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Lack of Compatibility of Histological Staining Methods With Proteomic Analysis of Laser-Capture Microdissected Brain Samples

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The anatomical complexity of the brain presents a challenge for the analysis of changes in gene and protein expression. Laser-capture microdissection (LCM) is a technique that is precise enough to dissect single cells within a tissue section. Protein expression in tissues obtained by LCM has been studied by Western blot and two-dimensional (2D) gel electrophoresis. However, it is not known whether histological staining techniques interfere with protein recovery and resolution on 2D gels. The goal of this study was to determine the effects of staining procedures on protein extraction and separation. LCM samples of rat brain striatum obtained after histological staining were compared with unstained, LCM-captured tissue and fresh-frozen, unstained, manually dissected samples. Specimens subsequently underwent protein extraction and 2D gel electrophoresis under identical conditions. Our results indicated that histological staining of the tissue greatly reduced protein recovery from LCM-captured samples. However, fixation and LCM without histological staining did not significantly affect protein recovery from brain tissue. These results indicate that LCM of fixed, unstained brain tissue could be used to dissect discrete brain regions for proteomic analysis. Histological staining of neural tissue should be avoided, because it interferes with protein recovery. LCM appears to be a promising tool for the study of localized protein changes underlying brain plasticity. (J Biomol Tech 2002;13:258–264)

KEY WORDS: histological staining, laser-capture microdissection, protein recovery, neural tissue, 2D gel electrophoresis.

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The anatomical complexity of the central nervous system makes characterization of biochemical changes in small regions very difficult. Powerful and precise techniques are needed to study protein changes in specific cell groups. Laser-capture microdissection (LCM) is a new technique that is precise enough to dissect single cells within a tissue section.[1,2] LCM has already been successfully applied to the study of messenger RNA (mRNA) expression in tissues.[3–5] In many cases, however, global analysis of protein expression levels may be preferable. Recent studies have demonstrated a lack of correlation between mRNA and protein levels under some circumstances.[6,7] Protein expression in tissues obtained by LCM has been studied by Western blot[8] and two-dimensional (2D) gel electrophoresis,[9–11] but the technique has never been applied to the study of neural tissue. Histological staining of the tissue sections is generally used to guide the LCM process. However, recent findings have suggested that some staining methods may have detrimental effects on subsequent protein separation techniques.[12,13]

The goal of this study was to test the effect of conventional neural staining methods (cresyl violet, hematoxylin–eosin [H&E], and toluidine blue) on protein recovery using a broad-based cellular extraction technique coupled to 2D electrophoresis-based proteomic analysis of brain tissue. In addition, we tried two stains not conventionally used in neural tissue: chlorazol black E and Sudan black B. Chlorazol black E was chosen because it is an anionic dye, whereas cresyl violet, hematoxylin, and toluidine blue are

cationic. Therefore, chlorazol black should interact with a different subset of tissue proteins. Sudan black B was chosen because it is a neutral lipid dye and should interact only minimally with proteins.

MATERIALS AND METHODS

Materials

Complete protease inhibitor cocktail was purchased from Roche (Indianapolis, IN); immobilized pH gradient (IPG) strips and 6% to 15% GelChips came from Proteome Systems, Inc. (Woburn, MA); urea and Tris were from Fisher Scientific (Pittsburgh, PA); and SYPRO ruby was obtained from Bio-Rad (Hercules, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Animals

Male Sprague-Dawley rats were housed in groups of three in cages on a 12-h light/dark cycle with ad libitum access to food and water. They were sacrificed by halothane overdose. Brains were then quickly dissected and snap-frozen in isopentane at −30°C and stored at −80°C until use. Sections (8 μm) were cut in a Leica (Bannockburn, IL) CM 1850 cryostat at −16°C, mounted on polylysine-coated glass slides, and stored at −80°C until used.

Tissue Staining and Dissection

The brain region known as the striatum (caudate/putamen)[14] was used for study. Unfixed, unstained control caudate/putamen tissue was manually dissected inside the cryostat using a #11 scalpel blade. Adjacent sections were fixed by immersion in 70% ethanol for 30 s. Five different staining protocols were tested. Nissl staining consisted of immersion in 0.05% w/v cresyl violet (1 min) followed by two rinses in water. H&E staining consisted of sequential immersions in Mayer's hematoxylin (30 s), water (2 × 10 s), and 0.05% w/v eosin (5 s), followed by two rinses in water. Sudan black B staining consisted of immersion in 0.3% w/v Sudan black B in 70% ethanol (10 min) followed by a rinse in 70% ethanol. In all cases, sections were then dehydrated through graded ethanols (70%, 95%, 100%; 15 s each), cleared in xylene (1 min), and air-dried. Toluidine blue staining was performed by immersion of slides in 0.5% w/v toluidine blue O in acetate buffer, pH 3.8 (10 s), followed by a 100% ethanol rinse. Chlorazol black E staining consisted of immersion in 1% w/v chlorazol black E in 70% ethanol (2 min) followed by two 95% ethanol rinses. In both cases, sections were then dehydrated in 100% ethanol (2 × 15 s), cleared in xylene (1 min), and air-dried. Protease and phosphatase inhibitors (Complete protease inhibitor cocktail and 0.1 M Na orthovanadate) were included in all aqueous solutions. Fixed and stained sections were then microdissected at room temperature using a PixCell II (Arcturus Engineering Inc., Mountain View, CA) laser-capture microdissector. Standard laser parameters were spot size, 30 μm; power, 40 mW; pulse duration, 5 ms; time between pulses, 400 ms. In all cases, the complete caudate/putamen was captured for subsequent processing.

Sample Preparation and 2D Gel Electrophoresis

Dissected tissues (four to six striatal sections, 25 μg total protein) were solubilized in 150 μL of ProteoPrep cellular and organelle membrane extraction reagent (Sigma). Samples were reduced for 1 h in 5 mM tributyl phosphine and then alkylated for 1.5 h using 15 mM iodoacetamide. After centrifugation at 21,000 × *g* for 5 min, all insoluble material was discarded. Protein concentration was determined using the Bradford protein assay. Samples were stored at −20°C before isoelectric focusing (IEF). IPG strips (11 cm, pH 3–10; Proteome Systems, Inc.) were rehydrated with 25 μg of protein in 180 μL of protein sample solution for 8 h at room temperature. The rehydrated strips were focused on the Proteome Systems IEF cell. The voltage was set at 500 V for 3 h, 1000 V for 2 h, and then 6000 V for 14 h. Focused IPG strips were equilibrated in sodium dodecyl sulfate (SDS) equilibration buffer containing 3 M urea, 2.5% (w/v) SDS, and 50 mM Tris/acetate buffer, pH 7.0, for 20 min. The equilibrated strips were then loaded onto 1-mm 6% to 15% SDS–polyacrylamide gels (GelChips; Proteome Systems, Inc.) for further separation in the second dimension. Conditions for the second dimension were 25 mA/gel for 30 min and then 40 mA/gel until the bromophenol blue dye front reached the end of the gel.

Gel Staining and Imaging

Gels were fixed for 30 min in a solution containing 10% methanol and 7% acetic acid. After fixation, gels were stained in 50 mL of SYPRO ruby overnight in the dark. Before scanning, gels were washed in 10% methanol and 7% acetic acid. The gels were scanned in an Alpha Innotech (San Leandro, CA) MultiImage light cabinet using ChemiImager software. Gels were analyzed using the ImagepIQ image analysis program (Proteome Systems, Ltd., Sydney, Australia). Spot numbers

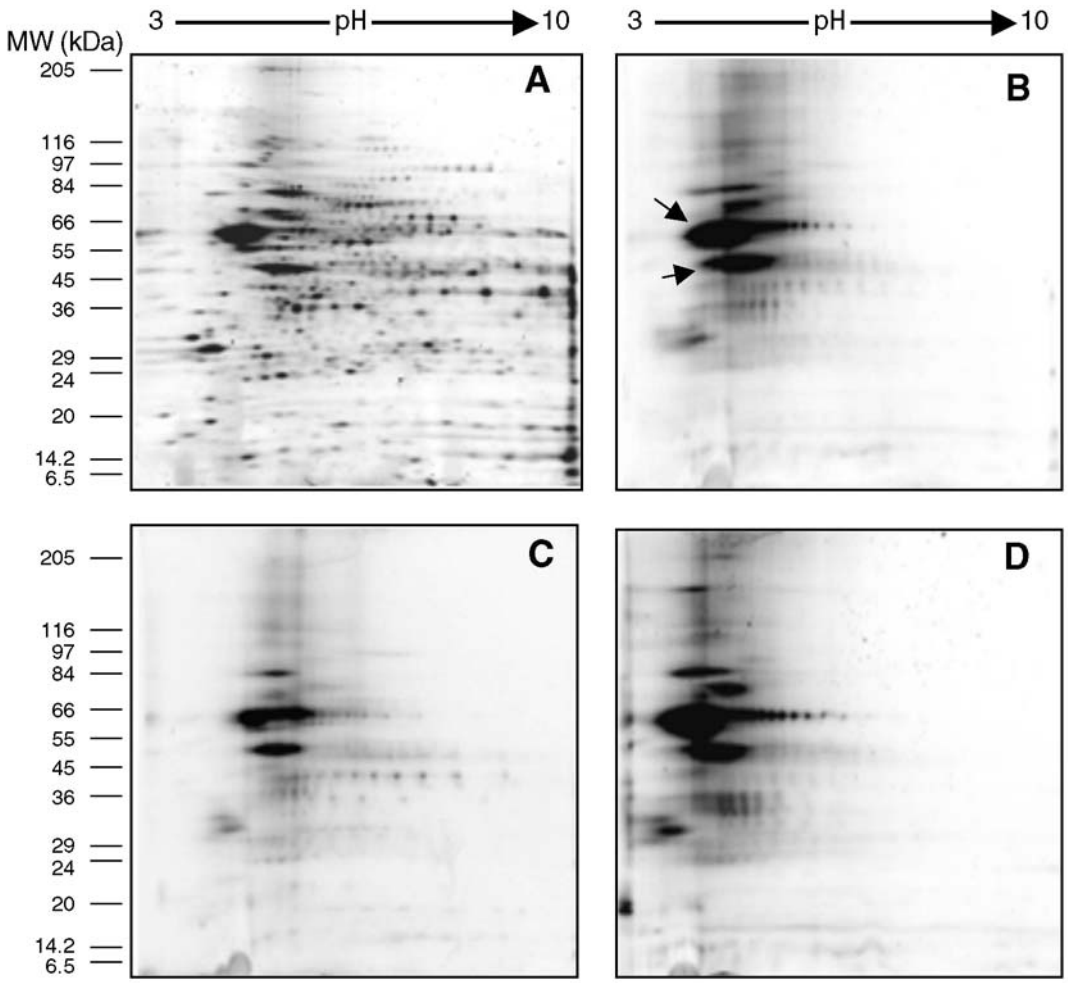

FIGURE 1

The effect of histological staining on 2D gel-based protein separation. Samples were processed and proteins extracted as described in the text; 25 μg of total protein was then loaded onto IPG strips and separated by 2D gel electrophoresis as described. **A:** Unfixed, unstained, manually dissected sample. **B:** Nissl-stained LCM sample. Arrows indicate major protein aggregates. **C:** H&E-stained LCM sample. **D:** Toluidine blue-stained LCM sample. Gels are representative of three independent experiments.

were compared using one-way analysis of variance, with $p < 0.05$ required for statistical significance.

RESULTS AND DISCUSSION

Chlorazol black E staining did not permit satisfactory anatomical resolution because the staining was too dark. Sudan black B staining turned out to be too light. Modifications of dye dilution or staining time did not produce any improvement in these results. Therefore, neither stain was tested further.

For the three other stains (H&E, cresyl violet, toluidine blue), protein expression patterns of stained and laser-captured caudate/putamen cells were compared with those obtained from fresh frozen unfixed and unstained sections. Staining protocols were optimized in an attempt to minimize the interaction of the various dyes with proteins while enabling satisfactory staining. A 10-fold dilution of the standard concentration of both cresyl violet and eosin stains was used. Dye dilution has been suggested by other investigators as a way of limiting detrimental effects of staining on protein extraction.[13] We used a pH 3.8 buffer for toluidine blue because toluidine blue has been reported to bind preferentially to nucleic acids rather than protein when prepared and used at a pH less than 4.0.[15] As shown in Figure 1, cresyl violet, H&E, and toluidine blue staining of tissues caused a significant decrease in the number of protein spots observed in

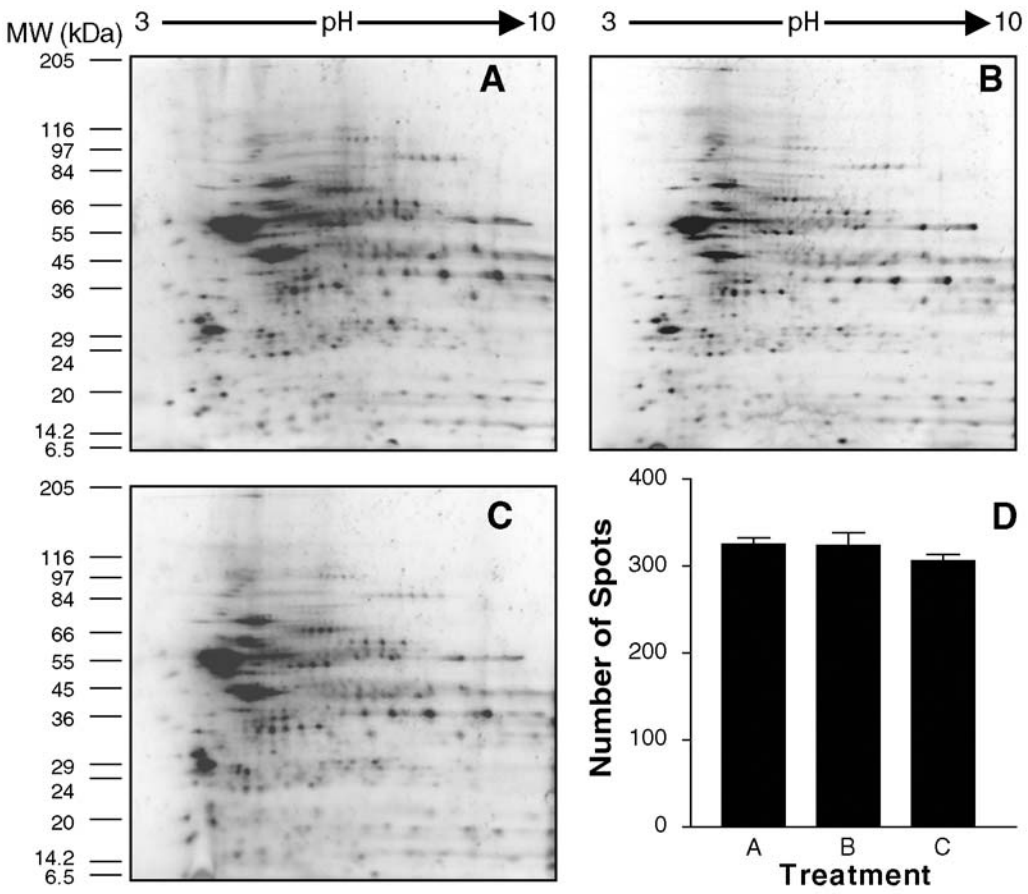

FIGURE 2

The effect of tissue fixation and LCM on 2D gel-based protein separation. Samples were processed and proteins extracted as described in the text; 25 μg of total protein was then loaded onto IPG strips and separated by 2D gel electrophoresis as described. **A:** Unfixed, unstained, manually dissected sample. **B:** Ethanol-fixed, manually dissected sample. **C:** Ethanol-fixed LCM sample. **D:** Number of spots detected on each gel. Results are expressed as mean ± standard error of the mean for three independent experiments. No statistically significant difference was observed between any of the treatment groups.

2D gels from stained tissues as opposed to control samples. In the case of cresyl violet and toluidine blue, proteins appear to be aggregated, with most of the staining being concentrated in two major spots (Fig. 1B, arrows).

To determine whether these detrimental effects were due to the histological staining procedures or to tissue fixation and LCM, 2D gels obtained from unfixed, manually dissected tissue were compared with gels of proteins extracted from fixed, manually dissected tissue, and fixed, unstained, laser-captured material. Figure 2 shows that fixation as recommended for the LCM protocol[1] (30 s in 70% ethanol) had a minimal effect on protein extraction and recovery (Fig. 2A, B). Similarly, LCM did not significantly alter protein recovery (Fig. 2C). There was no significant difference between the number of spots detected on each set of gels (Fig. 2D). On the gels obtained from unfixed, manually detected samples, 325 ± 7 distinct spots where detected, versus 323 ± 15 spots for the fixed, manually dissected samples and 306 ± 8 spots for the fixed, laser-captured ones. Thus, the decrease in protein spots observed in tissue derived from stained specimens can be attributed to the histological staining procedure itself.

In a recent paper, Craven et al. also described detrimental effects of hematoxylin–eosin and toluidine blue staining of kidney samples on protein recovery on 2D gels.[13] They described changes in the relative abundance of various proteins and a detrimental effect on protein focusing following both stains, but the effects observed by those investigators were less dramatic than the effects observed in the present study. However, it should be noted that they used less intense

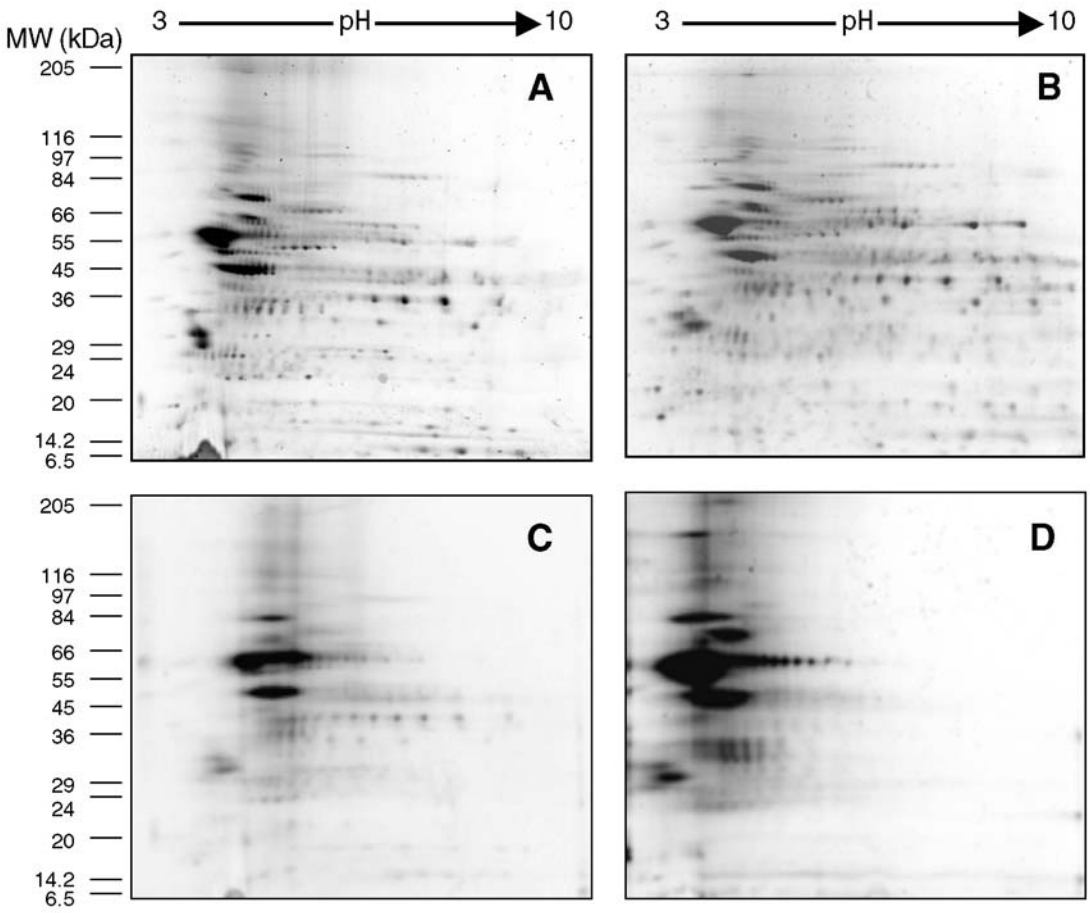

FIGURE 3

The effect of reducing histological staining intensity on 2D gel-based protein separation. Samples were processed and proteins extracted as described in the text; 25 μg of total protein was then loaded onto IPG strips and separated by 2D gel electrophoresis as described. **A:** H&E-stained sample, staining protocols used by Craven et al.[13] **B:** Toluidine blue-stained sample, staining protocols used by Craven et al.[13] **C:** H&E-stained sample, protocol described herein. **D:** Toluidine blue-stained sample, protocol described herein. Gels are representative of three independent experiments.

staining protocols than the ones used in our study, with longer stain differentiation and lower stain concentration for H&E and toluidine blue, respectively. To further investigate this discrepancy, H&E and toluidine blue staining protocols used by Craven et al. were tested on brain tissue sections. H&E stain was differentiated for a longer time as reported by Craven et al. (70% ethanol for 30 s, 100% ethanol for 1 min, xylene for 2 × 5 min, instead of 70%, 95%, 100% ethanol for 15 s each, xylene for 1 min). Toluidine blue was used at a 0.025% w/v dilution for 5 s by Craven et al., instead of 0.5% for 10 s. These protocol modifications enabled a good protein separation on 2D gels (Fig. 3A, B versus 3C, D). However, the staining intensity obtained with these protocols was no longer sufficient to allow visualization of the stain on an uncoverslipped slide under the LCM microscope. Figure 4 shows that, under the LCM microscope, sections stained using the protocols of Craven et al. (Fig. 4B, D) are similar to unstained sections (Fig. 4A), whereas staining is clearly visible when our more intense staining conditions are used (Fig. 4C, E). Thus, it appears that brain tissue requires more intense staining than kidney to provide stain visualization sufficient to effectively guide LCM. These staining requirements dramatically interfere with protein recovery on 2D gels.

CONCLUSIONS

In this study, we investigated the effect of histological staining followed by LCM of brain tissue on the 2D gel

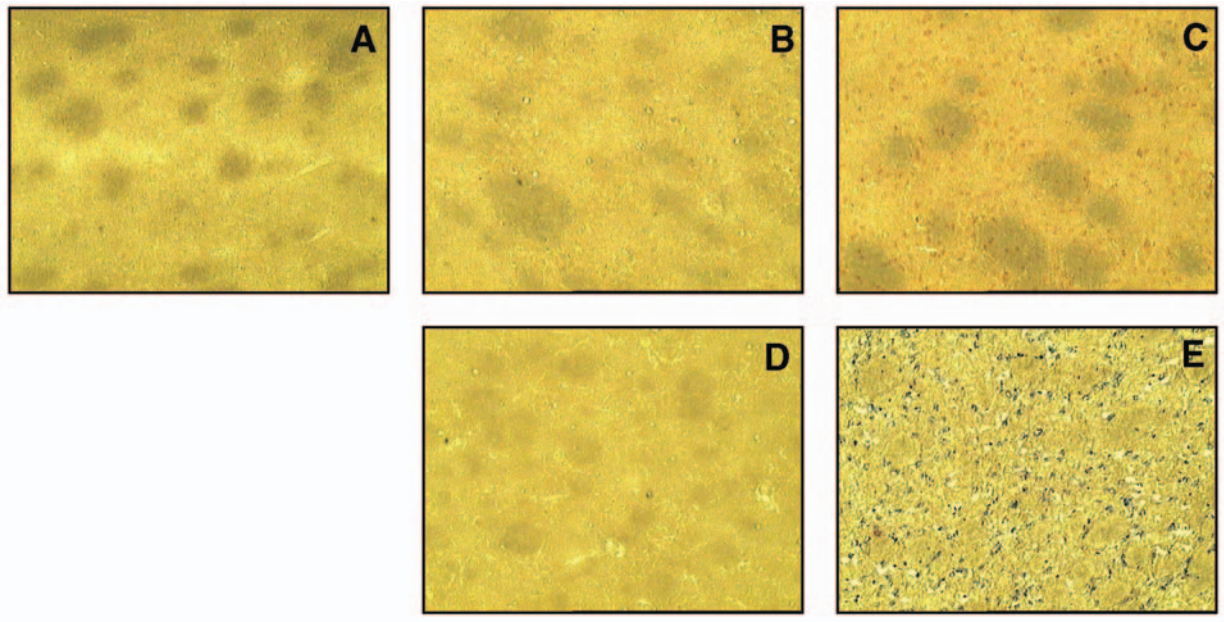

FIGURE 4

The effect of reducing histological staining intensity on anatomical resolution under the LCM microscope. Samples were processed as described in the text and visualized under the LCM microscope (200× magnification). **A:** Unstained section. **B:** H&E-stained section, low-intensity protocol. **C:** H&E-stained section, high-intensity protocol. **D:** Toluidine blue-stained section, low-intensity protocol. **E:** Toluidine blue-stained section, high-intensity protocol. Low-intensity protocols result in gels shown in Figure 3A and B, whereas high-intensity protocols result in gels shown in Figure 3C and D.

electrophoresis-based separation of proteins. Our results showed that histological staining methods optimized for efficient visualization under the LCM microscope greatly reduce protein recovery and are thus not suitable for 2D gel-based proteomic analyses of brain tissue. However, histological staining may still be useful to guide LCM for other tissues in which less intense stains can be visualized. Moreover, our results indicated that LCM without histological staining may be a feasible approach for the study of localized protein changes underlying brain plasticity. Alternatives to histological staining, such as navigated LCM,[16] in which an adjacent stained section is used to guide the dissection of the unstained specimen, may prove feasible for these types of studies.

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