it may be due to several reasons:

Dye concentration is too low. The user has to increase the antibody concentration.

High background. The investigator has to decrease the antibody concentration.

Poor penetration. The user has to enhance the concentration of Triton.

In order to moderate photobleaching, the investigator should use low-intensity light levels in the microscope to image a sample (section or coverslip). The samples should be stored in the dark when not in use.

During neuron quantification using the NIS-Elements software, observe the following:

Eliminate the background for image acquisition.

Carefully adjust the "Clean," "Smooth," and "Separate" filter settings.

The thresholding step of each different channel (H 33342, MAP2, and TH^+) requires cautious attention in order to avoid false positives (or negatives).

Define the boundaries of the objects. The exclusion of objects outside of these boundaries is a critical consideration for achieving the best results for stringent identification of neurons.

During the analysis of cell morphology using the MetaMorph package:

Calibrate the length.

Cautiously establish threshold.

Define the boundaries of the region of interest.

If the error message "IMA cannot perform measurements" is observed, go to "Integrated morphometry analysis," select "Source," and choose the option "Low pass."

During quantitation of neurite features using the Imaris FilamentTracer module:

To avoid background and noise, special care has to be taken with the introduction of the manual parameters, i.e., neurite length and size.

Identical grid dimensions should be utilized for parity.

Anticipated Results

This unit is meant to help investigators evaluate cell (neuron) phenotype by providing basic tools and strategies to quantitate number of neurons and neurite architecture. Moreover, changes in cell morphology can also be determined.

Staining

Applying these staining methods, we observe that TH^+ neurons colocalize with the expression of MAP2 neurons and the respective nuclei within the brain (SN region). This protocol can yield staining with high resolution and should provide the user with accurate and reliable cell number (neurons, astrocytes, etc.) for statistical analysis.

Neuron quantification

Once the images are background corrected, they should be properly thresholded (Fig. 12.33.8). The variability of the estimated final neuron number is very low when compared with other software programs, and cells in the background can still be visualized (even after colocalization), consequently facilitating the exclusion of objects that do not meet the criteria for cell size.

The expected number of TH^+ counts within the SN is ~ 2000 in control tissue sections (average of 11 sections), but is significantly decreased in rotenone-treated rats (~1200 TH-immunoreactive neurons). The number of TH-immunopositive cells is ~1500 in vehicle-culture cells and ~500 for cells treated with rotenone (Table 12.33.1).

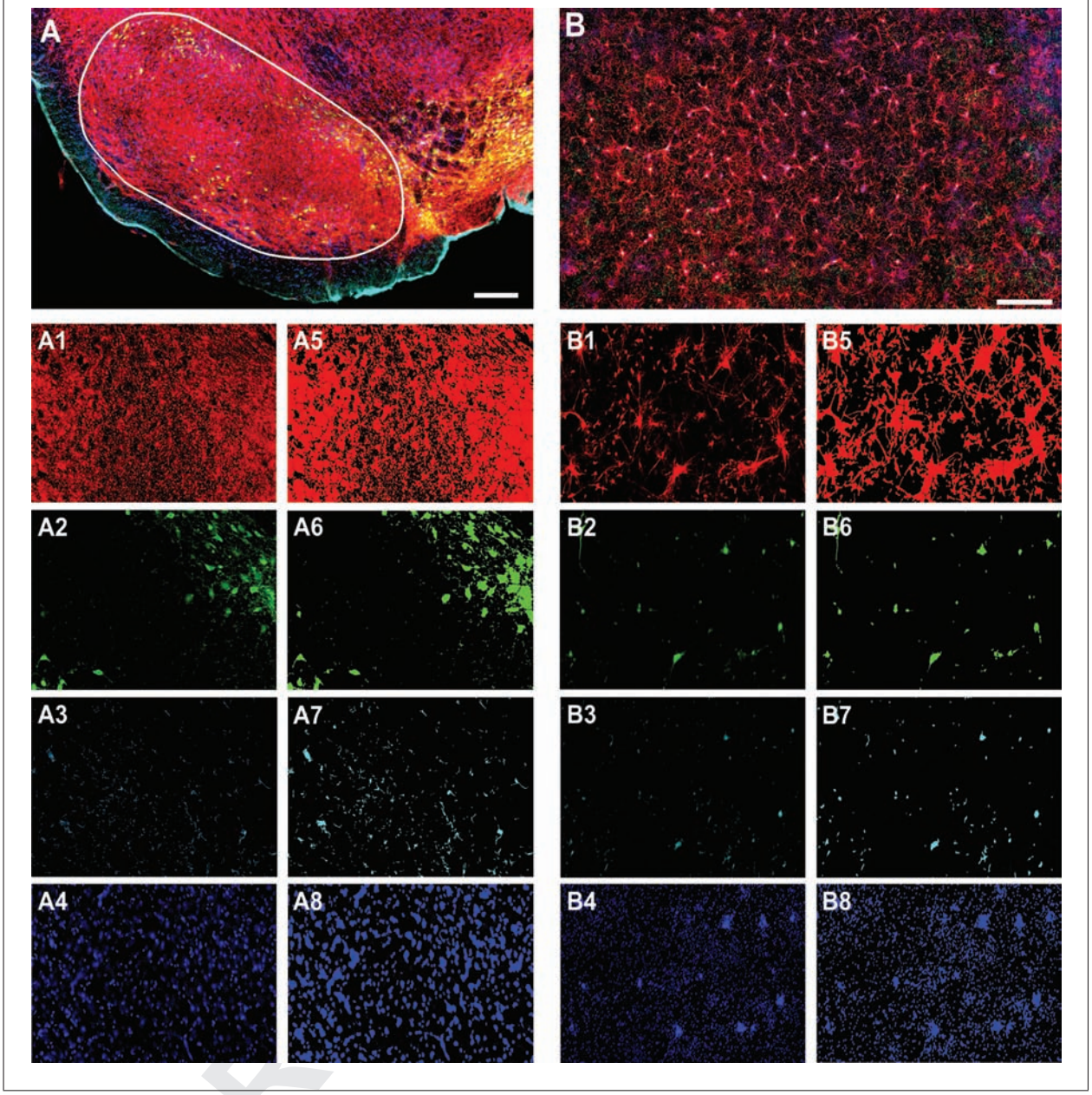

Figure 12.33.8 ROI delineation and image threshold adjustment. Pictures depict an example of region of interest delineation, which is defined prior cell calculation, for both in vivo (**A**) and in vitro (**B**). Neurons (MAP2 and TH^+), astrocytes ($GFAP^+$), and nuclei are illustrated before (A1 to A4) and after (A5 to A7) threshold regulation in the rat SN brain region as well as primary ventral midbrain cultures (B1 to B8). Scale bar in A, 250 μm; in B, 1000 μm. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/cy1233*.

GFAP-positive cells obtained within the SN are ~2100 and ~2800 for vehicle and rotenone groups, respectively (Table 12.33.2).

Neurite analysis

Neurites were evaluated using the Imaris tracing algorithm, which allows for easy semi-automatic tracing through reconstructed 3-D image stacks using a skeletonization algorithm (see Videos 2 and 3 at *http://www.currentprotocols.com/protocol/cy1233*). Control treatment should consistently yield high neurite length (sum) compared to toxin-treated neurons for both in vivo (~214 versus 135) and in vitro (~96 versus 80) studies. Also, the number of neurite segments and neurite branches are provided (Tables 12.33.5 and 12.33.6).

Table 12.33.5 Neurite Quantification in the SN of Rats[a]

Sample	Sum neurite length		Neurite segments		Neurite branches	
	Vehicle	Rotenone	Vehicle	Rotenone	Vehicle	Rotenone
#1	270	79	9	2	4	1
#2	227	138	7	4	3	2
#3	199	147	6	4	3	2
#4	167	155	5	5	3	2
#5	205	156	7	4	3	2

[a]Evaluation is carried out in 5 adult animals per group.

Table 12.33.6 Neurite Quantification in Primary Neuron Cultures[a]

Sample	Sum neurite length		Neurite segments		Neurite branches	
	Vehicle	Rotenone	Vehicle	Rotenone	Vehicle	Rotenone
#1	111	80	5	2	2	1
#2	93	79	4	2	2	1
#3	98	92	4	2	2	1
#4	93	80	4	3	2	1
#5	83	67	3	2	1	1

[a]Data presented correspond to the average values obtained from 5 rats per group.

Time Considerations

Immunohistochemistry for fluorescence labeling

Initial 30 min for rinsing, 5 hr of incubation in 1% Triton X-100, 30 min washing, 1 hr in blocking solution, 72 hr incubation in primary antibody solution, 1 hr extra incubation in primary antibody solution at room temperature, 30 min for rinsing, 2 hr incubation in secondary antibody solution, 20 min washing, 5 min for nuclei staining, 30 min for washing, and variable time for mounting, depending on sample number (as described here: 1 to 2 hr).

Immunocytochemistry

Initial 30 min for fixing, 30 min washing, 30 min in blocking solution, 24 hr incubation in primary antibody solution, 30 min for rinsing, 2 hr incubation in secondary antibody solution, 20 min washing, 5 min for nuclei staining, 30 min for washing, and 1 to 2 hr for mounting.

Cell counting estimation

For both in vivo and in vitro analysis using the motorized stage approach and the NIS-Elements package, the time per individual sample required for cell quantitation is ~3 min.

Morphological cell evaluation

As an average, the amount of time per individual section taken for changes in cell morphology is around 5 min.

Neurite length quantification

The duration of the analysis depends mainly of: (i) the size of the ROI and, (ii) if dendritic spines are also included in the study.

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