



Research article

Modification of AgNOR staining to reveal the nucleolus in thick sections specified for stereological and pathological assessments of brain tissue

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ABSTRACT

Background: Various stains have been devised to reveal degenerative or reactive cell phenotypes, or the disintegrative and/or neuropathic lesions associated with Alzheimer's, Parkinson's, and Pick's diseases, Down's syndrome, or chemical toxicity. Utilization of silver staining has allowed researchers to elucidate neural pathways promoting a greater understanding of the functional connections between brain regions. All of these methods employing silver can be characterized as 'directed staining technologies'.

New methods: The argyrophilic proteins (AgNOR) staining protocol was modified to stain nucleoli in thick sections prepared for stereological evaluation of brain tissue. Nucleoli appeared as black dots against a pale amber background. Tissue sections were counterstained with Toluidine Blue, or reduced-strength Tyrosine Hydroxylase immunohistochemistry to facilitate visualization of basic cellular morphology and regional nucleus identification. Here, we present a modified method for nucleolar staining in free-floating thick sections of brain embedded in a gelatin matrix. The modifications in our procedure include incubation in HCl to denature ('unravel') the DNA, a bleaching step to reduce non-specific background silver staining, and counterstaining with Toluidine Blue or reduced-strength tyrosine hydroxylase immunohistochemistry.

Comparison with old methods: Prior to the development of immunohistochemistry, silver staining was used primarily to identify pathological profiles and trace axon pathways; however, in many cases, a combination of silver staining and immunohistochemistry are required to fully visualize pathomorphology. The mechanism of these stains requires the binding of silver ions to cellular components and the subsequent reduction of the ions to metallic silver. Dilutions of TH primary antibody were evaluated to maximize identification of neurons and the nucleolus amongst the soma and processes present in the thick section. The use of stereology as a tool to estimate cell number has become increasingly prevalent in neuroscience experiments. As requirements for the preparation of experimental tissue have been refined, researchers have begun to use thicker sections, between 40 to 80 microns, to increase the number of optical planes available for analysis. These thick sections require modified staining protocols to assure complete penetration of stains throughout the tissue section.

Conclusions: This method is particularly useful in nucleolar identification for Stereology, and automated counting methods. Use of the nucleolus avoids some of the problems associated with use of the nucleus. The nucleolus is smaller than the nucleus and is less susceptible to transection during sectioning. It has a higher density than the nucleus and is easier to visualize. It is generally darker staining than the immunohistochemical reaction product that provides the identification marker for the cells to be counted. Examples of the method in several brain sections of the rat are shown, though the method has been also proven in other mammalian models.

1. Introduction

Silver stains have been developed and modified to identify cellular components of the nervous system including neurons, astrocytes, and

microglia, and subcellular structures including nucleic acids and proteins, in both normal and pathological states. Various stains have been devised to reveal degenerative or reactive cell phenotypes, or the disintegrative and/or neuropathic lesions associated with Traumatic Brain

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Injury, Alzheimer's, Parkinson's, and Pick's diseases, Down's syndrome, or chemical toxicity [1, 2, 3, 4, 5, 6]. Utilization of silver staining has allowed researchers to elucidate neural pathways promoting a greater understanding of the functional connections between brain regions. All of these methods employing silver can be characterized as 'directed staining technologies' [7]. Prior to the development of immunohistochemistry, silver staining was used primarily to identify pathological profiles and trace axon pathways; however, in many cases, a combination of silver staining and immunohistochemistry are required to fully visualize pathomorphology. The mechanism of these stains requires the binding of silver ions to cellular components and the subsequent reduction of the ions to metallic silver [8].

The nucleolus is the site of ribosome biosynthesis, and is involved in important aspects of chromosomal segregation and cell cycle control. The nucleolar organizing region (NOR) is a chromosomal region that contains ribosomal genes and associates with the nucleolus following nuclear division [9]. NORs contain acidic proteins that can be stained using silver methods. These argyrophilic proteins are referred to as "AgNORs". Several nucleolar proteins have been identified as components of the nucleolar particles [10]. Nucleolin, a nuclear phosphoprotein comprising up to 10% of nucleolar protein, is involved in ribosome biogenesis, ribosomal deoxyribonucleic acid (rDNA) transcription, ribosomal ribonucleic acid (rRNA) maturation, and in the maintenance of chromosome structure.

Stereology is a set of rigorous methods designed to estimate the number, size and shape of biological objects. The use of stereology as a tool to estimate cell number has become increasingly prevalent in neuroscience experiments. It requires the researcher to create sections and staining then choosing representative samples for quantification and morphometric analysis. The method presupposes well defined staining and tissue stain penetration [11]. As requirements for the preparation of experimental tissue have been refined, researchers have begun to use thicker sections, between 40 to 80 microns, to increase the number of optical planes available for analysis. These thick sections require modified staining protocols to assure complete penetration of stains throughout the tissue section [12, 13]. The use of automated counting methods require the optical integration and definition of "what is a cell?" This is accomplished by the stain marking on-off-on signal in the form of visual contrast, with the ability to separate neurons from other extracellular objects that may appear as neurons. The ability to stain the Nucleolus with a dark pigment, in addition to the typical histological staining, increases the hit rate for correct neurons by increasing the contrast of the on-off-on signal [14, 15]. In short, the AgNOR staining marks true neurons and defines them easier for the automation to pick them up, mitigating the error rate, and maximizing the accuracy of the Nucleolar Method for identifying cells, which is the Gold Standard for cell identification [16].

Pathological analysis of multiple types of cancers has revealed an elevation of AgNOR proteins as a consequence of aberrant cell duplication. The quantity of AgNORs in interphase cancer cells can be extrapolated to yield information on therapeutic efficacy, and the combination of AgNOR counts and histological information can be used to classify patients into low and high-risk groups for multiple types of cancers such as multiple myeloma, and pharyngeal and prostate cancers [17, 18, 19] AgNOR proteins have been implicated and used to identify microglia in humans with schizophrenia, and neurons in the raphe nucleus in humans with depression, and suicide [20, 21].

Here, we present a modified method for nucleolar staining in free-floating thick sections of brain embedded in a gelatin matrix. The modifications in our procedure include incubation in HCl to denature ('unravel') the DNA, a bleaching step to reduce non-specific background silver staining, and counterstaining with Toluidine Blue or reduced-strength tyrosine hydroxylase immunohistochemistry. Our modified protocol, was used to evaluate TH + neurons in substantia nigra, pars compacta, in an experimental rat model of Parkinson's disease [22].

2. Methods

The silver method for staining nucleolar organizer regions in cytological smears and fixed carcinoma tissue was adapted to stain thick, free-floating sections. The stain labels a subcellular organelle; therefore, some sections were lightly counterstained to visualize basic cellular morphology. Unless specified, all chemicals were purchased from Fisher (Fisher Scientific, Fairlawn, NJ).

2.1. Animal care statement

All experiments were performed in compliance with the NIH Guide for the Care and Use of Animals and were approved by the University of Kansas Medical Center Institutional Care and Use Committee.

2.2. Animals and husbandry

Male Long-Evans rats (90–100 days old) were bred in-house (breeding stock obtained from Harlan, Indianapolis, IN). Rats were housed in a temperature- and humidity-controlled animal facility with a 14:10-h light–dark cycle (on at 06:00 h) with ad libitum access to water and chow AIN-93G (Teklad, Indianapolis, IN). Rats were weighed and handled regularly. Litters were culled to 8 pups on postnatal day 1 and weaned on postnatal day 20. Rats were housed four per cage from weaning until lesion surgery, after which they were kept in single housing until euthanized to ensure undisturbed recovery [22].

2.3. Embedding and sectioning

Consistent with all the mammalian models, the rat brains were *immediately treated overnight with 20% glycerol and 2% dimethylsulfoxide* to prevent freeze-artifacts, and multiply embedded in a gelatin matrix using MultiBrain™ Technology. After curing, the block was rapidly frozen by immersion in isopentane chilled with crushed dry ice, and mounted on the freezing stage of an AO 860 sliding microtome (American Optical, Buffalo, NY). The MultiBrain™ block was sectioned in the coronal plane at 60 μ . Sections were collected sequentially into 24 containers filled with antigen preserve solution (50% Ethylene glycol, 49% PBS pH 7.0, 1% Polyvinyl Pyrrolidone). Sections not stained immediately were stored at -20 °C.

2.4. Modified silver nucleolar staining

Sections were rinsed three times for 3 min each in deionized water, and incubated in 1M HCl for 1 h at 37 °C to denature the nucleolar material. This reaction was arrested by incubation in sodium borate buffer, pH 8.5, for 10 min at room temperature (RT). Sections were rinsed three times for 3 min each in deionized water, and incubated in silver solution (see formula below) for 13 min at 37 °C. Sections were rinsed three times for 3 min each in deionized water, bleached in potassium ferricyanide/sodium borate solution (see formula below) for 1 min, and fixed in 1% sodium thiosulfate for 1 min.

2.5. Titration of silver nitrate quantity

The silver solution in the original procedure requires a large quantity of silver nitrate that makes the stain expensive. We titrated the silver nitrate concentration to determine whether a reduced quantity would provide both adequate staining and reduce the cost of the procedure. The titration was performed as percentages of the original quantity: 100%, 75%, 50%, 25%, and 10%.

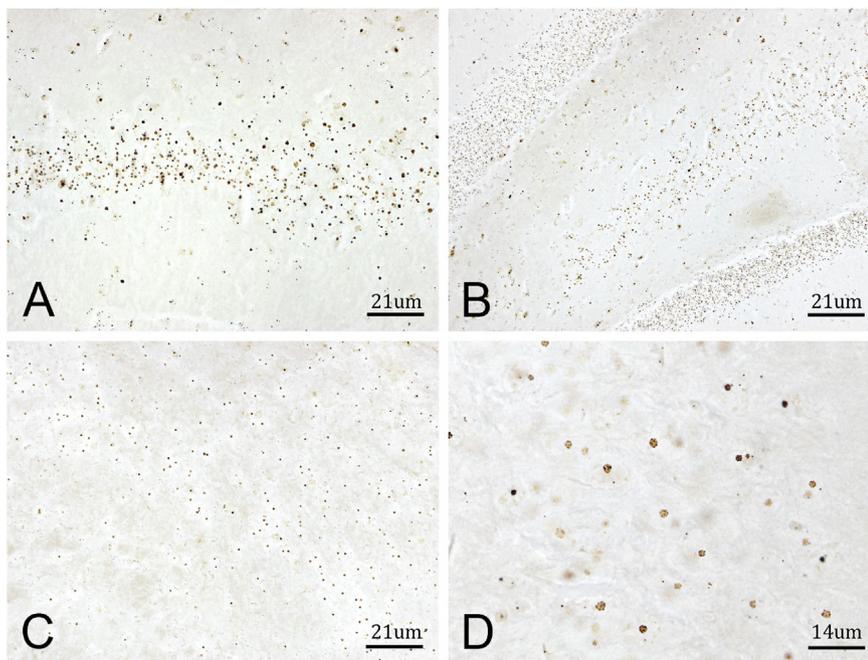


Figure 1. The transitional zone between CA1 and CA3 of rat hippocampus was easily observed due to the difference in nucleolar size between the regions (Figure 1A). Nucleoli in CA1 were, on average, 1.635 ± 0.055 microns in diameter. CA3 was marked by a greater width of the pyramidal layer and larger nucleoli, on average, 2.584 ± 0.069 microns. Within the dentate gyrus, nucleoli of the granule cells appeared to be the smallest observed, on average, 1.115 ± 0.046 microns (Figure 1B). In substantia nigra, pars compacta, one prominent nucleolus was observed per cell (Figure 1C). Measurement of nucleolar size revealed nigral nucleoli were 2.679 ± 0.070 microns in diameter. At high magnification nuclei appear amber in color (Figure 1D).

2.6. Toluidine Blue counterstain

Sections were silver stained as described above, mounted on subbed slides, and allowed to air-dry. Slides were rehydrated for 3 min in the following solutions: 95% EtOH; 95% EtOH + Formaldehyde; 95% EtOH; 70% EtOH; dH₂O X2; and incubated in 1% Toluidine Blue for 2 min at RT. Following counterstaining, sections were rinsed for 1 min in dH₂O, and 1 min in 70% EtOH. Slides were dehydrated for 3 min in the following solutions: 95% EtOH X2; 100% EtOH X2; EtOH/Xylene 1:1; 100% Xylene X2; and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

2.7. Reduced-strength tyrosine hydroxylase immunohistochemistry

To maximize antibody penetration, tissues were stained free-floating. Sections were rinsed three times for 5 min each. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 15 minutes. Following rinses, sections were permeabilized by treatment with 0.3% Triton X-100 for 30 min at RT. Sections were transferred directly to primary antibody and incubated overnight at RT. Following rinses, sections were incubated in biotinylated secondary antibody (1:238) for 1 h at RT. Sections were rinsed and incubated in ABC reagent (Vector Labs, Burlingame, CA) for 2 h at RT. To visualize the immunoreaction product, sections were treated with diaminobenzidine tetrahydrochloride + hydrogen peroxide (0.17g DAB + 50 µl 30% H₂O₂ in 1000 ml TBS, Sigma-Aldrich, St. Louis, MO) for 10 min at RT. Sections were rinsed, mounted on gelatinized (subbed) glass slides, air-dried, dehydrated in an ethanol series, cleared in xylene, and coverslipped with Permount.

A dilution series was performed on TH antibody concentrations to optimize visualization of immunoreactive neurons but prevent excessively dense staining that could occlude visualization of the nucleolus amongst the soma and fibers of the thick section. The dilutions of the primary antibody were 1:6,000, 1:12,000, 1:18,000, and 1:24,000 (Figure 4). The concentration of the secondary antibody was held constant at 1:238.

3. Measurement of nucleolar size

Images of various brain regions were captured using extended depth of field (EDF) at 40X. An image of a stage micrometer was captured at the

same resolution and used to calibrate the measurement scale in Photoshop CS5 (Adobe Systems, San Jose, CA). Regional images of nucleoli were opened in Photoshop, the ruler tool was used to measure nucleolar diameter of 25 random particles, and the results were recorded into Prism 5.0d (GraphPad Software, La Jolla, CA). In Prism, "Column Statistics" was used to calculate the mean and standard error of the mean.

Rinse solution:

25 mM Tris-buffered saline (TBS) with 0.3% Triton X-100 (TX).µ.

Silver Solution:

Part 1: 0.9 g gelatin, 450 µl Formic acid, 75 ml dH₂O.

Part 2: 49.5g AgNO₃, bring to 75 ml with dH₂O.

Add formic acid just prior to mixing, and incubate in a 37 °C water bath for 15 min.

Bleach Solution:

Stock: 10 g K₃Fe(CN)₆, 1.25 g Na₂B₄O₇, bring to 500 ml with distilled H₂O.

Working: Dilute stock solution 1:4 with distilled H₂O.

Toluidine Blue Solution:

Stock: 0.055 g Toluidine Blue O, 55 ml 1M Acetic acid. The solution is made fresh daily.

Working: 55 ml stock + 500 ml distilled H₂O. Filter through coarse paper. pH = 4.50–4.55.

4. Results

4.1. Characteristic AgNOR staining across rat brain regions

Silver staining of the nucleolus revealed a heterogeneous distribution of nucleolar architectonics' (sizes and shapes among the nucleoli) in different regions of rat brain. The transitional zone between CA1 and CA3 of hippocampus was easily observed due to the difference in nucleolar size between the regions (Figure 1A). Nucleoli in CA1 were, on average,

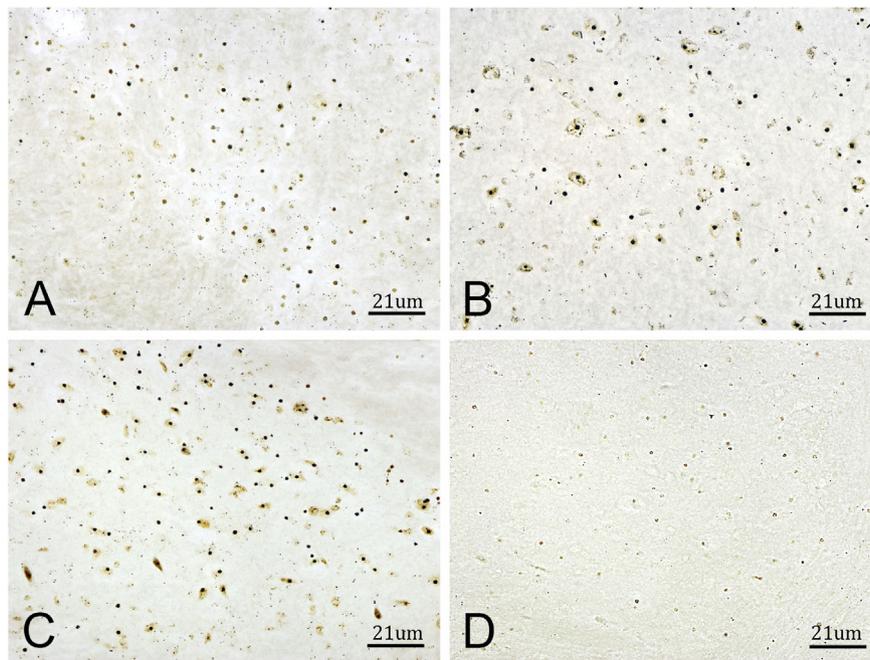


Figure 2. In the CA1, and CA3 of rat hippocampus, silver quantity was titrated to evaluate whether lower concentrations of silver nitrate produced adequate staining at a lower cost. Titrations were performed as a percentage of the original: 100% (Figure 2A), 75% (Figure 2B), 50% (Figure 2C), and 25% (Figure 2D, demonstrating a clear loss of integrity).

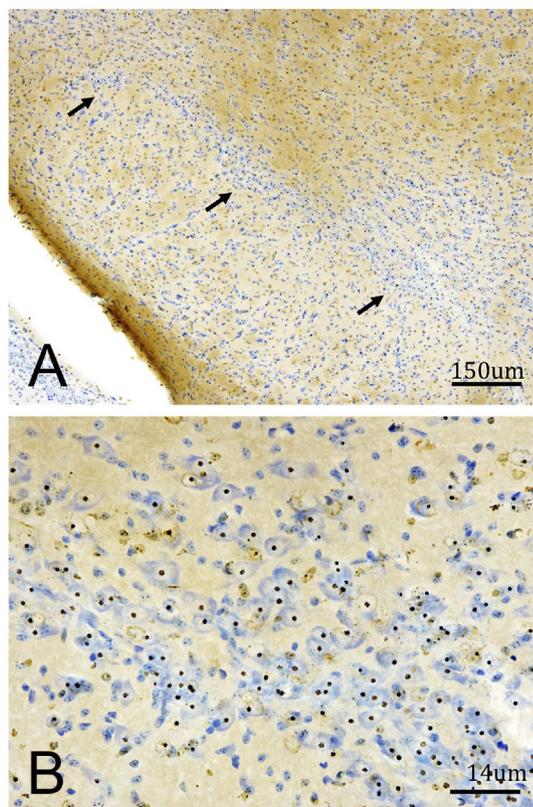


Figure 3. Toluidine blue was used successfully with AgNOR staining to visualize the boundaries of rat substantia nigra, pars compacta (Figure 3A) bordered superiolaterally by the Ventral Tegmental Area, superiolaterally by the Substantia Nigra Pars Lateralis, and transversoinferiorly by the Pars Reticulata. At higher magnification, neurons in substantia nigra, pars compacta are observed with a single nucleolus (Figure 3B). Arrows indicate the border of SNPC.

1.635 \pm 0.055 microns in diameter. CA3 was marked by a greater width of the pyramidal layer and larger nucleoli, on average, 2.584 \pm 0.069 microns. Within the dentate gyrus, nucleoli of the granule cells appeared to be the smallest observed, on average, 1.115 \pm 0.046 microns (Figure 1B). In substantia nigra, pars compacta, one prominent nucleolus was observed per cell (Figure 1C). Measurement of nucleolar size revealed nigral nucleoli were 2.679 \pm 0.070 microns in diameter. At high magnification nuclei appear amber in color (Figure 1D).

4.2. AgNOR staining - silver titration

The original AgNOR staining procedure called for a large quantity of silver nitrate that made the stain expensive to perform. We titrated the silver quantity to evaluate whether lower concentrations of silver nitrate produced adequate staining at a lower cost. Titrations were performed as a percentage of the original: 100% (Figure 2A), 75% (Figure 2B), 50% (Figure 2C), and 25% (Figure 2D). We determined the original concentration of silver nitrate could be reduced by 50% with no effect on staining integrity.

4.3. AgNOR staining - Toluidine Blue counterstain

The AgNOR stain imparts relatively little color to basic cell components; therefore, a counterstain was applied to allow determination of location. Delineation of the boundaries of the neuronal nucleus to be evaluated is critical for the accuracy of stereological studies. Toluidine blue was used successfully with AgNOR staining to visualize the boundaries of substantia nigra, pars compacta (Figure 3A). At higher magnification, neurons in substantia nigra, pars compacta are observed with a single nucleolus (Figure 3B).

4.4. AgNOR staining - tyrosine hydroxylase counterstain

The concentration of TH antibody routinely used in immunohistological studies (in striatum) produced staining that was too dense to distinctly identify neuronal cell bodies. Dilutions of TH antibody were evaluated over a four-fold range: 6,000 (Figure 4A); 12,000 (Figure 4B);

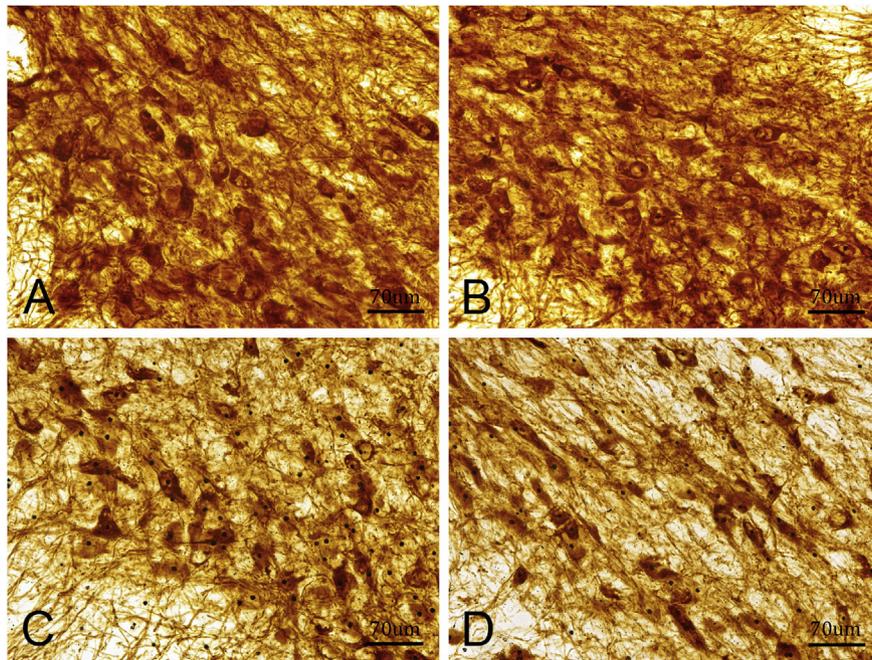


Figure 4. In the rat SNPC dilutions of TH antibody were evaluated over a four-fold range: 6,000 (Figure 4A); 12,000 (Figure 4B); 18,000 (Figure 4C); and 24,000 (Figure 4D) (Bar represents 70µ). Evaluation of the staining revealed the best combination of AgNOR/TH staining was using a dilution of 1:24,000.

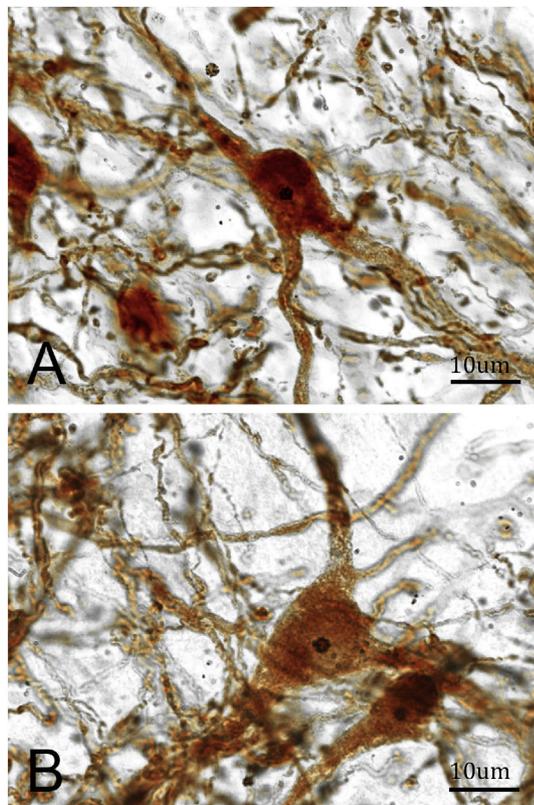


Figure 5. In the rat SNPC, a unique aspect of the stereology performed in this laboratory is the use of an additional 36X magnifier between the microscope and digital camera. This additional magnifier increases 100X images to 3600X (Bar represents 10µ) and allows a clearer visualization of the nucleolus (Figure 5A). In some cases, single neurons visualized at 100X magnification were unresolved multiple neurons when viewed at 3600X (Figure 5B) Resolution can be compared grossly in Figure 7.

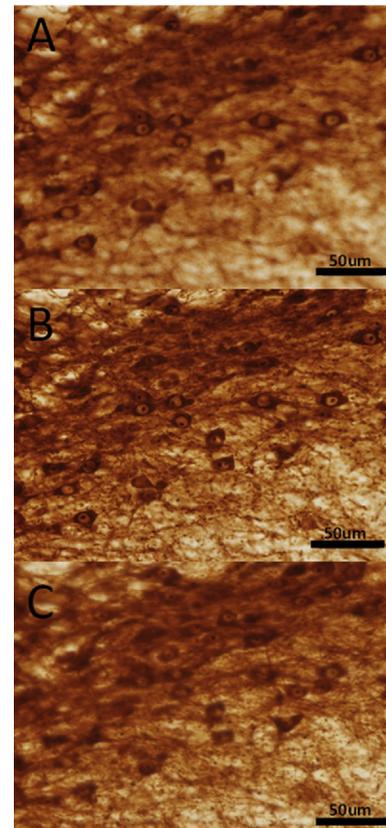


Figure 6. The tissue of the Rat SNPC at three different depths: A is the top of the tissue, or 0µ, B represents 17 µ through the stained tissue and C represents the bottom of the tissue, or 34µ through the tissue, as measured with a Heidenhain Linear Encoder, Schaumburg, IL., to demonstrate proper stain penetration of the tissue in thick sections (sectioned at 60µ and after dehydration and preparation, yield 34µ thickness).

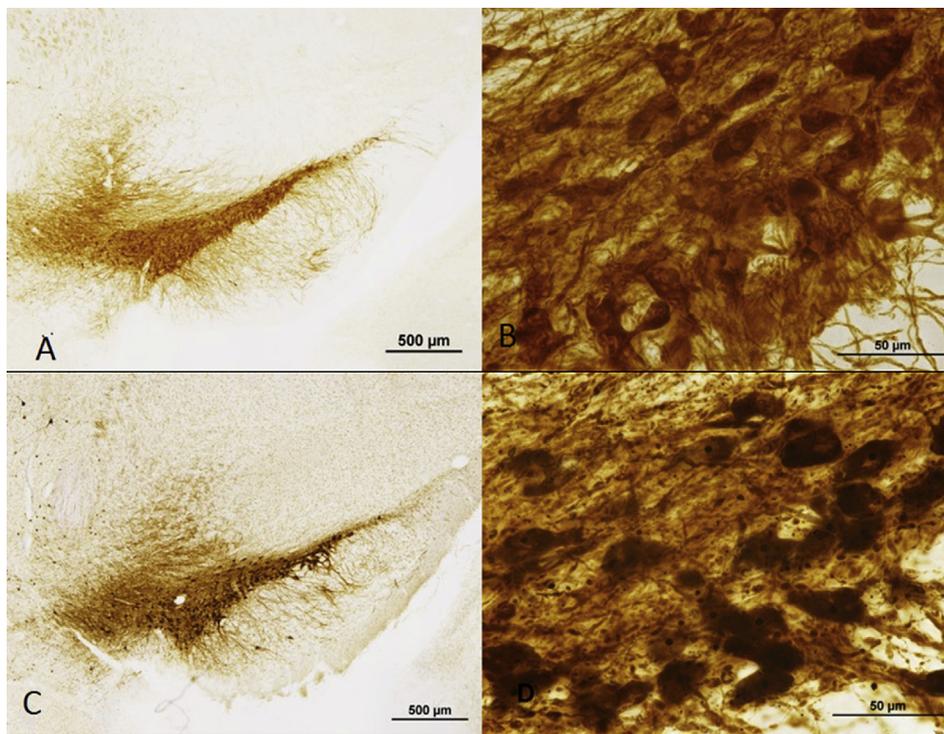


Figure 7. Figure 7A, 7B show Th stained sections and Figures 7C, 7D show the adjacent slides with AgNOR in the rat SNPC, demonstrating the potential for unresolved neuron identification.

18,000 (Figure 4C); and 24,000 (Figure 4D). Evaluation of the staining revealed the best combination of AgNOR/TH staining was using a dilution of 1:24,000.

4.5. Use of the AgNOR stain in a stereological assessment of TH + neurons

Brain sections stained with AgNOR/TH were evaluated by stereology to determine the number of TH immunoreactive neurons in a model of Parkinson's disease. A unique aspect of the stereology performed in this laboratory is the use of an additional 36X magnifier between the microscope and digital camera (Nikon E800 Eclipse microscope, IMI Tech color camera). This additional magnifier increases 100X images to 3600X and allows a clearer visualization of the nucleolus (Figure 5A). In some cases, single neurons visualized at 100X magnification were unresolved multiple neurons when viewed at 3600X (Figure 5B).

This modified staining protocol produced black nucleolar staining against a pale amber background (provided by the TH staining of the body of the soma, dendrites and axon). To aid in the visualization of basic cellular morphology, sections were counterstained with Toluidine Blue. This allowed identification of the regional neuronal nucleus, and specifically, the cellular neuronal nucleus that contained the silver-stained nucleolus.

To delineate the regional nucleus, substantia nigra, pars compacta, for evaluation in stereological studies, reduced-strength tyrosine hydroxylase immunohistochemistry was used as a counterstain (Figure 1). This facilitated identification of the boundaries of the regional nucleus; however, due to the density of neuronal cell bodies and processes, and the likelihood of a high-density of immunoreaction product when focusing through the thickness of the section during the stereological procedure, the intensity of the immunostaining was reduced. A dilution series of the primary antibody was evaluated from 1:6,000 through 1:24,000, and the highest dilution was selected as satisfactory to allow both delineation and identity, without obscuring cells in other focal planes. Figure 6 demonstrates the stain penetration through the thick sections at three levels (6A, top, 6B, middle, 6C bottom of Z axis). In combination with tyrosine hydroxylase (TH) staining, AgNOR has been

successfully used to streamline the stereological process in counting TH positive cells in the substantia nigra of Parkinson's Disease (PD) human tissue and animal models. By having the very dark and punctate nucleolar staining in combination with the diffuse cytoplasmic TH staining results are more accurate and achieved at a greatly accelerated pace.

5. Discussion

Historically, staining and quantification of AgNOR particles was hampered by non-standard protocols that produced differential results that could not be compared. The International Committee on AgNOR Quantitation established guidelines for staining to allow better comparisons of data across laboratories. The variance of cellular identification can cause large differences in the estimation of neuronal number across laboratories [23].

Pathological analysis of multiple types of cancers has revealed an elevation of AgNOR proteins as a consequence of aberrant cell duplication. The quantity of AgNORs in interphase cancer cells can be extrapolated to yield information on therapeutic efficacy, and the combination of AgNOR counts and histological information can be used to classify patients into low and high-risk groups for multiple types of cancers such as multiple myeloma, and pharyngeal and prostate cancers [17, 18] AgNOR proteins have been implicated in aberrant microglia in schizophrenia, and neurons in the raphe nucleus in humans with depression, and suicide [20, 21].

As stereological methods mature, there has been a shift in the 'counting unit' from the nucleus to the nucleolus to quantify cells that come into focus in the counting frame. Recent studies specify use of the nucleolus in the description of counting methods [22, 24]. Use of the nucleolus avoids some of the problems associated with use of the nucleus. The nucleolus is smaller than the nucleus and is less susceptible to transection during sectioning. It has a higher density than the nucleus and is easier to visualize. It is generally darker staining than the immunohistochemical reaction product that provides the identification marker for the cells to be counted [25]. With counting becoming ubiquitous in morphometry, ease of delineation has become paramount to cellular

identification. Identification of the Substantia Nigra Pars Compacta (SNPC) is essential in Parkinson's Disease research endpoints, and the AgNOR facilitates ease of nuclear identification and the Nucleator Method is enhanced by the increased definition of the nucleolus. Figure 7A, 6B show Th stained sections and Figures 7C, 7D show the adjacent slides with AgNOR. Automated counting can be seen as a significant advance in design-based stereology, as it will significantly increase throughput and allow for faster evaluation and larger sample sizes, but has limits in identifying neurons, which can be partially alleviated by the application of AgNOR, which can increase the detection rate of neurons. This is a result of more contrast, facilitating use of optical recognition from automated counting methods. These methods require counting rules that have high contrast to identify neurons from surrounding similarly appearing staining. AgNOR will greatly increase accuracy and speed in the process, both manual and automated.

Declarations

Author contribution statement

Ahmad, S.O.: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Baun, J.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tipton, B., Tate, Y.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Switzer III, R.C.: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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