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An investigation of the problem of two-layered immunohistochemical staining in paraformaldehyde fixed sections

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Abstract

In sections of paraformaldehyde fixed brain tissue, stained using immunohistochemical methods, the distribution of staining within the sections is not uniform. Whilst stained cells are seen at the top and bottom surfaces, the central thicknesses of the sections contain little or no immunoreactivity. This presents a major problem for quantification, as each section contains a population of cells that is not visualized by the staining method. Following extensive investigation of this phenomenon, we report that the failure of full thickness, immunohistochemical staining is not a failure of the immunohistochemical methodology *per se*, nor is it related directly to the thickness of the sections used. Rather, the problem lies in the chemistry of the tissue itself, and originates during fixation of the tissues using paraformaldehyde-based perfusion methods, which render the cell membranes impermeable to one or more components of the staining protocol. We show that this impermeability is affected by addition of membrane-disrupting agents to the fixative, and by a reduction of exposure to paraformaldehyde during fixation. The present investigation contributes to the development of new fixation protocols, optimised for use in both immunohistochemical methods and morphometric analyses.
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Keywords: Fixation; Immunohistochemistry; Staining artefact; Two-layer staining; Paraformaldehyde

1. Introduction

We observe that in sections prepared from rat brain, using standard methods of fixation and processing, immunohistochemical staining does not produce staining through the full thickness of the section. Rather, a two-layered distribution of staining is seen, such that the upper and lower thicknesses of the section are stained, and the central thickness of the section contains little or no immunoreactivity. The visual appearance of this artefact is demonstrated in Fig. 1. Two-layered staining is observed using antibodies against a wide range of antigens, including tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), choline acetyl-transferase (ChAT), dopamine and cyclic AMP-regulated phosphoprotein, molecular weight 32 (DARPP32) (Hemmings and Greengard, 1986), neuronal nuclear antigen (NeuN) and β-galactosidase, and in sections of brain from other species such as mouse and marmoset. Two-layered staining is not observed using conventional histochemical methods such as cresyl fast violet, or hematoxylin and eosin, in which stained cells are observed throughout the section thickness. The protocols used in our laboratory for fixation, cutting and immunohistochemical staining of brain tissues are typical of those used in many laboratories working with animal models of neurodegeneration and examples of similar protocols are widespread in the literature (Choi-Lundberg et al., 1997; Deacon et al., 1998; Deglon and Aebischer, 2002; Karlsson et al., 2002; Kirik et al., 2000; Mandel et al., 1997; Palfi et al., 2002; Sortwell et al., 2001).

As a result of this artefact, quantitative analysis of cell numbers in brain sections is hampered by the presence of an unknown and variable portion of each section which is unstained. This is a serious problem for all methods of cell counting, which will inevitably underestimate the true number of cells in the section. It is a particular problem for stereological analysis using the optical dissector method, which relies on partitioning of the section thickness for random sampling in three dimensions. Other workers have attempted to apply stereological counting methods in such sections by treating the top and

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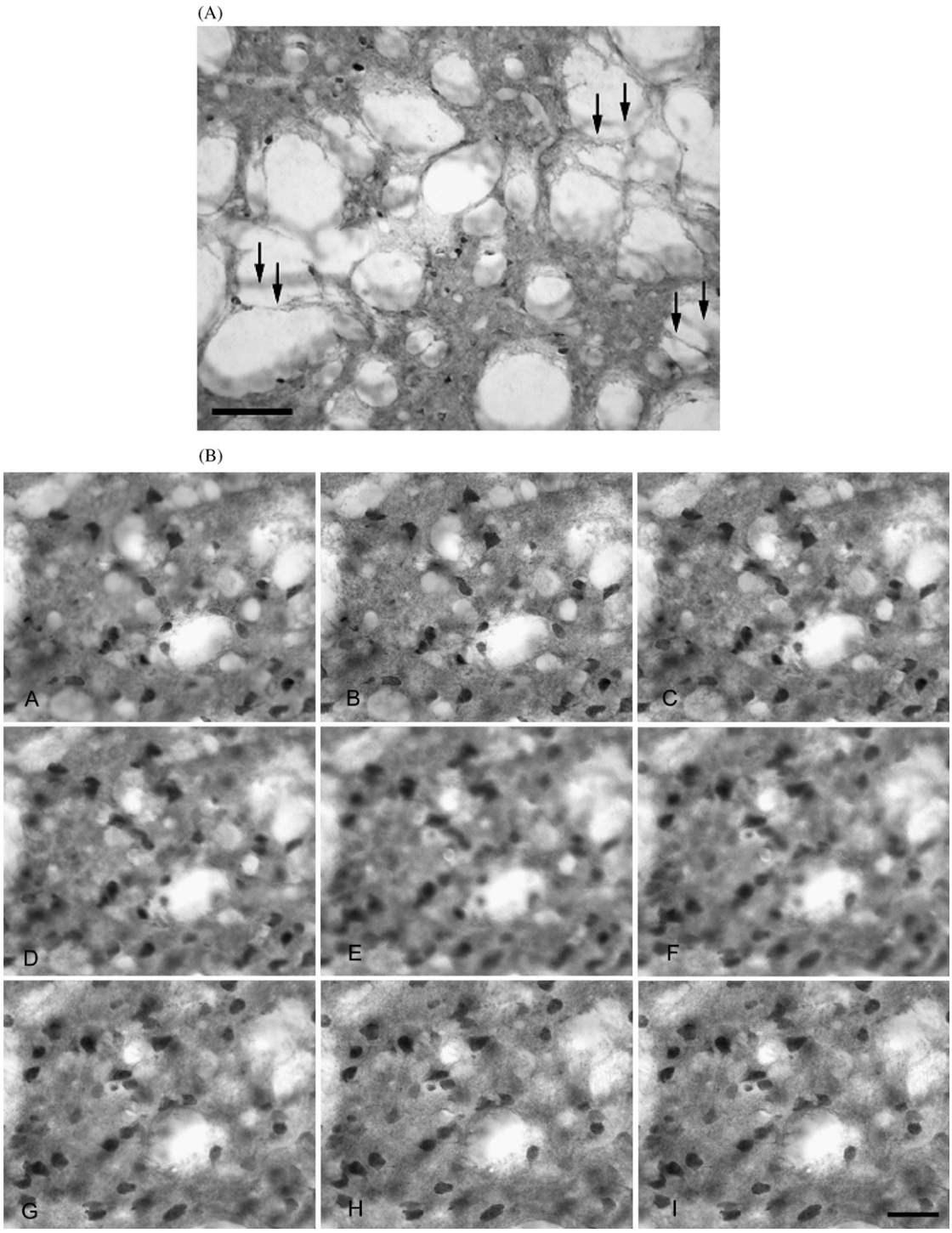

Fig. 1. (A) Photomicrograph of a 60 μm thick section of rat striatum stained immunohistochemically for DARPP32. The top surface of the section is well stained, showing DARPP32 cell bodies and their dendritic fields. The bottom surface of the section (out of the plane of focus) can also be seen. Where the unstained axon bundles pass through the section, the unstained middle thickness of the section can be seen as a gap between the top and bottom layers of staining. Arrows indicate areas of clear two-layered staining. Scale bar is 0.1 mm. (B) Micrographic sub-sectioning of a 40 μm section of rat striatum from experiment 1 stained for DARPP32. Each image represents a 3 μm depth of section and images are arranged sequentially from the top (A) to the bottom (I) of the section thickness. Note that whilst the top and bottom three levels of the section (A–C and G–I, respectively) contain stained cells, in the central three levels of the section (D–F) there are none, and only out of focus cells above and below the plane of focus are visible. Scale bar is 20 μm.

bottom stained portions of the section as two separate entities for counting purposes (Calhoun et al., 1996; Jinno et al., 1998; Ungerstedt and Arbuthnott, 1970; Van de Berg et al., 2003). However, the reason for the lack of full thickness staining is as yet unknown, and until the nature of the problem is fully investigated, it is not certain that accurate cell counts are possible in these sections, using even the most stringent approach. The present work investigates the causes of the fail-

ure of full thickness, immunohistochemical staining in thick sections.

2. Materials and methods

2.1. Animals

Adult, female Sprague-Dawley rats were used for all experiments (Harlan OLAC, Bicester, Oxon, UK). All rats weighed 200–250 g at the time of first surgery. Animals were housed under standard conditions with free access to food and water. All experiments were conducted in accordance with requirements of the UK Animal (Scientific Procedures) Act, 1986. Some of the experimental data were generated using animals from other experiments either published or in press (Torres et al., 2005, 2006).

2.2. Fixation and histology

Rats were terminally anaesthetized by intraperitoneal injection of 1 mg/kg sodium pentobarbitone, then perfused transcardially with 100 ml of phosphate buffered saline (PBS) at pH 7.4, followed by 250 ml of 4% paraformaldehyde in PBS delivered over 5 min. The brains were removed from the skull and post-fixed by immersion in the same fixative solution for 4 h, then transferred to 25% sucrose in PBS. After equilibration in the sucrose solution, coronal sections were cut on a freezing stage, sledge microtome at a thickness of 60 μm and stored in 0.1 M, Tris-buffered saline, pH 7.4 (TBS) at +4 °C prior to staining. All stains were carried out on a one in six series of sections.

A series of sections from each brain was stained using the general neuronal stain cresyl fast violet as follows. Sections were mounted onto gelatine coated microscope slides and allowed to dry at room temperature overnight. Slides were then dehydrated in an ascending series of alcohols: (70, 95, and 100% ethanol), then immersed for 30 min in a 50/50 (v/v) mixture of chloroform and ethanol. Slides were re-equilibrated to water via 5 min immersion (with agitation) in 95%, and 70% ethanol then distilled water. Staining was carried out by 5 min immersion in cresyl fast violet solution (5% in 0.1 M sodium acetate buffer, pH 3.5). Differentiation of the stain and dehydration was carried out in an ascending series of alcohols (70, 95, and 100% ethanol), after which section were cleared in xylene, and cover-slipped using DPX mounting medium.

Immunohistochemistry was carried out on free-floating sections. Within each experiment, all sections were stained at the same time, using the same solutions of antibodies and ensuring that incubation times and washes were the same for each brain. The standard protocol is as follows: sections were thoroughly washed in Tris-buffered saline (TBS). Endogenous peroxidase enzyme activity was quenched using a 10 min immersion in 3% hydrogen peroxide/10% methanol in distilled water, followed by washing and re-equilibration in TBS. After a 1 h pre-incubation in a solution of 3% normal goat serum/0.1% Triton X-100 in TBS, sections were incubated in primary antiserum (see below) in 1% normal goat serum/0.1% Triton X-100 for 60 h at +4 °C. After thorough washing, a biotinylated, the secondary antibody, in 1% normal goat serum in TBS was applied for 3 h. The sections were then washed for 30 min before application of 10% strepavidin–biotin–horseradish peroxidase solution (Dako) in TBS for 90 min, followed by thorough washing and equilibration to 0.05 M Tris non-saline solution at pH 7.4. The horseradish peroxidase label was revealed by 10 min incubation in a 0.5% solution of diaminobenzidine tetrahydrochloride (Sigma chemicals, UK) in Tris non-saline containing 0.3 μl/ml of hydrogen peroxide. Sections were finally mounted on gelatine-coated microscope slides dehydrated in an ascending series of alcohols, cleared, and cover-slipped using DPX mountant.

The primary (monoclonal) antibodies and dilutions used were: β-galactosidase (Promega), 1:6000; NeuN (Chemicon), 1:5000; tyrosine hydroxylase (Chemicon), 1:2000; DARPP32 (the kind gift of Professors Hemmings and Greengard, Cornell University), 1:30,000. Each batch of immunohistochemical staining included positive control tissue, and a negative control sample in which the primary antibody was omitted. Secondary anti-mouse antibody (Dako) was applied at a concentration of 1:200.

2.3. Morphometry

Because of the gross nature of the staining artefact observed, morphometric analysis was aimed at detecting the presence or absence of cells in the central thickness of the section, rather than at direct quantification of cell numbers. The distribution of staining within the sections thickness was measured by estimating the proportion of the section thickness that contained stained cells or not, an approach similar to that used by previous workers (Andersen and Gundersen, 1999). Using a Leica (UK) DMRE microscope with a stepping stage, cells were counted at different levels of focus, stepwise through the depth of the section. In each of five sections, 20 fields were selected randomly by delineation of the structure to be counted, and stepwise movement within the selected area to obtain a random sample. Cells were counted using a high power (×100) objective and a 10 × 10 eyepiece graticule counting grid. Starting at the surface of the section, the number of stained cells in the plane of focus was counted. The stage was then raised 1 μm at a time and at each step the number of stained cells was recorded. Another, quicker method of assessing penetration of staining was to use the stepping stage to make a simple tally count of the number of steps through the section that contain stained cells. In each of five sections, a single field (usually striatum) was randomly selected and examined using the high power (×100) objective. Starting at the surface of the section, the stage was raised 1 μm at a time and at each step the presence or absence of stained cells in focus was noted. These data were then expressed as the percentage of the tissue thickness at the top and bottom of the section that contained staining and the proportion of the section in the middle, which did not.

2.4. Adenoviral vectors

Some of the rat brains used in this study had been injected with a viral vector and stained immunohistochemically for

the transgene product. The recombinant, LacZ containing adenoviral vector (RAd36) used has been previously described (Gerdes et al., 2000). Briefly, the vector is a first generation, replication defective, E1, E3 deleted, type 5 adenovirus vector into which has been inserted the *Escherichia coli* LacZ gene. The inserted gene is driven by a 1.4 kb fragment from the major–immediate–early, murine cytomegalovirus promoter (MIEmCMV) (Zermansky et al., 2001). Vectors were grown using a complementing HEK-293 cell line (derived from human embryonic kidney) and purified by caesium chloride gradient centrifugation to a titre of 4×10^{10} IU/ml (Southgate et al., 2001).

2.5. Surgery

All surgery was performed under gaseous anaesthesia (2–3% isoflurane in Oxygen/Nitrous oxide). Animals were placed in a stereotaxic frame and cannula placements carried out using the co-ordinates of Paxinos and Watson. Viral vectors RAd36 containing the marker gene LacZ was delivered via a 30-gauge cannula connected to a 10 μl Hamilton syringe in a micro drive pump set to deliver at 1 μl/min. A volume of 3 μl was delivered over 3 min with 3 min allowed for diffusion of the injected solution into the brain before careful withdrawal of the cannula. The RAd36 was used at a titre of 1×10^6 IU/μl. The stereotaxic co-ordinates were $A = +0.6$, $L = \pm 3.0$, $V = -4.5$, with the nose bar set at −2.3 mm below the interaural line (Paxinos and Watson, 2003).

2.6. Experimental modifications to the standard protocol

The initial hypothesis tested was that the poor penetration of immunohistochemical staining was the result of a poor interaction of thick sections of tissue with one or more of the staining solutions used. On this basis, modifications to the standard IHC protocol, to prolong or improve exposure to the solutions involved were investigated.

2.6.1. Experiment 1: demonstration of the two-layered artefact

The brains from three normal rats were fixed and stained using the standard protocol (above). Sections were cut at 40 and 60 μm and 1 in 6 series of each stained with cresyl violet. Two further series of each were stained immunohistochemically for DARPP32 and NeuN. Morphometric analysis was carried out to determine the distribution of stained cells through the thickness of the sections.

2.6.2. Experiment 2: modification of the diaminobenzidine (DAB) solution

Free-floating, 60 μm sections from 3 rat brains were stained immunohistochemically for DARPP32 using the standard protocol up to, but not including the DAB step. The DAB reaction was then carried out using one of the following modifications intended to either improve the penetration of solutions into the section or to extend the time available for diffusion of the solutions used into the sections.

- *Addition of a surfactant to the DAB solution*. DAB solution with 0.05% Triton X-100 added. (Final wash prior to the DAB in TB containing 0.05% Triton X-100.) 5 min reaction time.
- *Dilution of the DAB solution to enable extension of the reaction time*. The standard DAB solution was diluted using Tris buffer. (a) ×2 dilution; 10 min reaction time. (b) ×5 dilution; 25 min reaction time.
- *Pre-incubation of sections in non-activated DAB*. Sections were pre-incubated in DAB solution without hydrogen peroxide, then transferred to the standard DAB solution: (a) 5 min pre-incubation; 5 min reaction time. (b) 20 min pre-incubation; 5 min reaction time.

2.6.3. Experiment 3: modification of the secondary antibody and strepavidin–biotin–HRP complex steps

Free-floating, 60 μm sections from 4 rat brains were used. Animals had received injections into the striatum of a viral vector containing the marker gene LacZ (see methods above). Striatal sections from each animal were stained immunohistochemically for β-galactosidase using the standard protocol, with the following modifications:

- *Extension of the secondary antibody incubation time*. Incubation in the secondary antibody was extended from 3 h to overnight.
- *Extension of the ABC incubation time*. Incubation in the ABC solution was extended from 2 h to overnight.
- *Extension of both the secondary antibody and ABC incubation times*. Sections were incubated overnight in the secondary antibody and overnight in the ABC solution.

2.6.4. Experiment 4: post-sectioning modification of cell membranes

To test whether the barrier to staining might be the cell membrane itself, sections were treated with known membrane permeabilising agents. Free-floating, 60 μm sections from 4 rat brains fixed using the standard protocol were used. As in experiment 2, the animals had received injections into the striatum, of a viral vector expressing the marker protein β-galactosidase. Striatal sections from each animal were stained immunohistochemically for β-galactosidase using the standard protocol following pre-treatment with known membrane permeabilising agents.

- *Pre-treatment with acetone*. Sections were washed in distilled water, and then placed in 100% acetone for 10 min. 3 × 5 min washes in water before re-equilibration to TBS.
- *Pre-treatment with the detergent Tween 20*. A solution of 0.2% Tween 20 (v/v) in TBS for 1 h, followed by replacement of the TX-100 in the blocking and primary antibody steps with 0.2% Tween 20.
- *Heat treatment*. Microwave treatment of sections in a solution of phosphate buffered saline. Sections were brought to the boil on high power, then kept boiling for 5 min (low power), and cooled to room temperature.

- *Delipidisation of the sections with chloroform/ethanol*. Using a standard delipidising method for histochemical methods: sections were mounted onto gelatine coated microscope slides and dried overnight. Dehydration was carried out in an ascending series of alcohols to 100% ethanol, then sections were immersed for 30 min in a solution of 50% chloroform/50% ethanol before rehydration in descending alcohols to water before re-equilibration in TBS. Immunohistochemistry was then carried out on slide mounted sections.

2.6.5. Experiment 5: modification of the fixative to affect cell membrane permeability

The brains from three normal rats were perfusion fixed using modifications of the standard paraformaldehyde fixative:

- Standard 4% PFA containing 0.2% (v/v) Triton X-100 (PFA/TX-100);
- 0.1 M PBS/4% PFA diluted with 20% (v/v) methanol (PFA/methanol).

Perfusion was carried out using the standard method and after removal from the skull, brains were post-fixed in the perfusion-fixative for 4 h. Sections were cut at 60 μm thickness and stained immunohistochemically with NeuN antibody using the standard protocol. To assess the distribution of cells within the section thickness, counts of positively stained cells in the corpus striatum and pre-frontal cortex were carried out using a fixed-area counting frame on five sections per brain. The total number of stained cells seen at different depths from the surface of the section was noted.

2.6.6. Experiment 6: investigating the influence of paraformaldehyde exposure

Three concentrations of fixative, 1, 2 and 4% paraformaldehyde were prepared using a stock solution of 20% PFA diluted in PBS. For each fixative three rats were perfused and the brains processed using the standard protocol. Sections were stained immunohistochemically for NeuN and histochemically using cresyl violet. Cell counts were carried on the NeuN stained sections through the depth of the section in the striatum of five sections from each brain and the number of stained cells were recorded at each level.

3. Results

3.1. Experiment 1: demonstration of the two-layered artefact

Fig. 2 shows how, when using the standard protocol, in DARPP32 stained sections there was a bi-phasic distribution of stained cells through the section thickness in both 60 and 40 μm thick sections. In each case, stained cells were only observed within 5–6 μm of the section surface, independent of section thickness. Taking into account the tissue shrinkage, which occurs during subsequent dehydration of the sections for cover-slipping, this corresponds to a depth of staining into the original section of just 10–15 μm from each surface. Stained

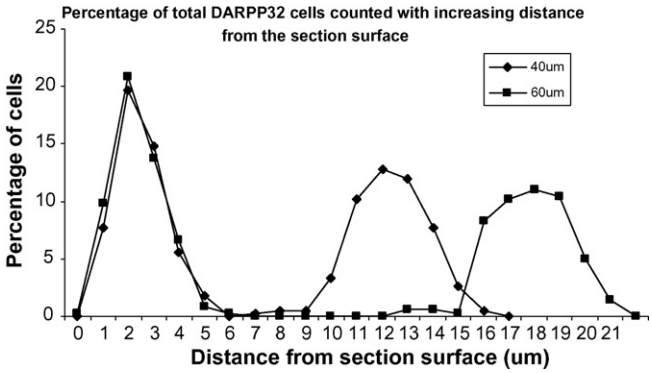

Fig. 2. Counts of DARPP32 positive cell bodies in the striatum of a normal rat. Cells were counted at 1 μm depth intervals through the thickness of the sections. Data are represented as the percentage of the total number of all cells counted that were found at each depth interval. In both 40 and 60 μm thick sections, stained cells are seen only within a thickness of 5–6 μm from the section surface. (Note that, due to dehydration in alcohol, there was shrinkage of the 40 and 60 μm sections to 15–17 and 19–21 μm, respectively.) The broad spread of the distal peaks (on the right hand side of each plot) relative to the proximal peak (on the left hand side of each plot) is an artefact resulting from counting from one surface, in samples of slightly varying thickness.

cells in sections stained for the neuronal nuclear marker NeuN had a similar bi-phasic distribution, whereas counts of sections stained histochemically with cresyl fast violet showed an even distribution throughout the section (Fig. 3).

3.2. Experiment 2: modifications to the DAB protocol

Three modifications were used to improve the penetration of the DAB solution into the sections. Firstly, the addition of a surfactant (Triton X-100) to the DAB solution. Secondly, dilution of the DAB solution, to allow the incubation time to be prolonged. Thirdly, pre-incubation of sections in "non-activated" DAB solution (containing no hydrogen peroxide) prior to the addition of the hydrogen peroxide substrate, to allow a greater

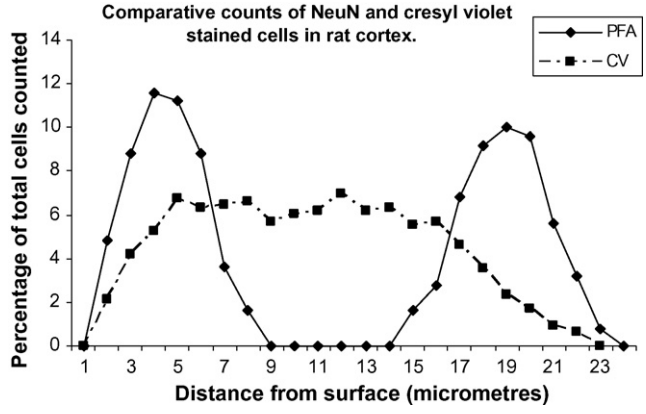

Fig. 3. Counts of NeuN and cresyl violet stained cells in 60 μm sections of the prefrontal cortex of normal rats. Cells were counted at 1 μm depth intervals through the thickness of the sections. Because of differences in the final section thickness with each method, data are represented as the percentage of the total number of all cells counted which were found at each depth interval. NeuN stained cells show the characteristic bi-phasic distribution with depth, whilst the cresyl violet positive cells are distributed uniformly throughout the section.

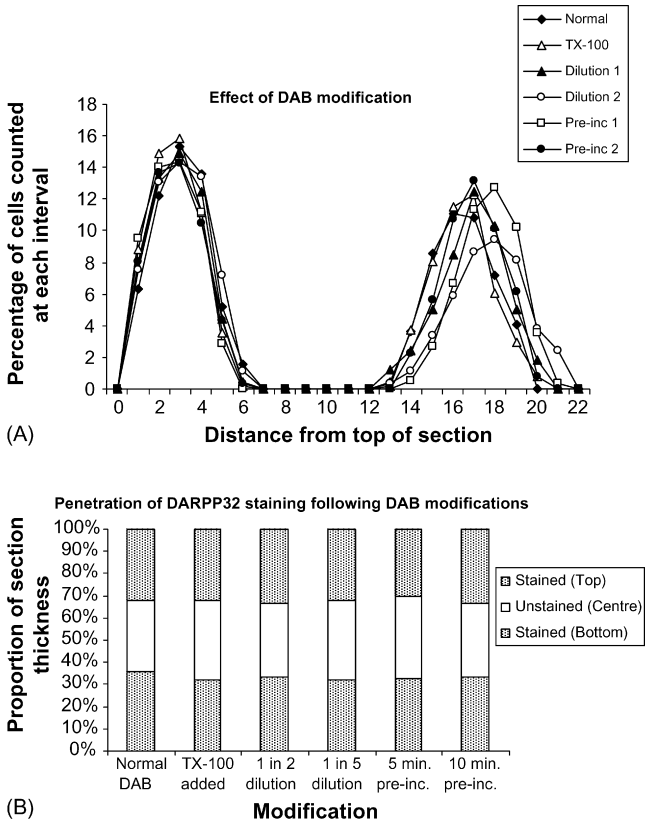

Fig. 4. (A) Counts of DARPP32 positive cell bodies in the striatum of a normal rat from experiment 1. Cells were counted at 1 μm depth intervals through the thickness of the sections. None of the modifications to the DAB staining protocol was able to modify the distribution of IHC staining through the section thickness. (B) Data from the same sections expressed as the proportion of the section thickness containing stained cells for each condition. Each bar can be viewed as a representation of the staining in the *z*-axis, showing the relative depth of stain penetration from each surface (grey) and the amount of unstained tissue in the central thickness of the section (white).

length of time for the DAB to penetrate into the section prior to the colour reaction.

Fig. 4 shows the distribution of stained cells throughout the section thickness for the different conditions of staining used. The results show that for all conditions of DAB, the distribution of DARPP32 positive cells within the thickness of the tissue sections was similar. Neither extension of the DAB pre-incubation time, nor of the DAB reaction itself, produced a change in the penetration of staining. Nor did the addition of the TX-100 despite its surfactant properties.

3.3. Experiment 3: modifications to the secondary antibody and streptavidin–biotin-complex steps

To ensure that enough time was allowed for the both the secondary antibodies, and the streptavidin–biotin–HRP-complex to diffuse into the section, we investigated the effects of extending the secondary antibody incubation time from 3 h to overnight, or the ABC/HRP incubation time from 2 h to overnight, or both. Fig. 5 shows both the depth of staining observed as the proportion of the total section thickness and the proportion of the section thickness in the middle of the section that was unstained

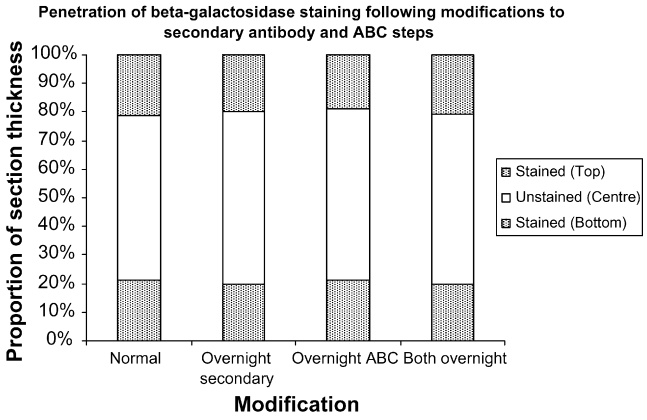

Fig. 5. Data from experiment 2 showing the proportion of the section thickness containing β-galactosidase stained cells following modifications of the secondary antibody and/or ABC steps of the IHC protocol. Each bar can be viewed as a representation of the staining showing the relative depth of stain penetration for each surface and the amount of unstained tissue in the central thickness of the section. None of the modifications to the protocol were able to affect the depth of stain penetration.

in each condition. None of the modifications used produced an effect on the depth of stain penetration observed.

Note that the apparent depth of penetration of the β-galactosidase staining in the striatum in experiment 2 was less than that observed with DARPP32 in experiment 1. All antibodies exhibit a clear two-layer artefact but there slight variations in the depth of staining are seen with each. The cells demonstrated in the β-galactosidase stained sections were exclusively small glial cells. DARPP32 stains the larger, output neurons of the striatum, and this may account for the increased depth of staining seen when using this antibody. An important observation was made in the sections from this experiment. Some areas of the sections contained blood vessels in which the vascular endothelium had been transduced by the viral vector. In all cases where this was observed, the β-galactosidase staining had the same distribution in the blood vessels as in the striatal parenchyma itself (Fig. 6). Namely, immunoreactivity was seen only at the top and bottom of the surfaces of the section, even though all of the solutions used in the immunohistochemical protocol had full access to the vascular endothelium via the lumen of the blood vessel.

3.4. Experiment 4: post-sectioning modification of cell membranes

It was hypothesised that treatment of sections with solvents or detergents prior to staining might permeabilise the tissue and allow better penetration of the IHC stain. Free-floating sections were pre-treated with either acetone, the detergent Tween-20, or heat treated using an antigen retrieval protocol involving heating to 100 °C. A further set of sections was mounted on microscope slides and delipidised by exposure to chloroform/ethanol.

The results obtained are summarised in Fig. 7, which shows the proportion of the section thickness that was stained or unstained for each condition. None of the free-floating modifications had an effect on penetration. Full thickness staining was

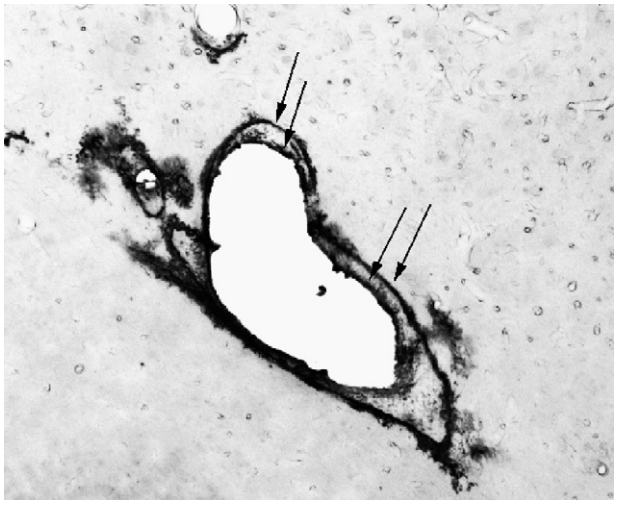

Fig. 6. β-Galactosidase staining of blood vessel in the ventral striatum adjacent to the corpus callosum from experiment 2 (60 μm section). The vascular endothelium has been transduced with the LacZ bearing adenoviral vector. However, the endothelium is stained only in the top and bottom surfaces of the section (arrows) whilst the vascular endothelium in the central thickness of the section is unstained, despite unhindered access of the staining solutions.

observed on the slide-mounted, delipidised sections. However, in these sections, the quality of the staining was greatly reduced, fewer stained cells were observed, and these had an altered distribution in the sections. The intensity of staining much also much reduced, and such sections could not be used routinely for immunohistochemical staining or for the purposes of quantitative analysis.

3.5. Experiment 5: modification of the fixative to affect cell membrane permeability

In this experiment, membrane-disrupting agents were used during the fixation process in order to preserve the cell membranes in a state that might allow full penetration of IHC staining. Rat brains were prepared using 4% paraformaldehyde perfusate to which was added either the detergent Triton X-100 or methanol and sections were processed for NeuN IHC using the standard protocol.

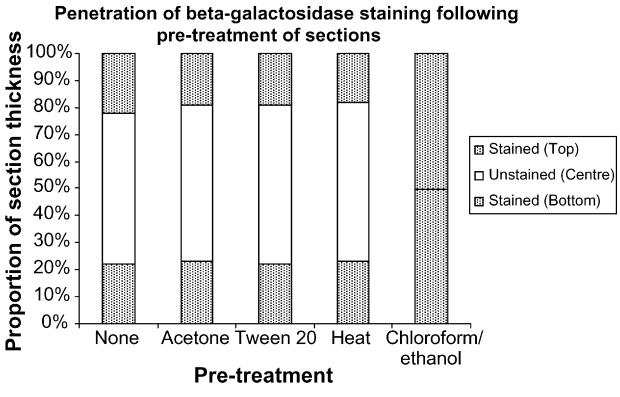

Fig. 7. Data from experiment 3 showing the proportion of the section thickness containing β-galactosidase stained cells following pre-treatment of sections with membrane permeabilising agents. Each bar can be viewed as a representation of the staining showing the relative depth of stain penetration for each surface and the amount of unstained tissue in the central thickness of the section. Only the chloroform/methanol modification of the protocol was able to affect the depth of stain penetration.

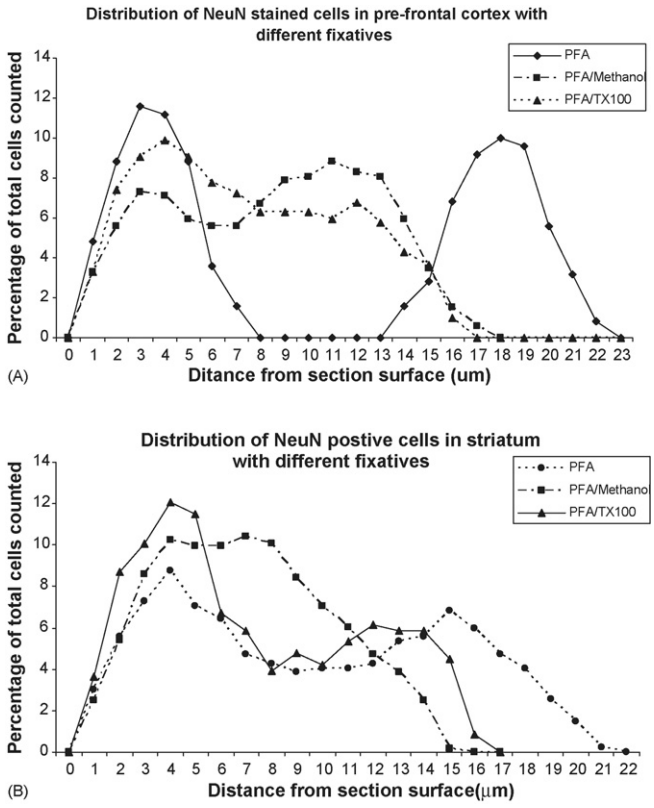

Fig. 8. (A) Plot of the number of NeuN positive cells in prefrontal cortex, counted at increasing distance from the surface of the surface in sections from brains perfused with different fixatives (experiment 4). Note that cells in sections from the PFA fixed brains show the familiar two-peaked distribution. However, in sections from the brains fixed using methanol or TX100 containing fixatives the two-peak profile is dramatically reduced indicating successful staining of cells in the central thickness of the section. (B) Plot of the number of NeuN positive cells in corpus striatum, counted at increasing distance from the surface of the surface in sections from brains perfused with different fixatives (experiment 4). Note that cells in the PFA and PFA/TX100 fixed brains have a slight two-peaked distribution though this is not as pronounced as that seen in prefrontal cortex sections.

Fig. 8 shows the distribution, of NeuN positive cells through the section thickness in sections of pre-frontal cortex or the corpus striatum for each condition. Sections from both PFA/methanol and PFA/TX100 fixed brains contained NeuN positive cells throughout the section thickness whilst sections from the PFA-only fixed brain did not (Fig. 8A). Both methanol and TX100 were effective in permeabilising cell membranes in the PFC. However, in the corpus striatum, only the PFA/methanol fixed brain showed full thickness staining in the striatum. Sections from the PFA/TX100 fixed brain had a similar distribution of stained cells to that seen in the PFA-only fixed brain (Fig. 8B).

In the striatum, there were significantly more cells in the PFA/methanol treated sections when compared to the PFA-only treated sections ($F(2, 49) = 8.69$; $p < 0.001$). In the pre-frontal cortex, there were significantly greater numbers of cells in both

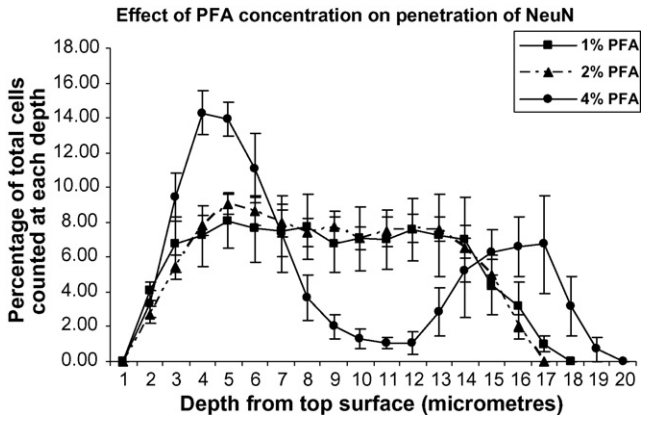

Fig. 9. The effect of PFA concentration on the penetration of NeuN staining (experiment 5). The plot shows the percentage of the total number of NeuN stained cells counted at 1 μm intervals with increasing depth from the top surface of the section. Cell counts were carried out on five sections through the dorsolateral cortex from three rat brains for each condition. Note that whilst cell counts are distributed throughout the thickness of the section in the 1% PFA and 2% PFA conditions in the 4% PFA condition we see the bi-phasic plot characteristic of the two-layer staining. Error bars are S.E.M.s.

the PFA/methanol and the PFA/TX100 when compared to the PFA only group ($F(2, 52) = 24.10$; $p < 0.001$).

3.6. Experiment 6: investigating the influence of paraformaldehyde fixation

To test whether the barrier to staining might result from over-exposure to paraformaldehyde, perfusions were carried out using either 1, 2 or 4% paraformaldehyde in PBS buffer. Counts from NeuN sections showed that as previously, the brain fixed using the standard protocol (4% PFA) had the two-layer staining artefact. In sections from the 1% PFA and 2% PFA fixed brains this artefact was not present and staining was observed through the full thickness of the section (Fig. 9) and an effect of paraformaldehyde concentration on the penetration of NeuN staining was clearly demonstrated.

4. Discussion

In the present paper, we have described the problem of full thickness immunohistochemical staining in thick sections of brain tissue, with particular reference to perfused paraformaldehyde fixed brains. We have demonstrated the two-layer staining artefact, using simple methods involving focusing through the thickness of sections using a high magnification microscope objective and either plotting the numbers of cells counted at each depth or recording the levels in which stained cells are observed.

Our primary hypothesis was that the poor penetration of immunohistochemical staining might result from poor interaction of the tissue with one or more of the staining solutions involved, due to the thickness and/or the hydrophobic nature of the sections. On this basis, modifications to the standard IHC protocol, to prolong or improve exposure to the solutions involved were investigated. As the shortest step in the procedure, and one which contained no surfactant compounds in the solution, the DAB step of the immunohistochemical protocol was an obvious candidate for the source of the problem. However, the failure of any modification of this step to alter the two-layer staining effect is striking, and the root cause of the two-layer staining artefact must be occur at an earlier stage in the staining protocol. However, in experiment 2, extension of the incubation times of the secondary antibody and/or ABC steps, were also ineffective at increasing the depth of penetration of the IHC staining.

These results, together with the observed distribution of β-galactosidase staining in the blood vessels seen in experiment 3, indicated that the problem of full thickness staining (FTS) was not a penetration problem *per se*. That is, the problem did not arise from a failure of one or more solutions used to diffuse into a thick section of tissue. We therefore concluded that some component of the tissue, was impermeable to at least one of the steps in the IHC protocol, and that the cell membranes were a likely candidate We hypothesised that only cells near the surface of the section, in which the membrane had been cut during sectioning were able to take up the immunohistochemical stain. This hypothesis is consistent with our observations that: (a) Cresyl violet and other histochemical stains give full thickness staining (Fig. 3). Not only are the cresyl violet molecules small, relative to the immunoglobulins used in the IHC, but sections for cresyl violet are routinely delipidised by dehydration and immersion in chloroform. (b) In β-galactosidase stained sections two-layer staining of blood vessels was observed, despite the full access of all solutions to the vascular endothelium. (c) Slight differences in the depth of penetrations depending on the antibodies used, probably due to differences in the size, shape and orientation of the cells being stained. Thus, larger cells will on average, penetrate more deeply into the section from the cut surface and display a greater apparent depth of staining.

In experiment 5, post-sectioning treatments of tissues with solvents and detergents were unable to produce full thickness staining except in one condition in which a chloroform/ethanol mixture was used to "delipidise" the sections. However, a dramatic deterioration in the quality of the staining was observed. Addition of membrane-disrupting agents such as methanol and Triton X-100 to the fixative solutions was successful in increasing the depth of penetration of NeuN staining in both the cortex and striatum. However, the differences between these two structures seen in the extent of penetration was puzzling. In the standard protocol, fixation is a two-step process consisting of perfusion of the brain *in situ* via the ascending aorta, followed by removal of the brain from the skull and post-fixation by immersion in the same fixative. This protocol has the potential therefore to produce differential fixation in different regions of the brain, and this might explain the differential effects of methanol and TX100 observed in cortex and striatum. Replenishment of both paraformaldehyde and TX100 during the post-fixation period relies on diffusion of these substances from the surface of the brain. Thus, in the pre-frontal cortex, which is close to the surface of the brain, replenishment of these substances will be more efficient than in the much deeper striatal tissue. This might explain

why, in the pre-frontal cortex we see greater effectiveness of the TX100, due to better access of TX100 in the cortex than in the striatum resulting in better permeabilisation of the membranes. In contrast there is a larger two-layer effect in the PFC than in the striatum in the PFA-only fixed brains, which we hypothesise is due to better access of PFA in the cortex than in the striatum having the opposite effect of decreasing membrane permeability.

As all counts were carried using the same sampling protocol, and over an equivalent sampling area, counts could reasonably be compared between treatments (Fig. 10). In the striatum, there were significantly more cells in the PFA/methanol treated sections when compared to the PFA-only treated sections. In fact, the methanol treated striata contained 27% more stained cells than the PFA only fixed brain, providing us with an estimate of the number of striatal cells that are unstained in the central thickness of 60 μm sections using the standard method of fixation. In the prefrontal cortex there were significantly greater numbers of cells in both the PFA/methanol and the PFA/TX100 when compared to the PFA only group, and the differences were even greater than in the striatum; sections from brains prepared with either of the modified fixatives contained at least 2.5 times the number of stained cells than those from PFA only treated brains.

Thus, it is not the thickness of the section itself, which is a barrier to penetration, but elements within the section, most likely the plasma membranes of the cells themselves, which are

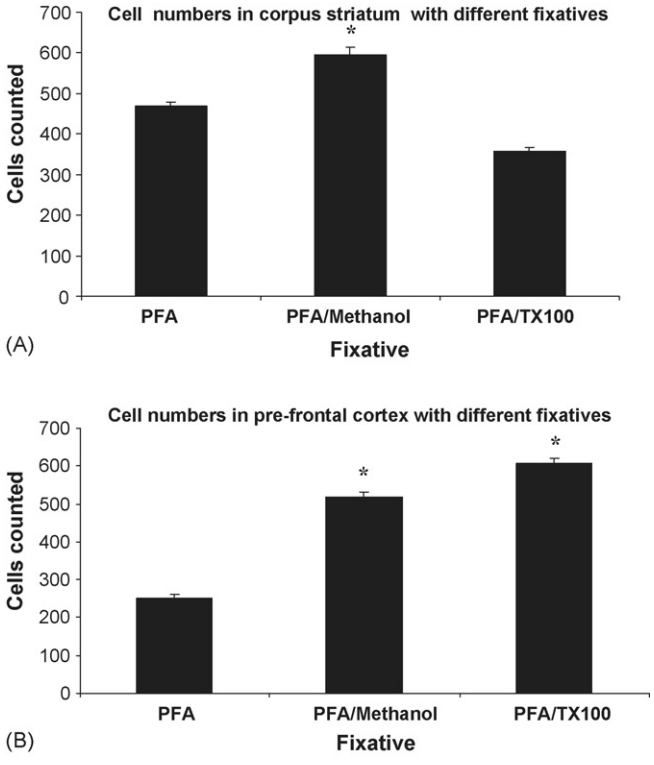

Fig. 10. Experiment 4: Using the same method of sampling, the number of NeuN stained cells counted in the pre-frontal cortex (A) and striatum (B) in sections from brain fixed using different fixative modifications. Counts made in the pre-frontal cortex on PFA/methanol or PFA/TX100 modifications are higher than those obtained from PFA fixed sections. Asterixes indicate a significant difference form the standard PFA fixed brain. Error bars are S.E.M.s.

impermeable to the immunohistochemical stain. There are several strands of evidence in support of this conclusion. Firstly, in sections stained for the marker gene β-galactosidase, blood vessel walls expressing the gene exhibited two-layered staining, despite the fact that all of the solutions used had access to the vascular endothelium via the lumen of the blood vessel. Secondly, full thickness staining of sections is obtained with non-immunohistochemical methods such as cresyl violet or haematoxylin and eosin. In comparison to the antibodies used in immunohistochemistry, dye molecules are relatively small and therefore more likely to overcome barriers to penetration. In addition, prior to staining with histological methods, sections are routinely delipidised using solvents such as chloroform and methanol, which disrupt the structure of cell membranes. Thirdly, we have shown that full thickness staining can be obtained by modification of the standard 4% paraformaldehyde to include membrane-disrupting agents such as methanol or Triton X-100, or by reduction of the concentration of paraformaldehyde to either 1 or 2%.

The nature of the barrier to immunohistochemical penetration is at present unknown. Four percent paraformaldehyde should more properly be considered as 4% formaldehyde as the paraformaldehyde polymer (in theory) dissociates to the monomeric formaldehyde at low concentrations. Formaldehyde fixes tissue by cross-linking a wide range of tissue components including proteins and lipids, both of which are present in cell membranes. Aldehydes are known to be progressive fixatives such that the degree of cross-linking attained is proportional to the fixation time. Widespread use of 4% paraformaldehyde arose in the 1980s when immunohistochemical methods became available. Four percent paraformaldehyde was substituted as a purified version of the then standard fixative, 10% formalin. Commercially produced formalin contains methanol and other impurities which can adversely affect the antigenicity of tissue sections, and which are eliminated in solutions freshly prepared from paraformaldehyde. However, paraformaldehyde is difficult to dissolve in water and may persist in polymeric form in solution (Bancroft and Stevens, 1990). This may or may not be an issue in the current work but experiments to determine the stability and reproducibility of 4% paraformaldehyde solutions would be informative in this regard.

The problem of full thickness staining is not widely discussed in the scientific literature, though several other workers have commented on the problem in work centred on other investigations. Van de Berg et al. analysed sections of rat brain, stained immunohistochemically for parvalbumin, by counting cells at five different depths in the section thickness. They reported that the central thickness (33%) of the section contained no stained cells. They went on to use an optical dissector method of counting, using only the stained portions of the section as if these were two thinner stained sections contained within the a thicker slice of tissue (Van de Berg et al., 2003). A similar approach was adopted by Calhoun et al. to count synaptophysin immunoreactive boutons in rat hippocampus (Calhoun et al., 1996) and by Jinno et al. investigating rat hippocampal GABAergic neurons (Jinno et al., 1998). Kirik et al. commented on two-layered staining in sections of rat's brain in which they were counting

TH positive cell numbers in dopamine transplants and they too counted the stained portions using the optical dissector and made corrections to their calculations to account for this (Kirik et al., 2001).

Our results suggest that the barrier to staining is the cell membrane itself (or some component of the extra-cellular matrix closely associated with the cell body). We propose that only those cells, which have had their membranes or processes cut during sectioning are able to take up the immunohistochemical staining and that cells uncut during sectioning remain unstained. If this is so, then it is possible that stained portions of the section may contain intact cells that are unstained, and that counting methods that concentrate on the stained thickness of the section will continue to underestimate true cell numbers. Whilst the problem of full thickness staining is primarily a histological issue, it is one that has special implications for stereological methods, firstly, because it would be desirable to use the optical dissector method in fully stained thick sections. Secondly, because if the barrier to antibody penetration is associated with the cell membrane then even the most rigorous application of three-dimensional stereological counting will fail to produce an accurate estimate of cell numbers.

Other workers have made detailed studies of the range of artefacts, which are encountered during the cutting and staining of tissue sections. Gardella et al. looked at shrinkage of tissue sections in the *z*-axis during cutting and staining of either vibratome, cryostat or celloidin cut sections (Gardella et al., 2003). They reported an artefact similar to that reported here, namely that more cells were observed at the surfaces of the sections than in the central thickness, a problem which they attributed to greater compression of the section surfaces during dehydration. However, this is apparently not the same artefact as described in the current paper. The stain used was thionine, a small molecular weight dye, which was able to penetrate the sections easily and produce staining of cells in the full thickness of the section. Whilst we report a complete absence of staining in the central thickness of the section, they reported only differential distribution of stained cells in the *z*-axis. In a systematic investigation of tissue shrinkage artefacts, Dorph-Petersen et al. reported reduced cell counts in the central portion of thick sections, also stained using thionine. However, they concluded that the observed artefact was caused by differential shrinkage within the sections, such that the surfaces of the section were more compacted during processing (Dorph-Petersen et al., 2001).

In the modern neuroscience laboratory the failure to obtain full thickness staining with immunohistochemical methods is a serious problem. Our results suggest that, using conventional methods of fixation and staining, immunohistochemically stained sections contain an unknown and variable portion of the section thickness in which there are no stained cells. Additionally, the analysis of histological material increasingly calls for the use stereological methods and the optical dissector method of cell counting. Whilst the thick section used in the present work ought to be ideally suited, such methods, cannot adequately be carried on sections stained in this way.

It is our experience that the two-layer artefact described here, is observed by many laboratories in the neuroscience field (personal communications with a large number of workers over many years). As a consequence, many laboratories that aspire to carry out unbiased stereological sampling on their material feel unable to do so. The fixatives and perfusion protocol used by our laboratory are standard methods, which are widely used in the neurosciences. If our conclusions were correct, then we would expect that any laboratory using such protocols is likely to experience two-layer immunohistochemical staining on thick sections. Some workers may be unaware of the two-layer effect because they use sections of less than 40 μm thickness. In such sections unstained cells at the centre of the section would be easy to overlook. Alternatively, it is possible that some laboratories, assuming that the problem stems from the section thickness, have attempted to solve the problem by cutting still thinner sections. Our results show that, depending on the size and shape of the cells being stained, cells might still be missed even in relatively thin sections using immunohistochemical protocols.

We conclude that modification of the widely used, paraformaldehyde-based, perfusion protocol will be necessary in order to achieve full thickness staining with immunohistochemical methods. The task may not be an easy one. Ideally, a modification of the fixative is required which enables the same perfusion protocols to be used, and more importantly, does not necessitate major modification of the staining method. Any new protocol should ideally be as versatile as the present one in terms of the techniques, which can be carried out on the tissue. In our laboratory tissues preserved using the standard fixation protocol can be stained immunohistochemically using more than 30 different antibodies, as well as a number of histochemical stains. Whilst the addition of membrane-disrupting agents and/or the reduction of the concentration of paraformaldehyde, are obvious modifications to try, there are pitfalls to consider. Membrane-disrupting agents such as methanol also have fixative properties and have the potential to adversely affect the immunohistochemical detection of antigens within the tissue, particularly when using monoclonal antibodies, which target a single tissue epitope. Reduction of the concentration of paraformaldehyde may have the same effect, altering the antigenicity of compounds of interest so that they are undetectable by IHC. Clearly the chemical and anatomical preservation of the tissue must not be lost in the pursuit of membrane permeability. We may therefore need to consider the use of compound fixatives, in which the concentration of PFA is reduced, and other fixative agents such as glutaraldehyde, picric acid or zinc chloride are added to maintain levels of fixation. We are currently investigating such options.

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