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Dual use of immunohistochemistry for film densitometry and light microscopy

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Abstract

In the present study, we applied the principles of immunoblotting and light microscopy immunohistochemistry to develop a combined methodology that allows obtaining optical density data in films, as well as morphological and protein distribution data on slides using the same brain tissue section, thus maximizing the data obtained from a single sample. This is especially important when experiments are performed using very valuable or unique tissue samples, which is a very common case in the study of the human brain.

The ideal methodology should combine the possibility of measuring levels of expression of a marker, and the capability to map accurately the distribution of that marker in the region of interest. To achieve this, two things are required: first, the technique needs to be sensitive enough to obtain optical density or intensity measurements of the marker, and second, a good preservation of the tissue is needed for the study of distribution patterns and morphological analysis.

Here we show that our combined methodology produced reliable results for different tissue preservation conditions (fresh-frozen and fixed tissue), in different species (rat and human), in different brain areas (substantia nigra and striatum), and for the detection of different markers (tyrosine hydroxylase and μ -opioid receptor). This methodology also combines the accuracy of optical density data acquisition in film with obtaining histological slides from the same sample.

In summary, the methodology proposed here is very versatile and does not require the use of specialized equipment, other than the routine equipment present in an anatomy laboratory.

Keywords

optical density; frozen tissue; fixed tissue; striatum; substantia nigra; tyrosine hydroxylase; μ -opioid receptor

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INTRODUCTION

Immunohistochemistry allows studying the pattern of expression of specific markers in tissues including the central nervous system. This technique has been used for decades to study the expression of neurotransmitters, enzymes, and other molecules in the brain in health and disease. However, immunohistochemistry has been mostly used to study staining patterns, or for the quantification of the number of cells. The use of immunohistochemistry in neuroscience to obtain data on the levels of expression of a marker in the brain has been scarce, and very different methodologies have been applied with different levels of success. The main efforts to obtain standardized methodologies for the use of immunohistochemistry as a tool to analyze the levels of expression of a marker have been done in the cancer research field, where an array of different methodologies have been developed (see e.g. Ermert et al., 2001; Luongo de Matos et al., 2010). These methodologies include both, techniques to measure the levels of a marker in a specific cell, as well as the levels of a marker in a tissue area, although the implementation of these methods in the neuroscience field has not been as extensive. The reasons for this include problems with signal-to-noise ratio, and sensitivity for the detection of markers that can be expressed in very small areas, or in relatively low levels. Early on, a method of choice in the neuroscience field was radioactive immunohistochemistry, with different variations of this technique, from simply tagging the primary antibody with a radionuclide, to more sensitive methods that used radionuclide-labeled secondary antibodies (see e.g. Raisman-Vozari et al., 1991; Blanchard et al., 1993; Pepin et al., 1996; Dentresangle et al., 2001; Eastwood et al., 2001). However, these techniques have been progressively abandoned due to the need of special permits, dedicated radioactivity-approved spaces, the long exposure time needed, and the increasing difficulty of finding suppliers for radionuclide tags for immunohistochemistry. Other methodologies that have been tested include the measurement of immunofluorescence intensity, and the density of light microscopy chromogens such as diaminobenzidine (DAB) or other chromogens (see e.g. Mausset-Bonnefont et al., 2003; Smichth et al., 2004; Smith et al., 2005; Blackbeard et al., 2007). All these methodologies can yield semiquantitative data on the expression of a marker in the brain, although there are differences in sensitivity and other parameters that must be considered (see e.g. Eastwood and Harrison, 1995; Ermert et al., 2001).

The ideal methodology should combine the possibility of measuring levels of expression of a marker, and the capability of accurately map the distribution of that marker in the region of interest. To achieve this, two things are required: first, the technique needs to be sensitive enough to obtain optical density or intensity measurements of the marker, and second, a good preservation of the tissue is needed for the study of distribution patterns and morphological analysis.

Radioactive immunohistochemistry possesses great sensitivity, but requires a very lengthy process. For this methodology, first films are exposed for a period of time that ranges from days to weeks, depending on the marker studied (see e.g. Blanchard et al., 1993; Pepin et al., 1996; Dentresangle et al., 2001; Eastwood et al., 2001). After films are exposed, if anatomical studies are desired, the tissue sections have to be incubated with an emulsion (e.g. silver emulsion), which normally requires from several days to weeks of incubation (see e.g. Raisman-Vozari et al., 1991; Blanchard et al., 1993; Eastwood et al., 2001).

Immunofluorescence methods have the advantage of being faster for data collection, although they generally present variable stability and sensitivity (Ermert et al., 2001), and the signal from the fluorophores becomes degraded during observation due to photobleaching (see e.g. Ermert et al., 2001). In addition, autofluorescence is a major problem when studies are conducted in adult brain tissue. Another recently developed

methodology is the use of near-infrared technology, for which near-infrared dyes are coupled to secondary antibodies (Loebke et al., 2007; Wang et al., 2011). This methodology can reduce or eliminate autofluorescence issues, since most of naturally-occurring autofluorescent molecules emit below the near-infrared wavelength (which ranges between 700–1400nm). However, this methodology requires specialized equipment, such as xenon lamps instead of the traditional mercury lamps for fluorescence, and customized filters (Loebke et al., 2007; Wang et al., 2011).

The use of light microscopy chromogens such as DAB, DAB-nickel, and other similar chromogens, has the advantage of a better signal stability, since these are normally permanent preparations, which can be stored long-term without loss of signal. The caveats of this methodology arise from the way the samples are analyzed, rather than from a lack of signal, or problems with signal stability. For brightfield microscopy densitometry, many parameters have to be controlled, including, exposure time, light source stability, camera settings, and the choice of permanent mounting medium (e.g. different mounting media possess different light refraction properties).

In our search for a sensitive and stable method that could combine obtaining semi-quantitative data, and allow the morphological analysis of the same section, without the caveats of the above mentioned methodologies, we have developed a method that combines the use of film densitometry and brightfield microscopy. In the present study, we show how this technique can be used in brain tissue that has been preserved in different ways (fresh-frozen and fixed tissue). We also tested the capability of this technique for the detection of different markers and in different species.

2. MATERIALS AND METHODS

2.1 Brain samples used in this study

The histological sections used in this study were obtained from two different sources:

2.1.1 Human brain tissue—All the human brain samples used in this study were obtained from the Stanley Research Institute Neuropathology with permission from their selection committee (Torrey et al., 2000). All experimental procedures were also approved by the University of Alabama at Birmingham Institutional Review Board and in accordance with The Code of Ethics of the World Medical Association and the Declaration of Helsinki. This tissue consisted of slide-mounted 14-micron thick coronal sections through the human substantia nigra (SN) of an adult control subject provided as a test case. Prior to arrival to our laboratory, tissue was preserved by rapid freezing after dissection, sectioned using a cryostat, and sections were preserved at -80°C .

2.1.2 Animal brain tissue—Adult rat brain tissue used in this study consisted of free-floating coronal sections at the level of the striatum and substantia nigra, which were obtained from a stock of spare tissue available in our laboratory. This stock was spare control (untreated) rat brain tissue from a set of experiments performed previously in the University of Maryland at Baltimore with the approval of the Institutional Animal Care and Use Committee (IACUC), protocol number 0705010, in accordance with the National Institutes of Health guidelines regarding the care and use of animals for experimental procedures. The use of this spare tissue for the present study is also in accordance with the University of Alabama at Birmingham IACUC guidelines.

Brain tissue was obtained from rats deeply anesthetized using a mixture of ketamine and xylazine (5 mg ketamine + 1 mg xylazine/100 g body weight), and sequentially perfused with a saline solution (0.9% sodium chloride), and 4% paraformaldehyde in 0.1M phosphate

buffer (PB), pH 7.4. Brains were immediately removed from the skull, postfixed overnight in fixative.

2.2 Antibodies

2.2.1 Antibodies used in this study—To test our technique we used the following antibodies: 1) mouse monoclonal anti-tyrosine hydroxylase (Millipore, Billerica, MA, USA; MAB5280, diluted 1:500), in rat substantia nigra; 2) mouse monoclonal anti-tyrosine hydroxylase (Sigma-Aldrich, St Louis, MO, USA; T2928, diluted 1:80,000), in human substantia nigra; 3) rabbit polyclonal anti- μ -opioid receptor (Immunostar, Hudson, WI, USA; 24216, diluted 1:2000) in rat striatum. See Table 1.

2.2.2 Antibodies specificity—Detection of tyrosine hydroxylase in human brain tissue was achieved using a mouse monoclonal anti-TH manufactured by Sigma-Aldrich (clone TH-16) was used. This antibody was raised in mouse against purified rat tyrosine hydroxylase and recognizes an epitope in the N-terminal region between aminoacids 40–152 of human TH. For the detection of tyrosine hydroxylase in rat brain tissue, an anti-tyrosine hydroxylase antibody manufactured by Millipore (clone 2/40/15) was used. This antibody was raised in mouse against purified tyrosine hydroxylase from a rat pheochromocytoma, and its specificity is routinely assessed by western-blot by the manufacturer. The immunohistochemical detection of μ -opioid receptor in rat brain tissue was achieved using a polyclonal antibody raised in rabbit against a synthetic peptide corresponding to aminoacids 384–398 predicted from cloned rat μ -opioid receptor 1. The specificity of this antibody was determined by the manufacturer by immunolabeling of transfected cells, western-blot and immunoisolation studies.

2.3 Tissue processing

Fresh-frozen tissue (human substantia nigra), was collected directly on slides and stored at -80°C . Prior to immunohistochemistry the tissue was fan-dried for 45 minutes at room temperature (RT), fixed in 4% paraformaldehyde in PB for 30 minutes at RT, and immunohistochemistry was performed in a humid chamber. See Table 1.

To test how the storage method affected the results of our technique, fixed tissue (rat striatum and substantia nigra) was cryoprotected using 30% sucrose in PB, frozen in dry ice and sectioned on a cryostat. Sections were collected free-floating in PB and then, two different procedures were followed. **1)** On-slide immunohistochemistry: sections were immediately mounted on superfrost slides, allowed to dry for 30 minutes at RT and stored at -20°C until use. In this case, immunohistochemistry was performed on slides in a humid chamber. **2)** Free-floating immunohistochemistry: sections were stored at 4°C in PB with 0.02% sodium azide until use. These sections were processed for immunohistochemistry as free-floating, and mounted on-slides prior to develop the reaction. See Table 1.

2.4 Immunohistochemistry for film and light microscopy

In the present work, we developed an immunohistochemistry protocol to obtain densitometry data and light microscopy labeling from the same tissue sections. The immunohistochemical procedure was carried out in two different manners: On-slide immunohistochemistry (see **2.4.1**), or free-floating immunohistochemistry (see **2.4.2**). All the immunohistochemical experiments included negative controls consisting on the omission of the primary antibody, but following the same exact procedure in all the other steps.

2.4.1 On-slide immunohistochemistry—Prior to starting the procedure, fresh-frozen tissue (human) was fan-dried for 45 minutes, and fixed in 4% paraformaldehyde in PB for

30 minutes at RT. Fixed tissue (rat) was fan-dried for 30 minutes at RT. In all cases the slides were thoroughly rinsed in 0.01M phosphate buffered saline (PBS), pH 7.4 (4×5 minute), followed by blocking the endogenous peroxidase using 5% hydrogen peroxide diluted in PBS (30 minute at RT). After that, the slides were rinsed in PBS (4×5 minute) before preincubation with 10% normal serum [horse (S-2000) or rabbit (S-1000) serum, Vector Laboratories, Burlingame, CA, USA] in PBS containing 0.3% triton x-100 (PBS-T) for 1 hour at RT. The appropriate antibody dilution (see section 2.2) was prepared in a solution containing 3% normal serum in PBS-T, and allowed to incubate for 19 hours at RT. After that, the slides were rinsed in PBS (4×5 minute) and incubated for 45 minutes at RT with a biotinylated secondary antibody diluted 1:400 (horse anti-mouse, Vector Laboratories BA-2001; or goat-anti rabbit, Vector Laboratories, BA-1000) prepared in a solution containing 3% normal serum in PBS-T. An avidin-biotin-peroxidase (ABC) kit (Vector Laboratories, PK-6100) was used to prepare a 1:100 stock solution. Part of this solution was stored for later use, while the other part was further diluted to 1:2500 and immediately applied to the slides, allowing them to incubate for 45 minutes at RT. After that, the slides were rinsed in PBS (4×5 minute) and transferred to a darkroom. In the darkroom, the slides were placed between two transparent plastic films in a Kodak Biomax cassette (Kodak, Rochester, NY, USA), and incubated with Immun-Star HRP substrate kit (Bio-Rad, Hercules, CA, USA; 170-5041) for 5 minutes at RT, as per manufacturer instructions. Following this, Kodak Biomax XAR films (Kodak; 1660760) were exposed for varying times, ranging from 2 to 15 minutes, depending on the antigen to be detected. Films were developed using Kodak GBX developer and fixer (Kodak; 1900943 and 1901859, respectively).

Once films were obtained, the slides were carefully removed from the cassette and rinsed in PBS (4×5 minute) at RT. This was followed by reincubation with the remainder 1:100 ABC stock solution for 45 minutes at RT. After that, the slides were rinsed in PBS (4×5 minute) and developed for light microscopy using a 3-3' diaminobenzidine peroxidase substrate kit (Vector Laboratories; SK4100), for 2–10 minutes depending on the antigen to be detected. In some cases, the slides were counterstained using thionin or hematoxylin. Sections were dehydrated in a graded series of ethanol, cleared in xylene and coverslipped with Eukitt (Electron Microscope Sciences, Hatfield, PA, USA; 15322).

2.4.2 Free-floating immunohistochemistry—The incubation steps were identical to the on-slide immunohistochemistry (see section 2.4.1), with some differences in the incubation scheme. In this case, sections were incubated free-floating in wells through the first ABC incubation (dilution 1:2500). After that, sections were rinsed in PBS (4×5 minute) followed by two rinses (5 minute each) in 0.025M PB, mounted on superfrost slides, and fan-dried for 1 hour at RT. Then, sections were rehydrated in PBS, and developed for film as above (see section 2.4.1). The subsequent steps were identical to the on-slide procedure.

2.5 Film digitization and densitometry analysis

Films were digitized using a flatbed scanner at a resolution of 600dpi. Scans were imported into Image Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD, USA). The two-step calibration feature of the software was used to create a standard curve for optical density (OD) quantification, the region of interest (ROI) was selected, and OD data was obtained. In addition, light microscopy slides were also scanned for side-by-side comparison with the films.

2.6 Light microscopy and image acquisition

Slides were studied and photographed using a Nikon Eclipse 50i brightfield microscope (Nikon, Tokyo, Japan) equipped with a Nikon DS-Fi1 color digital camera. Photomontage and lettering were done using Corel Draw 12 software (Corel, Ottawa, Canada).

3. RESULTS AND DISCUSSION

In the present work, we describe a methodology that takes advantage of the principles of two well known techniques: film immunoblotting, which is routinely used to obtain optical density data for quantification of proteins; and light microscopy immunohistochemistry, which is used for the study of the distribution pattern of different markers, as well as for the study of morphological features and cell counting. To ensure that this combined methodology presented adequate accuracy and could be widely used in the neuroscience field, we tested it for the two commonly used methods of tissue incubation for immunohistochemistry, on-slide incubation using a humid chamber, and free-floating incubation in wells.

3.1 Performance in on-slide incubation

The on-slide incubation method produced good results in all our tests with tissue sections 14–16 μm thick. In the case of fixed rat tissue, sections for this method of incubation were first collected free-floating, and immediately mounted on slides. In the case of fresh-frozen tissue (human), sections were collected directly on slides. No differences in the performance of this technique were observed between the two types of tissue (fixed versus fresh-frozen), obtaining in both cases reliable and repeatable results (see Figures 1 and 2).

3.2 Performance in free-floating incubation and tissue thickness

Since free-floating immunohistochemistry is normally used in tissue sections of a wide range of thicknesses, we tested our technique in rat fixed tissue sections of thickness ranging from 16 to 50 μm (see Table 1). The free-floating technique used (see methods) produced similar results to the on-slide protocol in sections of 16 microns in thickness stained for TH (not shown). However, thicker tissue sections (20, 30 and 50 microns) presented increased problems to obtain a good film signal. On the other hand, after reincubation for DAB, the staining observed in these thicker sections was comparable to the staining obtained for thinner (16-micron thick sections), as well as to the staining obtained for the on-slide procedure. This indicates that this was not due to a failure of the immunohistochemical protocol, but rather a problem for obtaining optimal exposure in films for thicker sections.

In summary, these results (presented in 3.1 and 3.2) show that this dual method to quantify immunolabeling in film, and obtain light microscopy preparations, yields enough quality for morphological studies (e.g. cells counts, and mapping of protein distribution), works properly with different types of tissue preservation, and in different species. However, tissue thickness is a crucial parameter to obtain optimal results. We show here the results of testing this technique to detect tyrosine hydroxylase in the same brain area (substantia nigra/ventral tegmental area) of two different species (rodent and human). Representative images of the results obtained are shown in Figures 1 and 2. Tyrosine hydroxylase is expressed by the dopamine-producing neurons of the substantia nigra compacta (SNc) and the ventral tegmental area (VTA). Our experiments showed highly specific results, both in the rat and human tissue. In both cases, the anti-TH antibodies clearly delineated the SN/VTA complex on film (Figures 1A, 2A). Light microscopy images further showed the specificity of the immunolabeling (Figures 1B, 2B), in both cases showing that TH cells are only present in the SNc, while the SNr contained TH positive processes (Figures 1C, 2C). Morphological preservation was enough to distinguish clearly cell bodies, both in the fixed (rat) and fresh-

frozen (human) tissue, allowing the observation of cell bodies and processes (Figures 1C, 2C).

3.3 Performance of the technique in the detection of smaller structures

We further tested the capabilities of this dual detection technique by using an antibody against the μ -opioid receptor in rat striatal sections. The purpose of this test was to check the capability of this technique to obtain densitometry data from smaller structures within a region. The μ -opioid receptor is expressed in discrete areas called “striosomes” within the striatum, which makes this an ideal marker to test the resolution of the technique. In the representative data shown in Figure 3A, four striosomes of different sizes and morphology were selected and analyzed for different parameters. Our results showed that the film labeling for μ -opioid had enough resolution to obtain densitometry data from individual striosomes (Figure 3A). In addition, after reincubation for light microscopy the striatal sections showed the expected pattern of striosome distribution within the striatum (Figure 3B). The labeling was highly specific with μ -opioid labeling clearly delineating the darkly stained striosomes from the unlabeled matrix (Figure 3C).

4. CONCLUSIONS

Our tests demonstrate that this technique will be useful for a wide variety of tissue preservations, from frozen to fixed tissue, to obtain quantitative and morphological data from the same sample, combining the accuracy of optical density data acquisition in film, with obtaining histological slides from the same sample. We also show here that this technique has enough sensitivity not only for the study of large brain regions (e.g. the SN/VTA), but also to obtain data from small cytoarchitectonic compartments, such as the striosomes of the striatum.

It is worth to note that this combined methodology does not require any specialized equipment and for this reason will be readily usable in a wide variety of laboratories. In addition, any image processing/analysis software with the capability of measuring density/intensity can be used for the analysis of the films obtained. Finally, this technique has a potential use to obtain densitometry data on the expression of small molecules such as neurotransmitters (e.g. GABA, dopamine), that cannot be quantified by the use of western-blot, and currently require the use of specialized equipment (e.g. high performance liquid chromatography).

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References

- Blackbeard J, O'Dea KP, Wallace VCJ, Segerdahl A, Pheby T, Takata M, Field MJ, Rice ASC. Quantification of the rat spinal microglial response to peripheral nerve injury as revealed by immunohistochemical image analysis and flow cytometry. *J Neurosci Methods*. 2007; 164:207–17. [PubMed: 17553569]
- Blanchard V, Raisman-Vozari R, Savasta M, Hirsch E, Javoy-Agid F, Feuerstein C, Agid Y. Cellular quantification of tyrosine hydroxylase in the rat brain by immunautoradiography. *J Neurochem*. 1993; 61:617–26. [PubMed: 8101560]
- Dentresangle C, Le Cavorsin M, Savasta M, Leviel V. Increased extracellular DA and normal evoked DA release in the rat striatum after a partial lesion of the substantia nigra. *Brain Res*. 2001; 893:178–85. [PubMed: 11223005]

- Eastwood SL, Cotter D, Harrison PJ. Cerebellar synaptic protein expression in schizophrenia. *Neuroscience*. 2001; 105(1):219–29. [PubMed: 11483314]
- Eastwood SL, Harrison PJ. Decreased synaptophysin in the medial temporal lobe in schizophrenia demonstrated using immunoautoradiography. *Neuroscience*. 1995; 69(2):339–43. [PubMed: 8552231]
- Ermert L, Hocke C, Duncker HR, Seeger W, Ermert M. Comparison of different detection methods in quantitative microdensitometry. *Am J Pathol*. 2001; 158:407–17. [PubMed: 11159179]
- Loebke C, Sueltmann H, Schmidt C, Henjes F, Wiemann S, Poustka A, Korf U. Infrared-protein detection arrays for quantitative proteomics. *Proteomics*. 2007; 7:558–64. [PubMed: 17309101]
- Luongo de Matos L, Trufelli DC, Luongo de Matos MG, da Silva Pinhal MA. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark Insights*. 2010; 5:9–20. [PubMed: 20212918]
- Mausset-Bonnefont AL, de Seze R, Privat A. Immunohistochemistry as a tool for topographical semi-quantification of neurotransmitters in the brain. *Brai Res Brain Res Protoc*. 2003; 10:148–155.
- Pepin JL, Levy P, Garcin A, Feuerstein C, Savasta M. Effects of long-term hypoxia on tyrosine hydroxylase protein content in catecholaminergic rat brainstem areas: a quantitative autoradiographic study. *Brain Res*. 1996; 733:1–8. [PubMed: 8891241]
- Raisman-Vozari R, Hirsch E, Javoy-Agid F, Vassort C, Savasta M, Feuerstein C, Thibault J, Agid Y. Quantitative autoradiography of tyrosine hydroxylase immunoreactivity in the rat brain. *J Neurochem*. 1991; 57(4):1212–22. [PubMed: 1680158]
- Schmitt O, Preusse S, Haas SJP. Comparison of contrast, sensitivity and efficiency of signal amplified and nonamplified immunohistochemical reactions suitable for videomicroscopy-based quantification and neuroimaging. *Brain Res Brain Res Protoc*. 2004; 12:157–71. [PubMed: 15013467]
- Smith PD, McLean KJ, Murphy MA, Wilson Y, Murphy M, Turnley AM, Cook MJ. A brightness-area-product-based protocol for the quantitative assessment of antigen abundance in fluorescent immunohistochemistry. *Brain Res Brain Res Protoc*. 2005; 15(1):21–9. [PubMed: 15878147]
- Wang C, Wu C, Popescu DC, Zhu J, Macklin WB, Miller RH, Wang Y. Longitudinal near-infrared imaging of myelination. *J Neurosci*. 2011; 31(7):2382–90. [PubMed: 21325505]

- We show a combined methodology for protein quantification and morphology analysis
- We obtain films for optical density and slide immunolabeling from the same section
- Can be used in fresh-frozen and fixed brain tissue
- Allows optical density analysis of large and small brain regions
- Yields good quality for morphological analysis and cell counts.

Figure 1. TH immunolabeling in the substantia nigra of the rat

A) A screen capture of the densitometry analysis shows the film image of the section as well as the calibration curve, and the data obtained for the region of interest marked in the image (object 1). Data obtained in this case included area, density (mean, maximum, minimum and sum), and integrated optical density (IOD). **B–C)** The same section was photographed after development for light microscopy (see methods section 2.4.1). In **B** a scan of the whole section is shown for side-by-side comparison with the image obtained in film. This DAB developed image shows a strong specific staining for TH in the SN/VTA. Boxed area in **B** corresponds with the high magnification image shown in **C**, where the presence of strongly labeled TH positive neurons and processes are observed in the SNc (black arrows), while processes are observed in the SNr (arrowheads). This figure shows the versatility of this technique to obtain densitometry quantitative data, as well as high detail images of immunolabeling from the same section. Note the optimal preservation of the morphology of cells and processes in **C**.

ctx: cortex; hip: hippocampus; SNc: substantia nigra compacta; SNr: substantia nigra reticulata; SN/VTA: substantia nigra/ventral tegmental area.

Scale bars: 2 mm in **B**, 200 μ m in **C**

Figure 2. TH immunolabeling in the substantia nigra of the human

In these images, film data acquisition including calibration curve and densitometry data (A), are presented together with light microscopy images from the same section obtained after DAB development (B–C). **A)** Screen capture image of the film with the region of interest (object 1) analyzed for densitometry. Data obtained in this case included area, density (mean, maximum, minimum and sum), and integrated optical density (IOD) **B)** This low magnification photograph shows the scanned image obtained from the slide after developing with DAB, for comparison with the film image shown in A. The boxed area in B corresponds with the high magnification image shown in C. **C)** Despite of being fresh-frozen tissue, both, quantification data, and detailed images of neurons (arrows) and processes (arrowheads) can be obtained from the same sample.

SNc: substantia nigra compacta; SNr: substantia nigra reticulata; SN/VTA: substantia nigra/ventral tegmental area.

Scale bars: 5 mm in B, 500 μ m in C

Figure 3. μ -opioid receptor immunolabeling in the striatum of the rat

A) Screen capture image of the quantification of several striosomes stained for μ -opioid receptor. In this image, objects 1–4 represent different striosomes that were selected to obtain densitometry data and other parameters, such as the size of the striosome (area). Calibration curve and the data obtained are also shown in this image. Data obtained in this case included area, density (mean, maximum, minimum and sum), and integrated optical density (IOD) **B)** General view of the same section after developing with DAB showing the distribution of the μ -opioid immunoreactive patches. Boxed area in B corresponds with the high magnification image shown in C. **C)** Detail of an immunolabeled striosome (empty arrows) showing the specificity of the staining. μ -Opioid appears as a “patchy” expression in the rat striatum, with dark staining corresponding with the striosomes surrounded by the lightly stained or unlabeled matrix. These images show the capability of this technique to produce enough definition for detail quantification of small regions of interest such as the striosomes.

ctx: cortex; CPu: caudate-putamen.

Scale bars: 2 mm in B, 100 μ m in C

TABLE 1

Specimens and antibodies used in the study

SPECIMENS	BRAIN AREA	ANTIBODIES	METHOD	TISSUE THICKNESS
Human fresh-frozen	Substantia nigra	Tyrosine hydroxylase	On-slide	14 microns
Rat fixed	Striatum	μ -opioid receptor	On-slide	16 microns
Rat fixed	Substantia nigra	Tyrosine hydroxylase	On-slide	16 microns
Rat fixed	Striatum	Tyrosine hydroxylase	Free-floating	16 microns 20 microns 30 microns 50 microns